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THE STRUCTURE, FUNCTION AND EVOLUTION OF CYTOCHROMES

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ABBREVIATIONS

CCP	Cytochrome *c* peroxidase
CD	Circular dichroism
CDNP	4-Carboxyl-2, 6-dinitrophenol
D.a.	*Desulfuromonas acetoxidans*
*D.d.*N.	*Desulfovibrio desulfuricans* Norway
DML	β, γ-Dimyristoyl L-α-lecithin
*D.v.*M.	*Desulfovibrio vulgaris* Miyazaki
N-MeIm	*N*-Methyl imidazole
NMR	Nuclear magnetic resonance
OM	Mitochondrial outer membrane
PMS	Pentamethylenesulfide
R	$\Sigma\|F_o-F_c\|/\Sigma\|F_o\|$ where F_o and F_c are the observed and calculated structure factors respectively
Redox	Reduction–oxidation. The redox potential is measured against the hydrogen electrode at pH 7. By convention the more positive the redox potential the more stable the reduced form.
RMS	Root mean square
SO	Sulfite oxidase
TFA	Trifluoroacetate
TFC	Trifluoromethylphenylcarbamate
THT	Tetrahydrothiophene
TML	Trimethyl lysine
TPP	*Meso*-tetraphenylporphyrinato-iron

I. INTRODUCTION

Cytochromes form a diverse group of proteins with only a few features in common. They all contain protoheme IX (Fig. 1) or one of its derivatives and function in electron transport, usually as components of a complex reaction pathway. They are found in nearly all forms of life, including bacteria, protozoa, yeasts and all higher organisms.

Most cytochromes are involved in energy transduction to produce ATP from the oxidation of metabolites or by photosynthesis. As such they are nearly always associated with membrane systems and often can be solubilized only by detergents. For example, the mitochondrial cytochrome *c* is a water soluble, peripheral membrane protein. However, the remaining mitochondrial cytochromes, notably cytochromes *b*, c_1 and cytochrome oxidase, are integral membrane proteins often part of multi-subunit complexes and are very difficult to isolate and characterize as individual protein species. Thus, of the mitochondrial cytochromes, only the soluble *c*-types have been characterized structurally.

The first *b*-type cytochrome to be well characterized is the microsomal cytochrome b_5. It is functionally quite distinct from the *b* cytochromes of mitochondria. Although also an

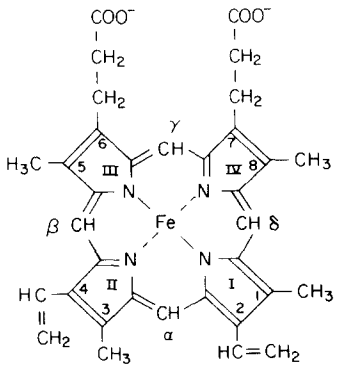

FIG. 1. Structural formula of the heme group. The peripheral positions of the pyrrole rings (I to IV) are numbered 1 to 8. The "front face" is defined as viewed with the peripheral positions increasing clockwise. Mesoporphyrin is the heme derivative in which the vinyl groups of protoporphyrin IX at positions 2 and 4 are replaced by ethyl groups.

integral membrane protein, it could be released by mild proteolysis and studied in a soluble form. Most of the remaining well characterized cytochromes are found in bacteria and can be isolated and studied in aqueous solution (see Meyer and Kamen, 1982).

The scope of this review will be limited to cytochromes whose high resolution crystal structures are known. These include several monoheme *c*-type cytochromes from eukaryotic and prokaryotic sources, two *b*-type cytochromes, b_{562} and b_5, the high spin *c'* cytochrome and two related multiheme c_3 cytochromes. The cytochromes will be divided on a structural basis into four major families and discussed separately. These are the cytochrome *c* family,* the cytochrome b_5 family, the four-α-helix *c'*-b_{562} family and the four-heme c_3 family.

The major properties and structural features of the cytochromes discussed in this review are summarized in Table 1. All cytochromes of Table 1 transfer electrons *via* a reversible change in the oxidation state of the heme iron between +2 and +3, but carry out no other catalytic function. All have at least one histidine coordinated to the iron atom. However, the mode of attachment and the nature of the sixth iron ligand varies among them. The redox potentials also show considerable variation among the cytochromes as do the environment and orientations of the heme groups.

The cytochrome *c* family has been thoroughly reviewed elsewhere (Dickerson and Timkovich, 1975; Salemme, 1977; Ferguson-Miller *et al.*, 1979; Meyer and Kamen, 1982). The b_5 family, the four α-helix family and the c_3 family, having been characterized structurally more recently, have not been reviewed extensively and will receive relatively broader coverage than the cytochrome *c* family.

TABLE 1. PHYSICAL PROPERTIES OF CYTOCHROMES

Cytochrome	Redox Potential (mV)	Mode of Heme Attachment	6th Ligand	Spin State	Degree of Heme Exposure
c	100 to 450	covalent	Met	low	nearly buried
b_5	20	non-covalent	His	low	slightly exposed
b_{562}	185	non-covalent	Met	low	partly exposed
c'	0 to 140	covalent	vacant	high	partly exposed
c_3	−340 to −280	covalent	His	low	3 nearly buried, 1 slightly exposed

II. MITOCHONDRIAL-TYPE CYTOCHROME *c* FOLD

1. *General Properties*

The mitochondrial type cytochrome *c* family is found in a wide variety of eukaryotic and prokaryotic organisms. The amino acid sequences of nearly 70 eukaryotic and about 32 bacterial cytochromes *c* have been determined. They fall into approximately eight classes as summarized in Table 2. The first two classes contain the cytochromes *c* from the mitochondria of eukaryotes and the cytochromes c_2 from bacteria. The members of these two classes are very similar structurally and spectroscopically and range from 97 to 129 amino acid residues in length. The remaining six classes are all bacterial, differing somewhat in their spectroscopic and structural properties and range from 79 to 99 residues in length.

The structures of nine mitochondrial-type cytochromes *c* have been determined to atomic resolution. Four of these are from eukaryotic sources and five from bacterial. Two of the latter belong to the c_2 class. The amino acid sequences of these nine cytochromes *c* are shown in Fig. 2.

With two exceptions, all mitochondrial-type cytochromes *c* contain a common covalent site of attachment of the heme group to the amino terminal half of the polypeptide chain. The

* "Cytochrome *c*" will refer to the highly conserved class of hemoprotein from mitochondria while "cytochrome *c*-type" will refer to any of the structurally related members from prokaryotic sources. The broader definition of cytochrome *c* to include *all* cytochromes with covalently bound heme will *not* be used. However many traditional names such as c_2, c_{556}, etc. will be retained.

TABLE 2. PROPERTIES AND CLASSIFICATION OF MITOCHONDRIAL-TYPE CYTOCHROMES *c*

Name of Subclass[a]	Source	Size (No. of Residues)	Dickerson Classification[b]	Redox Potential (mV)	Crystal Data	Function, Redox Partners, Other Comments
c	Mitochondria of Eukaryotes	102–112	M	~260	Tuna, 1.8A oxidized[c] 1.5A reduced[d] Bonito, 2.8A oxidized[e] 2.3A reduced[f] Horse, 2.8A oxidized[g] Rice, 2.0A oxidized[h]	Component of mitochondrial electron transport chain: oxidative phosphorylation. Electron donor: Complex III, bc_1 cytochrome *c* reductase. Electron acceptor: Complex IV, cytochrome *c* oxidase.
c_2	Mostly purple non-sulfur phototrophic bacteria; a few aerobic denitrifying bacteria	97–129 2 structural subclasses	L and M	250–400	*Rhospirilum rubrum* c_2[i], 2.0A oxidized *Paracoccus denitrificans*[j], 2.45A oxidized	Electron transfer component for cyclic photo-phosphorylation. Also, occasional aerobic electron transfer. In non-phototrophic bacteria interacts with *cd*-type nitrite reductase. This class forms continuously variable structural class with mitochondrial cytochromes *c*.
c_{551}	Mostly Pseudomonads, a few phototrophic non-sulfur bacteria	82–93	S	270–280 for Pseudomonads 100–400 for Phototrophic	*Pseudomonas aeruginosa* c_{551}[k], 1.6A oxidized, and reduced	In Pseudomonads, serves as electron donor to *cd*-type nitrite reductase or c_{551} peroxidase. Role equivalent to azurin with which it readily reacts. In phototrophic, serves in photophosphorylation.
c_{555}	Green photosynthetic bacteria	86–99	S	103–145	*Clorobium thiosulfatophilum* c_{555}[l] 2.7A oxidized	Phototrophic electron transport. Also, thiosulfate-cytochrome c_{555} oxidoreductase.

c_{554}	Eukaryotic algae, Cyanobacteria	83-89	S*	340-390	*Anacystis nidulans* c_{554}[m], 3.0A oxidized	Photosynthetic electron transport. Electron donor: membrane-bound cytochrome *f*. Electron acceptor: chlorophyl P700. c_{554} interchangeable with plastocyonine.
c_4	Pseudomonads, *Azotobacter*	~190 (diheme cytochrome)	S (each half)	325	*Pseudomonas aeruginosa* c_4[n], 5.0A, oxidized	Unknown function, produced under anaerobic growth together with azurin. Structure is two c_{551}-like folding domains which probably arose via gene fusion.
c_5	Pseudomonads, *Azotobacter*	87	S	320	*Azotobacter vinlandii* c_5[o], preliminary report	Function unknown, but possibly electron donor to cytochrome *o*.
c_{553}	*Desulfovibrio*	79	S	−220 to +50	None	Electron carrier for formate and lactate dehydrogenase. Transfers electrons from lactate to cytochrome c_3 or other enzymes involved in sulfur metabolism.

[a] These names will be used to differentiate the various classes of mitochondrial-type cytochromes *c*. Individual members may absorb light at slightly different wavelengths and be named differently in the literature.

[b] Dickerson (1980a) classified the various mitochondrial-type structures as L (long), M (medium), S (short) and S* (short, but distantly related to S).

[c] Takano and Dickerson, 1981a; [d] Tanako and Dickerson, 1981b; [e] Tanaka *et al.*, 1975; [f] Matsuura *et al.*, 1979; [g] Dickerson *et al.*, 1971; [h] Ochi *et al.*, 1983; [i] Salemme *et al.*, 1973; [j] Timkovich and Dickerson, 1976; [k] Matsuura *et al.*, 1982; [l] Korszan and Salemme, 1977; [m] Ludwig *et al.*, 1982; [n] Sawyer *et al.*, 1981; [o] Stout, 1978.

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                                                                5                   10                  15                  20
Tuna                                                  Gly Asp Val Ala Lys Gly Lys Lys Thr Phe Val Gln Lys  *  Cys Ala Gln Cys His Thr Val Glu
Bonito                                                Gly Asp Val Ala Lys Gly Lys Lys Thr Phe Val Gln Lys  *  Cys Ala Gln Cys His Thr Val Glu
Horse                                                 Gly Asp Val Glu Lys Gly Lys Lys Ile Phe Val Gln Lys  *  Cys Ala Gln Cys His Thr Val Glu
Rice      Ala Ser Phe Ser Glu Ala Pro Pro Gly Asn Pro Lys Ala Gly Glu Lys Ile Phe Lys Thr Lys  *  Cys Ala Gln Cys His Thr Val Asp
c2                                        Glu Gly Asp Ala Ala Ala Gly Glu Lys Val Ser Lys Lys  *  *  Cys Leu Ala Cys His Thr Phe Asp
c550                                Gln Asp Gly Asp Ala Ala Asp Gly Glu Lys Glu Phe Asn Lys  *  *  Cys Lys Ala Cys His Met Ile Gln
c551                                            Glu Asp Pro Glu Val Leu Phe Lys Asn Lys Gly Cys Val Ala Cys His Ala Ile Asp
c554                                  Ala Asp Leu Ala His Gly Gly Gln Val Phe Ser Ala Asn  *  Cys Ala Ser Cys His Leu Gly Gly
c555                                  Tyr Asp Ala Ala Ala Gly Lys Ala Thr Tyr Asp Ala Ser  *  Cys Ala Met Cys His Lys Thr Gly

                                                25                  30                  35                  40                  45
Tuna      Asn Gly Gly  *  *  *  *  *  *  Lys His Lys Val Gly Pro Asn Leu Trp Gly Leu Phe Gly Arg Lys Thr Gly Gln Ala Glu Gly
Bonito    Asn Gly Gly  *  *  *  *  *  *  Lys His Lys Val Gly Pro Asn Leu Trp Gly Leu Phe Gly Arg Lys Thr Gly Gln Ala Glu Gly
Horse     Lys Gly Gly  *  *  *  *  *  *  Lys His Lys Thr Gly Pro Asn Leu His Gly Leu Phe Gly Arg Lys Thr Gly Gln Ala Pro Gly
Rice      Lys Gly Ala  *  *  *  *  *  *  Gly His Lys Gln Gly Pro Asn Leu Asn Gly Leu Phe Gly Arg Gln Ser Gly Thr Thr Pro Gly
c2        Gln Gly Gly  *  *  *  *  *  *  Ala Asn Lys Val Gly Pro Asn Leu Phe Gly Val Phe Glu Asn Thr Ala Ala His Lys Asp Asn
c550      Ala Pro Asp Gly Thr Asp Ile Ile Lys Gly Gly Lys Thr Gly Pro Asn Leu Tyr Gly Val Val Gly Arg Lys Ile Ala Ser Glu Glu Gly
c551      Thr  *  *  *  *  *  *  *  *  Lys  *  Met Val Gly Pro Ala Tyr Lys Asp Val Ala Ala Lys Phe Ala  *  *  *  *  *
c554       *  *  *  *  *  *  *  *  *  Arg Asn Val Val Asn Pro Ala  *  Lys Thr Leu  *  Glu Lys Ala  *  *  *  *  *  *
c555      Met  *  *  *  *  *  *  *  *   *  *  Met Gly Ala Pro Lys Val Gly Asp Lys Ala Ala  *  *  *  *  *  *  *  *

                      50                  55                  60                  65                  70
Tuna      Tyr Ser Tyr Thr Asp Ala Asn Lys Ser  *  *  *  Lys Gly  *  *  Ile Val Trp Asn Asn Asp Thr Leu Met Phe Tyr Leu Glu Asn
Bonito    Tyr Ser Tyr Thr Asp Ala Asn Lys Ser  *  *  *  Lys Gly  *  *  Ile Val Trp Asn Glu Asn Thr Leu Met Glu Tyr Leu Glu Asn
Horse     Phe Thr Tyr Thr Asp Ala Asn Lys Asn  *  *  *  Lys Gly  *  *  Ile Thr Trp Lys Glu Glu Thr Leu Met Glu Tyr Leu Glu Asn
Rice      Tyr Ser Tyr Ser Thr Ala Asp Lys Asn  *  *  *  Met Ala  *  *  Val Ile Trp Glu Glu Asn Thr Leu Tyr Asp Tyr Leu Glu Asn
c2        Tyr Ala Tyr Ser Glu Ser Tyr Thr Glu Met Lys Ala Lys Gly  *  *  Leu Thr Trp Thr Glu Ala Asn Leu Ala Ala Tyr Val Lys Asp
c550      Phe Lys Tyr Gly Glu Gly Ile Leu Glu Val Ala Glu Lys Asn Pro Asp Leu Thr Trp Thr Glu Ala Asp Leu Ile Glu Tyr Val Thr Asp
c551       *  *  *  *  *  *  *  *  *  *  *  *  *  Gly  *  *  Gln Ala Gly Ala Glu Ala Glu Leu Ala Gln Arg Ile Lys Asn
c554       *  *  *  *  *  *  *  *  *  *  *  *  Asp Leu  *  *  Asp Glu Tyr Gly Met Ala Ser Ile Glu  *  Ala Ile Thr  *
c555       *  *  *  *  *  *  *  *  *  *  *  *   *  Trp  *  *  Ala Pro His Ile Ala Lys Gly Met Asn Val Met Val Ala Asn

                      75                  80                  85
Tuna      Pro Lys Lys Tyr Ile  *  *  *  *  *  *  Pro Gly  *  *  Thr Lys Met  *  Ile Phe Ala Gly  *  Ile Lys Lys  *  Lys Gly
Bonito    Pro Lys Lys Tyr Ile  *  *  *  *  *  *  Pro Gly  *  *  Thr Lys Met  *  Ile Phe Ala Gly  *  Ile Lys Lys  *  Lys Gly
Horse     Pro Lys Lys Tyr Ile  *  *  *  *  *  *  Pro Gly  *  *  Thr Lys Met  *  Ile Phe Ala Gly  *  Ile Lys Lys  *  Lys Thr
Rice      Pro TML Lys Tyr Ile  *  *  *  *  *  *  Pro Gly  *  *  Thr Lys Met  *  Val Phe Pro Gly  *  Leu TML Lys  *  Pro Gln
c2        Pro Lys Ala Phe Val Leu Glu Lys Ser Gly Asp Pro Lys Ala Lys Ser Lys Met  *  Thr Phe Lys  *  *  Leu Thr Lys  *  Asp Asp
c550      Pro Lys Pro Trp Leu Val Lys Met Thr Asp Asp Lys Gly Ala Lys Thr Lys Met  *  Thr Phe Lys  *  *  Met Gly Lys  *  *  *
c551      Gly Ser Gln Gly Val Trp  *  *  *  *  *  *  *  Gly Pro Ile Pro Met Pro Pro Asn Ala  *  *  Val Ser  *  *  Asp Asp
c554      Thr Gln Val Thr Asn  *  *  *  *  *  *  *  *  Gly Lys Gly Ala Met Pro Ala Phe Gly Ala Lys Leu Ser  *  *  Ala Asp
c555      Ser Ile Lys Gly Tyr Lys Gly  *  *  *  *  *  *  Thr Lys Gly Met Met Pro Ala Lys Gly Gly Asn Pro Lys Leu Thr Asp Ala

