The Binding Sites on Human Heme Oxygenase-1 for Cytochrome P450 Reductase and Biliverdin Reductase*  

Received for publication, January 29, 2003, and in revised form, March 5, 2003  
Published, JBC Papers in Press, March 6, 2003, DOI 10.1074/jbc.M300989200  

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Human heme oxygenase-1 (hHO-1) catalyzes the NADPH-cytochrome P450 reductase-dependent oxidation of heme to biliverdin, CO, and free iron. The biliverdin is subsequently reduced to bilirubin by biliverdin reductase. Earlier kinetic studies suggested that biliverdin reductase facilitates the release of biliverdin from hHO-1 (Liu, Y., and Ortiz de Montellano, P. R. (2000) J. Biol. Chem. 275, 5297–5307). We have investigated the binding of P450 reductase and biliverdin reductase to truncated, soluble hHO-1 by fluorescence resonance energy transfer and site-specific mutagenesis. P450 reductase and biliverdin reductase bind to truncated hHO-1 with $K_d = 0.4 \pm 0.1$ and $0.2 \pm 0.1 \mu M$, respectively. FRET experiments indicate that biliverdin reductase and P450 reductase reductase compete for binding to truncated hHO-1. Mutation of surface ionic residues shows that hHO-1 residues Lys$^{18}$, Lys$^{22}$, Lys$^{79}$, Arg$^{183}$, Arg$^{185}$, and Glu$^{127}$ contribute to the binding of cytochrome P450 reductase. The mutagenesis results and a computational analysis of the protein surfaces partially define the binding site for P450 reductase. An overlapping binding site including Lys$^{18}$, Lys$^{22}$, Lys$^{79}$, Arg$^{183}$, and Arg$^{185}$ is similarly defined for biliverdin reductase. These results confirm the binding of biliverdin reductase to hHO-1 and define binding sites of the two reductases.

Heme oxygenase oxidizes heme$^1$, a pro-oxidant and toxic species, to biliverdin, CO, and free iron (1). Each of the three metabolites formed in the reaction is thought to be physiologically important. Biliverdin is converted by biliverdin reductase to bilirubin, which must then be conjugated with glucuronic acid to be excreted (2). However, bilirubin, albeit toxic at high concentrations, has potent antioxidant properties that may contribute to the anti-inflammatory activity of the heme oxygenases (3, 4). Although still controversial, CO appears to be released, followed by dissociation of the biliverdin. Single turnover studies have shown that the rate-limiting step is the release of biliverdin, but in the presence of biliverdin reductase this step is accelerated, and one of the electron transfer steps becomes rate-limiting (17).

The electrons required for the catalytic turnover of heme oxygenase are provided by NADPH-cytochrome P450 reductase (1, 19, 20), a 78-kDa membrane-bound flavoprotein that incorporates one FAD and one FMN as prosthetic groups (reviewed in Ref. 21). Heterologous expression of the full-length P450 reductase followed by trypsinolysis to remove the 6-kDa N-terminal membrane-binding domain yields a soluble protein whose crystal structure has been determined (22). The electron flow in this protein has been established by studies with cytochrome c and cytochrome P450 as electron acceptors to proceed from the NADPH to the FAD and finally to the FMN prior to transfer to the acceptor heme group (23–25). A role for charge pairing in formation of transient electron transfer complexes between cytochrome P450 enzymes and P450 reductase is supported by site-directed mutagenesis studies (26, 30). However, Voznesensky and Schenkm (31, 32) have reported that electrostatic interactions can destabilize rather than stabilize complex formation. The role of electrostatic interactions of surface residues is thus not as well resolved in the case of P450 reductase-cytochrome P450 as it is in the interactions of, for example, of cytochrome P450 with cytochrome $b_5$ (e.g. see Ref. 29).

Rat biliverdin IX$\alpha$ reductase, the enzyme that reduces daily iron needs are met by absorption from the diet (8). As a result of these activities, heme oxygenase has been shown to have, inter alia, important anti-inflammatory (9) and antiatherosclerotic (10) functions.

