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Malate dehydrogenase: Isolation from *E. coli* and comparison with the eukaryotic mitochondrial and cytoplasmic forms

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Escherichia coli malate dehydrogenase has been isolated in homogeneous form by a procedure employing chromatography on DEAE-cellulose, 5'-AMP-Sepharose, and Sephacryl-200. It is composed of two identical polypeptide chains each of M_r = 32 500. Like porcine mitochondrial malate dehydrogenase, it is devoid of tryptophan, but otherwise it is not particularly more similar in composition to one of the eukaryotic isozymes than to the other. However, amino-terminal sequence analysis of the first 36 residues shows remarkable similarity of the bacterial and mitochondrial enzymes (69% identical residues) in contrast to the cytoplasmic form (27%). The two porcine heart enzymes are identical in 24% of the positions compared. These results clearly establish that all three forms of malate dehydrogenase have evolved from a common precursor and that the prokaryotic and mitochondrial forms have retained sequences that are much closer to the ancestral one than the cytoplasmic enzyme. These findings appear to further substantiate the endosymbiotic hypothesis for the origin of the mitochondrion.

Malate dehydrogenase (MDH), which catalyzes the reversible NAD^+-dependent conversion of L-malate to oxaloacetate, occurs in a broad spectrum of living organisms. In eukaryotes, there are two distinct forms of the enzyme, one associated with mitochondria (mMDH) and the other found in the cytoplasm (sMDH) (Banaszak & Bradshaw, 1975). Prokaryotes possess only a single form. However, from one species of bacteria to another, they appear to occur in either dimeric (*Escherichia coli*, Murphey et al., 1967b; *Pseudomonas testosteroni*, You & Kaplan, 1975) or tetrameric (*Bacillus subtilis*, *Bacillus stearothermophilus*, Murphey et al., 1967b) structures (Murphey et al., 1967a). Both eukaryotic isozymes are dimeric. The subunit size in all forms is 32 000-35 000 M_r (Banaszak & Bradshaw, 1975).

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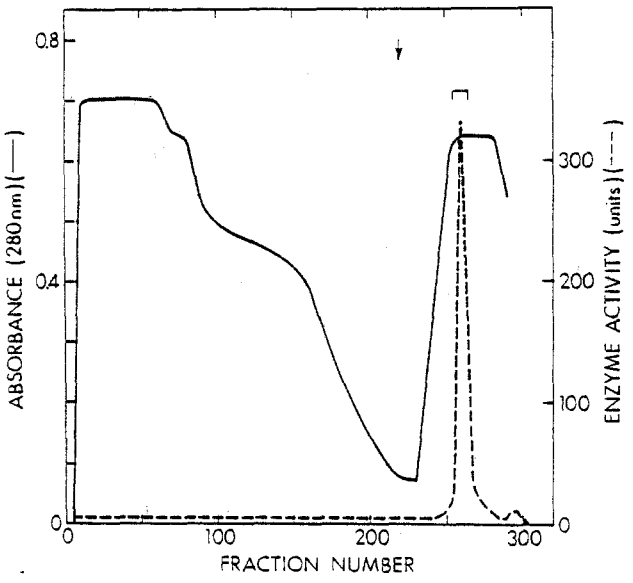

Isolation and characterization of the two MDH isozymes of pig heart have revealed little similarity except for molecular weight and subunit structure, suggesting that if they shared a common precursor, then the divergence must have occurred a long time ago. It was of interest, therefore, to isolate a prokaryotic version of the enzyme and to compare it with its eukaryotic counterparts. *E. coli* was selected as the source because it had been established that its MDH had a dimeric structure (Murphey et al., 1967b).