          90                  95                 100
Tuna      Glu Arg Gln Asp Leu Val Ala Tyr Leu Lys Ser Ala Thr Ser
Bonito    Glu Arg Gln Asp Leu Val Ala Tyr Leu Lys Ser Ala Thr Ser
Horse     Glu Arg Glu Asp Leu Ile Ala Tyr Leu Lys Lys Ala Thr Asn Glu
Rice      Glu Arg Ala Asp Leu Ile Ser Tyr Leu Lys Glu Ala Thr Ser
c2        Glu Ile Glu Asn Val Ile Ala Tyr Leu Lys Thr Leu Lys
c550      Asn Gln Ala Asp Val Val Ala Phe Leu Ala Gln Asn Ser Pro Asp Ala Gly Gly Asp Gly Glu Ala Ala
c551      Glu Ala Gln Thr Leu Ala Lys Trp Val Leu Ser Gln Lys
c554      Asp Ile Glu Gly Val Ala Ser Tyr Ala Leu Asp Gln Ser Gly Lys Glu Trp
c555      Gln Val Gly Asn Ala Val Ala Tyr Met Val Gly Gln Ser Lys
```

FIG. 2. Amino acid sequences of the nine cytochrome *c* molecules for which atomic resolution structures are available. The numbering scheme is based on the tuna sequence. From Dickerson and Timkovich, 1975; Dickerson, 1980a; and Meyer and Kamen, 1982.

common site consists of the peptide sequence -Cys-X-Y-Cis-His-. In the two exceptions the first cysteine is replaced by another residue (Val or Ala) leaving a single covalent bond between the protein and the heme. The fifth and sixth heme ligands are histidine and methionine, the latter occurring in the carboxyl terminal half of the polypeptide chain. All *c*-type cytochromes have similar tertiary structures and side chain packing arrangements around the heme. The heme group is deeply buried in all structures and is similarly oriented (see below).

2. *Eukaryotic Cytochromes* c

The eukaryotic cytochromes *c* are located in the intermembrane space of mitochondria and transport electrons from the cytochrome reductase b–c_1 complex (complex III) to the cytochrome oxidase a–a_3 complex (complex IV). Nearly all have a redox potential of 260 mV $\pm$ 10 mV and an isoelectric point of about 10. Their redox potentials are generally independent of pH from about pH 5 to 8 (Margalit and Schejter, 1973).

The crystal structures of mitochondrial cytochrome *c* from horse (Dickerson *et al.*, 1971), rice (Ochi *et al.*, 1983), bonito (Tanaka *et al.*, 1975; Matsuura *et al.*, 1979), and tuna (Takano and Dickerson, 1981a,b) have been determined to atomic resolution. The first two have been studied in the oxidized form only, at 2.8 and 2.0 Å resolution, respectively. The bonito and tuna structures have been determined in both the reduced and oxidized states, the former at 2.3 Å and 2.8 Å resolution, respectively and the latter at 1.5 and 1.8 Å resolution, respectively. The structures in the two oxidation states for both species are very similar, differing, in the case of tuna, only at intermolecular contact points and at the site of a buried water molecule. Reduced tuna cytochrome *c* contains one molecule per asymmetrical unit and has been refined to crystallographic *R*-factor of 0.173. The oxidized form contains two molecules per asymmetric unit and has been refined to an *R* of 0.208. The RMS displacement of the main chain and β-carbon atoms, after alignment of the reduced and oxidized structures, is 0.43 Å compared with an RMS displacement of 0.28 Å for the two independent oxidized molecules.

The structure of tuna cytochrome *c* is shown in Fig. 3. It contains five short α-helices, which comprise about 45% of the structure, but no β-sheet. The two longest helices contain 10 and 14 residues respectively, and occur near the amino and carboxyl termini. The two helices are in contact, their axes forming an angle of about 80°, and together form the most highly conserved feature of secondary structure among the nine *c*-type structures.

The heme group is deeply buried in a nearly hydrophobic environment with only the edge of pyrrole ring II (Fig. 1) and the adjacent thioether bridge lying on the protein surface. The

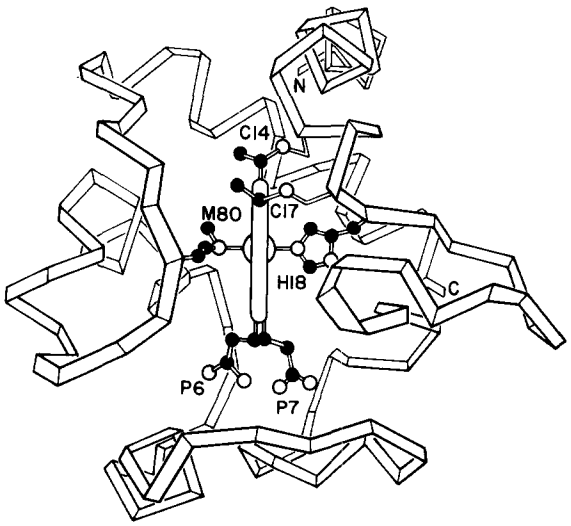

FIG. 3. Schematic representation of the structure of tuna cytochrome *c*. This molecule and the other two *c*-type cytochrome (Figs. 5 and 7) are viewed with the heme in the same orientation. Propionate-6 is located at the lower front (outer) position of the heme pocket and propionate-7 is at the lower back position. Reproduced by permission from Dickerson (1980a).

heme is attached covalently to the protein by thioether linkage from Cys 14 and Cys 17 to the heme vinyl groups at positions 2 and 4 respectively. Coordination bonds are also formed from the epsilon nitrogen of His 18 and the sulfur of Met 80 to the fifth and sixth coordination sites of the heme iron. The heme group is oriented so that its front face, defined as viewed in Fig. 1 with a clockwise numbering scheme, faces Met 80. The two ligands form bonds of high field strength and maintain the heme iron in the low spin state in both oxidation states. A hydrogen bond is formed from the delta nitrogen of His 18 to the carbonyl oxygen of Pro 30 (see Table 3) which probably stabilizes the orientation of the histidine ligand. A hydrogen bond between the hydroxyl of Tyr 67 and the sulfur of Met 80 is also present.

TABLE 3. HYDROGEN BONDING TO HEME PROPIONATES AND AXIAL LIGANDS IN MITOCHRONDRIAL-TYPE CYTOCHROMES *c*

	Tuna *c*	*R. rubrum* c_2	*P. denitrificans* c_{550}	*P. aeruginosa* c_{551}
Propionate-7 (inner) on pyrrole ring IV				
O1	Arg 38 N_η	Arg 38 N_η	Arg 38 N_η	Arg 67 N_η
O1	Tyr 48 O_η	Tyr 48 O_η	Tyr 48 O_η	
O1	H_2O (No. 3)			
O2	Trp 59 N_ε	Trp 59 N_ε	Trp 59 N_ε	Trp 75a N_ε
Propionate-6 (outer) on pyrrole ring III				
O1	Thr 49 N	Ser 49 N	Gly 49 N	Val 75 N
O2	Thr 49 O_γ	Ser 49 O_γ		H_2O
O2	Thr 78 O_γ	Tyr 46 O_γ	Thr 78 O_γ	
Heme ligands				
His 18 N_δ	Pro 30 O	Pro 30 O	Pro 30 O	Pro 30 O
Met 80 S_δ	Tyr 67 O_η	Tyr 57 O_η	Tyr 57 O_η	Asn 82 N_γ

The two heme propionates are buried. Propionate-7 on pyrrole ring IV is located in the lower rear portion of the molecule (Fig. 3). However, its environment is somewhat polar. It forms a salt bridge to Arg 38, which is also partially buried, and accepts hydrogen bonds from the hydroxyl of Tyr 48, the epsilon nitrogen of Trp 59 and a buried water molecule (Table 3). Propionate-6, on pyrrole ring III, is in a less polar environment, forming hydrogen bonds to the peptide nitrogen and gamma hydroxyl of Thr 49 and the gamma hydroxyl of Thr 78.

There are several classes of amino acid residues that are either invariant or highly conserved among the 60 eukaryotic *c*-type cytochromes. Six glycine residues are invariant and another four are highly conserved. All but two occur in tightly packed regions of the structure where there is no room for a side chain to extend from the alpha carbon. Five of these are at the third position of a type II reverse turn (Venkatachalam, 1968).

There are four invariant aromatic residues and three residues which are always aromatic. Two of the former, Tyr 48 and Trp 59, form hydrogen bonds to the deeply buried heme propionates as described above (Table 3). One of the latter, Tyr 67, forms a hydrogen bond to sulfur of Met 80 but is sometimes replaced by Phe. The remaining four aromatic residues appear to play a structural role, probably providing bulk and hydrophobicity for the heme environment in the protein interior.

Tuna cytochrome *c* is very basic with 18 positively charged residues, mostly lysines, and 7 negatively charged residues. Furthermore, many of the excess lysine residues are clustered around the mouth of the heme crevice giving rise to a pronounced charge asymmetry. These lysine residues are involved in interaction with the oxidase and the reductase of cytochrome *c* as discussed in Section VI.

There are twelve well resolved water molecules common to both oxidation states. Three of these are internal while the remaining nine lie on the protein surface. The most prominent internal water is about 5 Å from Met 80 and forms hydrogen bonds to Asn 52 N_δ, Tyr 67 O_η and Thr 78 O_γ (Fig. 4). In the oxidized form this water molecule is about 0.7 Å closer to Met 80 than in the reduced form. This shift of the water molecule is accompanied by an

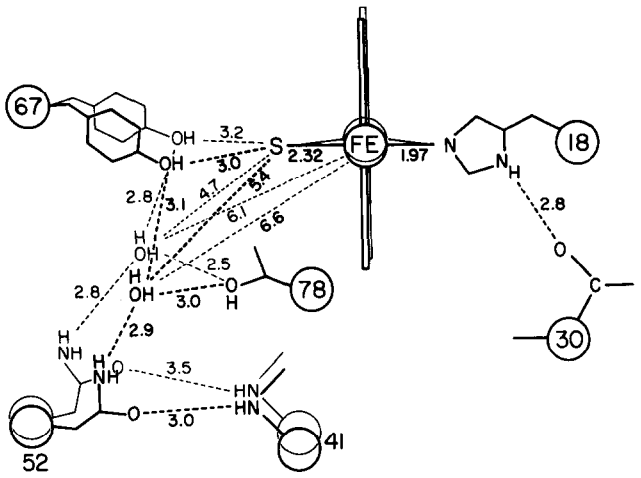

FIG. 4. Structural differences between oxidized and reduced tuna cytochrome *c*. The heme plane, a buried water molecule, the sulfur of Met 80, the main chain atoms of Pro 30 and Thr 41, and the side chain atoms of His 18, Asn 52, Tyr 67 and Thr 78 are shown with heavy lines for the reduced protein. Hydrogen bond distances in the reduced protein are also shown in heavy lines. The atoms of those groups in the heme pocket which move significantly upon oxidation including the heme group itself, the buried water, the main chain of Thr 41 and the side chains Asn 52 and Tyr 67 are shown with light lines. Hydrogen bond distances which differ significantly in the oxidized state are also shown in light lines.

alteration in the positions of some of the residues whose side chains are hydrogen bonded to it. Most notably the main and side chains of Asn 52 rotate (Fig. 4) causing a 1.6 Å change of position for the delta nitrogen (Fig. 4) and a slight inward movement of the second helix (at the bottom left of the molecule, as viewed in Fig. 3) which contains this residue. A few other side chains which are involved in hydrogen bonds to the buried water molecule or are packed against the heme, undergo shifts of about 0.4 Å. These conformational changes in the heme pocket are shown in Fig. 4.

Differences in the chemical properties of the reduced and oxidized forms of cytochrome *c* have been observed. A comparison of their relative resistance to thermal denaturation, protease digestion, surface denaturation and chemical modification indicates that the reduced form is more rigid and stable than the oxidized form (Dickerson and Timkovich, 1975). Also no eukaryotic cytochrome *c* has been found to crystallize isomorphously in both oxidation states. While the small observed differences in conformation between the two redox states might cause variations in crystal packing, it is difficult to understand how these differences can lead to pronounced changes in their relative stability. However, it is known that the methionine-to-iron bond is about 1000 fold weaker in the oxidized than in the reduced form of cytochrome *c* (Dickerson and Timkovich, 1975). If the pathway toward a partially unfolded and chemically more labile molecule includes breaking this bond then there would be a lower energy barrier for this kinetic pathway in the oxidized than in the reduced state. Such a kinetic difference would not be observed in the crystal structure studies.

The remaining eukaryotic cytochrome *c* structures are generally quite similar to the tuna protein. The sequence of bonito *c* (Tanaka *et al.*, 1975) differs from tuna *c* at only three places, residues 61, 62 and 66 (see Fig. 2). Horse *c* (Dickerson *et al.*, 1971) differs somewhat more from tuna, having 18 substitutions and one additional residue at the carboxyl terminus. It has three more lysine residues and four more glutamic acid residues, one of the latter replacing an aspartic acid. The more limited resolution of the bonito and horse structures precludes detailed comparisons with the tuna structure or analysis of the slight differences in chain fold.

Rice cytochrome *c* is substantially different from tune *c* in amino acid sequence (see Fig. 2). There are eight additional residues at the N-terminus. In addition, there are 43 substitutions in the remainder of the sequence including the replacement of two lysines by trimethyl lysine. Yet its main chain conformation is very similar to that of tuna (Ochi *et al.*,

1983). When the α-carbon atoms of the rice and tuna cytochrome *c* are aligned their RMS displacement is about 0.58 Å compared with an RMS displacement of about 0.22 Å between the α-carbons of the two independent oxidized tuna molecules (Takano and Dickerson, 1981a). Furthermore, all main chain hydrogen bonds are invariant for the two species and the internal hydrogen bonding pattern is nearly identical. The eight additional N-terminal residues of the rice protein are in an extended conformation and are wrapped around the C-terminal helix at the back of the molecule as viewed in Fig. 3. They are well away from the exposed heme edge and are unlikely to have any effect on electron transfer. The trimethyl lysine substitutions, at positions 72 and 86, lie on the left side of the molecule near the front, as viewed in Fig. 3. They are among those lysine residues clustered around the mouth of the heme crevice which are involved in interactions with redox partners (see Section VI).

3. *Prokaryotic Cytochromes* c_2

The prokaryotic cytochromes c_2 are found in many purple phototrophic bacteria and in two non-phototrophic bacteria (Meyer and Kamen, 1982). They appear to function as electron carriers in the cyclic process of photosynthesis in the phototrophic bacteria or in oxygen or nitrite respiration in the non-phototrophic bacteria. The redox potentials of the cytochromes c_2 are generally higher and more varied than those from eukaryotes, ranging from 250 mV–400 mV with an average of 345 mV. The midpoint potentials of several of them vary with pH over the neutral pH range. Their isoelectric points are generally lower than those of the eukaryotic *c*'s, averaging about 6.

The amino acid sequences of 14 such cytochromes are known and the crystal structures of two have been determined. One is cytochrome c_2 from *Rhodospirillum rubrum*, determined at 2.0 Å resolution (Salemme *et al.*, 1973), and the other is cytochrome c_{550} from *Paracoccus denitrificans*, determined at 2.45 Å resolution (Timkovich and Dickerson, 1976; Ambler *et al.*, 1981a). The amino acid sequences of both of these cytochromes are given in Fig. 2. Crystals of *R. rubrum* cytochrome c_2 reduced by dithionite showed no change in conformation. A schematic diagram of *R. rubrum* cytochrome c_2 is shown in Fig. 5 and a comparison of its α-carbon backbone with that of tuna cytochrome *c* is shown in Fig. 6.

The sequence alignments of the cytochromes c_2 relative to the mitochondrial *c*'s are based on a comparison of the known eukaryotic and c_2 structures (Dickerson, 1980a; Meyer and Kamen, 1982). One subclass of c_2 is virtually identical in length to the eukaryotic cytochromes c_2 while the other, including both *R. rubrum* c_2 and *P. denitrificans* c_{550},

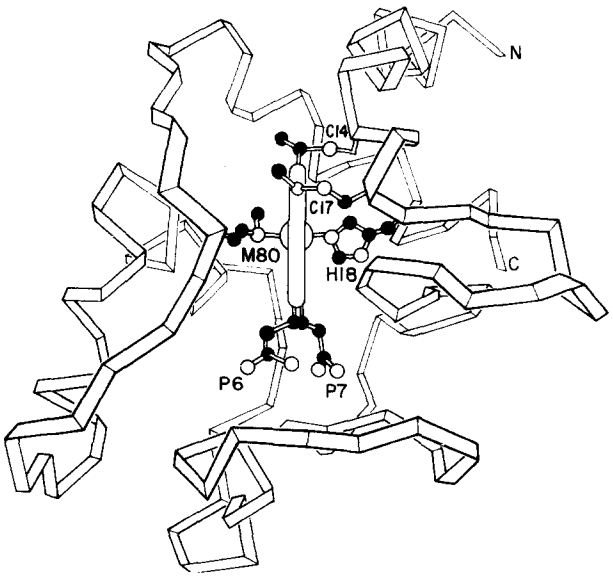

FIG. 5. Schematic diagram of *R. rubrum* cytochrome c_2. The molecule is oriented as in Fig. 3. Reproduced by permission from Dickerson (1980a).

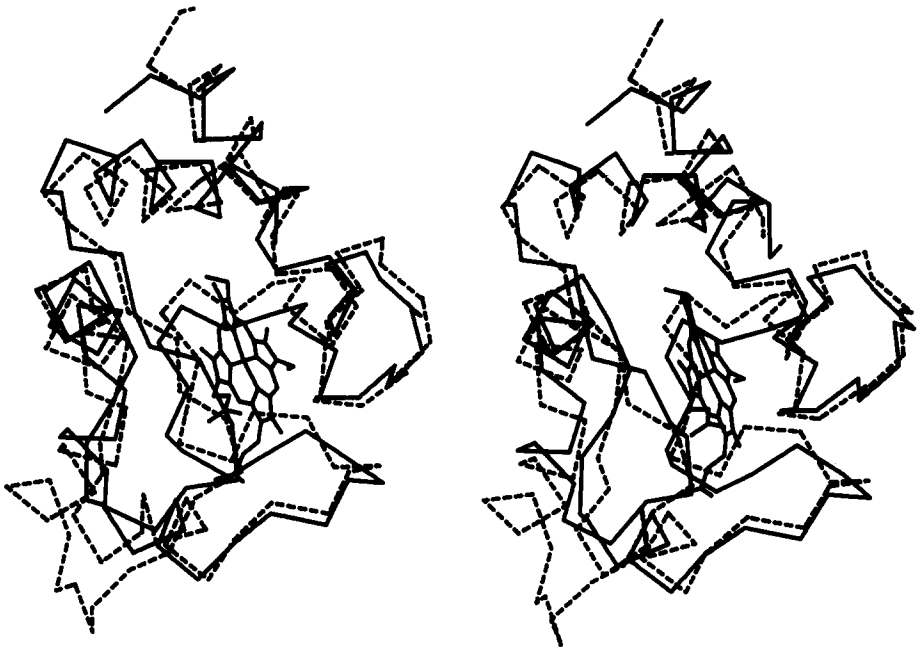

FIG. 6. Comparison of the α-carbon backbone diagrams of *r. rubrum* cytochrome c_2 (dashed lines) and tuna cytochrome *c* (solid lines). The heme groups of both molecules were superimposed manually on the MMS-X graphics system.

contains three or four short insertions in the sequence (see Fig. 2). The structures of *R. rubrum* c_2 and tuna *c* are remarkably similar (see Fig. 6). A short insertion in c_2 occurs after the second helix at the bottom (Fig. 5) and a longer one after the fourth helix in the lower left portion. *P. denitrificans* cytochrome c_{550} contains an additional six residue insertion shortly past the histidine ligand to the heme and a nine residue addition at the carboxyl terminus (see Fig. 2).

Most of the invariant or highly conserved residues of eukaryotic *c* are retained in the prokaryotic cytochromes c_2. The environments of the heme groups are very similar. The two heme ligands are identical. The delta nitrogen of the histidine ligand is hydrogen bonded to the carbonyl oxygen of the highly conserved Pro 30 (Table 3). The presence of proline at this location must be very important for the peptide fold and side chain packing near the histidine since it is the most highly conserved residue other than the heme ligands among the eukaryotic and prokaryotic *c*-type cytochromes. The hydrogen bond from Tyr 67 to the sulfur of Met 80 (Table 3) is also maintained in the two known c_2 structures. However, this residue is replaced by phenylalanine in one or two other c_2 sequences.

The heme propionates are buried in *R. rubrum* c_2 and *P. denitrificans* c_{550} and form hydrogen bonding patterns similar to those of eukaryotic *c*. Propionate-7, on pyrrole ring IV (in the rear in Figs 5 and 6), is hydrogen bonded to homologous tyrosine and tryptophan side chains in both bacterial proteins (Table 3). However, the salt bridge to Arg 38 of eukaryotic *c*, while maintained in c_{550}, is eliminated in c_2, that residue being replaced by asparagine. Neither of the prokaryotic cytochrome c_2 structures has been refined sufficiently for analogous water molecules to be detected near the propionates. Propionate-6 on pyrrole ring III (in the front in Figs 5 and 6) also forms hydrogen bonds with suitable groups in c_2 and c_{550} but not in such a conserved manner. In *R. rubrum* c_2 a hydrogen bond from propionate-6 to Tyr 46 replaces the one to Thr 78 in *c* although both hydrogen bonds to the homologous Ser 49 are maintained (Table 3). In *P. denitrificans* c_{550} the hydrogen bond from propionate-6 to Thr 78 is maintained but only the hydrogen bond to the peptide nitrogen of Gly 49 is retained.

The glycine residues which are highly conserved among the eukaryotic cytochromes *c* are not generally conserved among the c_2 cytochromes. This is probably because of the

numerous small changes in tertiary structure caused by insertions, deletions and amino acid substitutions, in spite of the overall similarities of folding patterns. The aromatic residues packed around the heme group, however, tend to be well conserved among the eukaryotic *c* and prokaryotic c_2 cytochromes. Within the two classes there are four positions which are always occupied by aromatic side chains, three of which are in contact with the heme. There are three other positions which are aromatic in all but one or two instances. A few of these aromatic residues are involved in hydrogen bonding to the methionine ligand or to the propionates but their bulk and hydrophobicity within the heme cavity is probably of greater importance since many other amino acids could substitute in hydrogen bond formation.

4. Pseudomonas *Cytochrome* c_{551}

The *Pseudomonas* cytochrome c_{551} class contains eight members of known sequence varying from 82 to 92 residues (Meyer and Kamen, 1982). Six of these, from various *Pseudomonas* strains or from *Azotobacter vinlandii*, have similar redox potentials, of about +280 mV. Two others, both from purple non-sulfur phototropic bacteria, have widely disparate potentials of greater than +400 mV or below +200 mV. The former are involved in nitrite reduction or aerobic metabolism while the latter are involved in cyclic photophosphorylation.

The crystal structure of cytochrome c_{551} from *Pseudomonas aeruginosa* has been determined at 1.6 Å resolution in both the oxidized and reduced states and refined to crystallographic *R* factors of 0.195 and 0.187, respectively (Matsuura *et al.*, 1982). Approximately 70 water molecules were located, eight of which are internal. Its amino acid sequence is given in Fig. 2 and its structure is shown schematically in Fig. 7. The α-carbon skeletons of c_{551} and tuna *c* are compared in Fig. 8.

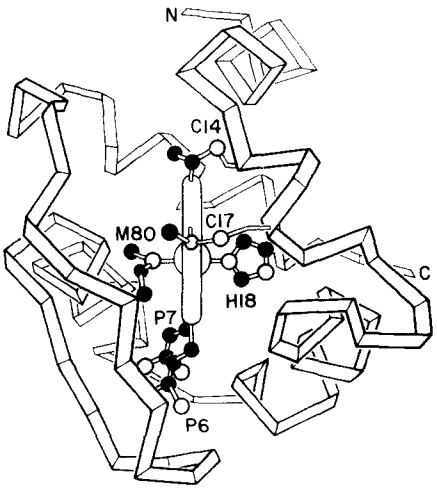

FIG. 7. Schematic diagram of *P. aeruginosa* cytochrome c_{551}. The orientation is the same as in Fig. 3. Reproduced by permission from Dickerson (1980a).

The major difference between the structures of mitochondrial cytochrome *c* and c_{551} is a 15 residue delection in the middle of the latter protein. This causes the residues equivalent to Thr 40 and Gly 56 of *c* to be joined at the back of the molecule (as viewed in Fig. 8) with the concomitant loss of the peptide loop and helix at the bottom covering the heme propionates (compare Figs 3 and 7). This loss is compensated by rearrangement of the polypeptide chain between the heme ligands so as to close the gap and maintain a similar non-polar environment for the heme. There is also a three residue deletion approximately at position 22 of *c* and one or two single insertions or deletions elsewhere in the sequence. These changes lead to a greater difference in polypeptide conformation between tuna *c* and c_{551} (Fig. 8) than between it and *R. rubrum* c_2 (Fig. 6).

FIG. 8. Comparison of the α-carbon backbone diagrams of *P. aeruginosa* cytochrome c_{551} (dashed lines) and tuna cytochrome *c* (solid lines). The heme groups of both molecules were superimposed manually on the MMS-X graphics system.

The relative orientations of the amino and carboxyl terminal helices are maintained in the c_{551} structure. A salt bridge between Lys 13 and Glu 90 stabilizing their interaction is also maintained.

The orientation and hydrophobic environment of the heme group of c_{551} is also similar to that of *c*. The environment is somewhat more polar, however. There is a buried water molecule in van der Waals contact with pyrrole ring II and with Pro 81 which is located next to the methionine heme ligand. It is linked to another water molecule near the protein surface as well as to two peptide carbonyl oxygens. The corresponding region in tuna *c* contains no water molecules.

In some instances interactions of the heme group or its ligands with certain side chains in tuna cytochrome *c* are replaced by functionally similar ones at structurally different positions. The hydrogen bond between the delta nitrogen of the heme ligand His 18 and the carbonyl oxygen of Pro 30 is maintained as it is with all *c*-type cytochromes structurally studied so far (Table 3). However, the hydrogen bond from Tyr 67 to the sulfur of Met 80 in tuna *c* is replaced by a hydrogen bond from Asn 82 on the opposite side of the heme cavity.

Propionate-7 on pyrrole ring IV (in the rear in Figs 7 and 8) is quite buried. Its charge is stabilized by a salt bridge to Arg 67, not to the homologous Lys 38 as in *c* (Table 3). This propionate also maintains a hydrogen bond to the epsilon nitrogen of Trp 75a. However, this tryptophan is not in a position equivalent to Trp 59 of tuna *c*. Propionate-6 on pyrrole ring III is more exposed to solvent, forming hydrogen bonds to the peptide nitrogen of Val 75 and to a buried water molecule.

Very few aromatic residues are in contact with the heme group in c_{551} compared with the larger cytochromes *c* and c_2. The three or four that are in contact do not occur at equivalent positions in tuna cytochrome *c*. On the other hand, the distribution of lysine side chains surrounding the exposed heme edge is similar.

The refined structure of reduced cytochrome c_{551} is virtually the same as the oxidized structure. It was determined from oxidized crystals which had been reduced by soaking in ascorbate solution. The r.m.s. difference between refined atomic coordinates in the two forms is 0.08 Å. Slight differences in main chain torsion angles occur at the edges of the heme crevice. A difference density map shows no change in the heme group or protein molecule upon reduction except for a slight movement of the water molecule next to pyrrole ring II away from the heme iron and from Met 80 S_γ. The movement might be significant, possibly indicating interaction of the water dipole with the positive charge of the oxidized heme group.

5. *Other Bacterial Cytochromes* c

(a) Chlorobium *cytochrome* c_{555}

The *Chlorobium* c_{555} class is found in green photosynthetic bacteria. Two sequences have been determined and are 86 and 99 residues in length (Meyer and Kamen, 1982). The midpoint redox potentials of the cytochromes c_{555} are much lower than the other mitochondrial-type *c* cytochromes, ranging from +103 to +140 mV. This class of cytochrome *c* reduces photooxidized bacteriochlorophyl during cyclic photophosphorylation and during sulfur metabolism.

The structure of c_{555} from *Chlorobium thiosulfatophilum* has been determined to 2.7 Å resolution (Korszun and Salemme, 1977). Its conformation is similar to the other mitochondrial-type cytochrome *c*'s, particularly cytochrome c_{551}. It shares with c_{551} the major 15 residue deletion at the bottom of the molecule from about positions 40 to 55 (see Figs 2, 3 and 7). Like c_{551}, the lower left part of the molecule (as c_{551} is viewed in Fig. 7) is folded closer to the heme propionates to maintain a hydrophobic environment for the heme group. Other features of the heme environment, such as the nature of the ligands and its position in the molecule are also very similar to c_{551}. At the present stage of the structure analysis there are no obvious reasons for the markedly lower redox potential for c_{555} compared to c_{551} or other *c*-type cytochromes (Korszun and Salemme, 1977).

(b) *Algal cytochrome* c_{554}

The Algal c_{554} subclass is spread widely among algae and blue-green bacteria. Its members are involved in electron transfer between membrane bound cytochrome *f* and chlorophyll P-700 at photocenter I. Eight sequences have been determined, containing from 83 to 89 residues and varying in midpoint potentials from +340 to +390 mV (Meyer and Kamen, 1982).

The structure of cytochrome c_{554} from the cyanobacterium *Anacystis nidulans* has been determined to 3.0 Å resolution (Ludwig *et al.*, 1982). Like *Chlorobium* c_{555} its pattern of deletions (Fig. 2) and overall polypeptide fold is similar to *Pseudomonas* c_{551}. In fact, the c_{551} coordinate were compared directly with the c_{554} electron density map as part of the structure analysis (Ludwig *et al.*, 1982).

There are two regions of the Algal c_{554} structure which differ markedly from *Pseudomonas* c_{551}. The polypeptide chain near Pro 30 turns away from the pathway followed in c_{551} so as not to be in position for a hydrogen bond to form from the peptide nitrogen of Pro 30 to the delta nitrogen of His 18 as it does in c_{551} (Table 3). Pro 30 is not conserved in some Algal sequences (Meyer and Kamen, 1982), so its apparent general requirement is relaxed in this class of mitochondrial-type *c* cytochrome.

The other region of discrepancy between the *A. nidulans* c_{554} and the *Pseudomonas* c_{551} conformations comprises a ten residue loop before the methionine heme ligand. Unfortunately, this loop is not yet completely defined in the *A. nidulans* structure (Ludwig *et al.*, 1982) so detailed comparison is not yet possible.

(c) Pseudomonas aeruginosa *cytochrome* c_4

P. aeruginosa cytochrome c_4 is a diheme protein of about 181 residues. The redox potentials of the two hemes are similar and average about 325 mV (Meyer and Kamen, 1982). The amino acid sequence (Ambler, 1981) indicates that the protein consists of two cytochrome molecules each about the size of *Pseudomonas* c_{551} joined covalently. An electron density map has been calculated at 5.0 Å resolution (Sawyer *et al.*, 1981). It shows two distinct domains, each about the size of a monoheme cytochrome and containing a heme group. The sequence and map support the idea that the diheme cytochrome arose by gene duplication.

6. *Heme Electron Distribution and Methionine Chirality*

In low spin ferric cytochromes the five electrons in the 3*d* shell of the iron atom are distributed among the three low-lying orbitals d_{xy}, d_{xz}, and d_{yz} (Fig. 9a) (Wüthrich, 1970).

The x and y axes of these orbitals lie in the heme plane while the z axis is normal to it (Fig. 9b). The symmetry of the electric field surrounding the iron atom is approximately tetragonal. The unpaired spin density is located principally in the d_{xz} and d_{yz} orbitals (Palmer, 1983). Since these latter orbitals are delocalized into the π-orbital system of the porphyrin ring (Fig. 9c), the spin density of the unpaired electron is spread to all the atoms of the heme plane (Wüthrich, 1970). The symmetry of the heme iron is not perfectly tetragonal, however. The heme ring constituents as well as the protein atoms making up the heme environment are asymmetrically positioned. This causes the energy levels of the d_{xz} and d_{yz} orbitals to split (Fig. 9a), resulting in an asymmetrical distribution of the spin density over the heme plane.

The unpaired electron spin density near the heme periphery gives rise to a very large hyperfine chemical shift in the NMR spectrum of the ring methyl protons of ferric heme proteins (Wüthrich, 1970; Redfield and Gupta, 1971). The chemical shifts for the four ring methyl groups for horse heart cytochrome c are given in Table 4 (Keller and Wüthrich, 1978a). These shifts generally place the peaks well outside the continuous envelope of overlapping proton resonances arising from the remainder of the protein and enable the distribution of electron spin density over the porphyrin plane to be estimated. In the case of

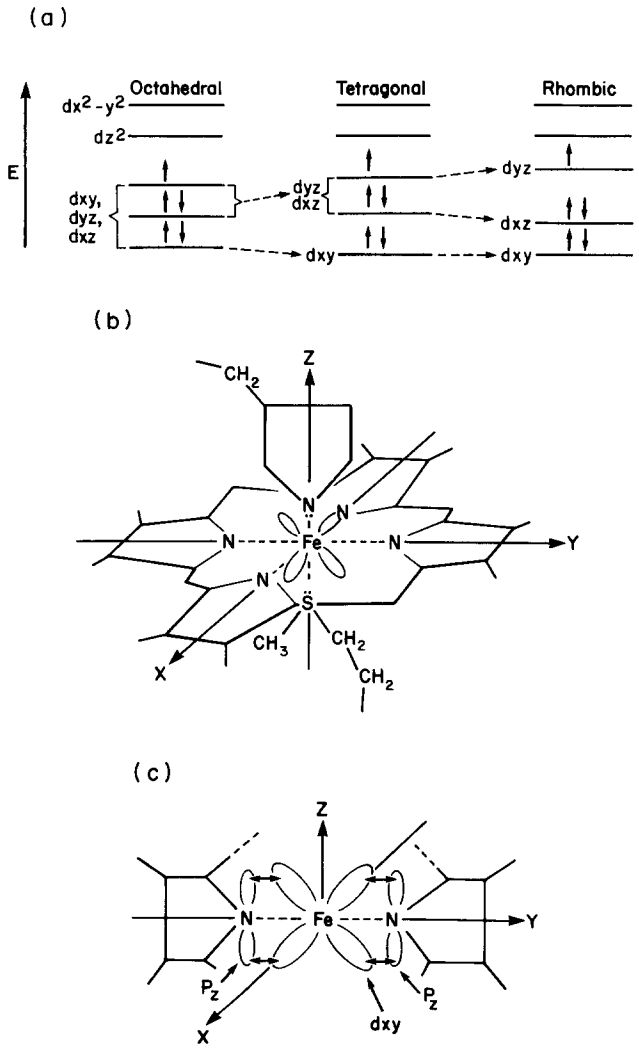

FIG. 9. (a) Energy level diagram for the $3d$ orbitals of the low spin diamagnetic ferric iron atom of the heme group in electric fields of octahedral, tetragonal or rhombic symmetry. Degenerate energy levels are indicated by left braces. Small vertical arrows indicate the directions of electron spin. The x and y axes are in the heme plane pointing toward the pyrrole nitrogran atoms and z is normal to the plane. (b) Orientation of the $3d_{yz}$ orbital of the iron atom with respect to the porphyrin plane and protein ligands. (c) Interaction of the $3d_{yz}$ orbital of the heme iron atom with the P_z orbitals of the pyrrole nitrogen atoms.

TABLE 4. SPECTROSCOPIC AND CONFORMATIONAL PROPERTIES OF MITOCHONDRIAL-TYPE CYTOCHROMES *c*

	Proton NMR Hyperfine Shifts (PPM)					
	Ring Methyl 1	Ring Methyl 3	Ring Methyl 5	Ring Methyl 8	Methionine Chirality (Ferrous)	695 nm CD Cotton Effect (Ferric)
Horse *c*[a,b]	3.9	27.3	6.9	31.7	R	neg.
S. cerevisia[c] *c* (Iso I)	7.9	30.6	11.2	33.9	R	neg.
S. cerevisia[c] *c* (Iso II)		29.8	11.9	33.1	R	neg.
C. krusei c[c]		29.8	10.9	32.4	R	neg.
R. rubrum c_2[d]	10.4	29.3	15.2	34.7	R	neg.
P. aeruginosa c_{551}[a,b]	21.1	9.6	28.8	14.4	S	pos.
R. gelatinosa c_{551} (Iso I)[e]	25.4	12.1	32.2	21.5	S	pos.
R. gelatinosa c_{551} (Iso II)[e]	29.6	10.8	35.6	17.2	S	pos.
P. stutzeri c_{551}[d]	22.1	12.3	31.9	17.6	S	pos.
P. mendocina c_{551}[d]	22.0	13.8	31.2	17.9	S	pos.
Euglena gracilis c_{552}[f]	9.5	29.0	13.8	35.0	R	neg.
