Stimulation of Cytochrome P450 Reactions by Apo-cytochrome b₅

EVIDENCE AGAINST TRANSFER OF HEME FROM CYTOCHROME P450 3A4 TO APO-CYTOCHROME b₅ OR HEME OXYGENASE *

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Many cytochrome P450 (P450)-dependent reactions have been shown to be stimulated by another microsomal protein, cytochrome b₅ (b₅). Two major explanations are (i) direct electron transfer from b₅ and (ii) a conformational effect in the absence of electron transfer. Some P450s (e.g. 3A4, 2C9, 17A, and 4A7) are stimulated by either b₅ or b₆ devoid of heme (apo-b₅), indicating a lack of electron transfer, whereas other P450s (e.g. 2E1) are stimulated by b₅ but not by apo-b₅. Recently, a proposal has been made by Guryev et al. (Biochemistry 40, 5018–5031, 2001) that the stimulation by apo-b₅ can be explained only by transfer of heme from P450 preparations to apo-b₅, enabling electron transfer. We have repeated earlier findings of stimulation of catalytic activity of testosterone 6β-hydroxylation activities with four P450 preparations, in which nearly all of the heme was accounted for as P450. Spectral analysis of mixtures indicated that only ~5% of the heme can be transferred to apo-b₅, which cannot account for the observed stimulation. The presence of the heme scavenger apomyoglobin did not inhibit the stimulation of P450 3A4-dependent testosterone or nifedipine oxidation activity. Further evidence against the presence of loosely bound P450 3A4 heme was provided in experiments with apo-heme oxygenase, in which only 3% of the P450 heme was converted to biliverdin. Finally, b₅ supported NADH-b₅ reductase/P450 3A4-dependent testosterone 6β-hydroxylation, but apo-b₅ did not. Thus, apo-b₅ can stimulate P450 3A4 reactions as well as b₅ in the absence of electron transfer, and heme transfer from P450 3A4 to apo-b₅ cannot be used to explain the catalytic stimulation.

P450 enzymes are widespread in nature and well known for their catalytic versatility (2–5). Interest in these mammalian enzymes is strong because of their critical roles in the metabolism of drugs, steroids, and carcinogens (2, 3, 5, 6). The most common mode of catalysis is mixed-function oxidation, which involves the use of both electrons and O₂ as substrates.

Electron input from pyridine nucleotides into P450s usually follows one of two general modes. The first, which is common in some bacterial and mitochondrial P450s, involves transfer of electrons from a flavoprotein to an iron-sulfur protein to P450 heme. The other major reaction, which is common in the microsomal P450s, involves electron transfer from a 2-flavin flavoprotein (NADPH-P450 reductase) into the P450 heme. Some P450-catalyzed reactions are stimulated by b₅, as first discovered in a series of studies in which NADH stimulated NADPH-dependent reactions in liver microsomes (7). The role of b₅ in some reactions was later reinforced in reconstitution (8–10) and immunochemical inhibition experiments (11). The occurrence and mechanism of b₅ involvement in P450-catalyzed reactions have been studied extensively in the past three decades (12–17). Some P450 reactions involve b₅, but others do not, even with the same P450 enzyme (10, 18). An added complexity is that NADPH-P450 reductase reduces b₅ efficiently as well as P450s, rendering the path of electron transfer difficult to establish in most cases. In general, direct electron transfer to ferric P₄₅₀ is inefficient because of the unfavorable difference in oxidation potentials (19, 20). Until recently, the generally accepted mechanism for b₅ stimulation has been introduction of an electron into the P₄₅₀ FeO₂⁺ complex, which is considered to have a much higher oxidation-reduction potential than the P₄₅₀ Fe³⁺/Fe²⁺ couple (19–22). Early studies with liver microsomes showed changes in the redox state of b₅ (7, 23). Other early experiments with b₅ substituted with redox-inactive metals were also used to argue in favor of an electron transfer mechanism. However, this issue has been difficult to address directly because of the kinetic complexities in mixing b₅ with P₄₅₀ in the P₄₅₀ FeO₂⁺ form (24–26).