Methods

Purification of E. coli *malate dehydrogenase*

Step 1: Ten pounds of frozen *E. coli* K12 cells, grown in enriched medium and harvested at three-fourths log phase, were thawed in 2.5 vol. of 0.02 M potassium phosphate, pH 7.0, containing 1 mM EDTA, 1 mM *o*-phenanthroline (OP), and 10 mM 2-mercaptoethanol (2-ME) and were disrupted by a single passage through a Mouton-Gaulin submicron disperser, chilled in ice, at 9000 psi. The homogenate was centrifuged for 60 min at 10 000 *g* and the supernatant brought to 45% saturation by the addition of solid $(NH_4)_2SO_4$. The precipitate was removed by centrifugation at 10 000 *g* for 30 min and the supernatant raised to 75% $(NH_4)_2SO_4$ saturation. The precipitate was removed by centrifugation at 10 000 *g* for 45 min and dissolved in 10 mM Tris, pH 7.5, 1 mM in EDTA, OP, 2-ME, and phenylmethylsulfonylfluoride. It was dialyzed against three changes of the same buffer.

Step 2: The dialyzed sample was applied to a column (50 x 6 cm) of DEAE-cellulose (Whatman DE-52) equilibrated in 0.01 M Tris, pH 7.5, with 1 mM 2-ME and 1 mM EDTA. As shown in Fig. 1, the column was washed with starting buffer until the A_{280} was <0.1 and was then eluted with a 2-liter linear gradient from 0-0.1 M KCl in the same buffer.

Fig. 1. Elution profile of the $(NH_4)_2SO_4$ precipitate of *E. coli* malate dehydrogenase on a column (50 x 6 cm) of DE-52 cellulose. The columm, equilibrated in 0.01 M Tris, pH 7.5, containing 1 mM 2-mercapto-ethanol and EDTA, was washed with starting buffer until the A_{280} was below 0.1 (arrow). The enzyme was eluted with a 2-liter linear gradient composed of the equilibrating buffer alone and with 0.1 M KCl. The column was developed at ~50 ml/h and the active fractions pooled as indicated.

Step 3: The active fractions from the DE-52 column were pooled, dialyzed against 10 mM Tris, pH 8.0, containing 1 mM 2-ME, EDTA, and 0.05% NaN_3, and loaded onto a column (20 x 2.4 cm) of 5'-AMP-Sepharose (Sigma) equilibrated in the same buffer. The column was washed with buffer until the A_{280} was <0.05. The enzyme was eluted as a sharp peak by the same buffer containing 0.2 M NaCl (Fig. 2).

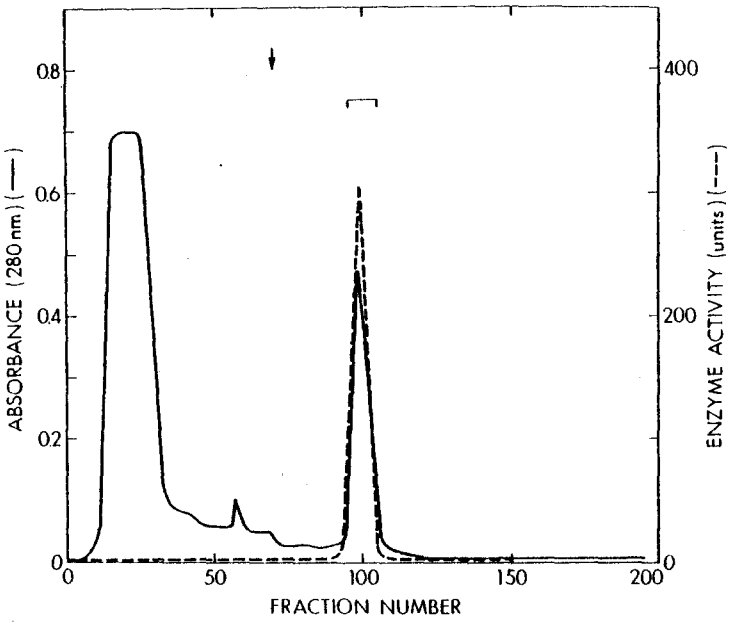

Fig. 2. Elution profile of the fractionation of the DE-52 pool on a column (20 x 2.4 cm) of 5'-AMP-Sepharose. The column was equilibrated with 0.01 M Tris, pH 8.0, containing 1 mM 2-mercaptoethanol and EDTA and 0.05% NaN_3. The column was washed until the A_{280} was below 0.05 (arrow) and then the enzyme was eluted directly with the same buffer containing 0.2 M NaCl. The active fractions were pooled as indicated.