Cyanobacteria c_{554}[g]					R	
P. mendocina c_5[h]	19.0	27.0	24.0	39.3	S	pos.
D. vulgaris c_{553}[i]	20.7	23.4	22.1	30.7	S	neg.
D. desulfuricans c_{553}[i]	14.3	29.7	20.3	36.8	S	neg.

[a] Keller and Wüthrich, 1978a; [b] Senn *et al.*, 1980; [c] Senn *et al.*, 1983a; Isozymes I and II; [d] Senn and Wüthrich, 1983a; [e] Senn and Wüthrich, 1983b; [f] Keller *et al.*, 1980; [g] Ulrich *et al.*, 1982; [h] Senn and Wüthrich, 1983c; [i] Senn *et al.*, 1983b.

horse heart cytochrome *c* (Keller and Wüthrich, 1978a) about 2% of the unpaired electron is localized on ring carbon atoms 3 and 8 (Fig. 1) while less than 0.5% are localized on ring carbons 1 and 5. This means the direction of maximum spin density for horse cytochrome *c* is along pyrrole rings II and IV as indicated in Fig. 10a. Keller and Wüthrich (1978a) note that pyrrole ring II, containing the extra spin density, is located at the solvent exposed edge of the heme and that this directionality of the electron orbital might facilitate electron transfer.

The opposite situation has been found for *P. aeruginosa* cytochrome c_{551} (Keller and Wüthrich, 1978b). In this case the chemical shifts for ring methyl protons 1 and 5 are much larger than for methyls 3 and 8 (Table 4). This means the axis of maximum spin density is oriented along pyrrole rings I and III (Fig. 10b), ninety degrees from that of mammalian cytochrome *c*. In both types of cytochromes pyrrole rings I and III are excluded from solvent. Since electron transfer for cytochrome c_{551} is as rapid as for horse cytochrome *c* the functional significance of the spin density distribution is not clear.

Through a series of NMR experiments involving nuclear Overhauser enhancements, saturation transfer and other techniques, Senn *et al.* (1980) were able to determine the positions of individual side chain protons with respect to the heme in solutions of reduced horse *c* and reduced *P. aeruginosa* c_{551}. By measuring the relative distances of the epsilon methyl groups of the methionine ligand to the heme *meso*-protons and to the gamma and delta methylene protons they showed that the methionine had R chirality in horse *c* and S chirality in c_{551}. These results are in agreement with the refined X-ray coordinates which are illustrated in Figs 10a and 10b. Senn *et al.* (1980) were also able to correlate the sign of the CD cotton effect of the 695 nm absorption band of the methionine ligand with its chirality when the iron is in the ferric state. Thus a combination of NMR and CD appears to provide a general method for determining the methionine chirality of cytochromes *c* of unknown structure in either oxidation state.

The study of the hyperfine shifts and the methionine chirality has been extended to other homologous proteins of the mitochondrial-type and c_{551}-type (see Table 4). The hyperfine shifts and the chirality and orientation of the methionine are consistently similar for all members of each homologous group, including cytochrome c_2 from *R. Rubrum*.

The direction of highest unpaired electron spin density is correlated with the direction in which the lone pair orbital of the sulfur atom is pointed (Senn *et al.*, 1980). Thus, in

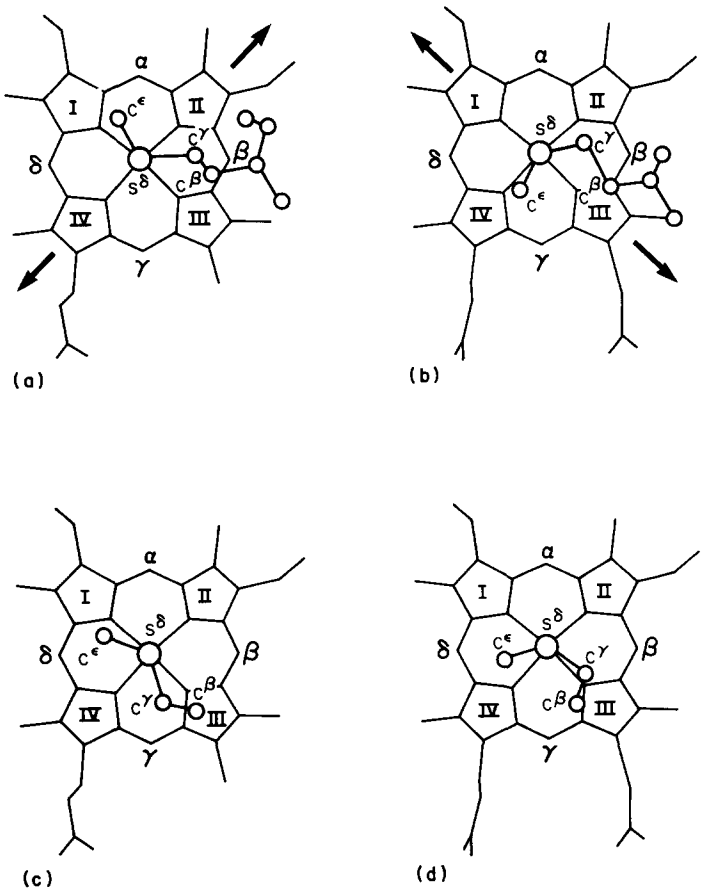

FIG. 10. Drawing of the heme group and axial methionine in various *c*-type cytochromes. The Roman numerals and Greek letters identify the pyrrole rings and *meso* carbon positions respectively. The heavy arrows in (a) and (b) indicate the major direction of the unpaired electron spin density. (a) Tuna ferrocytochrome *c*, coordinates from Takano and Dickerson (1981a). (b) *P. aeruginosa* ferrocytochrome c_{551}, coordinates from Matsuura *et al.* (1982). (c) *P. mendocina* ferrocytochrome c_5, methionine conformation and orientation from NMR data (Senn and Wüthrich, 1983c). (d) *Desulfovibrio vulgaris* ferrocytochrome c_{553}, methionine conformation and orientation by NMR from Senn *et al.* (1983b). Reproduced by permission from Senn and Wüthrich (1983c) and Senn *et al.* (1983b).

mitochondrial-type cytochromes *c* the lone pair is directed along pyrrole rings II and IV while in c_{551} it is pointed along rings I and III (see Figs 10a and 10b). If the polypeptide chains in the vicinity of methionine ligand are in very similar positions relative to the heme, as is the case for these two types of cytochromes (see Fig. 8), the direction of the lone pair will be determined primarily by the methionine chirality. This difference in chirality is also manifest by the difference in hydrogen bond pattern of the mitochondrial-type *c* and c_{551} described above where the methionine sulfur accepts a hydrogen bond from non-equivalent hydrogen bond donors from different directions in the two proteins (Table 3). The reason that the directions of highest unpaired spin density and the sulfur lone pair orbitals are correlated is that the lone pair of electrons destabilizes the d_{xz} or d_{yz} orbital parallel to it forcing the two paired electrons into the other (d_{yz} or d_{xz}) orbital perpendicular to it.

The hyperfine shifts and heme chirality of some algal-type cytochromes c_{554} (including *Euglena gracilis* c_{552}) are also shown in Table 4. Although their size and amino acid sequence resembles c_{551} (Fig. 2), the hyperfine shifts of the heme ring methyls and the chirality of the methionine ligand are close to those of the mitochondrial *c* and c_2 type. This means that the configuration of atoms surrounding the heme group of the c_{554} class must resemble closely that of the mitochondrial *c*-type cytochrome, despite the large sequence deletions. The 3.0 Å structure of the Algal c_{554} from *A. nidulans* (Ludwig *et al.*, 1982) resembles c_{551} in overall fold but detailed comparisons are not yet possible, especially near the methionine ligand (see

Section II.3). On the other hand, structural data from NMR (Keller *et al.*, 1980; Keller and Wüthrich, 1981) indicates many structural similarities between *Euglena gracilis* c_{552} and the mitochondrial-type cytochromes *c*.

The geometry of the methionine ligand and the heme electron distribution has been described for two other classes of cytochrome *c* whose X-ray structures are not yet known (Table 4). The chirality of the methionine of *P. mendocina* cytochrome c_5 is S in both oxidation states as it is in c_{551} (Senn and Wüthrich, 1983c). However, the hyperfine shifts more closely resemble those of the mitochondrial-type *c* class. The proximity of the methionine side chain atoms to the various heme *meso*-protons also appears to differ from either class (Senn and Wüthrich, 1983a). The proposed orientation of the methionine is shown in Fig. 10c. The S-chiral methionine is rotated about 70° from its orientation in c_{551} so that the lone pair sulfur orbitals are lined up with pyrrole rings II and IV.

Desulfovibrio c_{553} (Table 2) also has S chirality in the reduced state and hyperfine chemical shifts similar to the c_5 and to the mitochondrial-type *c* class (Table 4). The proposed methionine orientation in the ferrous protein based on the NMR experiments (Senn *et al.*, 1983b) is shown in Fig. 10d. However, the CD cotton effect at 695 nm indicates the methionine chirality has shifted to R in the oxidized state. This implies a rather large conformational difference between the oxidized and reduced proteins. Such a redox-dependent conformation change has not yet been observed in any cytochrome structure.

7. *Evolutionary Aspects*

(a) *Eukaryotic cytochromes* c

The cytochrome *c* molecule has been used in phylogenetic studies to trace the evolution of eukaryotes for a very wide variety of species over a several hundred-million year period (see Dickerson and Timkovich, 1975). The advantages of using cytochrome *c* are that its sequence is known for nearly 70 eukaryotic species and its structure for 4 species. Furthermore, the mutation rate is slow, the protein is ubiquitous in nature and has a standard biochemical role, there is only a single gene per organism (except for baker's yeast cytochrome *c*) and the gene is not transferable among eukaryotes (Schultz and Schirmer, 1979).

A matrix containing the number of amino acid differences between all pairs of the 60 known sequences (Dickerson and Timkovich, 1975) shows groupings of small differences among related groups. For example, mammals differ among themselves on average by about 7 residues, birds by 3 residues and yeasts by 32 residues. Distantly related species differ by 40–60 residues. Phylogenetic trees constructed from this difference matrix or from the related DNA difference matrix agree in general with trees constructed by more classical taxonomical methods.

The rate of evolution of eukaryotic cytochrome *c*, about 1% change in 20 million years, is slow compared with the hemoglobins, about 1% in 6 million years, or fibrinopeptides, about 1% in 1 million years. This variation can be understood by the highly conserved nature of the eukaryotic cytochrome *c* sequence which contains 28 invarient positions and 20 positions substituted by only one or two residues in one or two species. Only 9 positions are highly variable; thus only a small fraction of possible mutations would be able to produce a functioning molecule.

(b) *Prokaryotic cytochromes* c

The amino acid sequences of about 35 bacterial cytochromes *c* (Meyer and Kamen, 1982) and the eukaryotic cytochrome *c*'s have been aligned based on similarities of the known cytochrome *c* structures (Dickerson *et al.*, 1976). When the aligned sequences are compared they fall naturally into about six related classes distinct from one another (Dickerson, 1980a). The identities matrix for these six classes is shown in Table 5. It gives the number of sites occupied by identical amino acids in pairwise comparisons averaged either within one class or between two classes. The six classes of Table 5 are the same as those given in Table 2 except that the two c_2 subclasses are treated separately in Table 5, with eukaryotic

TABLE 5. IDENTITIES MATRIX FOR CYTOCHROME *c* FAMILY[a]

	L	M	S_1 (c_{555})	S_2(c_{554})	S_3 (c_{555})	S_4 (c_{553})
L (6: c_2; c_{550})[b]	50%	38%	18%	17%	16%	12%
M (9: *c*)[b]	38%	52%	19%	19%	15%	12%
S_1 (8: c_{551})[c]	18%	19%	44%	18%	14%	14%
S_2 (7: c_{554})[c]	17%	19%	18%	44%	18%	6%
S_3 (2: c_{555})[c]	15%	15%	14%	18%	47%	12%
S_4 (1: c_{553})[c]	12%	12%	14%	6%	12%	—

[a] Dickerson, 1980a; The cytochrome *c* classes are broken into the L, M and S structural categories of Dickerson (1980a). Average percentage identities of aligned residues are given. The quantities in parentheses are the number of sequences compared and the representative structures, except for S_4 where no structure is known.

[b] The L and M categories can be considered as subclasses of a single class (see Table 2).

[c] The small bacterial cytochromes are divided into four subclasses, S_1 through S_4, based on structural and functional similarities.

cytochrome *c* being included in the second class, and the c_4 and c_5 classes being omitted. The names of the six classes of Table 5 are based on the sizes of the representative structures and are, respectively, L (large), M (medium), and S_1–S_4 (small).

The six sequence classes of Table 5 are clearly defined since the identities within a class are distinctly greater than between classes. The one major exception is found in the comparison of the first two classes, L and M, which can be considered to be a single class with two distinct subclasses (Meyer and Kamen, 1982). The first three small structures, c_{551}, c_{554} and c_{555} (S_1, S_2 and S_3) are all quite similar and could be represented by a generalized structure. Yet their sequences are quite different and so should be regarded as separate classes. The spectroscopic studies of *Desulfovibrio* c_{553} (Senn *et al.*, 1983b) suggest that it also follows the general folding pattern of the other small cytochromes but is probably quite distinct in detail and should be labeled S_4.

Dickerson (1980b) has constructed a tentative phylogenetic tree shown in Fig. 11 relating various prokaryotic families based on their metabolism and the molecular structures and sequences of their cytochromes *c*. He proposes that the primordial cytochrome *c* ancestor molecule was of the small structural type and was involved in photosynthesis. As primitive organisms developed some branches gained insertions in their cytochrome *c* sequences, first to form the M structural subclass and then to form the L structural subclass. Others developed into the various small variants of Table 2 and Table 5.

The classification of the cytochromes *c* based on molecular size and sequence similarity, as in Table 2 and Table 5, gives rise to an anomalous distribution of certain genera among the classes. Ambler *et al.* (1979a,b) note that the cytochromes *c* of Rhodospirillaceae display a very wide variations of sequence with several having a striking resemblance to cytochromes *c* from organisms with quite distinct metabolic patterns. They suggest lateral transfer between species of genes coding for cytochrome *c* as an alternative explanation for the observed distribution of cytochrome molecules among various microorganisms.

III. CYTOCHROME b_5 FOLD

1. *Properties of Cytochrome* b_5

Microsomal cytochrome b_5 is located in the smooth endoplasmic reticulum of liver cells in a variety of mammals and avian species. The native protein is a monomer of about 16,000 daltons which is bound tightly to the membrane (Spatz and Strittmatter, 1971). The polypeptide chain is folded into two domains of unequal size separated by a flexible, probably unstructured peptide linkage of about 7 residues (Bendzko and Pfeil, 1983). The larger domain contains the heme group and, depending on the species, consists of approximately the first 86–90 residues from the amino terminus. This domain is quite

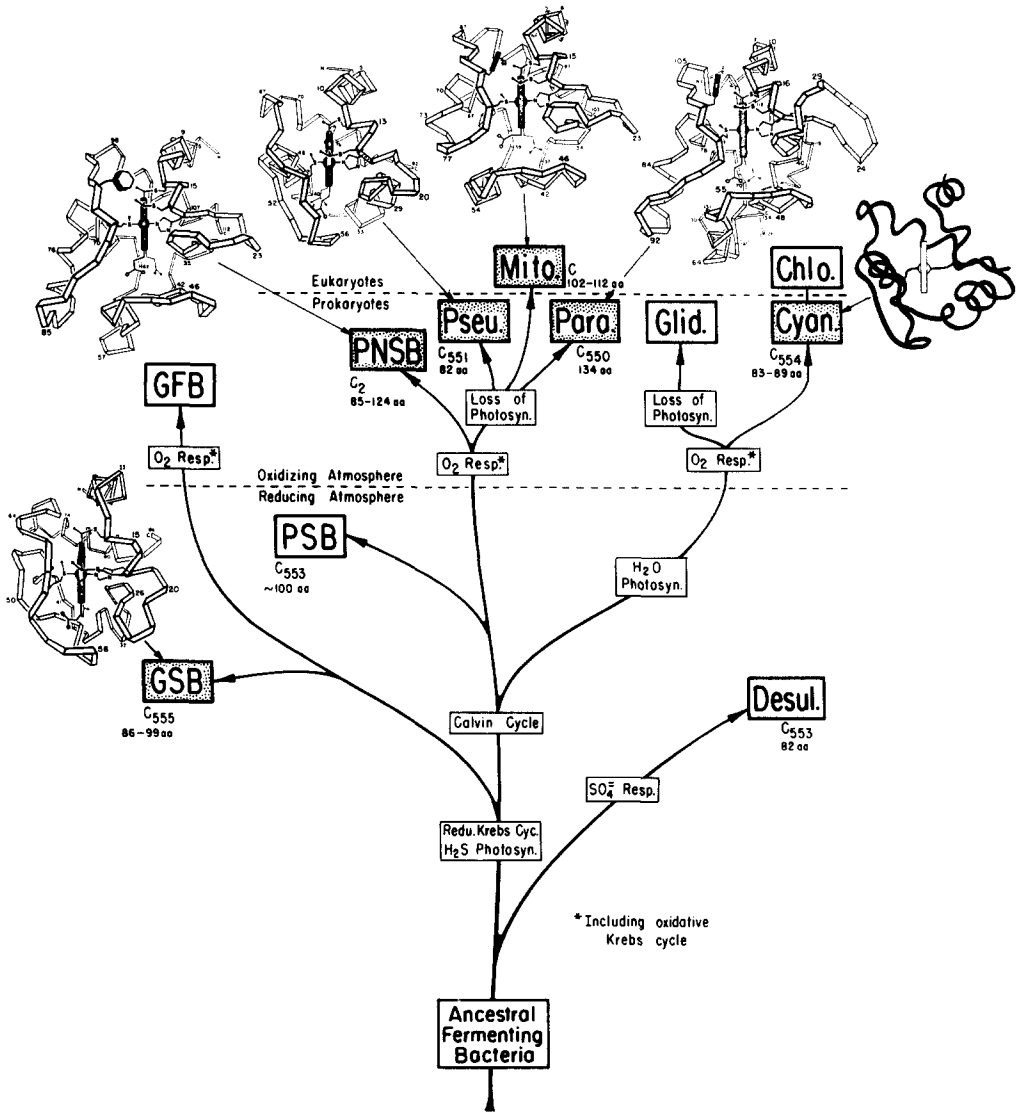

FIG. 11. Phylogenetic tree of the photosynthetic bacteria and their respiring descendants. Sulfate respiration is assumed to have arisen as a response to sulfate-releasing photosynthesis early on the primitive Earth, just as oxygen respiration arose as a consequence of oxygen-releasing photosynthesis at a later era. Oxygen respiration has evolved independently from photosynthesis at least three times: in the green, purple and blue-green photosynthetic bacteria. The known cytochrome *c* structures are shown. GSB = green sulfur bacteria (Chlorobiaceae); GFB = green filamentous bacteria (Chloroflexaceae); PSB = purple sulfur bacteria (Chromatiaceae); PNSB = purple nonsulfur bacteria (Rhodospirillaceae); Pseu. = *Pseudomonas*; Mito. = eukaryotic mitochondria; Para. = *Paracoccus*; Glid. = gliding bacteria related to cyanobacteria; Cyan. = cyanobacteria or blue-green algae; Chlo. = eukaryotic chloroplasts; Desul. = *Desulfovibrio*. Reproduced by permission from Dickerson (1980b).

hydrophylic and extends into the aqueous environment of the cytoplasm (Tanford, 1980). The smaller domain is quite hydrophobic, consisting approximately of the last 37 residues from the carboxyl terminus, and is responsible for attachment of the protein to the membrane. The known amino acid sequences of all cytochromes b_5 are shown in Fig. 12.

The heme binding fragment of cytochrome b_5 can be solubilized by treatment of microsomes with several different proteases. Trypsin yields the smallest fragment, containing residues 3–86, while chymotrypsin and carboxypeptidase Y cut the membrane bound molecule at position 93. The fragment of cytochrome b_5 used in the crystal structure analysis was probably produced by digestion with a lysosomal cathepsin contaminant in the microsomal preparation. It contained residues 1–93 but only residues 3–87, corresponding

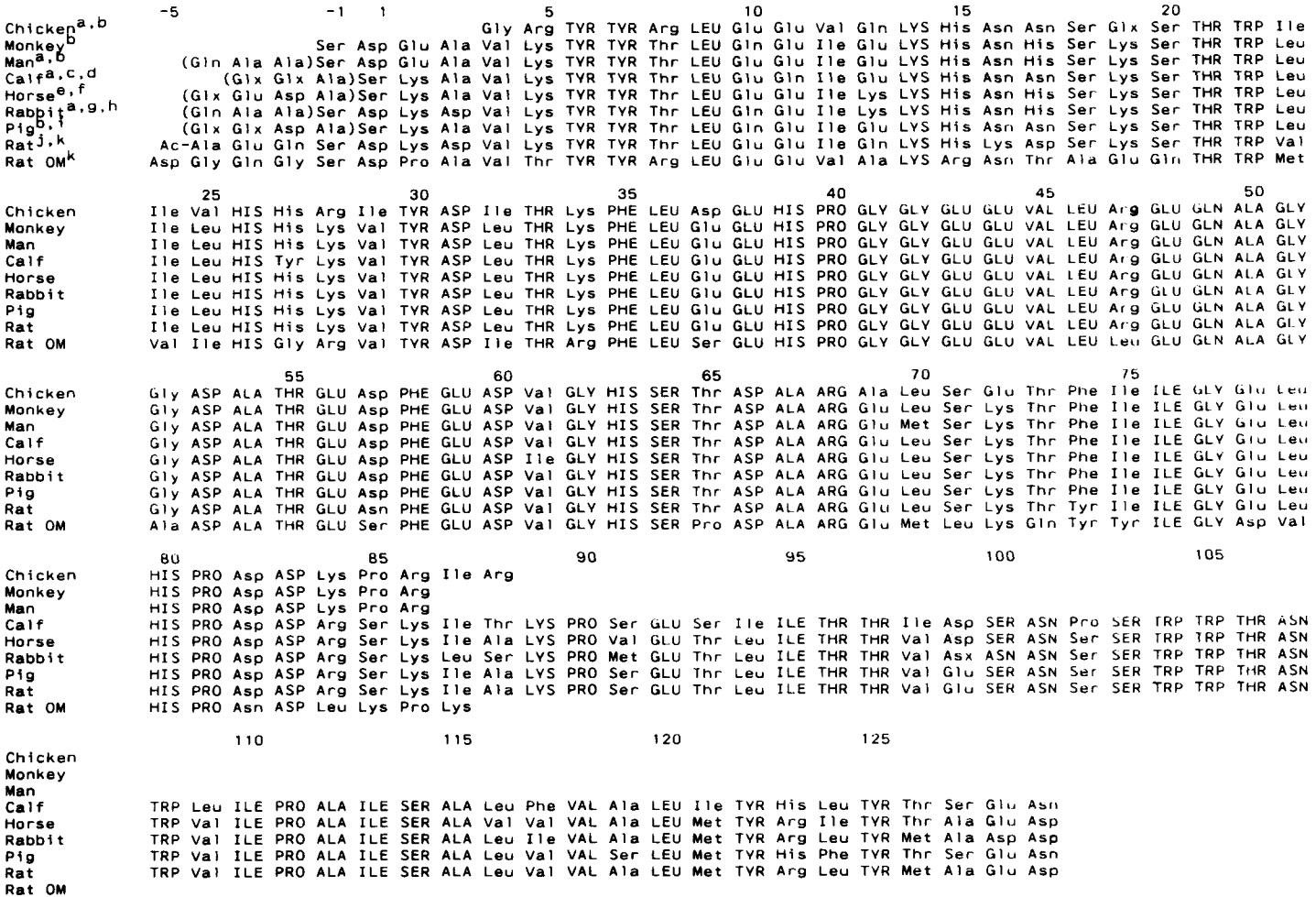

FIG. 12. Amino acid sequences of cytochrome b_5 from eight microsomal and one mitochondrial source. Included are the sequences of the membrane binding domain from 5 sources. Residues identical in all sequences are capitalized. Rat-OM refers to the mitochondrial outer membrane b_5 from rat. The numbering is based on that of calf (Ozols and Strittmatter, 1969) as employed in the crystal structure analysis (Mathews *et al.*, 1979a). The references are as follows: (a) Tsugita *et al.*, 1970; (b) Nobrega and Ozols, 1971; (c) Ozols and Strittmatter, 1969; (d) Fleming *et al.*, 1978; (e) Ozols *et al.*, 1976; (f) Ozols and Gerard, 1977a; (g) Kondo *et al.*, 1979; (h) Takagaki *et al.*, 1980; (i) Ozols and Gerard, 1977b; (j) Ozols and Heinemann, 1982; (k) Lederer *et al.*, 1983.

almost exactly to the trypsin resistant core, were visible in the electron density map (Mathews *et al.*, 1972; Mathews *et al.*, 1979a).

Cytochrome b_5 serves as an electron carrier in the NADH linked desaturation of fatty acids. The fatty acid desaturase system consists of three integral membrane proteins, NADH-cytochrome b_5 oxidoreductase (b_5 reductase), cytochrome b_5 and stearoyl CoA desaturase. The b_5 reductase also consists of two domains, one hydrophylic of about 33,000 daltons and the other hydrophobic of about 4000 daltons (Spatz and Strittmatter, 1973). The smaller domain anchors the protein to the membrane. The hydrophylic domain contains FAD and can be rendered soluble by protease treatment of microsomes with full retention of catalytic activity (Takasue and Omura, 1970). The desaturase is quite hydrophobic and apparently is buried in the membrane. It has a molecular weight of about 53,000 daltons and contains non-heme iron (Strittmatter *et al.*, 1974). Nothing is known of its tertiary or quaternary structure.

All three components of the fatty acid desaturase system can be solubilized by treatment with detergent. Intact b_5 and b_5 reductase each form aggregates of 10 to 20 molecules after removal of detergent. Both molecules can be added back to native microsomes or to artificial phospholipid vesicles such as β,γ-dimyristoyl L-α-lecithin (DML). The extent of binding of either protein is only limited by the surface area available on the vesicles. The complete fatty acid desaturase system can be reconstituted from the three purified protein components using DML vesicles (Enoch *et al.*, 1976).

The endoplasmic reticulum contains another cytochrome system, the NADPH-dependent cytochrome P450 hydroxylase, an inducible system involved in detoxification of a variety of drugs and other agents. The NADPH cytochrome P450 reductase, an amphipathic flavoenzyme containing both FMN and FAD, will substitute for cytochrome b_5 reductase in reconstituted DML vesicles to catalyze fatty acid desaturation (Enoch and Strittmatter, 1979). Cytochrome b_5 will also supply electrons to cytochrome P450 in a number of hydroxylation reactions (Hildebrandt and Estabrook, 1972; Imai and Sato, 1977).

A soluble form of cytochrome b_5 is found in erythrocytes where it serves to reduce methemoglobin (Hultquist and Passon, 1971). NADH cytochrome b_5 reductase is also present in soluble form, both molecules apparently being derived from the microsomal precursor by cathepsin digestion during erythroid development (Slaughter and Hultquist, 1979).

2. *Structure of the Heme Binding Domain*

The three dimensional structure of the heme binding fragment of bovine cytochrome b_5, comprising residues 1–93, was determined at 2.0 Å resolution (Mathews *et al.*, 1979a) and has been refined by the Hendrickson–Konnert restrained least squares procedure (Hendrickson and Konnert, 1980). The agreement factor R is 22% for data from 10 Å to 2 Å resolution (unpublished data). Only residues 3–87 were visible in the map and included in the refinement, the remainder being either disordered in the crystal or cleaved during crystallization.

The molecule is nearly cylindrical in shape and is about 35 Å high and about 30 Å in diameter. It consists of six short helices and a five stranded β sheet, as shown in Fig. 13. The β sheet divides the molecule into two entities each containing a hydrophobic core of aromatic and aliphatic side chains. The larger entity, about 60% of the molecule, contains four helices and is composed of the linear sequence from residues 21–78. It binds the heme group and is primarily involved in the electron transport function of the molecule. The smaller entity appears to serve a structural role only, although its functional importance is demonstrated by its retention in the cytochrome b_5 family (see below). It includes the first and last helices, contained in segments 3–20 and 78–87 and the hydrophobic side chains from the lower face of the pleated sheet surface.

The heme group is buried in the larger hydrophobic core with the propionic acid groups oriented toward the surface of the molecule (Fig. 13). Only parts of the propionate groups and the adjacent methyl groups (see Fig. 1) are in contact with solvent. Of the fourteen protein side chains in contact with the heme, eleven are hydrophobic and three are neutral polar. One of these is a serine whose hydroxyl group forms a hydrogen bond to a peptide

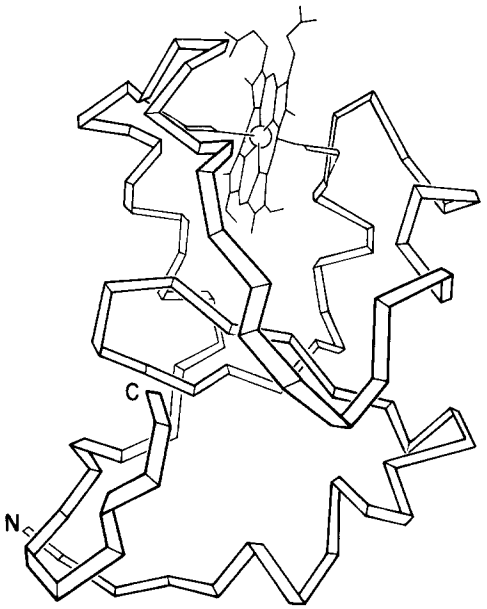

FIG. 13. Schematic diagram of the cytochrome b_5 heme binding domain.

carbonyl oxygen. The remaining two, His 39 and His 63 provide the coordination for the heme iron through their epsilon nitrogen atoms. Their protonated delta nitrogens form hydrogen bonds to main chain carbonyl oxygens. They are also tightly packed by hydrophobic side chains. His 63, for example lies with its plane against that of Phe 58.

One of the propionic acid groups extends into solution. The other bends back onto the molecule to form hydrogen bonds to the peptide nitrogen and side chain hydroxyl of Ser 64. This propionate group is almost completely excluded from solvent.

The heme group is oriented in the b_5 molecule so that the front face, defined in Fig. 1, is coordinated to the ligand with lower sequence number, His 39, while the back face is coordinated to the ligand with higher sequence numbers (Keller and Wüthrich, 1980; Mathews, 1980). This is opposite the situation in tuna cytochrome *c* (see Section II.2). NMR studies of ferricytochrome b_5 have shown that the heme binding is, in fact, disordered with about 10% of the population of heme groups facing His 63 (La Mar *et al.*, 1981).

The heme binding fragment of cytochrome b_5 is very acidic, containing 18 aspartate and glutamate residues plus two heme propionates and nine lysine and arginine residues. The excess acidic groups are concentrated around the heme crevice on the surface of the larger hydrophobic core. This region, essentially above the pleated sheet (as viewed in Fig. 13), has a net charge of -10 including the propionate groups, while the protein surface below the sheet has a net charge of $+1$.

The structure of ferrocytochrome b_5, reduced in the crystal by sodium dithionite, has been studied at 2.8 Å resolution by difference Fourier methods (Argos and Mathews, 1975). The conformation of the main chain and side chain atoms is virtually identical in the two forms. However, an electron dense peak in the reduced structure located near the solvent inaccessible propionate group indicates that a positive ion binds to the propionate upon reduction. This inferred binding has been interpreted to mean that in the oxidized protein the formal charge of $+1$ on the porphyrin core of the ferric heme is neutralized by the unshielded, solvent-inaccessible propionate group 7 Å away. In the reduced protein, the propionate would no longer be neutralized by the uncharged ferrous heme and would be able to attract a cation from solution. This interpretation has been supported by studies of the redox potential of cytochrome b_5 as a function of pH, ionic strength and temperature (Reid *et al.*, 1982), and of the pH dependence of the rate of reduction of cytochrome b_5 by $Fe(EDTA)^{-2}$ (Reid and Mauk, 1982).

3. *Evolutionary Relationships*

(a) *Other microsomal systems*

The complete amino acid sequences of cytochrome b_5 from five species have been determined as well as the sequence of just the heme binding fragment from three additional species. Seven of these species are mammalian and one avian. The sequence of the heme binding domain, residues 3-86 (Fig. 12), is highly conserved, averaging 94% identical between mammalian pairs and 78% between the avian and the individual mammalian sequences. Most of the variation in side chain type occurs on the protein surface and only two of the variable positions are involved in salt bridges. Fifty-seven of the residues in this segment are invariant among the eight species.

The five known sequences of the non-polar peptide which forms the membrane binding domain and flexible linkage are all from mammalian sources. Pairwise comparisons of the sequences of this segment, residues 87-129 (Fig. 12) indicate that about 77% of the residues are identical on the average compared with about 94% identical when mammalian heme binding domains are compared. The greater sequence variability of the non-polar peptide domain probably results from its small size resulting in relatively few intramolecular interactions of side chains in the folded domain and a relatively larger surface area which can interact non-specifically with the lipid surroundings.

(b) *Mitochondrial cytochrome* b_5

The mitochondrial outer membrane contains a cytochrome b_5-like hemoprotein of unknown function. It participates in an NADH-cytochrome *c* reductase activity which is insensitive to rotenone (Raw and Mahler, 1959) and therefore it is not part of the oxidative phosphorylation electron transport chain. The amino acid sequence of the heme binding fragment of rat outer membrane b_5 (OMb_5) released by proteolysis has been determined (Lederer *et al.*, 1983) and is shown in Fig. 12. The sequence of rat OMb_5 is about 58% identical to that of rat microsomal b_5 and it has about the same homology to the bovine microsomal b_5 sequence. This high degree of sequence homology between the microsomal and mitochondrial outer membrane proteins, with no insertions or deletions, suggests that their three dimensional structures are very similar.

Fourteen of the 57 species-invariant residues contained in the heme binding domains of the microsomal sequences are replaced in the OMb_5 sequence. Of these replacements, six are conservative. All but one of the remainder lie on the protein surface. The one exception is His-15 which is replaced by arginine. His-15 is buried in microsomal b_5 and is packed against Trp-22, which is invariant. This interaction is responsible for quenching of tryptophan fluorescence in microsomal b_5 and in flavocytochrome b_2 "core" (see below). Replacement of the tryptophan by arginine in the rat OMb_5 sequence is surprising, but the latter can be accommodated in the structure since the tip of the arginine side chain would extend into solution and could form a salt bridge to a nearby acidic side chain. The pi-electron system of the buried portion of the arginine could also interact with that of the adjacent Trp-22 to help stabilize this interaction (Morgan and McAdon, 1980).

(c) *Cytochrome* b_5 *family*

(i) *Flavocytochrome* b_2. Flavocytochrome b_2 is a tetrameric enzyme found in yeast mitochondria. It catalyzes the oxidation of lactate to pyruvate and transfers electrons to cytochrome *c*. Each subunit of 58,000 daltons contains both a non-covalently bound heme and an FMN group (Jacq and Lederer, 1974). Prolonged tryptic digestion of the molecule leaves a heme-containing fragment of about 11,000 daltons, called cytochrome b_2 "core" (Labeyrie *et al.*, 1967).

The amino acid sequence of cytochrome b_2 "core", Fig. 14, is homologous to that of cytochrome b_5 (Guiard *et al.*, 1974). Approximately 30% of the residues are identical (Table 6) if one single and one double deletion in the b_5 sequence are introduced (Fig. 14). The similarity in three dimensional structures implied by the sequence homology is supported by similarities in certain physical properties of the two molecules. The oxidation-reduction

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                                5                  10                  15                  20                  25
b5 (calf)          Ser Lys Ala Val Lys Tyr Tyr Thr Leu Glu Gln Ile Glu Lys His Asn Asn Ser Lys Ser  *  Thr TRP Leu Ile Leu
b2 (yeast)                     Lys Gln Lys Ile Ser Pro Ala Glu Val Ala Lys His Asn Lys Pro Asp Asp  *  Cys TRP Val Val Ile
SO (chicken)       Ala Pro Ser Tyr Pro Arg Tyr Thr Arg Glu Glu Val Gly Arg His Arg Ser Pro Glu Glu Arg Val TRP Val Thr His
NR (N. crassa)                    Asp Leu Glu Tyr Glu Ile Lys Gln Tyr His  ?  Asn  ?  Lys  ?  *  Thr TRP Leu Ile Leu

                               30                  35                  40                  45                  50
b5 (calf)          His Tyr Lys Val Tyr ASP Leu THR Lys PHE Leu Glu Glu HIS PRO GLY GLY Glu Glu Val Leu Arg Glu Gln ALA GLY
b2 (yeast)         Asn Gly Tyr Val Tyr ASP Leu THR Arg PHE Leu Pro Asn HIS PRO GLY GLY Gln Asp Val Ile Lys Phe Asn ALA GLY
SO (chicken)       Gly Thr Asp Val Phe ASP Val THR Asp PHE Val Glu Leu HIS PRO GLY GLY Pro Asp Lys Ile Leu Leu Ala ALA GLY
NR (N. crassa)     His Tyr Lys  ?  Tyr ASP Leu THR Lys PHE  ?   ?   ?  HIS PRO GLY GLY  ?  Glu

                               55                  60                  65                  70
b5 (calf)          Gly Asp Ala Thr Glu Asp PHE Glu Asp Val  *   *  Gly HIS Ser  *  Thr Asp Ala Arg Glu Leu Ser Lys Thr Phe
b2 (yeast)         Lys Asp Val Thr Ala Ile PHE Glu Pro Leu  *   *   *  HIS Ala  *  Pro Asn Val Ile Asp Lys Tyr Ile Ala Pro
SO (chicken)       Gly Ala Leu  *  Glu Pro PHE Trp Ala Leu Tyr Ala Val HIS Gly Glu Pro His Val Leu Glu Leu Leu Gln Gln Tyr
NR (N. crassa)

                          75                  80                  85                  90                  95
b5 (calf)           *   *  Ile Ile GLY Glu Leu His Pro Asp Asp Arg Ser Lys Ile Thr Lys Pro Ser Glu Ser
b2 (yeast)         Glu Lys Lys Leu GLY Pro Leu Glu Gly Ser Met Pro Pro Glu Leu Val Cys Pro Pro Tyr Ala Pro Gly Glu Thr Lys
SO (chicken)        *   *  Lys Val GLY Glu Leu Ser Pro Asp Gly Ala Pro Ala Ala Pro Asp Ala Gln Asp Pro Phe
NR (N. crassa)
```