Human heme oxygenase-1 (hHO-1) is a 33-kDa membrane-bound protein of 288 amino acid residues that binds heme and uses the bound entity as both the prosthetic group and substrate. A soluble form of hHO-1 (hHO-1$_{233}$) has been obtained by heterologous expression in *E. coli* of a truncated version of 265 amino acids that lacks the membrane binding domain (11). The crystal structures of hHO-1 truncated to a length of 233 amino acids (12), a rat homologue truncated to a length of 267 residues (13), and an inherently soluble full-length microbial heme oxygenase (14) have been determined. As recently reviewed, mechanistic studies have established that the transformation of heme to biliverdin is a three-step process that consumes three molecules of oxygen and seven electrons (15, 16). In the first step, ferric heme is oxidized to $\alpha$-meso-hydroxyheme in a reaction that consumes one molecule of oxygen and two electrons. The ferric $\alpha$-meso-hydroxyheme is then converted to ferrous verdoheme in a reaction that consumes a further molecule of oxygen and two electrons and releases CO. In the final stage of the reaction sequence, the verdoheme is converted to ferrous biliverdin with the consumption of a further molecule of oxygen and three electrons. The ferrous iron is then released, followed by dissociation of the biliverdin. Single turnover studies have shown that the rate-limiting step is the release of biliverdin, but in the presence of biliverdin reductase this step is accelerated, and one of the electron transfer steps becomes rate-limiting (17).
α-biliverdin to bilirubin, is a soluble cytosolic protein of 295 amino acid residues, three of which are cysteines. Mutation of one of these three residues, Cys73, was reported in one study to affect enzyme inactivation but in another to have no effect on activity (33, 34). The crystal structures of rat biliverdin IXα reductase (34, 35) and human biliverdin IXβ reductase (36) have been reported. The rat biliverdin IXα-reductase preferentially binds NADH at lower pH (pH ~6.7) but NADPH at higher pH (pH ~8.7). At physiological pH, the favored nucleotide cofactor is NADH. The protein has no redox-active prosthetic groups and thus appears to catalyze a direct hydride transfer from the pyridine nucleotide to the central carbon of biliverdin.

We report here an investigation of the surface sites and the electrostatic determinants within those sites of the interactions of hHO-1 (1) with rat P450 reductase and rat biliverdin IXα reductase. The results establish that ionic interactions contribute to the binding of these proteins and demonstrate that the binding sites for P450 reductase and biliverdin reductase either overlap or are allosterically linked.

**EXPERIMENTAL METHODS**

**Materials**—Ampicillin, isopropyl-β-D-thiogalactopyranoside, NADPH, adenosine, riboflavin 2'-AMP, and hemin were from Sigma. 7-Diethylamino-3-(4' -maleimidylphenyl)-4-methylcoumarin (CPM) was purchased from Molecular Probes, Inc. (Eugene, OR). Bl-21 (DE3) pLysS-competent cells were obtained from Stratagene (La Jolla, CA) and Q Sepharose™ fast flow from Amersham Biosciences.

**Enzymes**—Truncated hHO-1 (1) that retains amino acid residues 1–265 but lacks the C 3-terminal amino acids was primarily used in this study (11). In one set of experiments, we also employed hHO-1 (1) that consists of residues 1–233 rather than 1–265. This protein was expressed and purified as previously reported (38). hHO-1 (1) mutants were generated with the Stratagene QuickChange site-directed mutagenesis kit. Oligonucleotides were synthesized by Invitrogen, through the Cell Culture Facility of the University of California, San Francisco. Plasmid purifications and bacterial transformations were performed by standard procedures (39). Transformants were screened initially by ampicillin resistance and confirmed by sequence analysis. hHO-1 (1) and its mutants were expressed in BL(DE3)pLysS-competent cells. Purification and reconstitution with hemin were performed as previously reported (17, 40, 41). All of the experiments were performed in 0.1 M potassium phosphate buffer at pH 7.4 (standard buffer) unless otherwise specified.

**Preparation of CPM-labeled P450 Reductase and Biliverdin Reductase**—The fluorescent probe CPM reacts specifically with protein thiol groups to give thioether-coupled products. Modification of P450 reductase with CPM has been reported to have no effect on the activity of this enzyme (43). CPM was dissolved in dimethyl sulfoxide at a concentration of 20 mM. The labeling of P450 reductase was performed by incubating 10 μM P450 reductase with 100 μM CPM and 1 mM NADPH in the dark at ~25 °C for 3 h. NADPH was included in the labeling reaction to protect the cysteine residues near the NADPH binding site and to stabilize the enzyme. After the incubation, the mixture was passed through an Amersham Biosciences PD-10 desalting column pre-equilibrated with standard buffer to remove the unbound CPM. The labeled P450 reductase (P450 reductase-CPM) was concentrated to 0.4±0.1 μM P450 reductase-CPM. The dissociation constants for the binding of P450 reductase or biliverdin reductase to hHO-I265 and hHO-I233 were determined by FRET experiments.