In 1995, we reported that the reduction of ferric P₄₅₀ 3A4 required the presence of b₅ for maximum rates, as well as a substrate (testosterone) (27). The following year, in the course of doing what were intended to be controls for other experiments, we found that apo-b₅ was as effective as b₅ in stimulating P₄₅₀ 3A4-catalyzed testosterone 6β-hydroxylation and nifedipine oxidation (22). We extended this work and reported that P₄₅₀ 2C9 reactions were stimulated by apo-b₅ as well as b₅, but with P₄₅₀ 2E1, only b₅ was stimulatory (28). Other laboratories reported the stimulation of P₄₅₀s 17A (29) and 4A7 (30, 31) by apo-b₅. Recent work in our own groups has also shown stimulation of catalytic activities of P₄₅₀s 2A6, 2B6, 2C8, 2C19, and 3A5 by both b₅ and apo-b₅ (2). Some P₄₅₀s have

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‡ The abbreviations used are: P₄₅₀, cytochrome P₄₅₀ (also termed heme-thiolate protein P₄₅₀ by the Enzyme Commission (EC) 1.14.14.1) (1); P₄₂₀, cytochrome P₄₂₀ (spectrally detected denatured forms of P₄₅₀); b₅, cytochrome b₅ (EC 4.4.2 group); apo-b₅, b₅ devoid of heme; HPLC, high pressure liquid chromatography.

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not shown effects of b5 or apo-b5, i.e., 1A1, 1A2, 1B1, and 2D6.2

The conclusion regarding these experiments with apo-b5 is that electron transfer (to P450) by b5 is not involved in the stimulation (22, 29, 31). The effect of b5 varies depending upon the particular P450 3A4 system. Enhancement of some (but not all (27, 32)) catalytic activities is seen with either recombinant P450 3A4 or the enzyme purified from liver microsomes (33, 34). The extent of stimulation does vary in different systems, and the reconstituted systems with higher activities appear to show less stimulation (22, 35). However, the effect is usually 2–4-fold. Bacterial or baculovirus-based insect cell membranes in which P450 3A4 and NADPH-P450 reductase are simultaneously expressed have high catalytic activities (including testosterone 6β-hydroxylation activity) in the absence of b5 (25, 36), although b5 can be added to the membranes to further stimulate activity (37). The critical issue here is what the role of b5 is in liver (or other tissues), and studies with anti-b5 antibodies have shown a strong contribution of b5 to catalytic activity in liver microsomes, regardless of the mechanism (33, 35).

The conclusions reached with apo-b5 may or may not be relevant to microsomes. However, these stimulatory effects have been observed with numerous purified P450 preparations (3A4, 2C9, 17A, and 4A7) (22, 28–31), and conclusions about modulation of catalytic activities are not surprising in light of our current general understanding of protein-protein interactions. Some P450s have even shown catalytic stimulation by other P450s (28, 38). However, very recently, Guryev et al. (39) have questioned the conclusions of the apo-b5 studies (22, 29–31, 37). They repeated the experiments cited above with P450s 3A4 and 17A and also found stimulation of catalytic activities by apo-b5. Some transfer of heme to apo-b5 was also found; the issue of whether the source was the P450 itself (3A4 or 17A) or adventitiously bound heme was unresolved. These investigators also added heme to purified apo-b5 and apomyoglobin and characterized the binding as rapid and tight. A key experiment was the subsequent use of apomyoglobin as a heme scavenger in blocking stimulation by apo-b5 (39).

The results of Guryev et al. (39) are not in accord with the previously held general view about the lack of electron transfer in the stimulation of some reactions by apo-b5. Concerns involve the report of the presence of excess heme (30%) in the preparations and the relatively low fraction of transfer of heme (∼10%) (39). We address some of the issues raised in that work, those that bear directly on the issues of how much heme transfer contributes to the stimulation of P450 activities by apo-b5 and b5. Other experiments presented (39) do not directly address the question and are not considered here (e.g., binding to P450, truncations, mutants and use of alternate metals, and reduction in the absence of substrate); differences between apo-b5, b5, and derivatives may seem subtle but are known to have cooperative structural effects (40) that may not be readily interpreted.