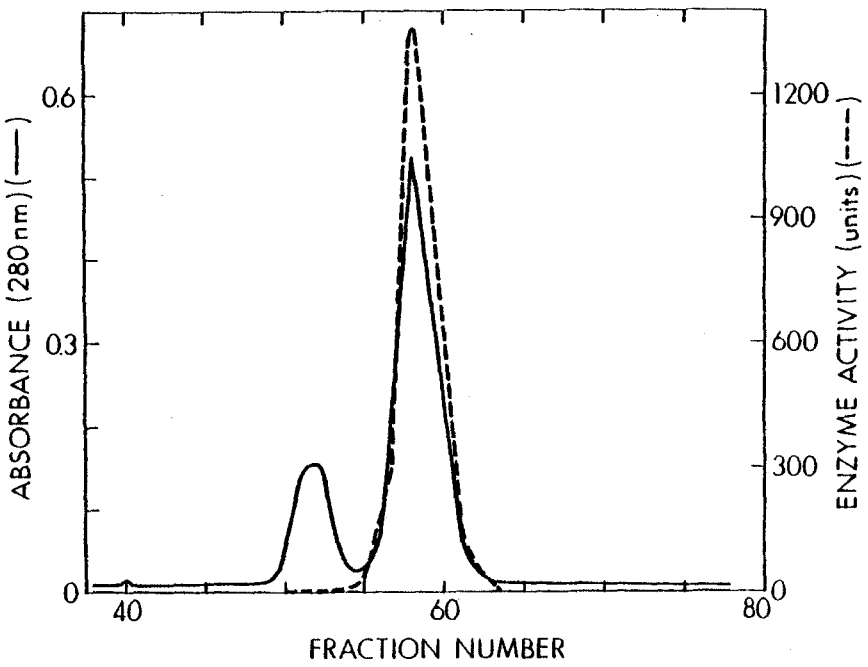

Fig. 3. Elution profile of the fractionation of *E. coli* malate dehydrogenase on a column (115 x 2.4 cm) of Sephacryl-200. The column was equilibrated in the same buffer as the 5'-AMP-Sepharose column (Fig. 2), and developed at 18 ml/h. The active fractions were pooled.

Step 4: The enzyme eluted from the affinity column was pooled and applied directly to a column (115 x 2.4 cm) of Sephacryl-200 equilibrated in the same buffer as the 5'-AMP-Sepharose column. As shown in Fig. 3, all of the activity applied was found in the second (major) peak eluted. These fractions were pooled and stored at -20°C.

Enzyme activity and protein concentration

E. coli malate dehydrogenase was assayed with 0.1 M malate and 2.5 mM NAD^+ as described previously (Glatthaar et al., 1974). Units were defined as μmol NADH/min and specific activity as units/mg of enzyme. An extinction coefficient, $E^{1\%}_{280nm} = 1.73$, was determined and used for estimating protein concentrations.

Gel electrophoresis

Purity and subunit molecular weight determinations were made on 15% polyacrylamide gels in 0.1% sodium dodecyl sulfate (SDS) run at 20 mA for 3-4 h as described by Laemmli (1970). Polyacrylamide electrophoresis in 12.5% gels in the absence of SDS was also used to assay homogeneity.

Amino acid analyses

The amino acid composition was determined by automatic analysis with a Durrum D-500 instrument in acid (6 N HCl) hydrolysates of 24, 48, and 72 h prepared at 110°C under reduced pressure. Half-cystine was determined as cysteic acid after performic acid oxidation (Moore, 1963), and tryptophan was quantitated spectrophotometrically (Edelhoch, 1967).