FIG. 14. Sequence alignment of the cytochrome b_5 family. The sequences are shown for cytochrome b_5 (Ozols and Strittmatter, 1969), flavocytochrome b_2 "core" (Guiard *et al.*, 1974), sulfite oxidase "core" (Guiard and Lederer, 1979a) and assimilatory nitrate reductase "core" (Le and Lederer, 1983). The last sequence is only partial but shows a striking similarity to microsomal cytochrome b_5. The 13 residues invariant in all members of the b_5 family are capitalized.

potentials are very close as are the visible absorption spectra. The fluorescence of the single tryptophan is quenched in both molecules (Labeyrie *et al.*, 1967; Huntley and Strittmatter, 1972) indicating that the conserved histidine at position 15, which is stacked against the face of Trp-22 in b_5, makes similar contact with the tryptophan in b_2 "core". Furthermore, there is an upfield-shifted methyl resonance in the NMR spectrum of b_2 "core" which is similar to one observed in cytochrome b_5 (Keller *et al.*, 1973). The latter has been assigned to the methyl group of Ile 76 of b_5 which is located above the indole ring of Trp-22. The most likely group in b_2 "core" to give rise to the resonance is the sequentially homologous Leu 76.

(ii) *Sulfite oxidase.* Sulfite oxidase is a dimeric protein obtained from liver cells of a number of species. It contains a molybdenum atom and a heme group in each 58,000 dalton subunit and transfers electrons to cytochrome *c* (Cohen and Friedovich, 1971). Like flavocytochrome b_2, it too can be digested with trypsin to produce a heme containing 11,000 dalton fragment, sulfite oxidase "core".

The amino acid sequence of chicken liver sulfite oxidase "core" (Fig. 14) is homologous to those of both cytochrome b_5 and cytochrome b_2 "core" (Guiard and Lederer, 1979a). The sequence identities are each about 30% with five and eight residues inserted or deleted in the respective comparisons (Table 6). The spectroscopic properties are also similar.

TABLE 6. IDENTITIES MATRIX FOR THE CYTOCHROME b_5 FAMILY[a]

	b_5 calf	b_5 rat OM	b_2 "core"	SO "core"	NR "core"
b_5 calf	...	60%	29%	29%	65%
b_5 rat OM	52/86		34%	35%	42%
b_2 "core"	26/92	30/89		30%	35%
SO "core"	27/92	32/92	27/89		32%
NR "core"	20/31	13/31	11/31	10/31	

[a] Guiard and Lederer, 1979a; Lederer *et al.*, 1983; Le and Lederer, 1983.

(iii) *Nitrate reductase.* A fourth member of the cytochrome b_5 family, assimilatory nitrate reductase, has recently been identified (Le and Lederer, 1983). The enzyme, isolated from *Neurospora crassa* contains three cofactors, FAD, heme and molybdenum, and has a total molecular weight of about 240,000 daltons. Chymotryptic digestion yields a heme-containing fragment of about 100 residues with an absorption spectrum similar to microsomal cytochrome b_5. The amino acid sequence of the first 39 residues of nitrate reductase "core" has been determined and is shown in Fig. 14. The similarities in sequence to the other members of the family are clear (Table 7). Interestingly, its sequence resembles that of cytochrome b_5 more closely than the others since 20 of 31 positions compared are identical, while only about 12 are identical for the other family members.