We considered a set of P450 3A4 preparations made since the earlier work (22, 28) because our purification methodology has been changed. In these preparations we account for nearly all of the measured heme as P450, although some key experiments were done with a preparation of one of the stocks in which 30% of the P450 had converted to cytochrome P420. Our earlier reported stimulatory effects of apo-b5 were fully repeatable, and we proceeded to focus on events that could occur in the time frame used to see the stimulations, e.g., reconstitution and assay. The transfer of 3–5% of the heme from P450 3A4 preparations does occur with apo-b5 or the enzyme heme oxygenase, with conversion of heme to biliverdin in the latter case. This fraction is attributed to trace cytochrome P420 and adds evidence to the view that heme oxygenase, which is normally involved in the physiological degradation of free heme, does not act directly on P450 heme. The lack of an effect of highly purified apomyoglobin as a heme scavenger and the inability of apo-b5 to support NADH-b5 reductase/P450 3A4-catalyzed testosterone 6β-hydroxylation provide more evidence against an obligatory role for heme transfer to apo-b5 in the stimulations of P450s.2

EXPERIMENTAL PROCEDURES

Chemicals—Biliverdin (IXa) was purchased from ICN (Costa Mesa, CA). Horse apomyoglobin (sequencing grade) was obtained from Sigma Chemical Co.

Spectroscopy and HPLC—UV-visible spectra were recorded using OLIS-Cary 14 and -Amino DW2 instruments (On-Line Instrument Systems, Bogart, GA). HPLC was done with a Spectra-Physics 8700 pump system and ThermoSeparation 6000 rapid scanning monochromator or a JASCO PU-980 pumping system (Japan Spectroscopic Co., Tokyo, Japan) and Chromatopac C-B3A detector (Shimadzu, Kyoto, Japan).

Enzymes—The original P450 3A4 Escherichia coli pCW expression vector (41, 42) was modified to include a C-terminal (His) tag (43). Purification was done using DEAE and Nii2 affinity chromatography as described by Hasea et al. (44). Rat NADPH-P450 reductase was expressed in E. coli (45) and purified as described elsewhere (46, 47). Rabbit liver b5 was prepared as described previously (48, 49) and used either as b5 (22), which had spectral properties similar to those reported previously (22). Rabbit liver NADH-b5 reductase was prepared as described previously (50) and had a specific activity of 24.3 μmol KFeCN reduced min−1 (nmol reductase)−1 (gift of Y. Imai; University Osaka Prefecture, Osaka, Japan).

E. coli-expressed human heme oxygenase (divoid of 23 residues at the C terminus) (51) was a gift of K. Auclair and P. R. Ortiz de Montellano (University of California, San Francisco, CA). The heme in the sample (36 nmol of enzyme) was converted to biliverdin by incubation with an equal concentration of NADPH-P450 reductase in the presence of 0.10 mM potassium phosphate buffer (pH 7.4) and an NADPH-generating system (vide infra). Spectral analysis indicated that the reaction was complete in the first minute. The mixture (in 3 ml) was applied to a 1.2 × 60-cm Sephadex G-10 column equilibrated with 50 mM potassium phosphate (pH 7.4) containing 0.10 mM EDTA and eluted with the same. The void volume fraction was detected by A280 measurements; the spectral analysis indicated the apparent presence of stable NADPH-P450 reductase flavin semiquinone. The sample was concentrated (to 1.9 ml) using an Amicon-Millipore ultrafiltration device equipped with a PM-10 membrane (Millipore Corp., Bedford, MA). Protein analysis indicated nearly complete recovery of the apo-heme oxygenase and NADPH-P450 reductase. This preparation of apo-heme oxygenase was used with hemin chloride, and a preliminary spectral analysis indicated that it was capable of forming biliverdin. NADPH-P450 reductase was added to the apo-heme oxygenase preparation in subsequent work with P450 3A4.

