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Apo-cytochrome b_5 as an indicator of changes in heme accessability: Preliminary studies with cytochrome P450 3A4

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Dedicated to Professor Alexander Ivanovich Archakov on the occasion of his 60th birthday

Abstract

Cytochrome P450s (P450 or CYP) are the largest family of hemeproteins yet characterized. X-ray crystallographic studies have shown that the heme of the P450 hemeproteins is buried in the interior of the protein molecule. Unexplored are answers to questions concerning the role of heme in the folding of newly synthesized apo-P450s and the factors that influence changes in heme accessibility following modification of the pattern of folding of the holo-P450s. We have carried out the present studies to measure changes in heme accessibility in P450s. This is an initial step to determining whether heme-binding confers structural and functional integrity and stability to a P450 molecule. Recently, we have shown that apo-high molecular weight cytochrome b_5 (apo-HMWb_5) is an efficient acceptor of heme when added to a preparation of purified recombinant CYP3A4. In the present work we have studied heme binding by apo-HMWb_5 when mixed with a number of different hemeproteins (myoglobin, hemoglobin, catalase, CYP4A1, CYP101, and CYP3A4). These hemeproteins differ in the location of the heme (i.e., surface or internal) allowing one to study changes in structure as measured by the process of heme transfer from one protein to another. It was found that heme transfer to apo-HMWb_5 occurs relatively rapidly from hemeproteins where the heme is located at or near the surface or when the hemeprotein is denatured. In contrast, heme transfer from P450s to apo-HMWb_5 occurs only following modification of the P450 structure with chaotropic agents. An exception is CYP3A4 where a measurable amount of heme is transferred to apo-HMWb_5 in the absence of denaturing agents. The preliminary results described here employs apo-HMWb5 as an indicator for assessing changes in heme-availability of P450s as the protein-folding of the molecule is altered.

Keywords: Cytochrome b_5; Apo-cytochrome b_5; Myoglobin; Hemoglobin; Catalase; Cytochrome P450cam; CYP4A1; CYP3A4; P450 structure; Chaotropic agents

1. Introduction

Coenzymes and prosthetic groups share a common role as obligatory partners in directing the functions of many different proteins. Heme is the one of the most widespread prosthetic groups used in nature where it serves as a component of many different types of hemeproteins that participate in electron transfer reactions, oxygen delivery and storage, catalysis of oxygen reduction, and the oxygenation of xenobiotics.

Hemeproteins can be subdivided into several groups depending on the substituents present on the porphyrin ring. Fe-protoporphyrin IX containing proteins are present in the highest abundance and include many different types of cytochromes, peroxidases and globins. Differences in the properties of these hemeproteins can be attributed to the presence of different distal and proximal ligands to the heme iron, as well as differences in the tertiary structure of the protein, and differences in the location (surface or internalized) of the heme in the three dimensional structure of the protein.

The optical and magnetic properties of heme has attracted a large number of investigations of hemeproteins because of the ease of applying physical methods (e.g., spectrophotometric) to the measurement of changes. Even so, many questions remain unanswered concerning the role of heme in the synthesis, stability, and function of hemeproteins. We have asked the question: ''Does heme play a role in the folding of the protein of P450s during its

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synthesis and how does heme stabilize the tertiary structure of P450 hemeproteins?''.

The present study was undertaken as a first step in studying the relationship of heme to the protein folding (structure) of the P450 hemeproteins. Recent crystallographic studies [1,2] have established that the heme of P450s is buried in the interior of the molecule. In contrast, heme is readily accessible (i.e., exposed to the surface) and exchangeable in myoglobin and b_5 [3–8]. Heme transfer from various hemeproteins to apo-myoglobin has shown that apo-myoglobin has a very high affinity for heme. Migration of heme between subunits of hemoglobin has been reported indicating a dynamic process of dissociation and reassembly of heme and globin [9].

Recently, we have shown [10] that apo-$HMWb_5$ is able to interact with heme associated with preparations of recombinant CYP3A4 resulting in the formation of holo-$HMWb_5$. In the present study we have extended these studies by measuring the formation of holo-$HMWb_5$ from apo-$HMWb_5$ as a probe to assess those conditions that increase the accessibility of heme present in P450 preparations. Although many studies have been carried out by others to measure the transfer of heme to apo-b_5, these studies suffer from the deficiency that they have used the truncated, soluble form of b_5 ($LMWb_5$) which is inactive in stimulating P450 catalyzed reactions and does not form a spectrophotometrically measurable complex with P450s [11]. This report describes the results of preliminary experiments in which P450s are subjected to treatment with chaotropic reagents and the transfer of heme to apo-$HMWb_5$ measured.