(iv) *The* b_5 *fold.* Guiard and Lederer (1979b) have analyzed the structural and functional implications of the striking homology of the three heme binding domains of microsomal cytochrome b_5, flavocytochrome b_2 and sulfite oxidase. When the comparison is extended to include the mitochondrial outer membrane b_5 and nitrate reductase "core", the homology between these domains from proteins of such diversity of origin, cellular location and physiological function is quite striking. The 13 residues invariant among the four classes of proteins are identified on a back-bone diagram of cytochrome b_5 in Fig. 15 and are listed in Table 7.

The most significant invariant residues (Table 7) are the two heme-linked histidines at positions 39 and 63 (using the b_5 numbering scheme). The invariant Phe 58 packs tightly against His 63. The carbonyl oxygen of Phe 58 also helps stabilize the orientation of His 63

TABLE 7. INVARIANT RESIDUES IN THE b_5-FOLD

Trp 22	Second hydrophobic entity, function unknown	
Asp 31	H-bond from Asp 31 O_γ to Thr 33 N	These two main-to-side chain H bonds slightly extend the β-sheet.
Thr 33	H-bond from Thr 33 O_γ to Ser 30 N	
Phe 35	Heme contact	
His 39	Fe ligand	
Pro 40	In tightly packed turn; packed against heme	
Gly 41	In tightly packed turn, crowded environment	
Gly 42	Gly 42 O to His 39 N_δ H-bond; disallowed region of Ramachandran diagram	
Ala 50	Tightly packed environment, unknown function	
Gly 51	Third position of type II reverse turn	
Phe 58	His 63 N_δ to Phe 58 O H-bond; phenyl ring packed against plane of His 63	
His 63	Fe ligand	
Gly 77	β-bulge[a]; disallowed region of Ramachandran diagram	

[a] Richardson, *et al.*, 1978.

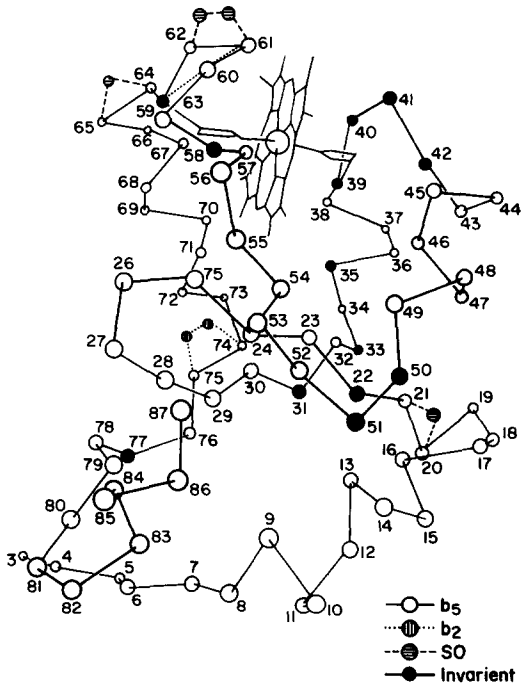

FIG. 15. Schematic diagram of the structure of cytochrome b_5 showing the locations of the 13 residues identical in all known microsomal and mitochondrial cytochromes b_5, cytochrome b_2 "core", sulfite oxidase "core" and nitrate reductase "core". The insertions and deletions in the sequences necessary for alignment are also shown. Insertions and deletions in the b_5 sequence are indicated by dotted and dashed lines for b_2 "core" and SO "core".

by accepting a hydrogen bond from its delta nitrogen atom. On the other side of the heme the invariant residues Pro 40, Gly 41 and Gly 42 are involved in a tight reversal of direction of the polypeptide chain. Pro 40 is in contact with the heme and the α-carbons of Gly 41 and Gly 42 are unable to accommodate side chains because of steric limitations. The carbonyl oxygen of Gly 42 also accepts a hydrogen bond from the delta nitrogen of His 39 to stabilize its orientation.

In general, the non-polar nature of the two hydrophobic entities separated by the pleated sheet structure appears to be retained in the b_5 fold. Ala 50 and Gly 51 are invariant and are located in a tightly packed environment. In the second hydrophobic entity Trp 22 is invariant. In b_5 and b_2 "core", at least, the spatial orientations of His 15, Trp 22 and an aliphatic side chain at position 76 are retained, indicating the generality of the packing of side chains in this region.

IV. 4-α-HELICAL CYTOCHROME FOLD

1. *Cytochrome* b_{562}

(a) *General properties*

Cytochrome b_{562} is a monomeric hemoprotein of about 12,000 daltons which is isolated from the cytoplasm of *E. coli* (Itagaki and Hager, 1966). Its function is unknown but it may serve as a soluble electron carrier. The iron is low spin in both the ferrous and ferric forms and the midpoint redox potential at neutral pH is about 185 mV (Pettigrew, 1983).

The amino acid sequence (Itagaki and Hager, 1968; Lederer *et al.*, 1981) is shown in Fig. 16. The protein contains relatively few aromatic residue; there are two histidines, two phenylalanines, two tyrosines but no tryptophan. The protein also contains no cysteine.

The heme group of b_{562} can be removed reversibly by treatment with acetone at low pH. A derivative of the protein has been prepared in which the heme has been replaced by mesoheme monosulfuric anhydride (Warme and Hager, 1970a). In this heme analogue one propionate of mesoheme (see caption to Fig. 1) has been converted to the sulfuric anhydride which is quite reactive toward lysine residues (Warme and Hager, 1970b). The activated propionate of the reconstituted complex reacts preferentially with Lys 47 to form a covalent adduct.

(b) *Molecular structure*

The structure of cytochrome b_{562} was solved initially at 2.5 Å resolution (Mathews *et al.*, 1979b; Lederer *et al.*, 1981) and has been partially refined at 2.0 Å resolution (Bethge and Mathews, 1984). Its structure is shown in Fig. 17. It consists of four nearly parallel α-helices which pack together with a left-handed twist of about 15° to 20°. The four helical segments A through D are made up, respecitvely, from residues 2–20, 23–42, 58–82 and 85–105 (Table 8). The loop between helices B and C contains four prolines but has no regular secondary structure. Of the three glycine residues, one, Gly 82, is in the third position of a type II reverse turn. The other two are located in helix C. One of these, Gly 64, is located in a tightly packed environment, close to the side chain of Tyr 102 and to one of the heme vinyl groups.

The two ligands to the heme iron are the delta sulfur of Met 7 and the epsilon nitrogen of His 102 (Table 8). The environment of the heme is asymmetrical. The front face, pointing toward Met 7, is in a very hydrophobic environment. The back face, attached to His 102, is rather exposed. Pyrrole rings I and II (see Fig. 1) are deeply buried with both vinyl groups lying in the interior of the helix bundle. However, one face of pyrrole rings III and IV and half the histidine ligand are exposed to solvent, along with the propionic acid groups. The delta nitrogen atom of the histidine ligand is also exposed, in contrast to most other heme proteins in which it is hydrogen bonded to a peptide carbonyl oxygen. In the crystal this nitrogen forms a hydrogen bond to Gln 103′ from a neighbouring molecule.

The charged side chains appear to be fairly evenly distributed over the protein surface. Unlike cytochromes b_5 and *c*, there are no obvious clusters of acidic or basic side chains. However, there are four acidic groups on the first three turns of helix A, on one side of the

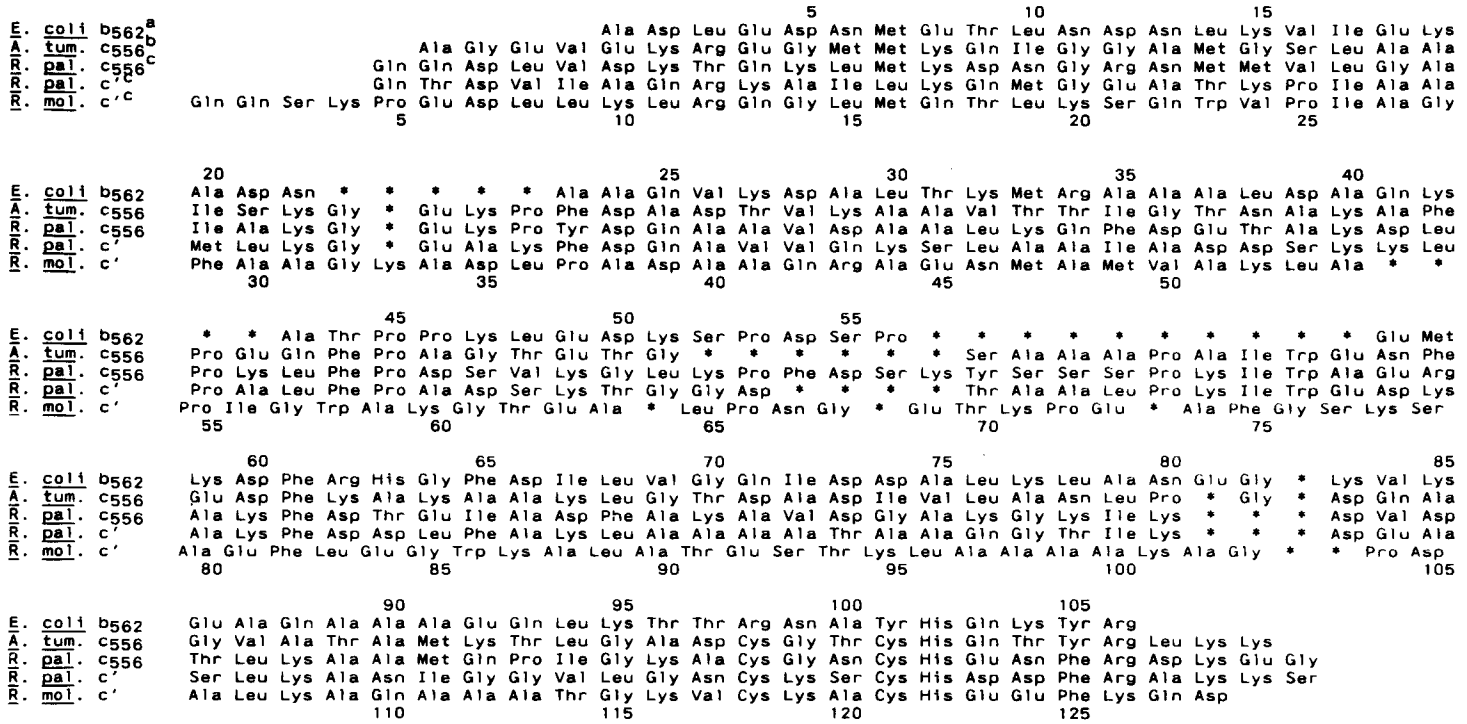

FIG. 16. Amino acid sequences of the 4-α-helical proteins cytochrome b_{562} from *E. coli* and cytochrome *c'* from *R. molichianum*. Homologous sequences from the monomeric *c'* from *R. pulastris* and the cytochromes c_{556} from *R. pulastris* and *A. tumefaciens* are also shown. The numbering at the top refers to cytochrome b_{562} while that at the bottom refers to *R. molichianum c'*. The references are as follows: (a) Itagaki and Hager, 1968; Lederer *et al.*, 1981. (b) Van Beeuman *et al.*, 1979. (c) Ambler *et al.*, 1981a.

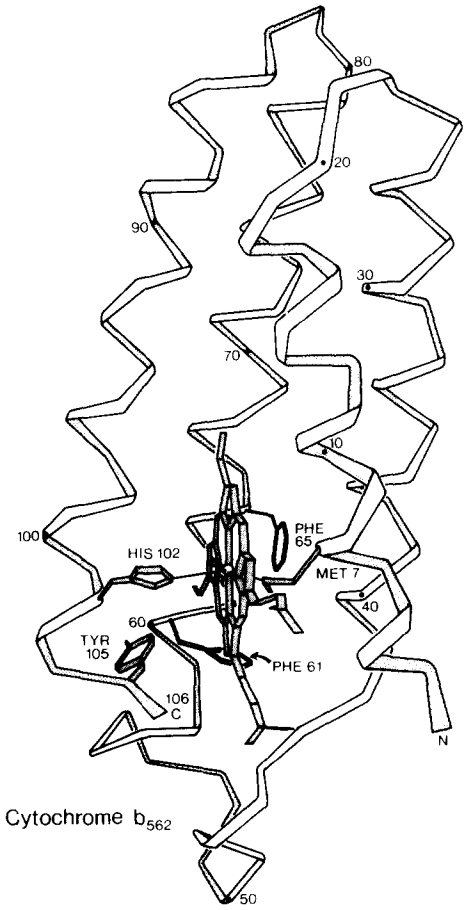

FIG. 17. Schematic diagram of cytochrome b_{562} from *E. coli*. Reproduced by permission from Weber *et al.*, 1981b.

partially exposed heme face, and four basic groups on the last three turns of helix D on the other side. In view of the importance of interactions between oppositely charged side chains in complexes between electron transport proteins (see Section VI), such dipolar clustering around the exposed heme edge and face of b_{562} may be of functional importance.

Both propionate groups extend into solution. Propionate-6 on pyrrole ring III could potentially form a salt bridge with Lys 47, which lies nearby (Table 8), as suggested by the chemical modification study of Warme and Hager (1970a). The other, propionate-7 is too far from any of the basic groups on helix D to form a salt bridge and can only interact with the solvent.

All six aromatic residues lie in the vicinity of the heme group. Both Phe 61 and Phe 65 point inward toward the heme with the latter lying roughly parallel to it near pyrrole ring II and vinyl-4 (see Fig. 17 and Table 8). Both the side chains of Tyr 101 and Tyr 105, as well as that of His 63 lie on the protein surface.

2. *Cytochromes* c′

(a) *General properties*

The cytochromes *c′* form a large and widespread class of cytochromes and occur in a variety of photosynthetic and denitrifying bacteria (Bartsch, 1978; Meyer and Kamen, 1982). They are usually dimeric and are made up of identical subunits of about 14,000 daltons. Their function is unknown but they are present in higher amounts in anaerobically grown cells than in aerobically grown cells. In photosynthetic bacteria, they are located in the periplasmic space but are not involved directly in photosynthesis.

TABLE 8. PROPERTIES AND INTERACTIONS OF *E. coli* CYTOCHROME b_{562} AND *R. molischianum* CYTOCHROME c'

(a) *Helices*

Segments	b_{562}	Residue (aligned with c')[a]	c'	Residue (aligned with b_{562})[a]
A	2–20	(1–15)	5–25	(10–24)
B	23–42	(26–43)	39–55	(40–57)
C	58–82	(58–79)	79–100	(79–100)
D	85–105	(84–105)	106–126	(104–125)

(b) *Heme contacts*

	b_{562}	c'
Vinyl-2 thioether bond	none	Cys 118 S_γ
Vinyl-4 thioether bond	none	Cys 121 S_γ
Heme ligand, front face	Met 7 S_δ	none
Heme ligand, back face	His 102 N_ε	His 122 N_ε
Propionate-6 salt bridge	Arg 47 N_η^b	Arg 12 N_η
Propionate-7 salt bridge	none	Lys 10′N_ζ^c
His ligand N_δ H-bond	none	none

(c) *Invariant*[d], *Important*[e] *or Notable*[f] *Residues*

b_{562}		c'	
Gly 82[e]	Type II reverse turn	Arg 12[d]	salt bridge to heme propionate
Gly 64[e]	tightly packed environment	Gly 61[d]	Type II reverse turn
		Thr 62[d]	Unknown function
Phe 61[f]	packed near heme; equivalent aromatic in c'	Gly 103[d]	Tightly packed in CD interhelical loop
Phe 65[f]	packed near heme; equivalent aromatic in c'	Gly 115[d]	Contact point between helices A and D
Tyr 101[f]	protein surface	Cys 118[d]	Covalent heme attachment
Tyr 105[f]	protein surface; equivalent aromatic in c'	Lys 119[d]	Unknown function
His 63[f]	protein surface	Cys 121[d]	Covalent heme attachment
		His 122[d]	Heme ligand
		Gly 14[e]	In contact with Gly 14′ (of dimer across 2-fold axis)
		Phe 82[f]	Equivalent aromatic in b_{562}
		Trp 86[f]	Equivalent aromatic in b_{562}
		Phe 125[f]	Equivalent aromatic in b_{562}

[a] Weber *et al.*, 1981b.
[b] Arg 47 salt bridge not observed in the crystal but could form with minor movements of the side chains.
[c] Salt bridge to Lys 10′ of 2-fold-related molecule.
[d] Invariant residues in dimeric cytochromes c'.
[e] Glycine residues in unfavorable conformation or tightly packed environment.
[f] Aromatic residues noted in the text.

The iron atom of the c' type cytochrome is high spin in both oxidation states and the reduced protein will bind carbon monoxide or nitric oxide (Weber *et al.*, 1981a). At very high pH the iron becomes low spin. The oxidation–reduction potentials range from about 0 mV to 150 mV. The amino acid sequences from twelve dimeric and one monomeric cytochrome c' have been determined (Ambler *et al.*, 1981b). The sequences of the dimeric c' from *Rhodospirillum molischianum*, whose structure is known, and of the monomeric c' from *Rhodopseudomonas palustris* are shown in Fig. 16.

The heme group in all cytochromes c' is attached covalently near the carboxyl terminus via thioether linkages between the two vinyl groups of the heme and two cysteine side chains. The sequence of the attached peptide is -Cys-X-Y-Cys-His- typical of c-type cytochromes.

(b) *Molecular structure*

The crystal structure of cytochrome c' from *Rhodospirillum molischianum* has been

determined to 2.5 Å resolution (Weber *et al.*, 1981a). The tertiary structure of the monomer is shown in Fig. 18. The monomer is composed of four nearly parallel α-helices, A through D, which pack with a left-handed twist of about 19°. The A helix extends from residue 5 to 25, the B helix from residues 39–55, the C helix from residues 79–100 and the D helix from residues 106 to about 126 (Table 8). The heme group is located about three-fifths of the way along the helix bundle from the doubly connected end and is partly shielded from solvent by the extensive BC loop from residues 56–78.

The heme group is covalently bound to the sulfur atoms of Cys 118 and Cys 121 through the vinyl groups at positions 2 and 4, respectively (Table 8). It lies roughly parallel to the A and C helical axes with pyrrole rings I and II (Fig. 1) located near the center of the helix bundle. The environment of the heme is quite asymmetrical. The front face, as viewed in Fig. 1, is tightly packed by side chains from helices A and B. The back face is partially exposed to solvent, particularly pyrrole rings III and IV and the propionate groups.

The fifth coordination site of the heme iron is occupied by the epsilon nitrogen of His 122. The histidine ligand is exposed to solvent, its δ nitrogen not making any hydrogen bonds to the protein molecule. The partially exposed heme face and exposed histidine ligand is a feature common to cytochrome b_{562}, as discussed below, and different from most other heme proteins.

The sixth coordination site of the heme iron is vacant, accounting for the high spin nature of the iron and its ability to bind small uncharged molecules. Met 16 lies close to the iron atom but the lone pair of electrons on the sulfur atom is directed away from it preventing the formation of a coordination bond.

Both heme propionates lie on the surface of the protein. The more exposed one,

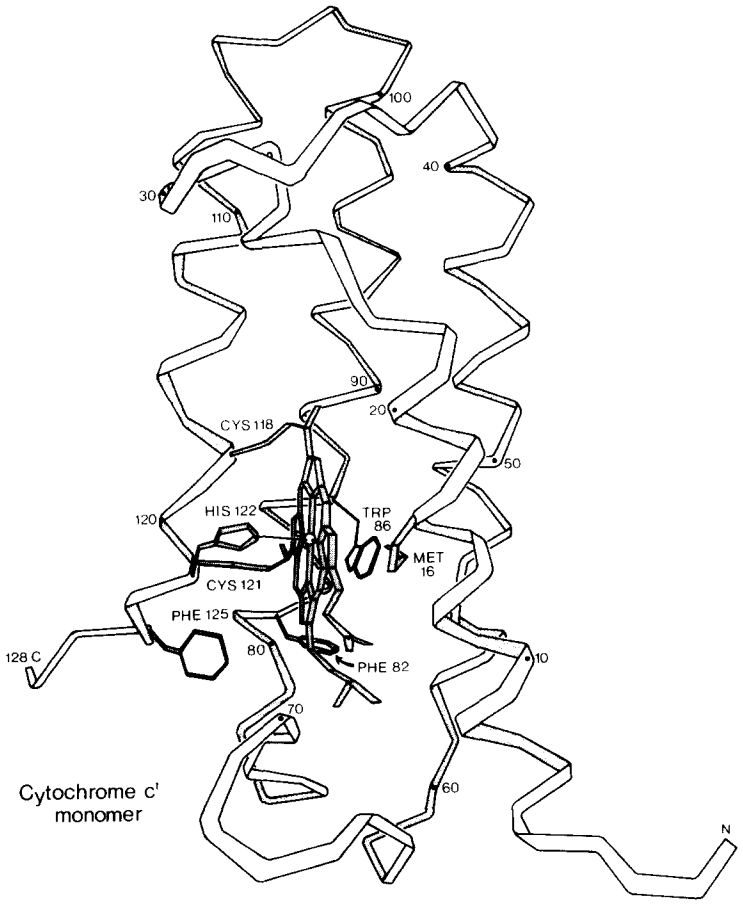

FIG. 18. Schematic diagram of the cytochrome c' monomer. Reproduced by permission from Weber *et al.*, 1981b.

propionate-7, makes contact with Glu 13 on helix A and Lys 10′ of helix A′ of the other subunit (Table 8). The other, propionate-6, forms a salt bridge with the invariant Arg 12 of helix A.

The cytochrome *c′* dimer is shown in Fig. 19. Helices A and B from each monomer form an antiparallel four-stranded bundle AA′BB′ with a left-handed twist similar to the monomer. Helices A and A′ make very close contact at a point where symmetrically related Gly 14 residues pack together. Most of the residues at the dimer interface are hydrophobic, with about five residues of helix A and three of helix B making contact across the molecular diad axis. In addition two pairs of salt bridges link the monomers.

There are approximately equal numbers of positive and negative side chains on the *R. molischianum c′* molecule and they are distributed evenly over most of the molecular surface. However, there is some clustering of positive charges near the exposed edges of the heme groups (Weber *et al.*, 1981a).

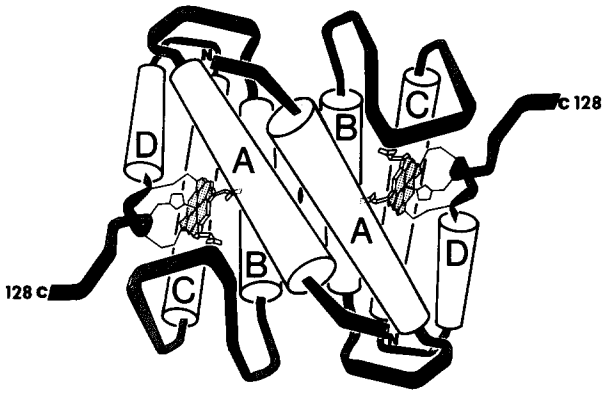

FIG. 19. Schematic diagram of the cytochrome *c′* dimer. Note the 4-α-helical configuration and left handed twist of the ABB′A′ helical interface. Reproduced by permission from Weber *et al.*, 1980.

(c) *Sequence conservation*

There are nine totally conserved residues among the 12 dimeric cytochrome *c′* sequences which have been determined (Ambler *et al.*, 1981b). They are Arg 12, Gly 61, Thr 62, Gly 103, Gly 115, Cys 118, Lys 119, Cys 121 and His 122 (see Table 8). Four of these interact directly with the heme group. Cys 118, Cys 121 and His 122 form the covalent and coordinate linkages to the heme group characteristic of *c* type cytochromes. Arg 12 forms the salt bridge to the less accessible propionate of pyrrole ring III. These four residues plus the conserved Lys 119, whose functional importance is not apparent, are also present in the monomeric cytochrome *c′*.

The three conserved glycine residues occur at sterically constrained positions in the three dimensional structure of *R. molischianum c′*. Gly 115 is located near the point of closest contact between helices A and D. Gly 103 lies in the tight CD interhelical loop region while Gly 61 occurs in a type II reverse turn. The role of Thr 62 is not known. In the monomeric cytochrome *c′* these last four residues are not conserved suggesting that if the overall structures are the same, a somewhat different arrangement of the helices and the interconnecting loops occurs.