Reconstitution of P450 3A4 and Assay of Testosterone 6β-Hydroxylation—The following order of mixing was used (all concentrations are for a final incubation mixture in 0.25 ml): P450 3A4 (20 nM), NADPH-P450 reductase (40 nM), b5 or apo-b5 (0–40 nM, usually 20 nM unless stated otherwise), apomyoglobin (10–40 nM, when indicated), sodium cholate (0.5 mM), and a phospholipid mixture (20 μM) consisting of a 1.1:1 (w/w) mixture of dilauroyl-sn-glycero-3-phosphocholine, dilauroyl-sn-glycero-3-phosphocholine, and bovine brain phosphatidylserine (34); these components were mixed in concentrated solution before dilution and allowed to stand for 10 min at room temperature, followed by the addition of potassium phosphate buffer (to 100 mM; pH 7.4), MgCl2 (to 5 mM), testosterone (to 200 μM), and an NADPH-generating system consisting of 5 mM glucose 6-phosphate, 0.5 mM NADP+, and 0.5 unit of glucose 6-phosphate dehydrogenase ml−1. Incubations proceeded for 10 min at 37 °C. Reactions were terminated, and the products were analyzed by HPLC (octadeylsilane column; A280 1.0 μmol min−1 flow rate; 64% CH3OH in H2O (v/v)).

Analysis of Heme and Biliverdin—Heme was estimated using the pyridine hemochrome method, with Δλ557–577 = 32.4 μM−1 cm−1 (52, 53). Biliverdin was extracted from incubations with acetone–HCl and analyzed by HPLC as described by Bonkowski et al. (54), using a Zorbax octadeylsilane column (3 μm; 6.2 × 80 mm; MacModd, Chadds Ford, PA) and both 670 and 405 nm measurements.
Analysis of Movement of Heme into apo-P450—The proposal of Guryev et al. (39) regarding the transfer of heme from P450 3A4 or heme adventitiously bound to P450 3A4 into apo-b5 was considered because that work showed that the incorporation of heme into apo-b5 was a rapid process. However, consideration of the known three-dimensional structures of prokaryotic P450s and P450 2C5 indicates that the heme prosthetic group is embedded inside of the protein (57, 58). Rapid loss of heme from P450s would appear to be highly unlikely, given the known thiolate axial ligation. A rapid exchange of P450 3A4 heme with its environment would make the purification of the holoenzyme nearly impossible.

If heme transfer to apo-b5 is the explanation for the stimulation of P450 3A4 reactions by apo-b5 (39), then the transfer must be rapid because the reconstitution/assay process is complete within 10–20 min. We restricted most analyses to this time frame (≤30 min). The spectra presented in Fig. 2 were recorded after incubations of all system components (except NADPH) under the normal reconstitution conditions. The spectra obtained with apo-b5 (Fig. 2, C and D) match those obtained in the absence of b5 (Fig. 2, A and B) and not those obtained with b5 (Fig. 2, E and F), as judged by the absence of the peaks at 424 and 550 nm. It should also be noted that the P450 spectrum was not decreased in the presence of apo-b5 (Fig. 2D).

However, the transfer of traces of heme into b5 might not have been detected in the design used in Fig. 2. We also used a protocol employed by Guryev et al. (39) in which a spectrum of an S2O42−-reduced mixture of P450 3A4/b5 was recorded versus S2O42−-reduced P450 3A4 (Fig. 3). In this assay, a small peak at 424 nm was detected when a mixture of apo-b5 and P450 3A4 was reduced, possibly indicating b5 formation. The absorbance of this peak is ~5% of that seen when a b5 solution was used at the same concentration.

We conclude that a finite but small amount of b5 might have been formed due to transfer of heme to apo-b5. In considering the results presented in Fig. 1, 5% incorporation of heme into apo-b5 cannot explain the stimulatory effects of apo-b5 because the amounts of b5 and apo-b5 needed to achieve a given amount of stimulation are nearly identical.

