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PROPERTIES OF THE TRYPTOPHAN RESIDUE IN RABBIT LIVER MICROSOMAL CYTOCHROME P-450 ISOZYME 2 AS DETERMINED BY FLUORESCENCE

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SUMMARY: Cytochrome P-450 isozyme 2 from rabbit liver microsomes fluoresces upon excitation at 295 nm due to the single tryptophyl residue (Trp_{121}) in the protein. The fluorescence spectrum, which is not altered by the presence of phospholipid or substrates, has a maximum at 335 nm, which suggests that the environment of the residue is hydrophobic. The fluorescence intensity decreases linearly with increase of specific content of the cytochrome preparations, and the holoenzyme was estimated to exhibit, at most, 6% as much fluorescence as the apoenzyme. This indicates that the fluorescence of the tryptophan is quenched by energy transfer to the heme. The distance between the tryptophyl residue and the heme was estimated to be less than 40 Å. From enhancement of the fluorescence by methanol and ethanol, 30 to 50% of the Trp residue was found to be accessible to these solvents. On the other hand, the accessibility to iodide and cesium ions, as estimated by quenching effects, is less than 14%. From such evidence, the tryptophyl residue is believed to be partly buried. Since Trp_{121} is conserved at or near the same position in all mammalian P-450's so far sequenced, the results obtained may be applicable to these related cytochromes as well.

Cytochrome P-450 enzymes bring about the activation of molecular oxygen or peroxy compounds in the monooxygenation of a large variety of lipophilic substances, including both physiologically occurring and foreign compounds (1). Although a number of such proteins have been purified and partially or completely sequenced, as reviewed elsewhere (2), much remains to be learned about structure-function relationships. A striking difference found in the first mammalian P-450's to be submitted to amino acid analysis was the presence of a single tryptophan residue in phenobarbital-inducible rabbit liver microsomal isozyme 2 and seven residues in 5,6-benzoflavone-inducible isozyme 4 (3). The occurrence of a single Trp residue in isozyme 2, which has recently been confirmed by sequencing with assignment to position 121 in the polypeptide chain (4), makes this protein of particular interest for fluorescence analysis.

Hemeproteins are generally non-fluorescent, which may be explained by the action of the heme as a sink to accept energy from excited aromatic amino acid residues in the protein (5). However, some hemeproteins have been reported to emit weak fluorescence, including hemoglobin (6) and rabbit P-450 isozymes 2

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and 4 (7), which indicates that in these proteins the distance between the heme and certain tryptophyl residues might be greater than the Förster critical distance for energy transfer (6). The observation that action spectra for the photodissociation of carbon monoxide-heme complexes of respiratory enzymes show a peak in the ultraviolet absorption region of the protein led to the proposal (8) that the energy of the excited amino acid residues was transferred to the heme and thus caused the dissociation (9).

In the present communication we describe the fluorescence properties of the Trp_{121} residue in rabbit liver microsomal cytochrome P-450 isozyme 2. The results may be pertinent to other forms of P-450 in view of the known conservation of a Trp residue at or near position 121 in all such mammalian cytochromes so far sequenced (2).

EXPERIMENTAL PROCEDURES

The isolation of rabbit liver microsomal P-450 isozyme 2 and the spectral methods used in the present work were described previously (3). d-Benzphetamine was kindly provided by the Upjohn Co.; cyclohexane was obtained from Aldrich, dilauroylglyceryl-3-phosphorylcholine from Calbiochem-Behring, and N-acetyl-L-tryptophanamide from Sigma. All fluorescence spectra were measured at 25°C in 25 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA, with use of an SLM-Aminco 8000 recording spectrofluorometer. Correction of fluorescence spectra for background, including Raman scattering, was done automatically by the instrument. A wavelength of 295 nm was selected for excitation of the tryptophyl residue in the cytochrome, and the concentrations of the samples to be analyzed were selected to give an A_{295} of less than 0.05. The emission bandpass was set at 2 nm and the excitation slit width at 8 nm.