2. Materials and methods

The chemicals used in the present study were purchased from the following sources: sodium cholate, 3-[(3-cholamidopropyl) dimethylammonio] - 1 - propanesulfonate (CHAPS), SDS, NADPH, phenylmethylsulfonyl fluoride (PMFS), Coomassie R-250, hemin, lyophilized horse skeletal myoglobin, 2×crystallized and lyophilized bovine hemoglobin, and crystalline bovine liver catalase suspension (catalog number C-100) (Sigma Chemical Co., St. Louis, MO); Ni–NTA–Agarose (Qiagen, Valencia, CA); Emulgen 913 (Kao Atlas, Japan); agarose, low temperature melting agarose, *iso*-propyl-β-D-thiogalactopyranoside (IPTG); and dithiothreitol (BRL); Bacto-Tryptone, Bacto-Peptone and Bacto-Yeast extract were purchased from Difco Laboratories, Detroit, MI.

3. Expression and purification of CYP3A4 and CYP4A1

E. coli were transformed with plasmids containing the cDNA for the designated proteins and grown as described [12]. Cells were disrupted and the recombinant P450s solubilized and purified as described [13]. The purified proteins were stored frozen at −70°C. It should be noted that recombinant P450s have been modified at the N-terminus of the expressed protein to facilitate high level expression in *E. coli*.

P450cam (CYP101) was purified from recombinant *E. coli* as previously described [14].

3.1. Preparation of apo-$HMWb_5$ and apo-myoglobin

Recombinant human holo-$HMWb_5$ containing a histidine tag at the N-terminal sequence was expressed and purified as previously described [15]. We use here the term $HMWb_5$ to designate the full length form (134 amino acids) of b_5 which contains the C-terminal membrane-binding segment and which is functional in reconstitution assays for the measurement of P450 activities [11]. Apo-$HMWb_5$ was prepared by removal of heme by acid–acetone treatment. Briefly, purified human holo-$HMWb_5$ (1000 nmol in 2 ml of 50 mM Tris–HCl buffer, pH 8.1), was added drop-wise to 50 ml of cold acetone (−20°C) containing 0.2% HCl, and the mixture was stirred for 30 min at 0°C. The white precipitate was recovered by centrifugation at 10 000 *g* for 15 min at 4°C, dried under a stream of nitrogen gas and homogenized in 1.0 ml of 100 mM potassium phosphate buffer, pH 7.4, containing 0.5% CHAPS, 1 mM EDTA, and 1 mM DTT. The sample was centrifuged following dialysis against three changes of 1000 ml of 0.1 M potassium phosphate buffer, pH 7.4, containing 0.1% CHAPS, 1 mM EDTA, and 1 mM DTT. The apo-$HMWb_5$ species showed a single band following SDS–PAGE and did not contain any heme absorbance in the Soret region following spectrophotometric analysis.

Apo-myoglobin was prepared from crystalline myoglobin by a similar procedure using the cold HCl–acetone extraction method for removal of the heme [16].

3.2. Spectral studies of interaction of apo-$HMWb_5$ with hemeproteins

Spectrophotometric measurements were carried out using an Aminco DW2a spectrophotometer modified by Olis.

3.3. Stop-flow experiments

Stop-flow experiments were carried out using an Aminco-Morrow Stopped-Flow Apparatus attached to an Aminco DW2 spectrophotometer linked to an IBM computer by a Strawberry Tree I/O board. Components of the reactions were prepared and mixed as described by Hargrove et al. [17] to minimize aggregation artifacts. Stock solutions of heme were prepared in 0.1 N NaOH and an aliquot was diluted in one syringe of the stopped-flow apparatus to the indicated concentration in 50 mM Tris–Cl

buffer (pH 8.0) containing 50 mM NaCl. Aliquots of the apo-proteins were diluted in the same buffer and placed in the other syringe of the stop-flow apparatus. The samples were mixed at 22°C and the absorbance change recorded. The absorbance changes were fitted to either a single- or two-exponential decay equation using the Microcal Origin 6.0 computer program

3.4. Analytical methods

The concentration of P450s was determined spectrophotometrically using a molar extinction coefficient of 91 $mM^{-1}cm^{-1}$ as described by Omura and Sato [18]. The concentration of b_5 was determined from the absolute absorption spectrum of the oxidized hemeprotein using a molar absorbance coefficient 117 $mM^{-1}cm^{-1}$ at 413 nm [19].

Protein concentrations were determined by the Micro BCA Protein Assay of Pierce. SDS–PAGE was carried out using a Bio-Rad Protean II (Bio-Rad) apparatus [20] and Protein Molecular Weight Standards (Gibco BRL).

4. Results

4.1. The interaction of heme with apo-HMWb$_5$

As first described by Strittmatter [6], apo-b_5 very efficiently binds heme stoichiometrically restoring the spectral properties of the native hemeprotein (i.e., a hemeprotein that does not bind carbon monoxide when in the ferrous state). In the present study (Fig. 1), the kinetics of interaction of heme with apo-HMWb$_5$ was measured spectrophotometrically using a stopped-flow apparatus as described in Section 3 and a calculated association rate constant (k_{on}) of $8.0\pm0.77\times10^6$ M^{-1} s^{-1} determined. Using the same conditions as described in the legend to Fig. 1, the rate constant for heme association to apo-myoglobin was determined to be $7.0\pm0.37\times10^7$ M^{-1} s^{-1}. Heme dissociation from holo-HMWb$_5$ (k_{off}) was measured by determining the increase of absorbance at 406 nm following the mixing of 2 μM apo-myoglobin with 10 μM holo-HMWb$_5$. A K_{eq} of 1.5×10^{13} M^{-1} was calculated from the ratio of k_{on}/k_{off} and is an index of the high affinity of heme for apo-HMWb$_5$. For comparison we determined the affinity of heme for apo-myoglobin to be 8.0×10^{13} M^{-1}.