A number of positions are identical in most of the sequences and substituted conservatively in the others. Most of these are in contact with the heme group and probably provide the hydrophobic bulk necessary to keep it in position. Since there is some variability in type they probably play no mechanistic role. One possible exception is position 126 which is always lysine or arginine. This residue is the only one, besides the histidyl heme ligand, which is located near the exposed face of the heme above pyrrole rings III.

It is somewhat surprising that Gly 14 is not conserved among the dimeric cytochromes *c′* since it is in an apparently key position at the dimer interface near the molecular two-fold axis

(Weber *et al.*, 1981a). The exact geometry of dimer formation must be somewhat variable within this class of molecules.

3. *Cytochrome* c_{556}

Cytochromes c_{556} form a special class of cytochrome with properties very similar to cytochromes c' and b_{562}. The proteins from two bacteria, *Rhodopseudomonas palustris* and *Agrobacterium tumefaciens*, have been characterized and their sequences determined (Ambler *et al.*, 1981b). Their sequences are shown in Fig. 16. The proteins are monomeric and have a molecular weight of about 13,000 daltons. The amino acid sequences are similar to those of the cytochromes c', especially near the carboxyl terminus. The heme group is bound covalently to the C terminal Cys-X-Y-Cys-His sequence typical of c-type cytochromes. The midpoint redox potential of the *Rps. palustris* c_{556} is 230 mV.

The absorption spectrum of c_{556} is typical of low spin c-type cytochromes except that the absorption maxima are shifted to longer wavelengths. Magnetic susceptibility and other measurements also show the iron to be low spin and therefore six coordinate (Moore *et al.*, 1982). Spectroscopic and NMR measurements indicate that the axial ligands of the heme are histidine and methionine. The only histidine present in either c_{556} sequence is the one corresponding to the conserved His 122 of the c' sequence. There is also a methionine in the two c_{556} sequences located at a position corresponding to Met 16 in *R. molischianum* c' (Fig. 15) which is near the unliganded heme iron. These similarities with cytochrome c' and b_{562} suggests that the c_{556} cytochromes have a very similar 4-α-helical three dimensional fold.

4. *Evolutionary Relationships*

(a) *Cytochromes* b_{562} *and* c'

Cytochromes c' and b_{562} have a very similar polypeptide chain conformation. In both proteins the heme group is situated about three fifths of the way along the divergent helical bundle from the doubly connected end. The heme group is located in very similar environments in the two proteins. The front face, viewed with the substituent positions increasing clockwise as in Fig. 1, lies against helices A and B in a very hydrophobic environment. A methionine side chain lies close to the heme iron on the front face making a coordinate bond to the iron atom in b_{562} but not in c'. The back face of pyrrole rings III and IV and the histidine heme ligand are exposed to solvent. Both propionate groups lie on the protein surface exposed to solvent, although the one on pyrrole ring III in c' is less exposed than in b_{562} because of interaction with the BC loop.

There are some major differences as well. The heme is bound covalently through thioether linkages in cytochrome c' but bound non-covalently in b_{562}. The heme iron is five-fold coordinated, high spin in c' but six-fold coordinated, low spin in b_{562}. The sulfur of a methionine side chain near the heme iron forms a coordination bond to the iron atom in b_{562} but not in c'. The less exposed heme propionate-6 of c' forms a salt bridge with an arginine sidechain in c' but not in b_{562}. Also, cytochrome c' is usually dimeric while cytochrome b_{562} is monomeric.

The three dimensional structures of the two proteins were compared directly in order to determine the extent of similarity and to assess their evolutionary relatedness (Weber *et al.*, 1981b). The two heme groups were superimposed on a molecular graphics display without the protein molecule being visible. The polypeptide α-carbon backbones were found to line up remarkably well, as shown in Fig. 20. By this procedure helices B, C, D and the first half of helix A were in nearly equivalent positions. Specifically 77 residues, segments 1–15, 26–43, 58–79 and 84–105 of b_{562} were found to be structurally homologous to segments 10–24, 40–57, 79–100 and 104–125 of c' (Table 8). Fourteen of these residues are identical in the two proteins. The primary differences in conformation occur in the loops connecting the helices. Cytochrome c' contains 22 more residues than cytochrome b_{562}. The extra residues occur at the N and C termini and the AB and BC loops.

The most striking similarity in structure is the near superposition of Met 7 and Met 16 in

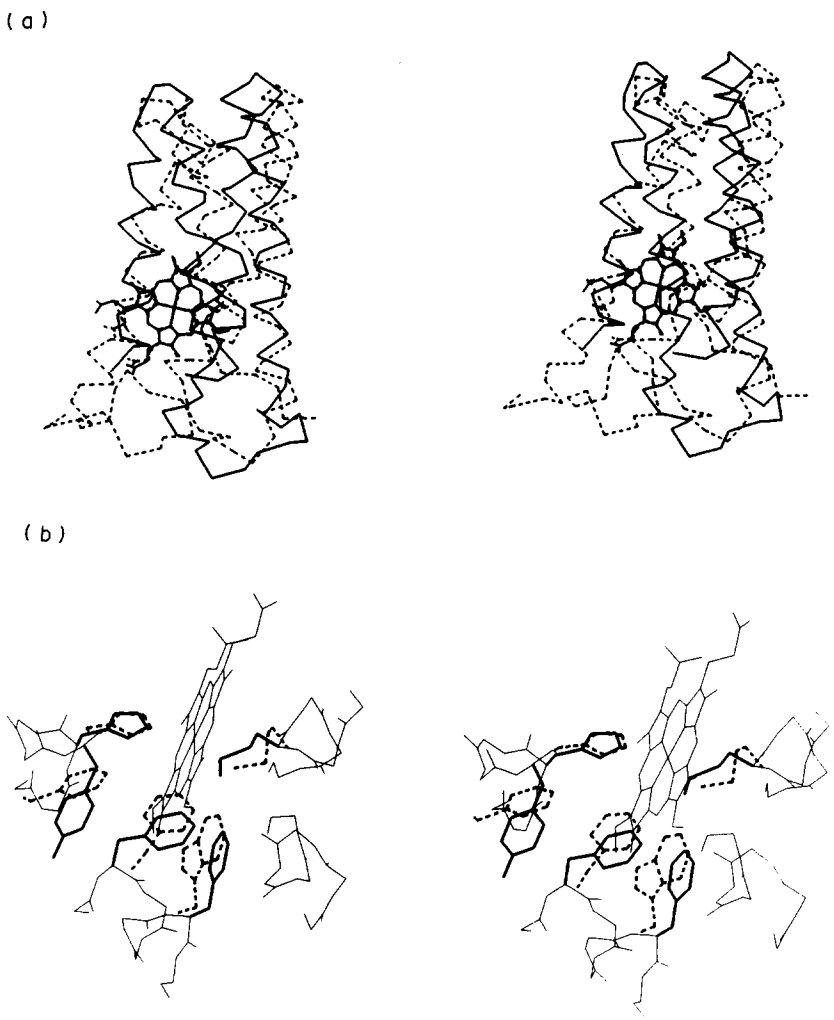

FIG. 20. Superposition of the c_α-diagrams of cytochrome b_{562} and the cytochrome c' monomer. The alignment of the two molecules is based solely on superposition of the two heme groups. Cytochrome b_{562} is solid while cytochrome c' is dashed. (a) Heme group and α-carbon chains. (b) Aromatic side chains common to the two molecules. Reproduced by permission from Weber *et al.*, 1981b.

cytochromes b_{562} and c' respectively. Yet, in contrast to b_{562}, Met 16 of c' is unable to coordinate to the heme iron atom. Examination of the two structures in Fig. 20 shows that the axes of the A helices of the two proteins diverge from one another to a greater extent than those of the other helices and that they are rotated with respect to each other slightly about their long axis. A small change in conformation of the helix backbone would be necessary to bring the sulfur atom of Met 16 close enough to form a bond to the iron atom. The alternative movement of the heme of c' to allow bond formation with Met 16 is prevented by its covalent attachment to helix D. The rigidity of the helices and the cooperativity of the side chain packing apparently make formation of this bond energetically unfavorable.

Three aromatic residues also share equivalent positions about the heme groups in cytochromes b_{562} and c'. These are Phe 61, Phe 65 and Tyr 105 in b_{562} and Phe 82, Trp 86 and Phe 125 in c', as shown in Figs 17 and 18. The aromatic character of Phe 125 is conserved in all the 13 cytochrome c' sequences and the identity of Phe 82 is conserved in all but two sequences. On the other hand, Trp 86 is not conserved. The only role these aromatic residues seem to play in the function of cytochromes b_{562} or c' seems to be to maintain a tightly packed hydrophobic environment about the buried portion of the heme.

(b) *Generalization*

The four-α-helical class of cytochromes is composed of three distinct subclasses. The

cytochrome b_{562} subclass is low spin with relatively high redox potential. The heme iron is six-fold coordinated with histidine and methionine ligands. The heme is bound non-covalently and the protein is monomeric. The cytochrome c_{556} subclass is also low spin, with a relatively high redox potential and is usually also monomeric. The heme is also six-fold coordinated with histidine and methionine ligands. However, the heme is bound covalently. In addition, the polypeptide chains of c_{556} are somewhat longer both at the chain termini and the AB and BC loop regions. The cytochrome c' subclass is high spin with relatively lower redox potential. The heme iron is five-fold coordinated with only a histidine ligand and the protein is usually dimeric. The heme is also bound covalently. The lengths of the polypeptide chain, and probably of the inter-helical loops as well, are similar to those of cytochrome c_{556}.

The amino acid sequences of the four-α-helical proteins are poorly conserved (Ambler *et al.*, 1981b). To maintain the maximum sequence identity, especially among residues thought to be functionally important, it is necessary to introduce four or five insertions or deletions in most of the sequences. All of these occur in the regions of the sequences presumed to be non-helical. The sequence of b_{562} is about 15% identical on average to the c_{556} and c' sequences. The c_{556} sequences average about 28% identical to the c' sequences and the c' sequences about 31% among themselves (Meyer and Kamen, 1982).

There is some question whether the structural similarity of cytochrome c' and b_{562} is a result of divergent evolution from a common ancestor (complicated perhaps by lateral gene transfer) or of convergent evolution to a stable supersecondary structure. Well known examples of divergent evolution are found in the mitochondrial cytochromes *c* (Dickerson and Timkovich, 1975) and the dehydrogenases (Rossmann *et al.*, 1975). Convergent evolution has been observed in the development of active sites of similar geometries in the serine protease families of trypsin and subtlisin (Kraut, 1977).

The four-α-helical motif has been observed in hemerythryn (Hendrickson *et al.*, 1975; Stenkamp *et al.*, 1978), tobacco mosaic virus coat protein (Stubbs *et al.*, 1977; Bloomer *et al.*, 1978) and apoferritin (Clegg *et al.*, 1980), as well as in cytochromes c' and b_{562}. There is little reason to believe that all of the above proteins of such diverse function and origin have evolved from a common precursor, particularly since the antiparallel 4-α-helical motif is an especially stable one (Sheridan *et al.*, 1982) and could very well have evolved independently a number of times. However, the close similarity in the relative heme locations, orientations and degree of solvent exposure in cytochromes b_{562} and c', as well as the correspondence of aromatic residues near the heme suggests that they may have evolved from a common molecular ancestor.

There is also some question whether the high spin dimeric form of cytochrome c' is the physiologically important form or an artifact of isolation (Moore *et al.*, 1982). No high spin spectrum of c' has been observed *in vivo* and antibodies against purified cytochrome c' are unreactive toward cell homogenates. It has been proposed that cytochrome c' exists *in vivo* as a complex with one or several other proteins (Dutton and Leigh, 1973) and is inaccessible to antibodies and possibly has an altered spectrum. Another proposal is that the native form of c' is monomeric, six-fold coordinated and low spin, possibly attached to membrane and that isolation and purification causes it to dimerize and for the coordination bond from the methionyl sulfur to the iron to break (Moore *et al.*, 1982).

Comparison of the amino acid sequences of cytochromes b_{562}, c_{556} and c', and consideration of the dimeric structure of cytochrome c' suggests that the latter possibility is unlikely and that the high-spin five-fold coordinated form is the naturally occurring one. First, Met 16 of *R. molischianum* cytochrome c' is not conserved in the other c' sequences. Considerable disruption of the present sequence alignment and hypothetical tertiary structures for these proteins would be required to position a methionine residue at the appropriate place to bind to iron. On the other hand, superposition of the methionine residue of the low spin c_{556} with those of b_{562} or *R. molischianum* c' occurs with minimal manipulation of the sequence alignment. Second, cytochrome c' from *Rhodopseudomonas palustris* is monomeric and high spin, indicating that dimerization is not required for the protein to be in the high spin form. Third, the contact area on the ABB'A' interface of cytochrome c' (see Fig. 19) is quite localized involving seven or eight hydrophobic residues

on each subunit. It seems unlikely that the structural changes that would lead to coordination of the heme by Met 16, at least in the case of the *R. molischinum* c', would perturb the surface of helix A sufficiently to destabilize the dimer. Finally, Arg 12, which forms a salt bridge with the less exposed heme propionate-6, is conserved in all the c' sequences but in neither of the c_{556} sequences. This suggests that the partial shielding and salt bridge formation of that propionate might be related to the high spin, 5-fold coordinated nature of the heme iron configuration.

V. MULTIHEME c_3-TYPE CYTOCHROMES

1. *General Properties*

The cytochromes c_3 are a distinct class of cytochromes found only in sulfate reducing bacteria. Their principal function is to transfer electrons from the enzyme hydrogenase to ferredoxin. The protein has been isolated from six species of *Desulfovibrio* and one of *Desulfuromonas* (Meyer and Kamen, 1982). The *Desulfovibrio* cytochromes contain four heme groups in a polypeptide chain of about 13,000 daltons. The *Desulfuromonas* cytochrome (sometimes called cytochrome c_7) contains three heme groups in 68 residues, but is otherwise very similar to the four-heme c_3 cytochromes. A preliminary crystal structure study of cytochrome c_7 has been reported (Haser *et al.*, 1979).

The redox potential for cytochrome c_3 is about -300 mV. The potentials of the four hemes are generally different from one another, having a spread of up to 235 mV. The reduction process usually follows four one-electron steps.

The amino acid sequences of cytochrome c_3 from seven species have been determined. On the basis of sequence alignment (Meyer and Kamen, 1982) and crystal structure analyses (Higuchi *et al.*, 1981) they fall into three sequence category typified by *Desulfovibrio vulgaris* Miyazaki (*D.v.*M.), *Desulfovibrio desulfuricans* Norway (*D.d.*N.) and *Desulfuromonas acetoxidans* (*D.a.*). The three sequences are shown in Fig. 21.

Each heme group is bound to the cytochrome c_3 molecule through thioether linkage to two cysteine side chains. In the first two categories of the four-heme c_3 cytochromes (*D.v.*M. and *D.d.*N.), the second heme group is attached to a pair of cysteines separated by four residues rather than the usual two as is common in most c cytochromes. This situation is true of the fourth heme in the *D.v.*M. category of c_3 as well but not of the *D.d.*N. category. On the other hand, the three heme *D.a.* cytochrome c_3 contains no cysteine pairs separated by four residues so that the best alignment with the other c_3's is achieved by deleting the second heme and its associated peptide (Meyer and Kamen, 1982).

The iron atom of each heme group is coordinated by two histidine side chains, as in cytochrome b_5, rather than the histidine and methionine side chain typical of most cytochromes with covalently-bound heme groups. Unlike cytochrome b_5, however, cytochrome c_3 is able to bind exogenous ligands such as carbon monoxide and imidazole to the hemes.

In the following discussion of the two crystal structures of cytochrome c_3 the amino acid numbering for the *D.d.*N. sequence is used and the heme groups are numbered sequentially from the amino terminus (Meyer and Kamen, 1982). This scheme will simplify the discussion and comparison of their structures.

2. *Molecular Structure of* D.d.*N. Cytochrome* c_3

Cytochrome c_3 from *Desulfovibrio desulfuricans* Norway contains 118 amino acid residues and four heme groups with a total molecular weight of 15,066 daltons. The structure has been solved at 2.5 Å resolution (Pierrot *et al.*, 1982). A schematic diagram of the molecule is shown in Fig. 22.

The structure is quite irregular with very few regions of secondary structure. There is one α helix 21 Å in length running from positions 84–101 and one short segment of two stranded β-sheet near the N terminus. There are 14 reverse turns, however, indicative of the numerous backbone contortions needed to bind four heme groups in such a small structure.

The polypeptide chain is folded into two distinct domains joined by a two-residue segment

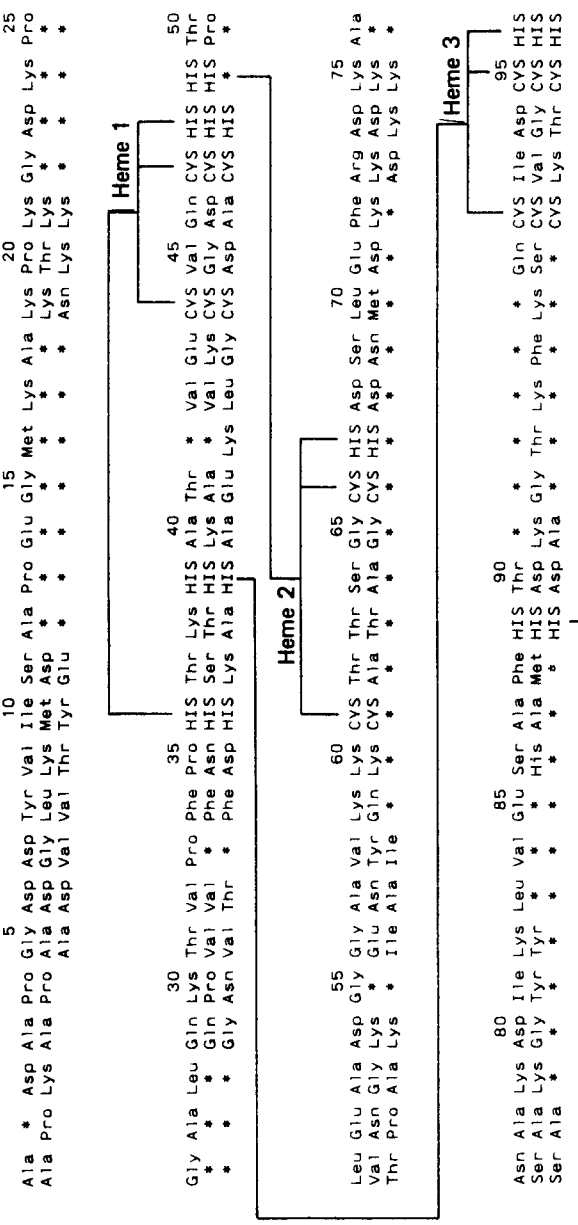

FIG. 21. Amino acid sequences of cytochrome c_3 from two *Desulfovibrio* species, *D. desulfuricans* Norway (Bruschi, 1981) and *D. vulgaris* Miyazaki (Shinkai *et al.*, 1980) and of cytochrome c_7 from *Desulfuromonas acetoxidans* (Ambler, 1971). The alignment is based on comparison of the two crystal structures (Higuchi *et al.*, 1981). The covalent and coordinate attachment sites of the four heme groups are indicated.

FIG. 22. Schematic diagram of the 4-heme cytochrome c_3 from *D. sulfuricans* Norway.

at positions 72–73. The amino-terminal domain is wrapped around the first heme and the carboxyl-terminal domain around the fourth heme. Hemes 2 and 3 lie in a large interdomain groove. Heme 2 interacts principally with side chains from domain 1 while heme 3 interacts mostly with domain 2, although domain 1 provides one of its ligands.

The four heme groups are each bound in a unique manner and have differing environments and degrees of solvent exposure. The first three heme groups along the chain are approximately perpendicular to one another, and the fourth is roughly parallel to the first, being inclined by about 35°. Each heme group has at least two aromatic residues in contact with it, besides the histidyl iron ligands, as well as a number of aliphatic side chains. The orientation of each of the histidine iron ligands is stabilized by a hydrogen bond from the N_δ proton of the histidine to an acceptor group on the protein, usually a backbone carbonyl oxygen or a side chain oxygen of serine, threonine or glutamine (Table 9).

The propionate groups on the hemes vary in their solvent accessibility as do the hemes themselves within the protein. Both propionates of the first heme interact with protein side chains, one with Lys 60 and the other with Tyr 8 (Table 9). The propionates of the others extend into solution. The overall exposures of the heme groups to solvent increases qualitatively in the order 1, 4, 3 and 2 (Table 9). At least one edge of each heme lies on the surface of the protein causing one cysteinyl thioether bridge to face the solvent.

The average separation of iron atoms is 14.1 Å ranging from 10.9 Å between hemes 1 and 3 to 17.3 Å between hemes 1 and 4. The charged residues are distributed fairly evenly over most of the protein surface. However, there is one region containing a pronounced cluster of basic amino acids, near the C-terminal end of the long helix.

3. *Molecular Structure of* D.v.*M. Cytochrome* c_3

The molecular structure of *Desulfovibrio vulgaris* Miyataki cytochrome c_3 has been determined to 1.8 Å resolution and refined to a crystallographic *R* factor of 0.176 (Higuchi *et al.*, 1984). It contains 107 amino acid residues and has a molecular weight of 13,995 daltons. A total of 47 water molecules were located, 12 of which are buried. The relative positions and orientation of the heme groups are very similar to those of *D.s.*N. c_3 as shown in Fig. 23a. The relative distances between iron atoms in *D.v.*M. c_3 differ from those of *D.s.*N. c_3 by 0.4 Å and

TABLE 9. INTERACTIONS OF HEME GROUPS AND LIGANDS WITH PROTEIN SIDE CHAINS OF CYTOCHROMES c_3 FROM *D. desulfuricans* NORWAY AND *D. vulgaris* MIYAZAKI[a]

	*D.d.*N.	*D.v.*M.
Heme 1		
Environment	least exposed	least exposed (114A^2)[b]
Fe	His 36, His 48	His 36, His 48
Vinyl-2	Cys 44	Cys 44
Vinyl-4	Cys 47	Cys 47
His 36 N_δ	Phe 34 O	Wat 25 O
His 48 N_δ	Lys 59 O	Wat 40 O
Propionate-6	Lys 60 N_δ	Cys 61 N
Propionate-7	Tyr 8 O_η	exposed
Heme 2		
Environment	most exposed	most exposed (154A^2)
Fe	His 49, His 67	His 49, His 67
Vinyl-2	Cys 61	Cys 61
Vinyl-4	Cys 66	Cys 66
His 49 N_δ	Thr 50 O_γ	Pro 50 O
His 67 N_δ	Leu 83 O	Ala 78 O
Propionate-6	exposed	Lys 90d N_ζ, His 86 Ne
Propionate-7	exposed	Lys 90d N_ζ, His 86 Ne
Heme 3		
Environment	next most exposed	next most exposed (127A^2)
Fe	His 39, His 96	His 39, His 96
Vinyl-2	Cys 92	Cys 92
Vinyl-4	Cys 95	Cys 95
His 39 N_δ	His 36 O	Asn 35 O
His 96 N_δ	Pro 108 O	Leu 108 O
Propionate-6	exposed	Lys 104 N_ζ
Propionate-7	exposed	Thr 38 O_γ
Heme 4		
Environment	next least exposed	next least exposed (116A^2)
Fe	His 89, His 115	His 89, His 115
Vinyl-2	Cys 111	Cys 111[c]
Vinyl-4	Cys 114	Cys 114[c]
His 89 N_δ	Ser 86 O_γ	Tyr 82 O
His 115 N_δ	Gln 29 O_ε	Wat 26 O
Propionate-6	exposed	Lys 19 N, Thr 81 O_γ, Thr 20 N, Thr 20 O_γ
Propionate-7	exposed	exposed

[a] The amino acid numbering is based on that of *D.d.*N. according to the sequence alignment of Higuchi *et al.*, 1981. Unmatched residues of *D.v.*M. are appended with letters.

[b] (Solvent accessibility of heme group calculated in Higuchi *et al.*, 1984).

[c] In *D.v.*M. cytochrome c_3 the cysteins bound to heme 4 are separated by four residues instead of two, and are located at positions 100 and 105 of that sequence.

the relative angles between heme planes by 9° on the average. The first three heme groups are perpendicular to each other as they are in *D.d.*N. c_3 but the first and fourth hemes are more closely parallel than in the case of *D.d.*N. c_3 (22° vs 35°).