**Preparation of hHO-I265 and hHO-I233**—The fluorescent probe CPM reacts specifically with protein thiol groups to give thioether-coupled products. Modification of P450 reductase with CPM has been reported to have no effect on the activity of this enzyme (43). CPM was dissolved in dimethyl sulfoxide at a concentration of 20 mM. The labeling of P450 reductase was performed by incubating 10 μM P450 reductase with 100 μM CPM and 1 mM NADPH in the dark at ~25 °C for 3 h. NADPH was included in the labeling reaction to protect the cysteine residues near the NADPH binding site and to stabilize the enzyme. After the incubation, the mixture was passed through an Amersham Biosciences PD-10 desalting column pre-equilibrated with standard buffer to remove the unbound CPM. The labeled P450 reductase (P450 reductase-CPM) was concentrated to 0.4±0.1 μM P450 reductase-CPM. The dissociation constants for the binding of P450 reductase or biliverdin reductase to hHO-I265 and hHO-I233 were determined by FRET experiments.

**Determination of the Binding Affinity of hHO-I265 with P450 Reductase and Biliverdin Reductase by Fluorescence Resonance Energy Transfer (FRET)**—The fluorescent probe CPM has an emission maximum at 455 nm and an excitation maximum at 385 nm. The UV-visible spectrum of hHO-I265 overlaps the fluorescence emission spectrum of CPM, so formation of a complex of hHO-1 with either P450 reductase-CPM or biliverdin reductase-CPM results in fluorescence quenching. Since hHO-I265 has significant absorption at 385 nm, preliminary experiments were performed to determine the optimal excitation wavelength. Based on the preliminary results, an excitation wavelength of 350 nm was used for all of the FRET experiments. All of the binding assays were performed on a SPECTRAmax™ M200 dual-scanning microplate spectrofluorometer (Molecular Devices Corp., Sunnyvale, CA). The concentration of P450 reductase-CPM or biliverdin re-
ductase-CPM was fixed at 0.05 μM, and that of hHO-1 was varied. All of the assays were carried out in standard buffer at room temperature (25 °C).

**Competitive Binding of P450 Reductase and Biliverdin Reductase to hHO-1**—The concentration of labeled protein was fixed at 0.05 μM, and that of hHO-1 was varied. The binding assay was performed in the presence of the competitor protein. RNase A was used as a negative control in these experiments. The percentage of quenching of the 455-nm emission was plotted against the concentration of hHO-1. To determine whether the inhibition of quenching caused by the competitor protein occurs in a dose-dependent manner, the concentration of the competitor protein was varied while the concentrations of hHO-1 and the labeled protein were held constant at 0.5 and 0.05 μM, respectively. The percentage of fluorescence quenching at 455 nm was recorded and plotted against the concentration of competitor protein. All of the experiments were performed in standard buffer at 25 °C.

**Electrostatic Potential Surface and Computer Modeling of the Docking Regions of hHO-1 and P450 Reductase**—GRASP (Graphical Representation and Analysis of Structural Properties) is a molecular visualization and analysis program (44). It was used to analyze and display the electrostatic properties of hHO-1 and P450 reductase. The program GRAMM (Global Range Molecular Matching) was used to predict the structure of the complex of hHO-1 with P450 reductase (45). The program was run at low resolution with the following parameters: mmode = generic, eta = 6.8, ro = 6.5, fr = 0, crang = grid-step, ccti = gray, crep = all, maxm = 1000, and ai = 20. The 20 best matches were used as the output. The most reasonable prediction was selected based on the mutagenesis studies and the electrostatic properties of the two proteins.