4.2. The transfer of heme from hemoglobin to apo-HMWb$_5$

As a prelude to studies of heme transfer from P450s to apo-HMWb$_5$ we carried out a number of studies measuring the transfer of heme from hemoglobin to apo-HMWb$_5$. Although hemoglobin possesses a high affinity for heme it can serve as an excellent donor of heme to apo-HMWb$_5$ (Fig. 2). However, in this case the kinetics of heme transfer are biphasic with rates of $k_{fast}=0.85$ min^{-1} and $k_{slow}=0.033$ min^{-1} (Fig. 2C). These biphasic properties have been reported [3] and are interpreted to reflect the differences in the properties of heme associated with the different subunits present in the quaternary structure of hemoglobin.

Of interest are the results of an experiment we carried

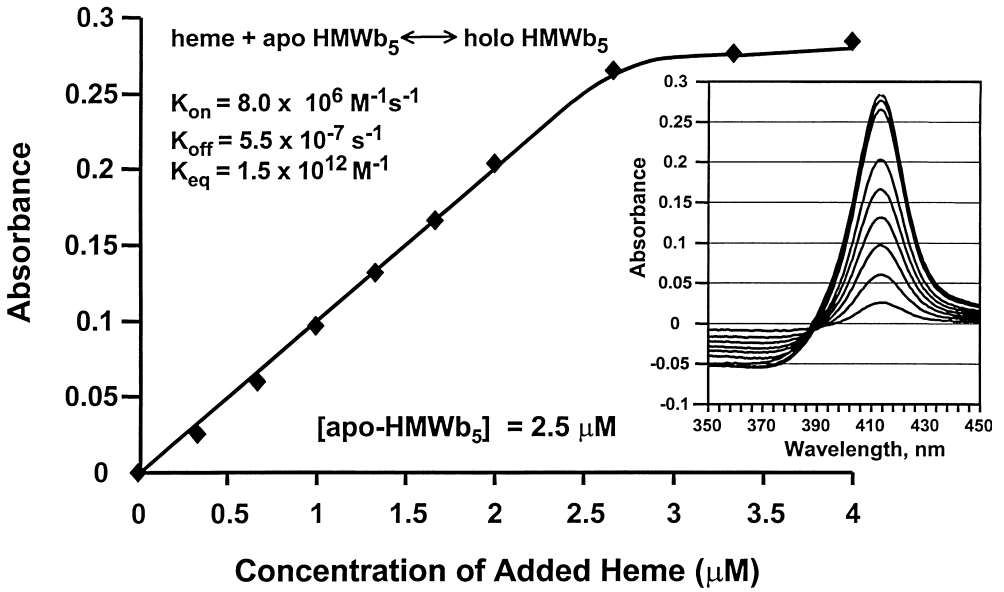

Fig. 1. The binding of heme to apo-HMWb$_5$. One syringe of the stopped-flow apparatus contained heme dissolved in 50 mM Tris–HCl buffer, pH 8.0, and 50 mM NaCl to give the concentrations indicated. The other syringe contained 2.5 μM apo-HMWb$_5$ dissolved in 50 mM Tris–HCl buffer, pH 8.0, containing 50 mM NaCl. The reactants were mixed and the kinetics of formation of holo-HMWb$_5$ measured at 413 nm at 25°C. The absorbance spectrum of the amount of holo-HMWb$_5$ formed was measured after each experiment (insert).