Lack of Function of Apo-b5 in NADH-b5 Reductase/P450 3A4-catalyzed Testosterone 6β-Hydroxylation—Some P450 reactions can be supported with NADH-b5 reductase and b5 substituted for NADPH-P450 reductase, with slower rates (59). In these systems, b5 is required because NADH-b5 reductase cannot directly transfer electrons to P450s, including P450 3A4 (55). We previously showed that P450 3A4 can catalyze b5-dependent testosterone 6β-hydroxylation in such a system (55) but that apo-b5 did not substitute for b5 (22).

This experiment was repeated with new proteins, and the results were identical (Fig. 4). If heme were transferred from P450 3A4 or anything in the P450 3A4 preparation to convert apo-b5 to b5, the apo-b5 should have been functional in this reaction. This is clearly not the case here, with the limit of ~5% contribution (Fig. 4).

Lack of Inhibition of P450 3A4-supported Reactions by Apomyoglobin—One of the critical arguments used by Guryev et al. (39) to support the view of the necessary transfer of heme from P450 3A4 to apo-b5 was the inhibition of apo-b5-stimulation of P450 3A4-catalyzed testosterone 6β-hydroxylation by apomyoglobin. Apomyoglobin rapidly binds heme in solution and was used as a scavenger. Guryev et al. (39) reported that apomyoglobin, added at the concentration of b5 or apo-b5, inhibited the stimulation of catalytic activity by apo-b5 but not by b5.

We examined this phenomenon with both testosterone 6β-hydroxylation and nifedipine oxidation because of the possible significance of the report (Fig. 5). The two P450 3A4 preparations used were those from Fig. 1, but in Fig. 5, B and D, we...
used a stock in which the P420 content was 30% of the total P450 plus P420 to investigate the possibility that potentially loosen binding of heme might facilitate the heme transfer process proposed by Guryev et al. (39).

The apomyoglobin:P450:apo-b5 ratios were varied from 0:1:1 to 2:1:1 (Fig. 5). No significant inhibition of basal, b5-stimulated, or apo-b5-stimulated testosterone 6β-hydroxylation or nifedipine oxidation was observed.

The clear difference with the result reported by Guryev et al. (39) is unexplained. Clearly one of the most significant differences in the systems studied here and in that work is the matter of the extra heme in the preparation reported by those authors (39). As indicated above, one of the stocks was picked to be one in which P420 was increased (probably due to use with repeated thawing and refreezing), but similar results were obtained (Fig. 5, B and D). Sigma sequencing grade horse apomyoglobin was utilized in our work (this is a standard for optimization of amino acid sequenators). Our work was all done with rabbit b5 and apo-b5 instead of human apo-b5 (39), although the stimulation of P450 activities by b5/apo-b5 has been demonstrated with proteins from varying sources (22, 29, 31), and the difference is not expected to account for the varying results.

Interaction of Heme Oxygenase with Heme Bound to P450

In the early literature involving the enzyme heme oxygenase (the enzyme that degrades free heme to biliverdin IXα and CO), the inference was often made that heme oxygenase induces heme oxygenase and decrease total P450 in rats, but the relationship of these events is not causal. We reported that par-
al. (63) reported that P450s 2B1 and 1A1 are substrates for rat heme oxygenase, with heme being converted to biliverdin. However, the P450 preparations contained P420, and the assays were done in the presence of detergents (Emulgen 911) in the absence of glycerol, conditions long known to convert P450 to P420 (64). Under these conditions, 35% of the added P450 2B1 was destroyed, and most (70%) was reported to be converted to P420 (64). Under these conditions, 35% of the added P450 2B1 was destroyed, and most (70%) was reported to be converted to P420 (64). However, the P450 preparations contained P420, and the as-

The analysis of heme loosely bound to heme proteins is not trivial, including P450s. Kutty et al. (63) reported that the Fe^{2+}-CO versus Fe^{3+} difference spectra of free heme, heme albumin (bovine), heme added to P450 2B1, and P420 had discernable spectra with wavelength maxima at 409, 414, 415, and 420 nm, respectively. However, we detected wavelength maxima for free heme, methemalbumin, and heme added to P450 3A4 at 413, 413, and 422 nm, respectively (using the peak finder or derivative function of the OLIS software). As discussed some time ago (64), release of heme from its thiol ligation results in the loss of a P450 spectrum (450 nm for Fe^{2+}-CO) and the appearance of a band in the region of 420 nm. The term P420 is rather operational, and spectra do not discern among P420 proteins with heme bound at various sites, inside and on the outside of proteins (see also Ref. 65 regarding the variability of spectra of P420 reconstituted from apo-P450 2B4 and heme).