**RESULTS**

**Determination of the Binding Affinity of hHO-1 for P450 Reductase**—To optimize the conditions for the FRET experiments to determine the binding affinity of hHO-1 for P450 reductase, the excitation spectrum of P450 reductase-CPM was determined with the emission wavelength at 450 nm. Based on the data in Fig. 1A, P450 reductase-CPM has a maximum excitation wavelength at 385 nm. Unfortunately, hHO-1, with a Soret maximum at 404 nm, has significant absorption at this wavelength (Fig. 1B). In order to minimize the absorption of hHO-1 at the excitation wavelength, the emission spectrum of labeled P450 reductase was measured at several dif-

![Fig. 4. A, docking model of P450 reductase and hHO-1 generated by GRAMM. B was obtained by rotating A by 180°. The docking interface is shown after detaching the two proteins and rotating them by 90 and 180°: hHO-1 (C–E) and P450 reductase (F–H). The electrostatic potential surfaces of the hHO-1 and P450 reductase are shown in color, where blue is electropositive, red is electronegative, and white is neutral.](image-url)
ferent excitation wavelengths (380, 350, 340, and 330 nm) at increasing distances from the maximum of 385 nm. As shown in Fig. 2, the excitation wavelength of 350 nm gave the best emission spectrum with the lowest background. In contrast, the emission intensities were too low upon excitation at 330 or 340 nm, and the background was relatively high. The FRET experiments were therefore carried with excitation at 350 nm.

Since the UV-visible absorption spectrum of hHO-1265 overlaps the emission spectrum of P450 reductase-CPM, the formation of the complex between these two proteins can be monitored by fluorescence quenching (Fig. 3A). Plotting the percentage of quenching at 435 nm against the concentration of the hHO-1265 comprising residues 1–265 gives a well behaved binding curve (Fig. 3B) from which a $K_d$ value of 0.4 ± 0.1 µM can be determined (Table I). When the same experiment was performed with hHO-1233 that had been further truncated to a length of 233 amino acids, the dissociation constant was found to be $K_d = 0.7 ± 0.1$ µM (Table I).

**The Electrostatic Potential Surfaces of P450 Reductase and hHO-1**—We used the GRASP program to define the electrostatic potential surfaces of P450 reductase (Fig. 4, F–H) and hHO-1233 (Fig. 4, C–E) (44). The surface of hHO-1 around the exposed heme is primarily positively charged, and that of P450 reductase in the vicinity of the FMN group is predominantly negatively charged. It has been reported that cytochrome P450 2B4 interacts with P450 reductase through complementary charge interactions (30). The crystal structure of the complex between the heme- and FMN-binding domains of cytochrome P450$_{2B4}$, a bacterial enzyme in which the heme-containing P450 domain and the FAD and FMN P450 reductase domains are part of a single polypeptide, shows that binding of the FMN domain occurs on the electropositive surface surrounding the proximal face of the heme domain (46). These results suggest that P450 reductase and hHO-1 may also interact through complementary electrostatic charges. The electropositive hHO-1 surface around the exposed heme is undoubtedly the ideal site for the binding of P450 reductase, since it provides a short electron transfer path from the flavins to the heme.

**Analysis of the Interface between hHO-1$_{265}$ and P450 Reductase by Mutagenesis**—The residues in hHO-1 involved in binding of P450 reductase should be relatively well conserved and solvent-accessible. We therefore aligned seven mammalian heme oxygenase amino acid sequences, including HO-1, HO-2, and HO-3 (accession numbers XP-036680, AAH02011, and O70453, AAA19130, XP_009946, P06762, and AAH10757). The relatively conserved, solvent-accessible and positively charged residues on the surface surrounding the exposed heme (Lys$_{18}$, Lys$_{22}$, Arg$_{35}$, Lys$_{39}$, Lys$_{179}$, Arg$_{183}$, and Arg$_{185}$) are labeled on the hHO-1$_{233}$ space-filling structure (Fig. 5A). Since P450 reductase is much larger than hHO-1, the interface may also cover some residues on the edge of hHO-1. We therefore also selected some residues close to Lys$_{18}$, Lys$_{22}$, and Arg$_{183}$ for mutagenesis. In this case, not only positively charged but also negatively charged residues were selected: Asp$_{12}$, Glu$_{15}$, Glu$_{19}$, Glu$_{23}$, Glu$_{127}$, Glu$_{190}$, Arg$_{198}$, and Glu$_{201}$ (Fig. 5B). Alanine scanning mutagenesis was performed for all of these residues, and the binding affinity of the mutant hHO-1$_{265}$ proteins with P450 reductase was measured by FRET.