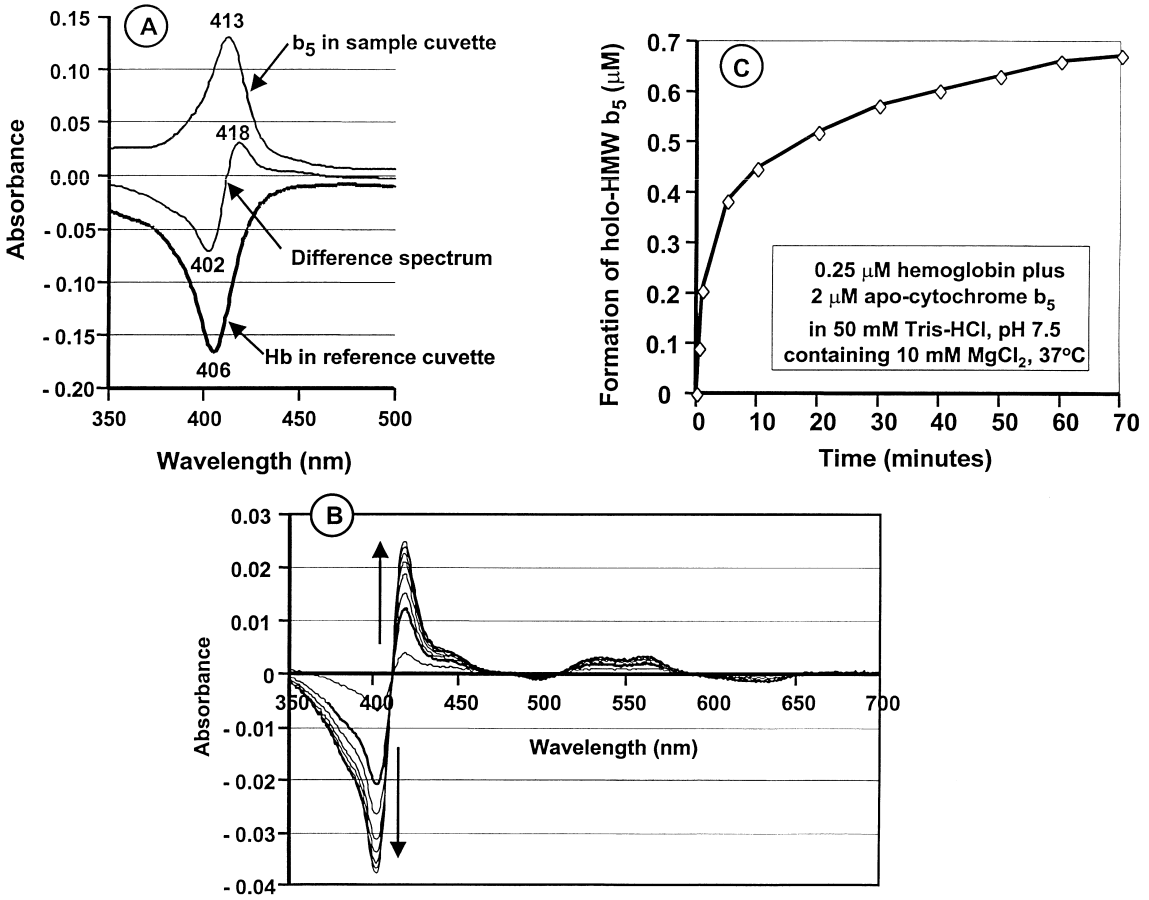

Fig. 2. The reaction of apo-HMWb$_5$ with bovine hemoglobin (Hb). Panel (A) Determination of the difference spectrum of holo-HMWb$_5$ minus hemoglobin. A 1 μM solution of holo-HMWb$_5$ in 50 mM Tris–HCl buffer (pH 7.5) containing 10 mM $MgCl_2$ was placed in the sample cuvette of the spectrophotometer. Buffer was placed in the reference cuvette and the spectrum recorded. In a second experiment a 0.25 μM solution of bovine hemoglobin was placed in the reference cuvette and buffer was placed in the sample cuvette and the spectrum recorded. The difference spectrum was determined by measuring the absorbance when 1 μM holo-HMWb5 was placed in the sample cuvette and 0.25 μM hemoglobin was placed in the reference cuvette. A molar absorbance coefficient of 66 mM^{-1} cm^{-1} was determined for the absorbance change at 418 minus 402 nm. Measurements were made using an Aminco DW2 Spectrophotometer as adapted by Olis. Panel (B) Spectral changes associated with the transfer of heme from bovine hemoglobin to apo-HMWb$_5$. A 2 μM solution of apo-HMWb$_5$ was mixed with 0.25 μM hemoglobin and placed in the sample cuvette of the spectrophotometer. Panel (C) The kinetics of absorbance change at 418 minus 402 nm during the transfer of heme from 0.25 μM hemoglobin to 2 μM apo-HMWb$_5$ as shown in panel (B).

out to determine the role of a direct interaction of apo-HMWb$_5$ with hemoglobin as a requirement for heme transfer. We placed 3 ml of a 3 μM solution of apo-HMWb$_5$ inside a dialysis membrane and soaked this for 40 h in 100 ml of a 2.5 μM solution of hemoglobin. Approximately 6% of the apo-HMWb$_5$ inside the dialysis sac was converted to holo-HMWb$_5$ indicating that heme is able to pass through the dialysis membrane but that only a small amount of heme transfer occurs without direct contact of the heme-donor and heme-acceptor.

4.3. The transfer of heme from catalase to apo-HMWb$_5$

In the present work we have used a number of different types of hemeproteins as potential donors of the heme group for apo-HMWb$_5$. These hemeproteins can be divided into two main groups based on the degree of heme exposure to the surface of the molecule. One group includes b$_5$, myoglobin, and hemoglobin while the other group consists of catalase and P450s. These hemeproteins differ from each other by size, oxidation–reduction potential, catalytic activity, the nature of the axial ligand of the heme, and the surface distribution of the electrostatic potential of the proteins.