Sequence analysis

Amino-terminal sequence analysis was performed in a Beckman 890C sequencer using the 0.33 M Quadrol program, a modification of the 0.1 M Quadrol program described by Brauer et al. (1975). All solvents and reagents were obtained from Beckman. Polybrene, which was added to the spinning cup with the protein sample to avoid extractive losses, was a gift from Abbott Laboratories. The phenylthiazolinones obtained were converted to the corresponding hydantoins by heating at 80°C in 1.0 N HCl for 10 min and were identified by thin-layer chromatography, gas chromatography, and high-performance liquid chromatography. The last two methods provided quantitative estimates (Thomas et al., 1981). The enzyme was carboxymethylated after mild reduction (Angeletti et al., 1971) before being analyzed.

Results

The *E. coli* malate dehydrogenase obtained by the procedure described herein was judged to be homogeneous by gel electrophoresis in the presence and in the absence of SDS. At a load sample of ~100 μg, only a very minor band comprising less than 1% of the total protein was evident in addition to the very intense main band, in the non-denaturing analysis. No contaminants were visible on the SDS gel at the same concentration. In the latter experiment, the *E. coli* protein migrated very slightly ahead of porcine heart mMDH (M_r = 33 080; R. T. Fernley, B. E. Glatthaar, M. R. Sutton, and R. A. Bradshaw, manuscript in preparation) consistent with the M_r = 32 500 calculated from the amino acid analyses (see below). The native protein has been reported by Murphey et al. (1967b) to be a dimer of identical subunits with a M_r = 61 000. The elution position on gel filtration columns and the subunit molecular weight determined from SDS-gel and amino acid analyses of the enzyme isolated in the experiments reported here suggest a M_r = 65 000, in excellent agreement with the earlier value.

The amino acid composition of *E. coli* MDH is shown in Table 1. The values shown are for the subunit polypeptide. The 314 residues produce a calculated molecular weight of 32 500 and an average residue weight of 104, reflecting the relative enrichment of lower-molecular-weight amino acids, e.g. glycine, alanine, etc. The enzyme is also distinguished by the low tyrosine (3) and tryptophan (0) content which is reflected in the $E^{1\%}_{280nm}$ = 1.73 determined.

Table 1. Amino acid composition of *E. coli* malate dehydrogenase[a]

	E. coli					Porcine	
	24 h	48 h	72 h	Average	Integer	mMDH[b]	sMDH[c]
Aspartic acid	23.6	24.3	24.2	24.0	24	24	39
Threonine	17.4	18.0	17.7	17.7	18	21	16
Serine	16.9	16.1	16.6	17.2	17	18	22
Glutamic acid	37.0	37.5	37.8	37.4	37	24	27
Proline	8.5	10.1	9.5	9.4	9	21	12
Glycine	37.0	37.6	37.5	37.4	37	28	23
Alanine	36.0	37.8	37.3	37.0	37	32	32
Valine	30.8	34.0	33.4	33.7	34	28	26
Half-cystine	2.9	--	--	2.9	3	8	5
Methionine	3.9	3.8	3.2	3.6	4	6	8
Isoleucine	12.7	14.7	14.5	14.6	15	23	19
Leucine	33.6	34.2	34.2	34.0	34	27	32
Tyrosine	2.8	3.5	3.4	3.2	3	5	8
Phenylalanine	10.0	10.0	10.0	10.0	10	11	11
Histidine	2.1	2.2	2.2	2.2	2	5	4
Lysine	20.7	21.8	21.9	21.5	22	25	31
Arginine	8.6	8.0	7.9	8.2	8	8	10
Tryptophan	0	--	--	--	--	--	5
TOTAL					314	314	330

[a]Residues/molecule of subunit
[b]From complete amino acid sequence (R. T. Fernley, B. E. Glatthaar, M. R. Sutton, and R. A. Bradshaw, in preparation). mMDH, mitochondrial malate dehydrogenase.
[c]From acid hydrolysates (Banaszak & Bradshaw, 1975). sMDH, cytoplasmic malate dehydrogenase.