All of the mutant proteins had a Soret maximum at 404 nm, $R_2$ ($A_{440}/A_{260}$) values in the range of 2.3–3.5 and at least 90% purity by SDS-PAGE. As shown in Table II, some of the mutants bind P450 reductase with 3–7-fold lower binding affinities. Most of the residues whose mutation to an alanine caused a significant decrease in binding affinity are positively charged and include Lys$_{18}$, Lys$_{22}$, Lys$_{179}$, Arg$_{23}$, Arg$_{48}$, Arg$_{185}$, and Arg$_{186}$ (Fig. 5, A and B). Only three, Glu$_{19}$, Glu$_{127}$, and Glu$_{190}$, are negatively charged. Mutation of the other residues has little or no effect on the P450 reductase binding affinity.

**Computer Modeling of the Docking of hHO-1$_{233}$ and P450 Reductase**—The GRAMP program is a geometric approach to identifying the docking interface between two proteins (45). In this program, the protein structure is simplified to digitized images on a three-dimensional grid, and the molecular surface complementarity is then estimated. Molecular details such as electrostatic charges are not considered. In the present study, the GRAMP program was run at low resolution, and the 20 best matches were examined. Among the 20 possible matches, all of which are quite different, the most reasonable model was selected based on the conclusion from the mutagenesis data that the binding site should be located around the exposed hHO-1 heme edge and close to Lys$_{18}$ and Lys$_{22}$ (Fig. 4, A and B). In this predicted complex, the surface of hHO-1$_{233}$ at the interface is primarily electropositive, and that of P450 is reductase electronegative. The proximal hHO-1 surface is close to the FMN domain of P450 reductase, from which the electrons are transferred to the heme. The model therefore satisfies the surface electrostatic requirements; the findings of the alanine scanning mutagenesis, and the known electron transfer properties of P450 reductase-hemoprotein interactions.

**Determination of the Binding Affinity of hHO-1$_{265}$ for Biliverdin Reductase**—Rat biliverdin reductase has three cysteines. Mutation of one of these three to an alanine was reported in one study to inactivate the enzyme (33), but in a second study it was found to have little effect on activity (34). Mutation of the other two cysteines did not alter activity. Biliverdin reductase was therefore labeled with the fluorescent probe CPM essentially as done for P450 reductase. Replacement of biliverdin reductase with the labeled biliverdin reductase in an otherwise standard heme oxygenase assay mixture showed that the labeled protein retained almost (86%) full activity. Although the thiol(s) labeled with CPM have not been identified, the present result is consistent with the report that mutation of the cysteine(s) does not alter catalytic activity (34).

Formation of the complex between biliverdin reductase-CPM and hHO-1$_{265}$ causes fluorescence quenching (Fig. 6A). A plot of the percentage of quenching at 455 nm against the concentration of hHO-1$_{265}$ yields a normal binding curve (Fig. 6B) from which $K_d = 0.2 ± 0.1$ µM is calculated (Table I). This value is comparable with that for the binding of P450 reductase (0.4 ± 0.1 µM). If the same experiment was performed with hHO-1$_{233}$ that had been further truncated to 233 residues, the dissociation constant was found to be $K_d = 0.5 ± 0.1$ µM (Table I).

The binding affinity of hHO-1 for biliverdin reductase has also been examined with the mutant proteins (Table II). The effect of alanine substitutions on the binding affinity is less...
marked for biliverdin reductase than P450 reductase. Nevertheless, the K18A, K22A, K179A, R183A, and R185A mutants have approximately a 3-fold weaker binding affinity for biliverdin reductase. These residues are located on one side of the heme binding crevice and overlap the proposed binding site for P450 reductase. The conversion of ferric heme to biliverdin, CO, and Fe^{2+} catalyzed by hHO-I requires the transfer of seven electrons, one at a time, from NADPH-cytochrome P450 reductase to cytochrome P450. Earlier single-turnover kinetic studies suggested that the rate-limiting step in the process of heme degradation, heme oxygenase catalyzes the oxidation of heme to biliverdin, CO, and free ferrous iron. This multistep transformation is supported by P450 reductase. The biliverdin product is subsequently reduced by biliverdin reductase to bilirubin. Earlier single-turnover kinetic studies suggested that the release of biliverdin from hHO-I was accelerated in the presence of biliverdin reductase (17). Here we have used a competition binding assay to determine whether the binding sites for P450 reductase and biliverdin reductase on hHO-I are the same, overlap, or are completely different. As shown in Fig. 7A, biliverdin reductase inhibits the binding of P450 reductase to hHO-I and Fig. 7B shows that this inhibitory effect is concentration-dependent. In contrast, RNase A, a negative control, has no effect on the binding of P450 reductase. Similarly, P450 reductase inhibits the binding of biliverdin reductase to hHO-I in a concentration-dependent manner (Fig. 7, C and D). These results indicate that the binding sites on hHO-I for biliverdin reductase and P450 reductase either directly overlap or are allosterically modulated. It is interesting in this context that the binding affinities for hHO-I for P450 reductase and biliverdin reductase are quite similar.