Inspection of the crystallographic structure of bovine catalase [21] indicates that a subunit of catalase has a partially exposed heme moiety which becomes buried upon tetramerization. When a solution of crystallized catalase (additionally purified by passage through a Sephadex G-25M column) is mixed with apo-HMWb$_5$ there is no spectrophotometric evidence of heme transfer to apo-HMWb$_5$ (Fig. 3) indicating that in this case the heme of catalase is inaccessible to apo-HMWb$_5$. However, when catalase is denatured by heating or lyophilization one sees

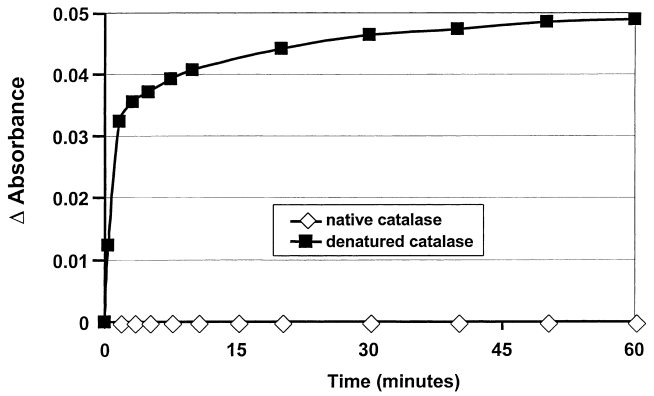

Fig. 3. The reaction of catalase with apo-$HMWb_5$. Experiments similar to those described in Fig. 2 were carried out using bovine catalase. A sample of a suspension of crystallized bovine catalase was further purified by chromatography with Sephadex G-25M and was diluted in 50 mM Tris–HCl containing 50 mM NaCl. An aliquot of this catalase solution (native catalase) was diluted to 0.25 μM and mixed with 1 μM apo-$HMWb_5$. Spectra were scanned and the changes in absorbance at 414 minus 378 nm determined. A sample of the catalase solution was placed in a water bath at 50°C for 30 min (heat denatured catalase) and an equivalent aliquot removed and mixed with apo-$HMWb_5$.

a rapid and efficient transfer of heme to apo-$HMWb_5$. As was seen in the case of heme transfer from hemoglobin (cf. Fig. 2), the heme transfer reaction from denatured catalase is biphasic with kinetic constants of $k_{fast}=18.8\pm1.9\ min^{-1}$ and $k_{slow}=0.7\pm0.03\ min^{-1}$.

4.4. The transfer of heme from P450s to apo-$HMWb_5$

In 1969, Muller-Eberhard et al. [22] reported that heme could be removed rather rapidly from P450cam (CYP101) when incubated with hemopexin or apo-myoglobin. It is of interest that this important observation has not been followed up in the intervening years since many questions remain unanswered about the mechanism of heme incorporation and removal from P450s.

When we incubated P450cam with apo-$HMWb_5$ for at least 2 h we were unable to detect any evidence for the formation of holo-$HMWb_5$. The reason for the difference in results may relate to the purity of the P450cam used in our experiment compared to the earlier studies by Muller-Eberhard et al. [22]. An examination of the three-dimensional structure of P450cam shows [23] that the heme is embedded in the interior of the molecule and may not be accessible to apo-$HMWb_5$ at the surface of the P450 using the experimental conditions we employed.

When purified CYP4A1 was incubated with apo-$HMWb_5$ there was no evidence of heme transfer (Fig. 4). However, addition of a low concentration of the chaotropic agent, guanidine hydrochloride, did initiate a slow transfer of heme from the P450 to apo-$HMWb_5$. This result suggests that a (small) perturbation of the structure of the P450 results in an alteration of the protein tertiary structure sufficient to allow some of the heme to become accessible to the apo-$HMWb_5$. Although the three-dimensional structure of CYP4A1 is not known we conclude that the heme group of this P450 isozyme is similar to other known structures of P450s where the heme is buried in the interior of the protein molecule. Similar results showing the inaccessibility of heme have been obtained (data not shown) when studying the transfer of heme from purified P450scc (CYP11A1) to apo-$HMWb_5$.

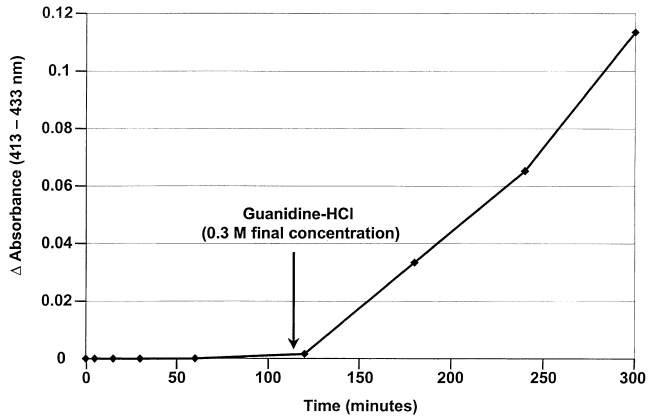

Fig. 4. The effect of guanidine–HCl on the transfer of heme from purified CYP4A1 to apo-$HMWb_5$. An aliquot of purified CYP4A1 was diluted to 1 μM in 50 mM Tris–HCl buffer (pH 7.6) containing 10 mM $MgCl_2$ and mixed with apo-$HMWb_5$ (2 μM final concentration). The change in absorbance at 413 minus 433 nm was measured spectrophotometrically. After 120 min an aliquot of 6 M guanidine–HCl was added to give a final concentration of 0.3 M. The sample was mixed and the change in spectra monitored.