If P450 3A4 were able to freely exchange heme with the medium or had excess heme that could be accessible to apo-b_{5} or apomyoglobin, then this should also be accessible to heme oxygenase, which would produce biliverdin in the presence of NADPH-P450 reductase and NADPH. We therefore decided to utilize heme oxygenase as a heme scavenger, similar to apo-

myoglobin. However, interpretation of the catalytic inhibition with high concentrations of heme oxygenase would be complicated by the competition of heme oxygenase with P450 for NADPH-P450 reductase, and we restricted our analysis to the destruction of P450 heme and formation of biliverdin. To increase the sensitivity of the assays, we converted the heme bound to hemoglobin into biliverdin in the presence of NADPH-P450 reductase and prepared apo-heme oxygenase us-

![Fig. 5. Lack of inhibition of P450 3A4-catalyzed oxidations by apomyoglobin with four different P450 3A4 preparations. The systems contained either no b_{5}, apo-b_{5} (■), b_{5} (□), or apo-b_{5} (△). A and B, testosterone β-hydroxylation; C and D, nifedipine oxidation. The same P450 3A4 preparation was used in A and C, and a different preparation was used in B and D.](image-url)

![Fig. 6. Lack of effect of incubation of P450 3A4 with apo-heme oxygenase on spectra. P450 3A4 (10.8 μM) was incubated (volume = 260 μl) with apo-heme oxygenase (nominal 7.3 μM), NADPH-P450 reductase (11.5 μM), potassium phosphate buffer (100 mM, pH 7.4), E. coli superoxide dismutase (2 μM), bovine erythrocyte catalase (800 IU ml⁻¹), and the NADPH-generating system for 20 min at 23 °C (α). The reaction was diluted with 1.74 ml of 0.10 M potassium phosphate buffer containing 1.0 mM EDTA, 40% glycerol (w/v), 0.5% sodium cholate (w/v), and 0.4% Emulgen 911 (w/v), and the mixture was divided into two cuvettes. CO was bubbled through the sample cuvette for 60 s, and solid Na_{2}S_{2}O_{4} was added to both cuvettes. The procedure was repeated as above without apo-heme oxygenase (b) or without NADPH (c).](image-url)

![Fig. 7. Formation of trace biliverdin from a P450 3A4 preparation in the presence of apo-heme oxygenase. Mixtures were prepared as described in the Fig. 6 legend and incubated for 30 min at 37 °C. A, HPLC of 140 pmol of standard biliverdin IXα; B, HPLC of 40% (200 μl) of a sample of the complete system containing P450 3A4 (3.5 nmol of P450), apo-heme oxygenase, NADPH-P450 reductase, and the NADPH-generating system; C, HPLC of 40% of an incubation similar to that in B but devoid of P450 3A4. The traces are at 405 and 670 nm (the same t_{0} values are offset 0.5 min in presentation due to the HPLC software).](image-url)
In incubation with P450 3A4 (Fig. 6), and only oxygenase system had little effect on the P450 spectrum after with a considerable amount of P420. A reconstituted heme system was formed after incubation with P450 3A4 (Fig. 6), and only ~5% of the heme was converted to biliverdin (Fig. 7), ruling out the presence of appreciable adventitiously bound heme and the use of intact P450 3A4 as a substrate by heme oxygenase. A further important argument against the necessity of heme oxygenase to apo-

Conclusions—The literature contains reports of at least four P450s for which catalysis is stimulated by apo-

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