**DISCUSSION**

The conversion of ferric heme to biliverdin, CO, and Fe^{2+} catalyzed by hHO-1 requires the transfer of seven electrons, one at a time, from NADPH-cytochrome P450 reductase to hHO-1. These electron transfers are thought to occur within transient hHO-I-P450 reductase complexes, in the same way that transfer of electrons from P450 reductase to cytochrome P450 is thought to occur. Here we have directly demonstrated by FRET the formation of a complex of P450 reductase with heme-bound hHO-I and have determined that its formation occurs with $K_d = 0.4 \pm 0.1 \mu M$ (Table I). This value is to be compared with the 10-fold higher affinity measured by the FRET technique for the binding of P450 reductase to cytochrome P450 2B4 ($K_d = 0.038 \mu M$) (47). At least part of the difference in these absolute values reflects the fact that binding to CYP2B4 was determined in incubations in which both proteins retain their membrane binding domains, whereas the present results have been obtained with soluble, truncated proteins from which the membrane-binding domain has been deleted. Comparison of the $K_d$ values for the binding of P450 reductase to hHO-I, lacking only the membrane-binding domain, or a further truncated hHO-I suggests that the 32-amino acid difference between the two proteins slightly decreases the ability of the protein to bind P450 reductase. The $K_d$ values indicate that the affinity for the hHO-I protein is 56% of that hHO-I (Table I). The catalytic activity of the shorter protein in the present work was determined to be 86% of that of the longer protein. Since the available crystal structure is that of hHO-I, the location of the additional residues in the structure is not known, although the present results suggest that their placement only modestly strengthens the binding of P450 reductase.

A single-turnover study of the kinetics of the hHO-I reaction indicated that the rate-limiting step in the in vitro assay was the dissociation of biliverdin from hHO-I (17). The addition of biliverdin reductase accelerated biliverdin dissociation and made one of the earlier electron transfer steps rate-limiting. The resulting inference that binding of biliverdin reductase to hHO-I might lead to either direct transfer of the
biliverdin from one protein to the other or to allosteric acceleration of its release is confirmed by the present FRET studies using CPM-labeled biliverdin reductase. Control experiments established that the binding of CPM to the sulphydryl groups of biliverdin reductase did not significantly decrease its catalytic activity. Quenching of the fluorescence of the CPM-labeled biliverdin reductase by truncated hHO-1265 directly demonstrates that the two proteins form a complex with $K_d$ = 0.2 ± 0.1 μM (Table I). This value is surprisingly similar to that for formation of the complex of P450 reductase with hHO-1265 ($K_d$ = 0.4 ± 0.1 μM). As found for the binding of P450 reductase, biliverdin reductase binds more tightly to hHO-1265 than to hHO-1233 (Table I). The biliverdin reductase binding affinity of the shorter hHO-1 protein is 45% of that to the longer version. The similar effect of the 32-amino acid difference between the two proteins on the binding of P450 reductase and biliverdin reductase is consistent with location of some of these residues in hHO-1 in the vicinity of the binding sites for P450 reductase and biliverdin reductase.

Analysis of the effect of unlabeled biliverdin reductase on the binding of labeled P450 reductase to hHO-1265, and conversely of the effect of unlabeled P450 reductase on the binding of labeled biliverdin reductase, establishes that a competition exists for binding of these two proteins to hHO-1. This competition can be explained by the existence of a common site for both proteins, two different but overlapping binding sites, or two distinct but allosterically linked sites. In principle, biliverdin reductase can potentially inhibit the turnover of hHO-1 by blocking formation of the requisite electron transfer complex between hHO-1 and P450 reductase. P450 reductase can also,
in principle, inhibit hHO-1 activity by decreasing the binding of biliverdin reductase and thereby slowing down product release.