4.5. The transfer of heme from CYP3A4 to apo-$HMWb_5$

We have recently published results [10] showing that incubation of P450 3A4 with apo-$HMWb_5$ results in the formation of holo-$HMWb_5$. These experiments were carried out using the conditions of catalysis required to obtain the maximal enzymatic activity of P450 3A4 during the 6β-hydroxylation of testosterone and the stimulation of this reaction by $HMWb_5$.

When a heme transfer experiment was carried out using buffer conditions similar to those described above (i.e., not the conditions required for enzymatic turnover of the P450) we also observed (Fig. 5) a time-dependent transfer of heme from the preparation of CYP3A4 to apo-$HMWb_5$. However, the amount of heme incorporated into apo-$HMWb_5$ was less than 10% of the heme present in the CYP3A4 preparation. Also, it should be noted that the rate of heme transfer is considerably slower than observed when measurements were made using the composition of the reaction medium needed for metabolic activity studies [10]. These results suggest that changes in P450 structure may occur during the cyclic function of CYP3A4 during metabolism. Additional studies are required before speculation on the mechanism of heme-release and transfer during the metabolic turnover of a P450 can be established.

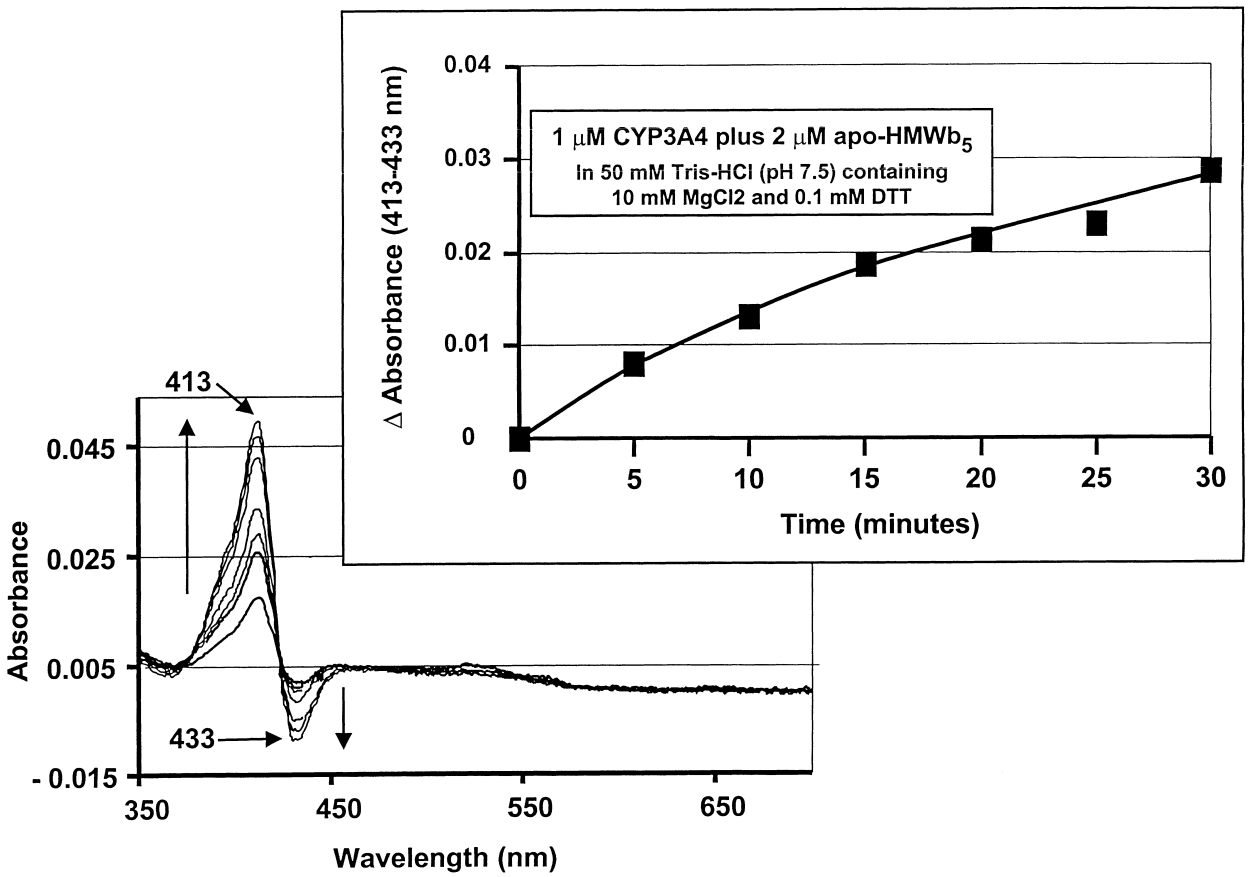

Fig. 5. The formation of holo-HMWb$_5$ from CYP3A4. A 1 μM solution of a purified recombinant CYP3A4 preparation was mixed with 2 μM apo-HMWb5 using a reaction medium of 50 mM Tris–HCl buffer (pH 7.5) containing 10 mM $MgCl_2$ and 0.1 mM dithiothritol (DTT) and the changes in absorbance at 413 minus 433 nm monitored.

4.6. The effect of guanidine–HCl on the spectral properties of CYP3A4 and the transfer of heme to apo-HMWb$_5$

A series of experiments were carried to determine changes in the structure of CYP3A4 by addition of the chaotropic agent, guanidine–HCl, as measured spectrophotometrically. We propose here that subtle modifications of the protein structure of this P450 CYP3A4 may be reflected by an increase in the rate of release of heme for transfer to apo-HMWb$_5$. As shown in Fig. 6 exposure of purified CYP3A4 to increasing concentrations of guanidine–HCl results in a series of sequential changes in optical