Chiral Orientation of Prosthetic Heme in the Cytochrome P-450 Active Site*

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The absolute orientation of the prosthetic heme group in the active site of a hemoprotein may influence its substrate selectivity and catalytic properties. The only method available until now to determine the chiral orientation of the heme in a hemoprotein has been a high resolution X-ray crystallography. The orientation of the heme in cytochrome P-450, therefore, is unknown because a crystallographic structure is not available for any form of this enzyme. We report here that the absolute configurations of the N-ethylprotoporphyrin IX adducts formed from the prosthetic hemes of cytochrome P-450 and hemoglobin during catalytic turnover of appropriate substrates are identical. The prosthetic heme in the inactivated cytochrome P-450 enzyme, therefore, has exactly the same orientation, relative to the fifth iron ligand, as the heme in hemoglobin. The approach described here can be used to determine the prosthetic heme orientation in other hemoproteins, including other cytochrome P-450 isozymes, for which X-ray structures are not available.

Major advances have been made in our understanding of the function, structure, and mechanism of cytochrome P-450 enzymes during recent years. The existence of multiple isozymes that differ in molecular weight, chromatographic and spectroscopic properties, tryptic peptide maps, terminal amino acid sequences, immunological reactivity, and substrate specificity is now well established (for reviews see Refs. 1-5). The structure and catalytic specificity of otherwise identical enzymes during recent years. The existence of multiple isozymes that differ in molecular weight, chromatographic and spectroscopic properties, tryptic peptide maps, terminal amino acid sequences, immunological reactivity, and substrate specificity is now well established (for reviews see Refs. 1-5). The structure and catalytic specificity of otherwise identical isozymes are identical. The prosthetic heme orientation in other hemoproteins, including other cytochrome P-450 isozymes, for which crystallographic structures are not available.

EXPERIMENTAL PROCEDURES

N-Ethylprotoporphyrin IX Regiosomers—The four N-ethylprotoporphyrin IX regiosomers formed in phenobarbital-pretreated rats injected with DDEP were isolated as the dimethyl esters and were purified as described previously (19). The procedure for isolation of the same N-ethyl isomers from nuclease isolated hemoglobin with ethylhydrazine has also been reported (24). The samples used in the present study were obtained by stirring hemoglobin with propyne (21) and with the 4-methyl analogue of DDEP (3,5-dicarbethoxy-1,4-dihydrocollidine) (23) are optically active. We have been unable, however, to determine the crucial degree of stereospecificity or the absolute orientation of the heme. Our recent discovery that hemoglobin reacts with ethylhydrazine to give the N-ethyl porphyrins obtained with DDEP (24) provides the key to this stereochemical impasse because the absolute stereochemistry of the hemoglobin adduct can be deduced from the known active site geometry of this hemoprotein. We report here the use of this novel approach to establish the absolute orientation of the prosthetic heme group in the rat cytochrome P-450 isozyme inactivated by DDEP. The chemical reactivity of hydrazine derivatives with heme (25), furthermore, suggests that heme adducts can be used to determine the prosthetic group stereochemistry in other hemoproteins for which crystallographic structures are not available.

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The abbreviation used is: DDEP, 3,5-bis(carbethoxy)-2,6-dimethyl-4-ethyl-1,4-dihydropropyridine.
the corresponding chlorozinc complexes for spectroscopic studies (18, 19).

Circular Dichroism Spectroscopy—The individual chlorozinc N-ethylprotoporphyrin IX isomers from cytochrome P-450 and from hemoglobin were dissolved in spectroscopic-grade chloroform. The concentration of each solution was adjusted to approximately the same value using a molar absorbance coefficient at 431 nm of 124,000 M\(^{-1}\) cm\(^{-1}\) (23). The circular dichroism spectra were recorded on a Jasco J-500A spectropolarimeter at 23 °C (2-cm cells, time constant 4, 2.5 cm/min). The spectra were corrected by subtraction of the baseline observed in the absence of the porphyrins.