RESULTS AND DISCUSSION

Fluorescence Spectrum of P-450 Isozyme 2: A typical fluorescence emission spectrum for P-450 isozyme 2 upon exposure to light at 295 nm, which is known to excite only Trp residues in proteins, is shown in the inset to Fig. 1. The peak has a maximum at 335 nm. Electrophoretically homogeneous preparations of this cytochrome varying in specific content from 6.7 to 16.4 nmol of P-450 per mg of protein, and thus differing in the amount of apoenzyme present, were examined. In all instances the emission spectra had the same shape with a maximum at 335 nm. Thus, the environmental state of the tryptophyl residue appears to be independent of the heme content of the preparation. As indicated in Fig. 1, the relative fluorescence at 335 nm (F_{335}) decreases in a linear fashion with respect to increasing specific P-450 content of the preparations. According to a linear least squares regression analysis, the relative fluorescence of 100% apoenzyme would be 13.9 ± 0.4 and that of 100% holoenzyme (with an apparent specific content of 19.1 ± 0.6 nmol of P-450 per mg of protein) would be zero. At the maximal specific content of 18.0, calculated from the molecular weight of 55,700 as determined from the amino acid sequence (4), the F_{335} value was estimated to be 6% that of the apoprotein. Thus, almost all of the observed fluorescence of the cytochrome must come from the apoenzyme in the

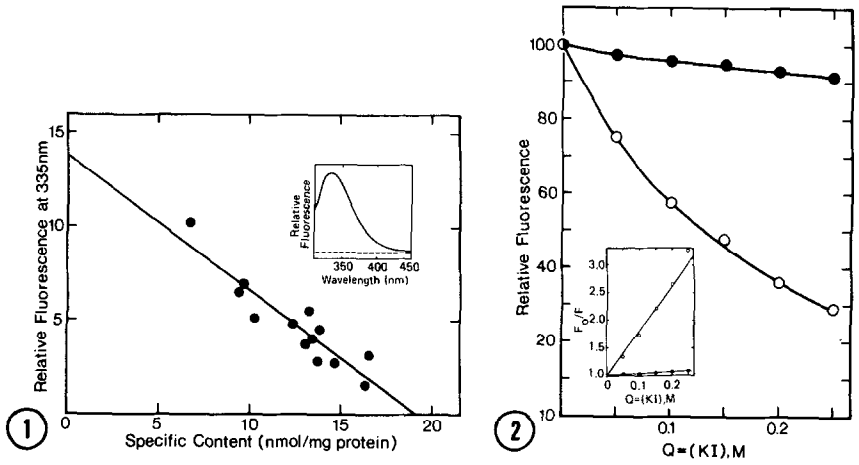

Fig. 1. Inset, fluorescence emission spectrum of P-450 isozyme 2 upon excitation at 295 nm. The P-450 concentration was 9.1 x 10^{-7} M and the specific content was 13.5 nmol per mg of protein.

Main figure, relationship between relative fluorescence intensity at 335 nm and the specific content of different P-450 isozyme 2 preparations. For comparison, all results are expressed per mg of protein per ml.

Fig. 2. Quenching by potassium iodide of fluorescence of P-450 isozyme 2 (•) with excitation at 295 nm and emission at 335 nm and of N-acetyltryptophanamide (o) with excitation at 295 nm and emission at 355 nm. KI was varied up to 0.25 M in the usual buffer mixture containing 5.4 x 10^{-7} M P-450 (specific content, 13.5 nmol per mg protein), or of 1.1 x 10^{-5} M acetyltryptophanamide. KCl, which has no quenching effect, was added to bring the sum of KI and KCl to 0.25 M. Inset, Fo/F vs. the iodide concentration.

preparations; until methods become available for the isolation of the apo- and holoenzymes completely free of each other, the value of 6% for the contribution of the latter should be considered provisional. In experiments not shown, phospholipid and substrates had no effect on the fluorescence of P-450 isozyme 2.