absorbance of the Soret band of oxidized CYP3A4. Of interest is the observation that low concentrations of guanidine–HCl (i.e., less than 0.5 M) had only a small effect in modifying the absorbance at 417 nm of oxidized P450 3A4. Higher concentrations of guanidine–HCl clearly had a major disruptive influence on the structure of CYP3A4. When experiments were carried out to determine the effect of low concentrations of guanidine–HCl (i.e., $<$0.5 M) on the transfer of heme from CYP3A4 to apo-HMWb$_5$, we observed (Fig. 7) an approximate six-fold increase in the rate of formation of holo-HMWb$_5$ when the reaction is carried out using 0.5 M guanidine–HCl. Independent experiments have determined (results not shown) that 0.5 M guanidine–HCl has no measurable influence on the ability of apo-HMWb$_5$ to serve as a heme acceptor.

5. Discussion

The data described in the present work shows that apo-HMWb$_5$ possesses a high affinity for heme (dissociation constant=6.6×10^{-12} M^{-1}) that is very similar to the affinity of other well-characterized high-affinity heme-acceptors such as apo-myoglobin (dissociation constant=$\sim 10^{-14}$ M^{-1}) [17]. In addition HMWb$_5$ possesses the hydrophobic membrane-binding domain required for interaction with P450s [11] and the reduced form of holo-HMWb$_5$ does not bind carbon monoxide permitting the use of this gas to monitor changes in the concentration of reduced P450s. These properties make apo-HMWb$_5$ a very important instrument for estimating changes in the accessibility of the heme of P450s as they undergo structural changes during catalytic turnover. It would be of interest to extend the elegant studies recently reported by Schlichting et al. [24] to a study of changes in heme-accessibility during P450cam function.

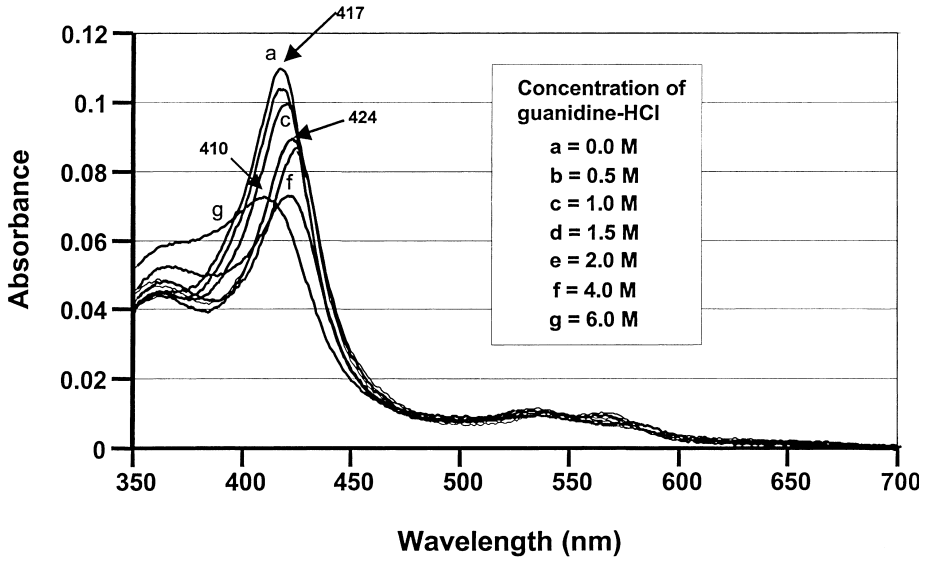

Fig. 6. Changes in the absorbance spectrum of CYP3A4 caused by exposure to varying concentrations of guanidine–HCl. A series of experiments were carried out where a 1 mM solution of purified recombinant CYP3A4 was diluted in a reaction medium of 50 mM Tris–HCl buffer (pH 7.5) containing 10 mM $MgCl_2$ and 0.1 mM dithiothritol (DTT) and the indicated concentrations of guanidine–HCl. The absorbance spectrum was then recorded (time=approximately 5 min).