The amino-terminal sequence of the first 36 residues of *E. coli* MDH, determined by automatic Edman degradation, is shown in Table 2. Unambiguous assignments were made for all positions except 26, 28, and 31, which were preliminarily determined to be serine residues by gas-liquid chromatography. However, the very low levels of dehydroserine observed did not allow more than tentative identification.

Discussion

The protocol for the purification of *E. coli* MDH is similar to that described by Murphey et al. (1967b) in the use of $(NH_4)_2SO_4$ precipitation, DEAE cellulose chromatography, and gel filtration. However, the use of affinity chromatography on 5'-AMP-Sepharose, as applied by Weininger and Banaszak (1978) to the isolation of porcine heart s- and mMDH, and the substitution of Sephacryl-200 for Sephadex G-100 eliminated the need for crystallization as the final purification step. The same affinity chromatography step has also been effectively applied by Wright and Sundaram (1979) to the purification of malate dehydrogenases from a number of thermophilic and mesophilic bacteria. The overall yield of enzyme from 10 lb of

Table 2. Amino terminal sequence of *E. coli* malate dehydrogenase

Cycle	Residue	Method of identification[a]	Cycle	Residue	Method of identification[a]
1	Methionine	T/G	19	Leucine	T/G/H
2	Lysine	T/G	20	Leucine	T/G/H
3	Valine	T/G	21	Lysine	T/G
4	Alanine	T/G	22	Threonine	T/G
5	Valine	T/G	23	Glutamine	T/G
6	Leucine	T/G/H	24	Leucine	T/G/H
7	Glycine	T/G	25	Proline	T/G
8	Alanine	T/G	26	(Serine)	T/G
9	Alanine	T/G	27	Glycine	T/G
10	Glycine	T/G	28	(Serine)	G
11	Glycine	T/G	29	Glutamic acid	T/G
12	Isoleucine	T/G/H	30	Leucine	T/G/H
13	Glycine	T/G	31	(Serine)	G
14	Glutamine	T/G	32	Leucine	T/G/H
15	Alanine	T/G	33	Tyrosine	T/G
16	Leucine	T/G/H	34	Aspartic acid	G
17	Alanine	T/G	35	Isoleucine	G/H
18	Leucine	T/G/H	36	Alanine	G

[a]Abbreviations: T, thin-layer (silica-gel) chromatography; G, gas-liquid chromatography; H, high-performance liquid chromatography.

frozen cells was ~100 mg (sp. act. = 350 units/mg), which represents about 5% of the activity recovered from the ammonium sulfate precipitation. This percent yield is comparable to the value of 6% reported by Murphey et al. (1967b). However, these workers started with twice as many cells and recovered only one-fourth as much enzyme. This can be explained, in part, by the fact that they overestimated the extinction coefficient, reported as $E_{280}^{1\%} = 3.39$, by a factor of 2. This may have been due to some contaminating protein in their preparation since they found 1.75 residues of tryptophan/subunit and the MDH, as isolated in this study, was devoid of this residue. This may also reflect the inaccuracies in the methods available for determining both the tryptophan content and extinction coefficients in the earlier studies. The remainder of their composition is in reasonable agreement with that reported here.