**RESULTS AND DISCUSSION**

The four regioisomers of N-ethylprotoporphyrin IX are isolated from phenobarbital-pretreated rats injected with DDEP but one of these, the isomer with the N-alkyl group on pyrrole ring A, is quantitatively the most important (19). The same four regioisomers are obtained in the reaction of ethylhydrazine with human hemoglobin except that here there is a strong preference for N-alkylation of the nitrogen in pyrrole ring C. The spectroscopic comparison of the porphyrins obtained from cytochrome P-450 and hemoglobin has been made with one pure regioisomer because the light rotatory properties of the N-ethylprotoporphyrin IX chromophore depend on the nitrogen which bears the N-alkyl group (see discussion below). The isomer alkylated on pyrrole ring C, shown by NMR to be free of contamination by other isomers (19), has been chosen for this purpose. The circular dichroism spectra of the ring C isomers derived from the two hemoproteins are identical in sign and amplitude (Fig. 2). The two N-ethyl porphyrins, therefore, not only have the same absolute configuration but also, in view of the identical molar ellipticity values, are of equal optical purity.

The absolute configuration of the heme prosthetic group in hemoglobin is that on the left in Fig. 1 (15). We have recently established that carbon radicals are liberated in the reaction of alkyl and aryl hydrazines with heme-bound oxygen and have shown that, at least in the case of phenylhydrazine, the phenyl group is covalently bound to the heme iron before it undergoes a 1,2-shift to one of the porphyrin nitrogen atoms (24, 26). The N-alkyl function in hemoglobin consequently is introduced from the side of the porphyrin to which molecular oxygen is bound. The ring C adduct from hemoglobin, therefore, can be assigned the R absolute configuration shown in Fig. 3. Alkylation of the cytochrome P-450 prosthetic heme during suicidal metabolism of DDEP and other substrates also requires that alkylation occur from the side of the heme which faces the activated oxygen species. The heme prosthetic group of the major isozyme induced by phenobarbital (an isozyme highly vulnerable to destruction) (19) consequently must, like hemoglobin, be oriented exclusively as shown on the left in Fig. 1. If the prosthetic group were present in two different orientations, the circular dichroism bands of the adduct should have been smaller in amplitude than those of the hemoglobin-derived porphyrin due to the inverse absorption of the opposite enantiomer. 2 For the same reason, the prosthetic heme must also have the same absolute orientation in all the other minor but still quantitatively significant hepatic isozymes destroyed by DDEP in phenobarbital-pretreated rats.

The importance of using a single pure regioisomer of the heme adduct in studies of this kind is emphasized by our finding that the circular dichroism spectra of the four isomers of N-ethylprotoporphyrin IX obtained from cytochrome P-450 are different (not shown). The sign of the strong circular dichroism peak at approximately 435–445 nm, for example, is positive for the zinc complexes of the isomers alkylated on rings A, C, and D (isomers II, III, and IV, respectively, in Ref. 19) but is negative for the isomer alkylated on ring B (isomer I). The signs and positions of other peaks in the spectra of the isomers also differ. The circular dichroism spectrum reported previously for the ring A isomer of N-(2-oxopropyl)-

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2 The two enantiomers of N-ethylprotoporphyrin IX, like all enantiomers, are expected to have circular dichroism spectra that are identical but of opposite sign.
protoporphyrin IX (21) obtained with propyne, however, is very similar to that of the ring A isomer of the N-ethyl structure obtained here. It can now be assigned the absolute stereochemistry expected if alkylation occurs from the same side as the addition of the N-ethyl in Fig. 3. The circular dichroism spectrum of the ring D isomer of N-(2-hydroxyethyl)protoporphyrin IX obtained with ethylene (20) is likewise virtually the same as that of the corresponding N-ethyl derivative (not shown) and must also have arisen by addition of the substrate to the heme from the same side.

The method developed for the present study is applicable not only to the study of cytochrome P-450 enzymes but also, if appropriate suicide substrates are used, to other hemoproteins that have a vacant coordination site (or an exchangeable ligand) on the prosthetic heme iron. The fact that N-alkylation occurs during the reaction of hydrazine derivatives with hemin itself (25) suggests that compounds of this class may be used to explore the orientation of heme in other hemoproteins, including the peroxidases and various carrier proteins.

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