Unexplained are the results showing heme-accessibilty with preparations of CYP3A4 [10]. Do these results indicate that the structure of recombinant CYP3A4 has a different heme environment from other P450s? Related to this question is the baffling mystery of differences in the rate of P450-heme turnover and P450-protein turnover in vivo as measured in liver microsomes. In the 1970s there were numerous reports describing in vivo experiments where heme synthesis and incorporation into P450s was determined using ^{14}C-aminolevulinic acid and P450-protein synthesis using ^{3}H-leucine. There was a discontinuity in the rates of P450-heme turnover (approximately 16 h) compared to the rates of P450-protein turnover (36–40 h) [25]. It was concluded from such experiments that there

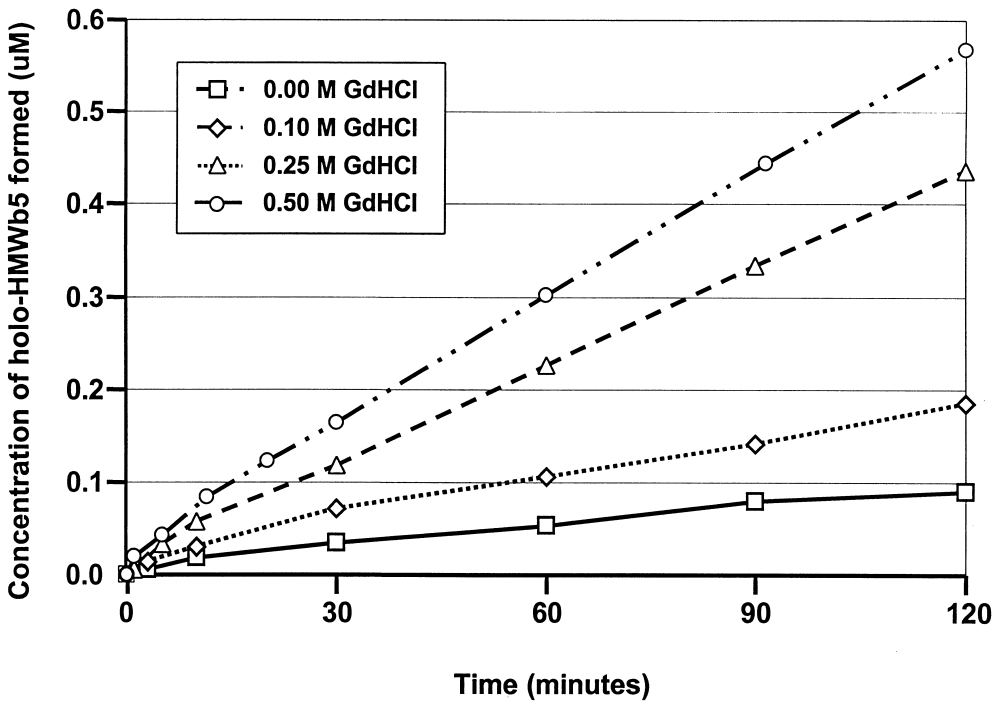

Fig. 7. The effect of increasing concentrations of guanidine–HCl on the rate of heme transfer from CYP3A4 to apo-$HMWb_5$. A series of experiments, as described in Fig. 6, were carried out at the concentrations of guanidine–HCl (GdHCl) indicated and the change in absorbance at 413 minus 433 nm measured. Using a molar absorbance coefficient of 66 mM^{-1} cm^{-1} the concentration of holo-$HMWb_5$ was determined.

was a reversible transfer of heme between different molecular species of microsome-bound P450s. These and other experiments point to the need for additional experiments to further examine changes in the structure of P450s as reflected by changes in heme-accessibility.

The apo-proteins for P450s are synthesized on ribosomes associated with the endoplasmic reticulum [26] while heme biosynthesis occurs in the matrix of the mitochondria. Unanswered is the question: ''How does heme migrate across the mitochondrial membrane to find the nascent P450 apo-protein?''. More important is the question: ''Does heme binding to the newly synthesized P450 apo-protein serve as a nucleus for directing the folding of the apo-protein as it becomes a P450 holoprotein?''. Answers to these questions remain to be fully explored. Future experiments will include the preparation of large amounts of apo-P450 by removing the heme with apo-$HMWb_5$ in order to fully characterize the mechanism of heme incorporation into P450s.

Many lessons have been learned from the plethora of studies reporting changes in heme-accessability and heme-incorporation into numerous different proteins. Apo-$HMWb_5$ now provides a new tool for the study of changes in P450 structure both in solution and under conditions of catalytic function.

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