The comparison of the *E. coli* MDH composition with that of the two eukaryotic MDHs of porcine heart is given in Table 1. The composition of the mitochondrial isozyme, calculated from the primary structure, is also characterized by the absence of tryptophan but differs markedly in glutamic acid, proline, cysteine, and isoleucine. Similar deviations with the cytoplasmic form are also evident. In fact, there is little compelling reason, on the basis of this evidence, to expect that the prokaryotic enzyme would be more similar to one or the other of the eukaryotic MDHs, if in fact it is similar to either. However, as shown in Fig. 4, an entirely different picture emerges from a comparison of the amino-terminal sequences of these three MDHs. Clearly, *E. coli* MDH shares an extensive number of identical residues with the mitochondrial isozyme that is considerably reduced in

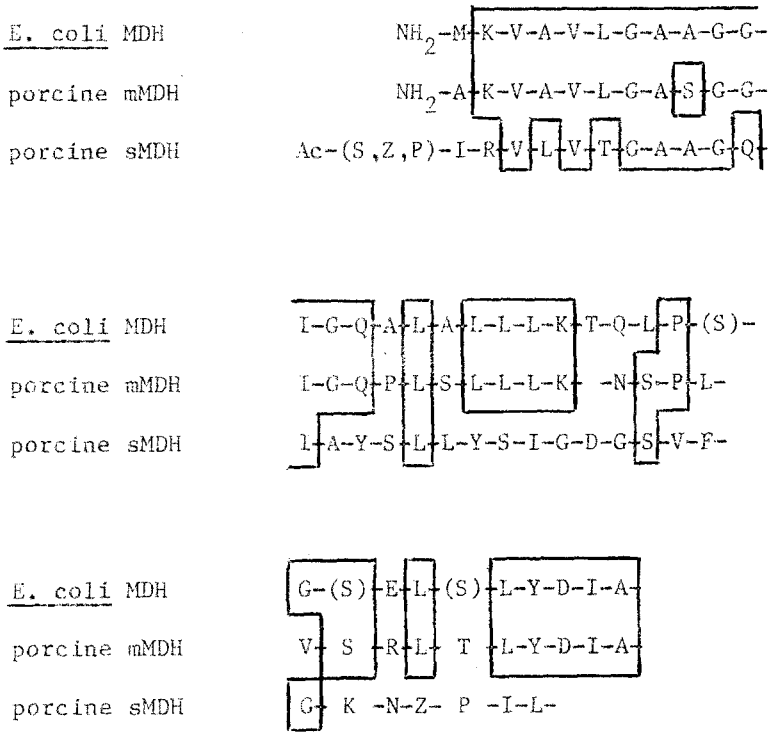

Fig. 4. Comparison of the amino-terminal sequences of *E. coli* porcine heart mitochondrial (mMDH) and porcine heart cytoplasmic malate dehydrogenase (sMDH). Residues identical in at least two of the segments are enclosed in boxes. Data taken from (mMDH) R. T. Fernley, B. E. Glatthaar, M. R. Sutton, and R. A. Bradshaw (manuscript in preparation) and (sMDH) R. A. Bradshaw, M. J. Wade, B. E. Glatthaar, G. R. Barbarash, and M. R. Sutton (unpublished observations).

a comparison with the cytoplasmic form. Furthermore, both the prokaryotic and mitochondrial MDHs commence at the same position and possess free α-amino groups whereas the cytoplasmic enzyme has three additional residues and an N^{α}-acetyl group. As summarized in Table 3, *E. coli* and porcine mMDH share 25/36 positions (69%) in contrast to the 9/33 identities characterizing the *E. coli*/porcine sMDH comparison. Interestingly, the two eukaryotic isozymes show a similar relatedness (8/33, or 24%). Although the quantitative aspects shown in Table 3 are derived from segments that represent only about 10% of each polypeptide and therefore could vary somewhat from those calculated from a comparison of the complete sequences, the two main conclusions, i.e. 1) that all three proteins show sufficient relatedness to suggest a common ancestral precursor and 2) that the prokaryotic form of the enzyme is much more similar to the mitochondrial isozyme than to the cytoplasmic one, are unlikely to be materially altered.