The conformation of the polypeptide chains of the two c_3's differ to a greater extent as shown in Figs 23b and 23c. *D.v.*M. c_3 contains ten reverse turns and three short helices, from residues 82–89, 91–97 and 104–109. The first two helices correspond closely to the 21 A helix of *D.s.*N. c_3 but are interrupted by a seven residue loop which extends to the second heme (Fig. 23). There is also a two stranded beta sheet connecting residues 8–10 to residues 31–34. This 11 residue hairpin loop corresponds to the 24 residue irregular loop partially connected by a beta sheet in *D.s.*N. c_3. The loop containing the two cysteinyl thioether linkages to the fourth heme is also different in *D.v.*M. since four residues are located between the cysteines rather than two.

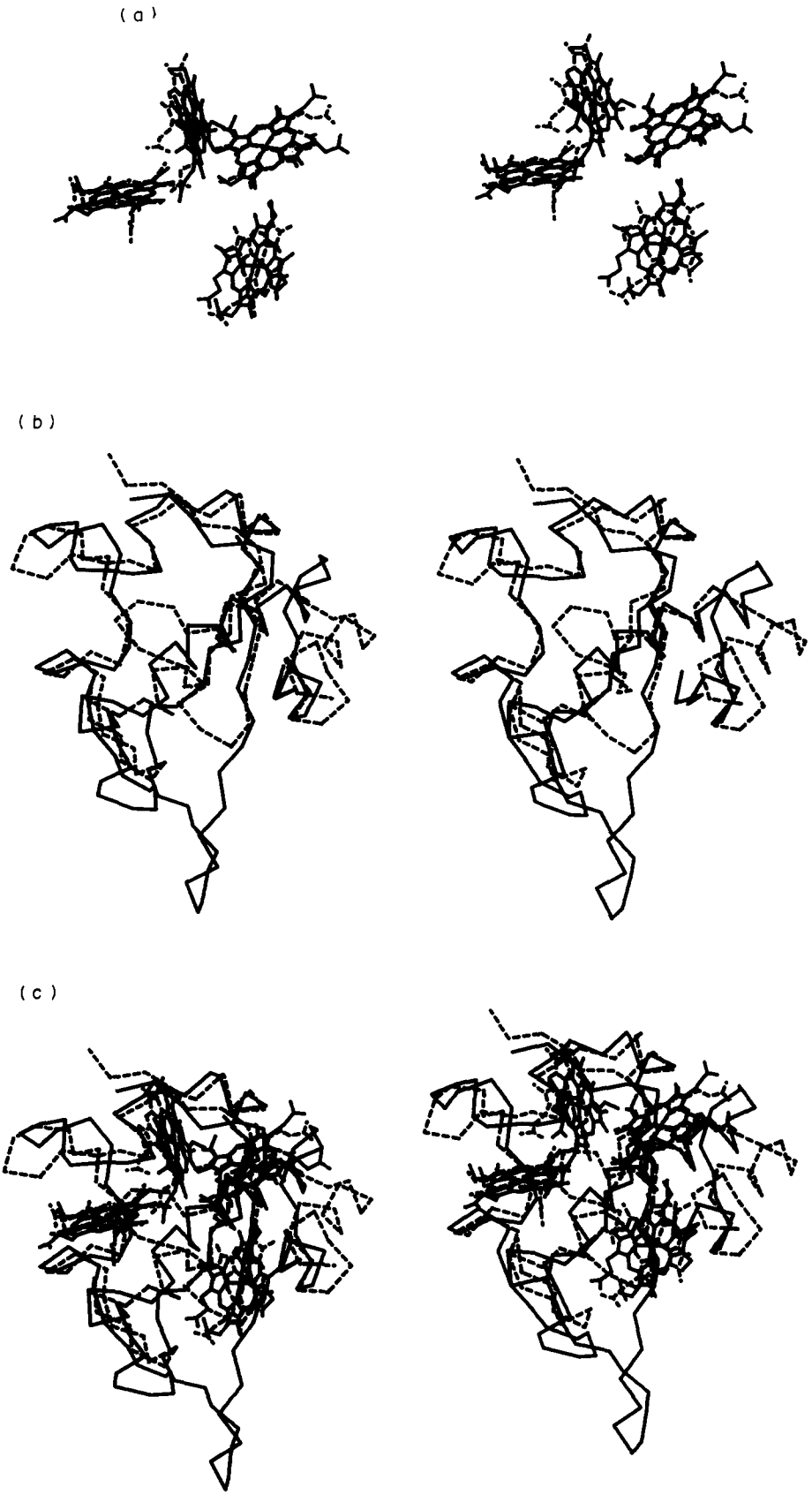

FIG. 23. Comparison of the structures of *D.d.*N. cytochrome c_3 (dashed lines) and *D.v.*M. cytochrome c_3 (solid lines). (a) Heme groups only. (b) Alpha-carbon backbone only. (c) Backbone atoms and heme groups.

The solvent accessibilities of heme groups 1, 3 and 4 in *D.v.*M. c_3 are about the same (119 A^2) while that of heme 2 is somewhat larger (154 A^3). All four heme groups have one thioether-containing edge exposed to solvent. Eight of the sixteen propionate oxygen atoms, including at least one from each heme, are hydrogen bonded to main or side chain nitrogens or hydroxyls (Table 9). Both carboxylate oxygens from propionate-6 on heme 4 and one carboxylate oxygen from propionate-6 and propionate-7 on hemes 1 and 3 respectively are totally buried. Thus, heme 2 in both *D.v.*M. and *D.s.*N c_3 is the most exposed, but there is some variation in the environments of the propionates of the remaining heme groups.

Five of the eight histidine ligands to iron atoms are hydrogen bonded through their delta nitrogens to main chain carbonyl oxygens (Table 9). The remainder are hydrogen bonded to bound water molecules which, in turn, are hydrogen bonded to main chain carbonyl oxygens. At least one histidine ligand for each heme group is also packed against the ring of a phenylalanine or tyrosine side chain, similar to cytochrome b_5. One of these, Phe 34, is stacked against His 39 of heme 3 and is also in contact with pyrrole ring IV of heme 1. This juxtaposition of stacked conjugated rings, illustrated in Fig. 24, may provide a pathway for intramolecular transfer between heme groups 1 and 3.

The amino acid sequences alignment of *D.v.*M. and *D.s.*N. cytochromes c_3 shown in Fig. 21 is based on comparison of their three dimensional structures (Higuchi *et al.*, 1981). Eight residues are conserved in this alignment besides the 24 invariant heme ligands. Except for two conserved glycine residues which may occur in tightly packed locations, none of these conserved residues has any obvious functional significance. However, a possible role of Phe 34 may be in intramolecular electron transfer as has already been discussed. The presence of aromatic groups near the heme groups of *D.s.*N. c_3 has also been noted. Two phenylalanines in the latter structure, Phe 34 and Phe 88, lie between hemes 1 and 3 and 2 and 4 respectively and were proposed to mediate electron transfer between these pairs (Pierrot *et al.*, 1982). The latter pair of hemes are separated by 16 Å between irons and Phe 88 is not conserved, being replaced by methionine in *D.v.*M. Hemes 1 and 3 on the other hand are separated by only 10 Å between irons and the intervening phenylalanine (Fig. 24) is conserved further supporting a functional importance to this structural arrangement.

4. *Evolutionary Relationships*

The first two categories of cytochrome c_3 of Fig. 21 are aligned on the basis of their structures (Higuchi *et al.*, 1981). The first category contains two strains of *D. vulgaris* and one of *D. gigas* and have an average sequence identity of 61%. The second category contains two strains of *D. desulfuricans* and one of *D. salexigens* with an average identity of 47%. The

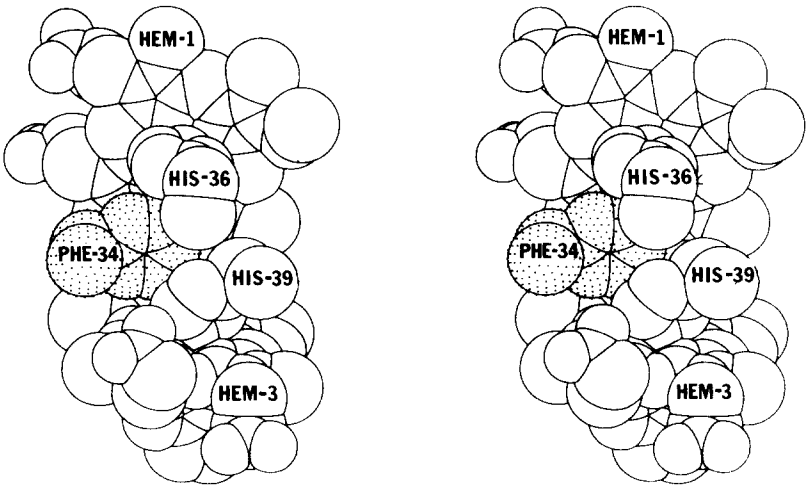

FIG. 24. Space-fitting model of the spatial arrangement of Heme 1, Heme 3, His 36, His 39 and Phe 34 in *D.v.*M. cytochrome c_3. The atoms of Phe 34 are dotted. Reproduced by permission from Higuchi *et al.*, 1984.

overall average of the two categories is about 32%. The degree of sequence homology among the cytochromes c_3 is low considering that 16 of the conserved residues serve to bind the four heme groups. The sequence of the three-heme *Desulfuromonas acetoxidans* (Fig. 21) has been aligned with other cytochromes c_3 (Meyer and Kamen, 1982). The alignment is based on the assumption that the peptide which binds heme 2 has been deleted, since the histidine ligands of the other three hemes can be aligned favorably (Meyer and Kamen, 1982). The *D. acetoxidans* then has an average identity of about 37% with the other sequences.

The similarity of the pattern of the heme ligands in the two halves of the c_3 amino acid sequences, particularly for the *D. vulgaris* category, has led to the suggestion that the four heme molecule arose by gene duplication of a two heme cytochrome (Meyer and Kamen, 1982). While this idea is consistent with the observation that *D.s.*N. cytochrome c_3 contains two folding domains separated approximately at residue 72 (Pierrot *et al.*, 1982), the folding of either half of the polypeptide chain is quite different from the other half in both c_3 molecules. Furthermore, the dispositions and modes of attachment of the heme groups are different in the two halves. Thus, if a gene duplication event were to have taken place, the stereochemical constraints of binding four heme groups in such a small molecule would have prevented preservation of a repeated folding pattern.

VI. INTERACTIONS OF CYTOCHROMES

1. *Cytochrome* c *with Cytochrome* c *Oxidase and Reductase*

The reactions of naturally occurring variants of eukaryotic cytochrome *c* with its oxidase or reductase have implicated the positively charged lysine residues around the exposed edge of the heme in the mechanism of interaction (Ferguson-Miller *et al.*, 1979). However, each of these variants has several substitutions making it impossible to identify unambiguously which residues are involved in the interactions. Within the last few years a number of derivatives of horse heart cytochrome *c* have become available containing specific singly-modified lysine residues. A comparison of the reaction kinetics or binding constants with the oxidase and the reductase of these derivatives against the native protein has led to a mapping on the cytochrome *c* molecule of the interaction surface.

Horse heart cytochrome *c* contains 19 lysine residues. Millett and colleagues (Ahmed *et al.*, 1978; Smith *et al.*, 1977, 1980) have prepared nine cytochrome *c* derivatives singly-modified at lysines with trifluoroacetate (TFA) and six singly-modified with trifluoromethylphenylcarbamate (TFC). Three of the substituted lysine positions are common to the two sets of derivatives. Both TFA and TFC neutralize the positively charged amino group of lysine but TFC is considerably more bulky than TFA.

The relative reaction rates of both of these sets of derivatives with cytochrome *c* oxidase and cytochrome *c* reductase are given in Table 10. Modification of Lys 13 produces a four- to seven-fold reduction in the electron transfer rates of cytochrome *c* with the oxidase (Smith *et al.*, 1977) and the reductase (Ahmed *et al.*, 1978). The lysine residues with the greatest effect on the rates are at positions 13, 72, 87, 25, 8 for the oxidase and 13, 72, 79, 87, 27 for the reductase (see Table 11) (Smith *et al.*, 1980). The remaining modified lysines are essentially ineffective. The effects of TFA and TFC are similar for the three derivatized sites in common, Lys 13, Lys 72 and Lys 79, indicating that charge neutralization is the dominant effect rather than bulk of the substituent.

Margoliash and colleagues (Ferguson-Miller *et al.*, 1978; Osheroff *et al.*, 1980) have prepared ten cytochrome *c* derivatives singly-modified at lysines by 4-carboxyl-2,6-dinitrophenol (CDNP). Six of those sites are common to those of the TFA and TFC derivatives. CDNP replaces the positive charge of lysine by a negative charge. The relative binding constants and initial reaction rates of these cytochrome *c* derivatives with cytochrome *c* oxidase (Ferguson-Miller *et al.*, 1978; Osheroff et al., 1980; Veerman *et al.*, 1983) and purified cytochrome *c* reductase (Konig *et al.*, 1980) are given in Table 10. The sites of six of these derivatives are common to the TFA and TFC derivatized sites. The effects of CDNP are qualitatively similar to those of TFA and TFC but much more pronounced. The greater effect

TABLE 10. STUDIES OF THE INTERACTION OF HORSE HEART CYTOCHROME *c* WITH ITS OXIDASE AND REDUCTASE BY CHEMICAL MODIFICATION OF LYSINE RESIDUES

	Cytochrome Oxidase						Cytochrome Reductase			
	TFA[a] (V/K_m)rel[b]	TFC[a] (V/K_m)rel[b]	CDNP[a] K_m(rel)[c]	CDNP[a] K_D(rel)[d]	CDNP[a] K_1(rel)[e]	Shielding Factors[f]	TFA[a] (V/K_m)rel[g,h]	TFC[a] (V/K_m)rel[g,h]	CDNP[a] K_1(rel)[i]	Shielding Factor[f]
Lysine Derivative										
5	—	—			—	4.1				4.9
7		—	1.7	10	4.8	3.4	—		2.0	2.9
8	—	2.4	13	30	—	7.7	—	1.7		4.4
13	4.9	6.9	66	133	240	8.9	4.2	3.8	38	5.3
22	1.1		—		—	0.5	1.0	—		0.8
25	2.7	—	2.3	13	7.3	3.7	0.9		1.7	2.4
27	—	1.6	10	23	14	2.5	—	2.1	11	2.1
39	—	—			—	1.2		—		1.3
53			—	—		2.0			—	1.9
55	1.0	—			—	2.0	1.0	—		2.0
60	—		1.3	1.3	1.6	1.2			1.2	1.5
72	3.1	2.7	33	66	114	4.9	2.1	2.1	—	1.8
73	—	—	6.7	20	—	4.9	—	—		1.8
79	1.9	1.8	—		—	3.3	2.5	2.3	—	4.4
86	—	—	27	33	34	8.6		—	19	10.4
87	2.8		23	33	20	7.0	2.0		23	9.1
88	1.4	—			—	2.4	1.5	—		4.0
99	0.9		—			1.8	1.0	—	—	1.8
100	—	0.9		—		1.8	—	1.0		1.7
Native (relative)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

[a] CDNP, 4-carboxy-2, 6-dinitrophenyl substituted; TFA, trifluoromethylcarbamoyl substituted; TFC, trifluoromethylphenylcarbomoyl substituted.
[b] (V/K_m)rel = (V/K_m)native/(V/K_m)derivative, relative reaction rates (Smith *et al.*, 1977, 1980).
[c] K_m(rel) = (K_m, derivative)/K_m, native), relative kinetic binding constant measured polarographically (Ferguson-Miller *et al.*, 1978; Osheroff *et al.*, 1980).
[d] K_D(rel) = (K_D, derivative)/K_D, native), relative dissociation constant, using Sepharyl G200 (Osheroff *et al.*, 1980).
[e] K_1(rel) = (K_1, native)/K_1, derivative), relative initial velocity (Veerman *et al.*, 1983).
[f] Shielding factor = (lysing reactivity of free cytochrome)/(lysine reactivity of complexed cytochrome) using acetic anhydride (Reider and Bosshard, 1980).
[g] Smith *et al.*, 1977.
[h] Smith *et al.*, 1980.
[i] König *et al.*, 1980.

TABLE 11. LYSINE RESIDUES OF CYTOCHROME *c* IDENTIFIED AS IMPORTANT FOR THE INTERACTION WITH CYTOCHROME OXIDASE OR REDUCTASE FROM CHEMICAL MODIFICATION STUDIES

Method [a]	Oxidase[a]	Reductase[a]	Peroxidase[b]
TFA, TFC [c]	13, 72, 87, 25, 8	13, 72, 79, 87, 27	—
CDNP[c]	13, 72, 86, 87, 8	13, 86, 87, 27	72, 86, 87, 13, 27
Shielding Factor [d,e]	13, 86, 87, 8, 72, 73	86, 87, 13, 8, 79, 5	(86, 87, 88)[e], (5, 7, 8)[e], (72, 73)[e], 13

[a] References given in Table 10. The lysine derivatives are listed in approximately decreasing order of involvement as estimated from Table 10.

[b] Kang *et al.*, 1978 (CDNP); Pettigrew, 1978 (shielding factor).

[c] Abbreviations are defined in the legend to Table 10. Note that neither a TFA or TFC derivative of Lys 86 nor a CDNP derivative of Lys 79 has been prepared.

[d] Shielding factor is defined in the text and the legend to Table 10.

[e] Residues within parentheses were not resolved. One to three residues within any group may have been shielded.

of CDNP is probably caused by the fact that it changes lysine by two net charges rather than one. The lysine residue with greatest effect on the reaction rates or binding constants are at positions 13, 72, 86, 87 and 8 for the oxidase and 13, 86, 87, 27 for the reductase (Table 11).

Reider and Bosshard (1980) have used differential chemical modification to identify the lysine residues of horse heart cytochrome *c* involved in interactions with the oxidase or reductase. The reactivity toward acetylation of each of the 19 lysine residues of free cytochrome *c* was compared to that of the intermolecular complex of cytochrome *c* with its oxidase or reductase. The shielding factor, which is the ratio of the lysine reactivity in the free and complexed forms, is given in Table 10 for each lysine of horse heart cytochrome *c*. The lysine residues afforded the greatest protection by cytochrome *c* oxidase are numbers 13, 86, 87, 8, 72, 73 and by purified cytochrome *c* reductase are numbers 86, 87, 13, 8, 79, 5 (Table 11).

All three studies of the cytochrome *c* interaction surface implicate lysine residues surrounding the exposed edge of the heme group. The approximate positions and relative importance of these residues are indicated schematically in Fig. 25. Lysines 13, 86 and 87 interact strongly with both the oxidase and the reductase as shown by all of the studies. Lys 72 appears to interact strongly with the oxidase but less strongly with the reductase. The remaining lysine residues of Fig. 25 appear to be less involved in these interactions with considerable variability in individual cases as indicated in Table 10.

The method of chemical modification of single lysine residues and the differential chemical

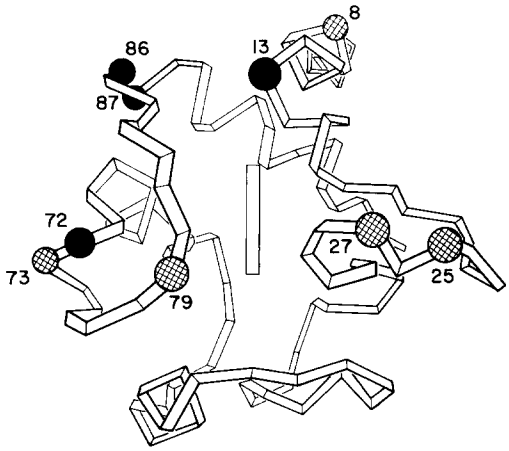

FIG. 25. Ribbon diagram of cytochrome *c* showing lysine residues involved in intermolecular complex with cytochrome *c* oxidase or reductase as inferred by chemical modification studies. Solid circles indicate strongly interacting lysines 13, 72, 86 and 87 while cross-hatched circles indicate the weaker interacting sites of lysines 8, 25, 27, 73, 79. Reproduced with modifications by permission from Eley and Moore, 1983.

modification method are complementary. The former evaluates the effects of individual side chains directly on the kinetic properties of the oxidation or reduction process, but might introduce conformational changes. Also, it cannot test all lysine residues. The latter method can examine all lysine residues, but does not measure directly the kinetic parameters.

The general agreement of the chemical modification studies supports the idea that the interaction of cytochrome *c* with its oxidase and reductase is largely electrostatic in nature (Smith *et al.*, 1981). The extremely high reaction rate of cytochrome *c* with its redox partners is close to being diffusion controlled (Ferguson-Miller *et al.*, 1978). This suggests that the dipole moment of cytochrome *c*, which is about 325 debye (Koppenol and Margoliash, 1982) is important to achieve proper orientation during the reaction. However, the variation of the dipole moment of the various lysine derivatives of cytochrome *c* is not sufficient to account for the large variation in reaction rates (Koppenol and Margoliash, 1982). This implies that specific interactions of certain lysine side chains, such as number 13, 72, 86 and 87 (Table 11), with specific negatively charged groups on its redox partners is important in the electron transfer complex.

2. *Cytochrome* b_5 *with Cytochrome* c

Cytochrome *c* can be reduced very rapidly by cytochrome b_5 *in vitro* and its rate of reduction has been used to assay cytochrome b_5 reductase in the presence of catalytic amounts of b_5 (Strittmatter, 1967). Cytochromes b_5 and *c* may function *in vivo* as physiological redox partners as well.

The intermolecular complex between cytochromes b_5 and *c* has been observed in solution by difference absorption spectroscopy in the Soret region (Mauk *et al.*, 1982). The complex is maximally stable between pH 7 and 8, midway between the isoelectric points of the two proteins. The association constant is about 10^7 M^{-1} when extrapolated to zero ionic strength and decreases rapidly with increased ionic strength. The rate of reduction of cytochrome *c* by cytochrome b_5 also varies inversely with ionic strength (Stonehuerner *et al.*, 1979).

Salemme (1976) has proposed a specific model for the complex between cytochromes b_5 and *c* in which the two molecules interact at their exposed heme edges (Fig. 26). The model is based on both charge and surface complementarity and results in the two heme groups lying nearly coplanar with their edges separated by about 8 Å. Electron transfer between the two hemes could take place by a tunneling mechanism (Hopfield, 1974; Salemme, 1977). The model makes use of the marked clustering of positively and negatively charged side chains on *c* and b_5 respectively, around the exposed heme edges. Specifically, lysyl residues 13, 27, 72 and 79 of eukaryotic cytochrome *c* are marched with Glu 48, Glu 44, Asp 60 and the exposed propionate-7 of the heme group of b_5 (Table 12).

Experimental verification of this model has come from kinetic studies using cytochrome *c* preparations which have had single lysyl residues modified chemically by TFA and TFC (Ng *et al.*, 1977). These studies have implicated lysyl residues 13, 25, 27 and 72 or 79 in the electron transfer mechanism. This suggests an additional interaction between Lys 25 and Glu 43 in the complex.

Further support for Salemme's model have come from NMR studies of the b_5–*c* complex in solution (Eley and Moore, 1983). The measured association constant for the complex is in approximate agreement with values of Mauk *et al.* (1982) extrapolated to similar ionic strengths. The line width of the resonance of TML 72 of *Candida krusei* cytochrome *c* is increased when complexed to cytochrome b_5 indicating a reduced mobility caused by involvement in the interface region. Also, one of the binding sites on horse cytochrome *c* for Cr(oxylate)$^{-3}$ determined by paramagnetic line broadening (Eley *et al.*, 1982) is eliminated in the complex with b_5. This site contains Lys 13 and Lys 72 and is consistent with the chemical modification studies.

The principles used by Salemme for proposing his model for the interaction of cytochrome *c* with b_5 are the maintenance of complementarity of charges and surface topography of the two proteins coupled with close approach of nearly coplanar prosthetic groups. These principles have been used for the construction of models for other redox complexes, as described below.