Site-specific mutagenesis studies were carried out to elucidate the binding site for P450 reductase on hHO-1265. Based on an alignment of known mammalian heme oxygenase sequences and the crystal structures of the human, rat, and bacterial enzymes, conserved surface-exposed charged residues surrounding the exposed heme edge were identified and were mutated to alamines. The effects of the mutations on the binding affinity of hHO-1265 for P450 reductase were then determined (Table II). These mutagenesis studies identified the positively charged residues Lys149, Lys153, Arg12, Glu15, Glu23, and Glu201, as sites whose mutation significantly increased the $K_d$ value for the binding of P450 reductase (Fig. 5, A and B). Mutation of a number of other residues, including Arg29, Lys149, Lys153, Asp12, Glu15, Glu23, and Glu201, had little or no effect on the binding of P450 reductase. In accord with the negative charged surface of P450 reductase in the vicinity of the FMN group, from which electrons are transferred to the heme group of acceptor proteins, most of the mutations that significantly decreased P450 reductase binding involved conversion of a positively charged side chain on hHO-1265 to a neutral side chain. Three mutations of carboxylate groups to neutral groups also decreased binding, however, presumably because the binding surfaces have localized regions of opposing charge despite the overall electrostatic potential of the surface (Fig. 4). Previous results have provided evidence that the binding of P450 reductase to CYP2B1 (26), CYP2B4 (29, 30), and CYP1A2 (28) is facilitated by interactions of positively charged residues on the hemoproteins with negatively charged carboxylic acid residues on P450 reductase (27). The present results with hHO-1265 are consistent with the findings for cytochrome P450 enzymes. One exception to this was the finding that the interaction of the reductase domain with the heme domain in cytochrome P450BM-3 is an enzyme in which both domains are normally part of a common polypeptide, is subject to repulsive rather than attractive ionic interactions (30). It was postulated that this reflected the fact that the two domains were already tethered together and did not depend on ionic interactions.

The mutagenesis results, combined with a computational analysis of the protein surfaces using the algorithm GRAMM, have been used to select a probable binding interface for the binding of P450 reductase to hHO-1265 (Fig. 4). GRAMM provides a docking view that ignores molecular level details and is therefore not accurate enough to identify and position the interactions of individual residues (18). Nevertheless, it can be used to determine the general binding site, location, and orientation of a docked protein. As shown in Fig. 4, a reasonable docking site for the small hHO-1 protein exists adjacent to the FMN domain of P450 reductase, a docking arrangement that places the heme and FMN groups in close proximity to each other.

Analogous site-specific mutagenesis studies have partially defined the binding site for biliverdin reductase on hHO-1265. The $K_d$ value for the binding of these two proteins was significantly increased (>5-fold) by K18A, K22A, K179A, R183A, and R185A hHO-1265 mutations (Table II). Mutations at all of the other hHO-1265 residues that were examined did not greatly alter the binding affinity for biliverdin reductase. Although the $K_d$ value for the binding of biliverdin reductase is comparable with that for P450 reductase, a small difference was observed in the maximum change observed in the alanine scanning mutagenesis results for these two proteins. The maximum change observed for biliverdin reductase was a factor of 3.9 for the K179A mutation, whereas the maximum change in the case of P450 reductase was a factor of 5.7 for the R198A mutation. This slight difference is probably not significant.

It has not been possible to model the binding of biliverdin reductase to hHO-1265 despite the fact that the crystal structures of both proteins are available. The alternatives provided by GRAMM could not be readily distinguished on the basis of the mutagenesis data. Nevertheless, inspection of Fig. 5 shows that the residues that are most critical for the binding of biliverdin reductase cluster along one edge of the site that is important for the binding of P450 reductase and include some of the same residues that are involved in the binding of P450 reductase. This overlap in the binding sites defined by mutagenesis explains the observation that the binding of biliverdin reductase competes with that of P450 reductase.

In summary, we have provided evidence here that P450 reductase and biliverdin reductase bind competitively to hHO-1265, have partially defined the residues involved in binding each of these two proteins, and have established that the binding sites for the two proteins partially overlap. The competitive nature of the binding of the two proteins, together with the evidence that biliverdin reductase accelerates catalytic turnover by promoting product release, could result in regulation of the activity of hHO-1 by the relative concentrations of the two proteins. However, it is to be remembered that the full-length hHO-1 and P450 reductase are membrane-bound, whereas biliverdin reductase is a cytosolic protein. Partitioning of the first two proteins into the membrane would effectively concentrate them and is likely to enhance their interaction at the expense of the interaction of hHO-1 with biliverdin reductase.

Acknowledgment—We thank Giselle Knudsen for help with the GRASP and GRAMM algorithms.

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