Table 3. Comparison of the amino terminal sequences of the malate dehydrogenases of *E. coli* and porcine heart

The lower left side of the matrix shows the number of identical residues per total positions compared. The extra three amino terminal residues of sMDH have not been included. The deletion introduced in the mMDH sequence corresponding to residue 22 in the *E. coli* protein has been treated as a non-identity. The values listed in the upper right side express the ratios as percentages.

	E. coli	Porcine	
		Mitochondrial	Cytoplasmic
E. coli	--	69	27
Porcine			
Mitochondrial	25/36	--	24
Cytoplasmic	9/33	8/33	--

The relationship observed between the bacterial and animal MDHs is very similar to that previously reported for superoxide dismutases (Steinman & Hill, 1973). In that study, comparison of the amino-terminal structures of two forms (Fe and Mn) from *E. coli* with the chicken liver mitochondrial enzyme (Mn) showed that 20 of 27 residues of the mitochondrial enzyme segment were identical to one or another of the two bacterial dismutases. The two *E. coli* enzymes were also related to each other to about the same extent. In contrast, the bovine erythrocyte superoxide dismutase, which contains Cu and Zn ions, did not show significant homology with any of the bacterial or chicken enzymes. Bridgen et al. (1975), from their sequence determination of the first 60 residues of *B. stearothermophilus* superoxide dismutase (Mn), found a similar relationship to the same eukaryotic enzymes.

As noted by Steinman and Hill (1973), the striking homology of the prokaryotic and mitochondrial forms of the same enzyme clearly suggests a common ancestor and appears to substantiate the endosymbiotic theory for the origin of mitochondria and chloroplasts. This theory proposes that these organelles arose from the internalization of specific prokaryotic cells by protoeukaryotes, surviving initially as intracellular symbionts and ultimately, through evolutionary change, adopting their present-day structure and function (Margulis, 1970). An important aspect of this hypothesis requires that much of the genome of the internalized prokaryote be transferred through subsequent evolutionary events to the nucleus, which now directs the synthesis of most mitochondrial proteins. The principal opposing theory suggests that there was a continuous development of eukaryotes from prokaryotes with the autogenic production of all intracellular organelles (Raff & Mahler, 1972; Uzzell & Spolsky, 1974). Support for the endosymbiotic hypothesis has been drawn from morphological and functional similarities between mitochondria/chloroplasts and prokaryotes and, more recently, from the extraordinary similarity in the primary structures of nucleic acid elements of the organisms/organelles

(Schwarz & Kössel, 1980; Phillips & Carr, 1981). However, Anderson et al. (1981), who have determined the complete sequence of the human mitochondrial genome, have suggested that 'the mammalian mitochondrial genetic system cannot generally be classified as either prokaryote-like or eukaryote-like' because of the distinct differences found in this genome (and its translation) and all other living organisms studied. Also Uzzell and Spolsky (1981) have argued that much of the structural data, used to construct phylogenetic trees (Schwartz & Dayhoff, 1978), that appear to strongly support endosymbiosis can be used equally well to support autogenesis.

The protein sequence data for the MDHs presented here do not, as with the results of Steinman and Hill (1973), resolve the mitochondrial origin controversy. However, they extend that study in that sMDH, as an evolutionary homolog of both the prokaryotic and mitochondrial forms, provides an 'internal evolutionary control' that is not complicated by either species or tissue variation. Except for the unlikely possibility that *E. coli* and porcine mitochondrial MDH evolved in a parallel fashion *after* the formation of eukaryotic cells and in a distinctly different manner than the porcine cytoplasmic enzyme, it must be concluded that the bacterial and mitochondrial forms more closely resemble the ancestral precursor, and that the cytoplasmic enzyme, for whatever reason, has undergone much more extensive mutational change. Thus, the mitochondrial and bacterial enzymes have retained their high degree of similarity over the same time period that another 'sibling' of the original ancestor gene has not. These developments seem more compatible with an endosymbiotic pathway than an autogenic one.

Acknowledgements

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