FIG. 26. Stereo diagram of the proposed intermolecular complex between cytochrome b_5 and cytochrome *c*. The planes of the heme groups are approximately parallel and the iron atoms are separated by about 16 Å. The α-carbon backbone chain are in light lines and the interacting side chain atoms are in heavy lines. The side chain orientations have been adjusted to enhance their interactions. The single letter codes for the interacting amino side chains are D = Asp, E = Glu and K = Lys.

3. *Cytochrome* b_5 *with Hemoglobin*

The role of the soluble cytochrome b_5–cytochrome b_5 reductase system in maintaining hemoglobin in the ferrous state in erythrocytes has been described earlier (Section III.1). The direct reduction of methemoglobin by cytochrome b_5 implies that a metastable complex must form between b_5 and the individual subunits of hemoglobin within in the tetrameric molecule. Complexes between cytochrome b_5 and intact hemoglobin or its isolated α and β subunits have been observed spectrophotometrically (Mauk and Mauk, 1982) and by isoelectric focusing (Righetti *et al.*, 1978). Studies of the kinetics of reduction of mutants of hemoglobin by cytochrome b_5 have shown the importance of hemoglobin Lys β66 and Lys β95 in the reduction process.

Poulos and Mauk (1983) have proposed a model for the complex of cytochrome b_5 with the α and β subunits of hemoglobin which is analogous to that proposed by Salemme for the b_5–*c* complex. In this model, cytochrome b_5 uses three or four glutamate or aspartate residues and the exposed propionate to interact with four or five lysine residues on the hemoglobin α or b chains (Table 12), including those implicated by the mutant studies. This model, shown in Fig. 27, neatly avoids a difficulty not present in the model for the b_5–*c* complex, namely, that the heme propionates of hemoglobin might repel the exposed propionate of cytochrome b_5. The model includes intervening lysine residues (Lys α61 and Lys β66), bridging the propionates of b_5 and hemoglobin (Table 12), providing hydrogen bond interactions but keeping the two like-charged groups separated. The heme planes of the globin subunits are approximately parallel to those of the cytochrome and are separated by about 7–8 Å as in the b_5–*c* complex model. Sufficient room on the surface of the hemoglobin

TABLE 12. SIDE CHAIN INTERACTIONS IN HYPOTHETICAL INTERMOLECULAR COMPLEXES BETWEEN ELECTRON TRANSFER PROTEINS

Cytochrome c	*Cytochrome* b_5[a]
Cyt *c*	Cyt b_5
Lys 13	Glu 48
Lys 27	Glu 44
Lys 72	Asp 60
Lys 79	Propionate

Hemoglobin	*Cytochrome* b_5[b]	
Hb.α	Hb.β	Cyt.b_5[c]
	Lys 59	Glu 48
Lys 56	Lys 61	Glu 44
Lys 60	Lys 65	Glu 43
Lys 90	Lys 95	Asp 60
Lys 61/propionate	Lys 66/propionate	Propionate

Cytochrome c	*Cytochrome* c *peroxidase*[c]
Cyt.*c*	CCP
Lys 13	Asp 37
Lys 27	Asp 79
Lys 72	Asp 216
Lys 86	Asp 37
Lys 87	Asp 34
Gln 12	Asn 87
Gln 16	Gln 86

Cytochrome c	*Flavodoxin*[d]
Cyt.*c*	Flavodoxin
Lys 13	Glu 120
Lys 25	Glu 65
Lys 27	Glu 62
Lys 79	Asp 58

[a] Salemme, 1976.
[b] Poulos and Mauk, 1983.
[c] Poulos and Kraut, 1980.
[d] Simondsen *et al.*, 1982.

tetramer exists to accommodate four cytochrome b_5 molecules at once, which is consistent with the observed stoichiometry of binding (Mauk and Mauk, 1982).

4. *Other Complexes with Cytochrome* c

(a) *Cytochrome* c *with cytochrome* c *peroxidase*

The kinetics of reaction of cytochrome *c* peroxidase with a number of cytochrome *c* derivatives substituted at single lysine residues by CDNP have been studied (Kang *et al.*, 1978). The lysine residues showing the greatest reduction in the reaction rate are, in decreasing order of effectiveness, at positions 72, 86, 87, 13, 27 (Table 11). Differential chemical modification (Pettigrew, 1978) of free vs CCP-complexed cytochrome *c* also implicate these residues in the interaction site (Table 11).

Poulos and Kraut (1980) have constructed a hypothetical model by computer graphics for the CCP–cytochrome *c* complex based on the known crystal structures of cytochrome *c* (Swanson *et al.*, 1977) and of CCP (Poulos *et al.*, 1980). Initially lysines 13, 27 and 72 of cytochrome *c* were paired with aspartates 37, 79 and 216 of CCP. Four additional pairs of interaction were then introduced after slight adjustments of the models. The interactions, shown in Table 12 include two pairs of neutral residues hydrogen bonded together. There are several non-polar contacts on the topologically complementary interacting surfaces of the two molecules. There is also an antiparallel positioning of the N-terminal helix of cytochrome *c* with a corresponding helix in CCP at the interface helping to stabilize the complex (Sheridan *et al.*, 1982). The edges of the two heme groups in the hypothetical complex are nearly parallel and separated by about 16 Å.

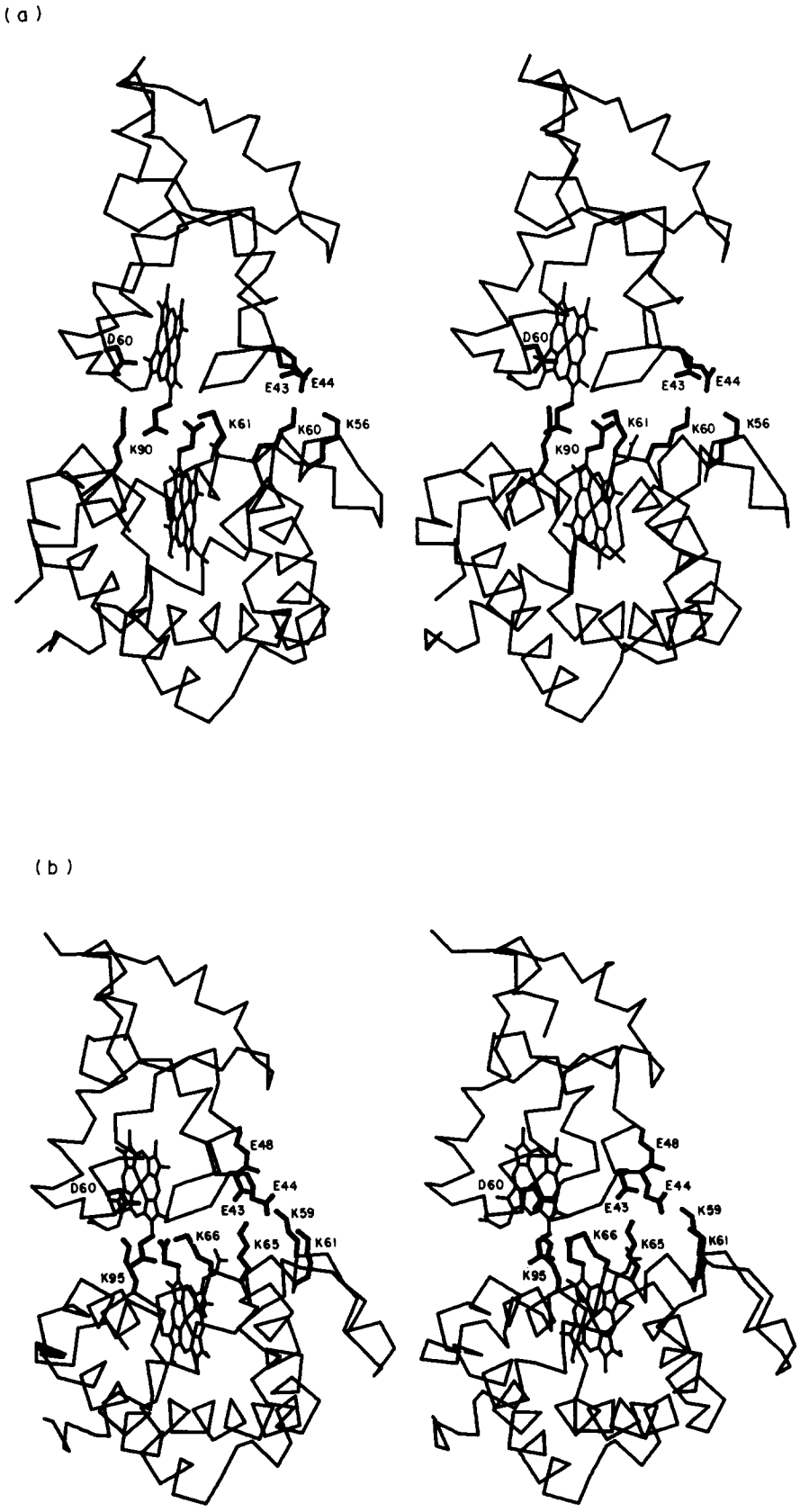

FIG 27. Stereo diagram of the proposed intermolecular complex between cytochrome b_5 and hemoglobin. (a) b_5–α-chain complex; (b) b_5–β-chain complex. The side chain orientations have been adjusted to enhance their interactions. The single letter codes for the interacting amino side chains are D = Asp, E = Glu and K = Lys.

(b) *Cytochrome* c *with Flavodoxin*

The kinetics and ionic strength dependence of electron transfer between the non-physiological partners cytochrome *c* and flavodoxin have been studied in solution (Simondsen *et al.*, 1982). The results show that the reaction rate varies inversely with ionic strength indicating that their interaction is electrostatic in nature.

A hypothetical model for the cytochrome *c* flavodoxin complex was constructed using computer graphics (Simondsen *et al.*, 1982). Lysine residues 13, 25, 27 and 79 of cytochrome *c* were paired with the cluster of uncompensated acidic residues Glu 120, Glu 65, Glu 62 and Asp 58, respectively (Table 12) near the FMN group of flavodoxin (Burnett *et al.*, 1974). The planar prosthetic groups are about 25° from being coparallel and are nearly in van der Waals contact at their solvent-exposed edges.

Experimental verification of any of the proposed complexes is badly needed. Co-crystallization of any complex would be the most direct approach. Other methods of analysis such as NMR or chemical crosslinking could also be used to study these complexes.

5. *Cytochrome* b_5 *with* b_5 *Reductase*

The interaction of cytochrome b_5 and cytochrome b_5 reductase has also been shown to involve charge complementarity. The neutralization of only seven lysyl residues of cytochrome b_5 reductase abolishes its ability to reduce cytochrome b_5 while similar modification of b_5 has no effect (Loverde and Strittmatter, 1968). However neutralization of Glu 43, Glu 44, Glu 48 and the heme propionate of b_5 reduced the rate of reduction approximately in proportion to the number of groups modified (Dailey and Strittmatter, 1979). Since the structure of cytochrome b_5 reductase is not known, no hypothetical model of a complex between it and cytochrome b_5 can be built. However, the models for the b_5–*c* and b_5–hemoglobin complexes serve as prototypes for such a model which is consistent with the experimental data.

VII. OXIDATION REDUCTION POTENTIAL

The redox potentials of the various cytochromes cover a range of nearly 750 mV (see Table 1). The maintenance of the redox potential of a particular cytochrome is one of the major functional roles carried out by the polypeptide moiety of the protein and is important for regulation of electron flow through the redox partners of the cytochromes.

There are three relatively independent, additive factors which govern the redox potential of the heme group (Marchon *et al.*, 1982). These are: (a) the nature of the axial ligands to the heme iron, (b) the inductive effects of constituents to the porphyrin ring and (c) the effect of the protein environment. The third factor includes the degree of solvent exposure and/or the polarity of the heme environment, the nature and distribution of charged groups about the heme and other perturbations of structure or electric field surrounding the heme group. The first of these factors, the nature of the heme ligands, is fixed within any class or subclass of cytochrome. The remaining factors determine the variations of redox potential within each class or subclass and depend on the protein structure.

A few of these factors can be studied individually by using model compounds. Table 13 shows the effects of variation of the fifth and sixth ligands on the redox potential of the heme. Three different series of model compounds are presented in which the 5th coordination site of the heme iron is occupied by histidine covalently attached to the heme group. Within each series only the nature of the 6th site is varied, being occupied by methionine or histidine analogues either covalently attached to the heme or present in excess amounts. The net effect of replacing one imidazole ligand by methionine is to raise the redox potential by approximately 160 mV. This result is consistent with the electron withdrawing power of methionine relative to the strong donor power of histidine, helping stabilize the ferrous heme (Moore and Williams, 1977).

The mode of attachment of the heme to the protein, i.e. the presence of unsaturated vinyl groups vs thioether linkage in the *b*-type and *c*-type hemes, respectively, is an inductive factor (b, above) and has a relatively smaller effect. The midpoint potential of Fe-protoporphyrin is

TABLE 13. REDUCTION-OXIDATION MIDPOINT POTENTIALS OF HEME COMPLEXES IN AQUEOUS SOLUTION

Heme	E_m at pH 7.0
Hemeoctapeptide[a] + imidazole	−0.21
Hemeoctapeptide[a] + acetylmethionine	−0.05
Bishistidine mesoheme[b]	−0.22
Histidine-methionine mesoheme[b]	−0.074
Fe-protoporphyrin[d]	−0.115
Fe-mesoporphyrin[d]	−0.158
Fe(C_5Im) (TPP) (N-MeIm)[c]	+0.018
Fe(C_5Im) (TPP) (THT)[c]	+0.185
Fe(C_5Im) (TPP) (PMS)[c]	+0.186
Cytochrome *c*	+0.26

[a] Harbury *et al.*, 1965. The heme octapeptide consists of residues 14–21 of cytochrome *c*, containing the covalently bound heme group, produced by peptic followed by tryptic digestion (Harbury and Loach, 1960). Excess imidazole or acetylmethionine yield His-His heme or His-Met heme model complexes, respectively.

[b] Warme and Hager, 1970b; Wilson, 1974. Bishistidine and histidine-methionine mesoheme are derivatives of mesoheme bissulfuric anhydride in which the sulfuric anhydride moiety attached to the heme propionates are replaced by the respective amino acids. Mesoheme is a derivative of protoheme IX in which vinyl groups at positions 2 and 4 (Fig. 1) are replaced by ethyl groups. The propionate chains connecting the histidine and/or methionine groups allow sufficient flexibility for the amino acid side chains to form stable ligands to the heme iron at the 5th and 6th coordination sites.

[c] Marchon *et al.*, 1982. Fe(C_5Im) (TPP) is a "tail porphyrin" derivative of *meso*-tetraphenylporphyrinato-iron (Mashiko *et al.*, 1978) in which imidazole is attached to one of the phenyl rings at the *ortho*-position by a flexible 5-carbon chain. The imidazole thus forms a very stable iron ligand at the 5th coordination position. The *N*-MeIm derivative has *N*-methyl imidazole at the 6th coordinate position and forms a His-His heme analogue. The THT and PMS derivatives have tetrahydrothiophene and pentomethylene sulfide attached at the 6th coordination site, respectively, and act as His-Met heme analogues.

[d] Cowgill and Clark, 1952; Shack and Clark, 1947. Fe-protoporphyrin and Fe-mesoporphyrin were measured at pH 9 and the results extrapolated to pH 7.

about 40 mV higher than that of Fe-mesoporphyrin (Table 13) in which the vinyl groups are replaced by ethyl groups (Fig. 1). However, the redox potential of the two eukaryotic cytochromes *c* having only a single thioether linkage (Moore and Williams, 1977) are negligibly different from those having both.

Another type of inductive effect on the redox potential of the heme is produced by hydrogen bond formation to the axial ligand. Doeff *et al.* (1983) studied the effect of adding 1,10-phenanthrolene to tetraphenylprophyrinato-iron 1 (TPP) complexed with imidazole or *N*-methyl imidazole. Increasing amounts of phenanthroline caused the redox potential of the imidazole-complexed TPP to fall by up to nearly 100 mV but had no effect on the *N*-methyl imidazole complex which cannot form a hydrogen bond. 1,10-phenanthrolene will not displace imidazole but will form a hydrogen bond with it. The decrease in redox potential with hydrogen bond formation is consistent with the decreased positive charge on the heme which would destabilize the reduced form. In the cytochromes *c*, c_3 and b_5 families the histidine ligands to the heme irons are hydrogen bonded to main-chain carbonyl oxygen atoms or well fixed water molecules so that variation of redox potential by variation of

hydrogen bonding is unlikely. However, in the four-α-helical cytochromes the histidine ligand is exposed to solvent so that regulation of potential by variation of hydrogen bonding to the histidine could occur under physiological conditions.

The effects of heme's ligands and mode of attachment are insufficient to account for the 400 mV or more difference between free or imidazole complexed heme and cytochrome *c*. Kassner (1972) found that the redox potential of the bis-pyridine complex of mesoheme methyl ester, when dissolved in benzene, was about 300 mV higher than that of the comparable complex of the free acid in aqueous solution. He attributed this difference to the destabilization of the singly-positively-charged ferric heme iron in a medium of low dielectric constant in the absence of a counter ion. The difference matches rather well the potential difference between model heme complexes using one histidine and one methionine ligand and cytochrome *c* (Table 13). Kassner (1973) computed the effect on the redox potential of burying a charged heme in the interior of a protein of low dielectric constant which in turn is dissolved in aqueous solution. He proposed that variation in the polarity of the heme environment could serve to regulate the redox potential of various cytochromes.

Stellwagen (1978) found that the redox potential of a series of heme proteins was unrelated to the distribution of polar or apolar atoms within the heme crevices but was linearly related to the fractional exposure of the heme group to solvent. He proposed that the degree of solvent exposure of the heme was the principle determinant of its redox potential. However, the collection of proteins in his data base included ones with methionine, histidine or water as the sixth heme ligand. Furthermore, more recently determined cytochrome structures contradict this generalization. Cytochrome b_{562} has a highly exposed heme, yet a midpoint potential only about 75 mV more negative than cytochrome *c*. The hemes of cytochrome c_3 (Higuchi *et al.*, 1984) have roughly the same exposed surface area as cytochrome b_5 (Stellwagen, 1978) yet are about 300 mV lower in potential. Thus, the protein portion of the cytochromes plays a much larger role in determining the redox potential than merely providing a non-aqueous environment for a portion of the heme.

The three factors controlling redox potential discussed above can explain reasonably well the 240 mV difference in potential between cytochromes b_5 and *c*. The heme of cytochrome b_5 has one histidine replacing methionine as ligand, two vinyl groups rather than thioether linkage and about 20% more exposed surface area (Stellwagon, 1978). The first factor accounts for about −110 mV, the second for about −40 mV and the third for about −60 mV {0.2x[−300 mV] (Kassner, 1973)} giving a total of about −210 mV. However, this method of estimation clearly fails for other cytochromes such as c_3 and b_{562}.

Moore and Williams (1977) have found a correlation between the redox potential of several cytochromes *c* and the chemical shifts of several methionine proton resonances. They attribute the potential variation to a slight stretching of the Fe–S bond lengths caused by stresses introduced by variations in the protein folding. However, none of the crystal structure analyses have shown significant Fe–S bond length variation.

Moore (1983) has proposed that the wide range of redox potentials among various *c*-type cytochromes, covering a range of 350 mV, can be related to electrostatic interactions between the propionate side chains and the positive charge on the ferric ion. The redox potential of *P. aeruginosa* cytochrome c_{551} varies with pH over the pH range 5–8, dropping by about 75 mV as the pH increases (Moore *et al.*, 1980). The pH dependence has been interpreted as arising from a group which ionizes with different pK_a values in the reduced and oxidized forms. The values are $pK_a^{red} = 7.3$ and $pK_a^{ox} = 6.2$. The visible absorption and NMR spectra of reduced c_{551} show perturbations of the heme environment which occur with a $pK_a = 7.3$. NMR spectra of the oxidized protein show analogous perturbations with a pK_a near 6.2. These effects on the redox and spectral properties of c_{551} have been attributed to one of the heme propionates. Other possibilities were eliminated by reference to the X-ray structure. Propionate-7 (in the back of the molecule as viewed in Fig. 7) is probably ionized in this pH range since it is bridged to Arg 67. Therefore, propionate-6 (in front in Fig. 7), which is somewhat accessible to solvent (Matsuura *et al.*, 1982), is likely to be responsible for this pH effect. Deprotonation of this group would help stabilize the oxidized protein by neutralizing its positive charge causing a lowering of the redox potential with increased pH.

In most mitochondrial cytochromes *c* propionate-7 is ionized at neutral pH since it forms an internal salt bridge with Arg 38 (Table 3). In some *c*-type cytochromes lacking Arg 38, or its equivalent, the pKa of this propionate lies between 6 and 8 and the cytochrome shows a potential variation of about 60 mV over this pH range with little variation of structure. This indicates the ability of a fixed propionate group to regulate redox potential. Thus, protein mediated variations in the electrostatic interactions of the heme propionate and the ferric ion could serve to control the redox potential of various *c*-type cytochromes.

The importance of the partially buried heme propionate in stabilizing the oxidized form of cytochrome b_5 (Argos and Mathews, 1975) has been discussed earlier (see Section III.2). The orientation and environments of the propionates in cytochromes b_{562}, c' and c_3 have been noted in Sections IV and V and summarized in Tables 8 and 9. Interactions of these groups with protein side or main chain atoms may be important for controlling the redox potentials of these cytochromes as well.

VIII. CONCLUSIONS

The four structural classes of cytochromes discussed here appear to be quite different from one another. Each forms a distinct family of proteins. They range from having little regular secondary structure and many chain reversals, like the mitochondrial *c* and the multiheme c_3 types, to being highly regular like the four-α-helical type cytochrome. Yet with the exception of the b_5 class, these classes have a common mode of covalently attachment of the heme to a peptide of the type Cys-X-Y-[Z-W]-Cys-His. The possibility exists that these three families, at least as far as the short heme-binding peptide is concerned, were derived from a common evolutionary source with perhaps several duplicate copies of this short gene sequence being generated and dispersed. However, the common peptide is so short, containing only three conserved residues, that its ubiquity may not be statistically significant and may have resulted from convergent evolution.

The lack of a covalently bound heme in cytochrome b_{562} suggests a genetic divergence within the 4-α-helical class of cytochromes, rather than its emergence of a separate protein family, since several structural features are still held in common with other members of the class. Whether or not the heme was attached covalently to the hypothetical precursor molecule is open to speculation.

The cytochrome b_5 family probably evolved independently of any of the families with covalently attached heme, since very few structural features are common. The same conclusion may be drawn about other structurally unrelated heme proteins such as the globins, catalase, or peroxidases. The b_5 fold is relatively stable in the absence of the heme (Huntly and Strittmatter, 1972) so that its covalent attachment would not be required for chain folding. Thus, there is no requirement for covalent attachment of the heme in the folding process for b_5. Cytochromes *c* or c_3, however, would probably not form stable apoproteins so that the covalent attachment of the heme would still be required for formation of the functioning molecule. The 4-α-helical fold would be intermediate since apocytochrome b_{562} probably can form a stable structure, although the heme is covalently attached in other members of the family.

The factors governing the redox potentials of the cytochromes do appear to be common to all classes. The heme is largely buried in a hydrophobic pocket, although the orientation of the propionates, histidine ligands and degree of solvent exposure varies widely. All cytochromes have at least one histidine ligand. The low potential cytochromes employ histidine for the sixth ligand while the high potential ones use methionine or maintain a 5-coordinate iron. The microenvironments of the propionates also appear to be important for the redox potential although the significance of their individual arrangements is not yet fully understood.

The mechanism of interaction with their redox partners also seems to be common to some cytochromes. Both cytochromes b_5 and *c* appear to interact with their physiological redox partners through charge complimentary and direct electron transfer between prosthetic groups. However, no actual complex has yet been studied crystallographically, and similar complex formation with the four-α-helical or c_3 classes have yet to be demonstrated.

The structural features of all four classes of cytochromes appear to be well established. The functional aspects of the structures must still be explored, particularly for the 4-α-helical and c_3 classes. There are still a number of classes of cytochromes of unknown structure, particularly the membrane bound *b*-type cytochromes found in mitochondria, chloroplasts and other systems. It will be interesting to learn the structural features and functional properties of these classes when the techniques of crystallization and structural analysis of such systems become more fully developed.

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