Quenching of Fluorescence of the Tryptophyl Residue by Iodide: The effects of ions which are known to quench the fluorescence of other proteins (10,11) were tested with P-450 isozyme 2. Figure 2 shows that the relative fluorescence intensity of the cytochrome decreased slightly with increasing KI concentrations. No P-420 was formed, even at the highest KI level used. In contrast, much more extensive quenching was observed with the model compound, N-acetyltryptophanamide, reaching about 50% at 0.13 M and 70% at 0.25 M iodide. As shown in the inset to Fig. 2, Fo/F, the ratio of the relative fluorescence in the absence of KI to that in the presence, was plotted against the iodide concentration. According to the Stern-Volmer equation (10), Fo/F equals 1 + $K_Q[Q]$ where [Q] is the concentration of the quencher and K_Q is the dissociation constant for the complex between the quencher and the tryptophyl residue. The K_Q values of iodide for the cytochrome and the model compound were estimated to be 0.38 and 8.0 M^{-1}, respectively. In experiments not shown, the quenching

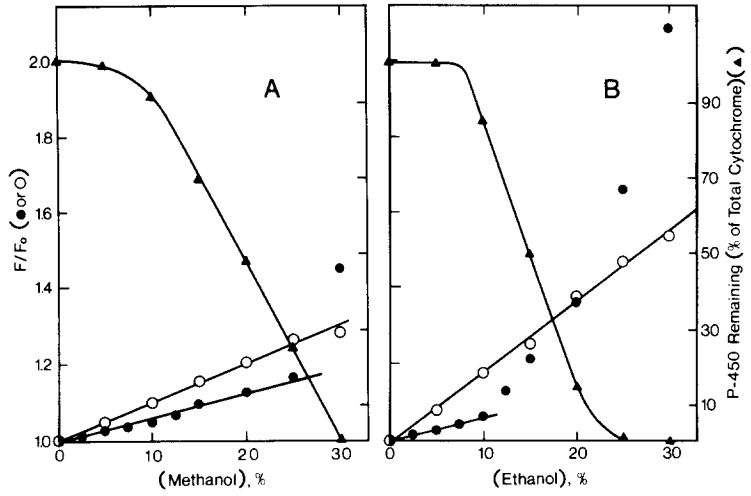

Fig. 3. Solvent perturbation of the fluorescence of 4.1×10^{-7} M P-450 isozyme 2 (•) and 7.2×10^{-6} M N-acetyltryptophanamide (o) in the presence of varying concentrations of methanol (A) or ethanol (B). The specific content of the P-450 was 13.5 nmol per mg protein. To the cytochrome in the usual buffer mixture, a solution containing up to 60% (v/v) of ethanol or methanol in the same buffer mixture was added. The solutions were mixed and allowed to stand at 25°C for 15 min before measurements were made. The excitation and emission wavelengths were as in Fig. 2. The specific content of the P-450 preparation (Δ), which changed due to P-420 formation, was determined in the presence of the alcohol concentrations indicated.

effect of CsCl at up to 1.0 M concentration on the tryptophyl fluorescence was examined. The fluorescence emission of acetyltryptophanamide at 355 nm was strongly quenched, with a K_Q value of 1.9 M^{-1}, whereas that of the tryptophyl residue of P-450 isozyme 2 at 335 nm was quenched only about 10% even at the highest CsCl concentration used.

Enhancement of Tryptophyl Fluorescence by Methanol and Ethanol: Some organic solvents such as ethanol, methanol, dioxane, and ethylene glycol have been reported to enhance tryptophan fluorescence (12,13). When perturbation of fluorescence is used to examine the solvent accessibility of the aromatic amino acids in a protein, the effects of conformational change need to be considered. As shown in Fig. 3, the fluorescence of both P-450 isozyme 2 and N-acetyltryptophanamide is increased in the presence of methanol or ethanol. No shift in the wavelength maximum of the emission peak of the model tryptophan-containing compound was observed in the presence of 30% methanol or ethanol, where the dielectric constant is considered to be 60 to 70 (14). Furthermore, the wavelength maximum of the fluorescence emission of the cytochrome remains at 335 nm in the presence of the alcohols, thus indicating that no sizeable conformational change is induced around Trp_{121}.

The results presented show that the fluorescence of the model compound increases linearly with increasing alcohol concentration over the range used and that of the cytochrome increases linearly over a narrower range and then

more extensively at higher levels of methanol and, especially, ethanol. Comparison of the slopes in the linear ranges permits the accessibility of the tryptophyl residue to the solvent to be estimated. The accessibility of the protein to methanol (ave. molecular diameter, 2.8 Å (15)) and to ethanol (probable ave. molecular diameter, 3.5 Å) was estimated to be 50 and 30%, respectively. In the range of alcohol concentrations which gave linear plots, more than 85% of the cytochrome remained as P-450 rather than P-420. At higher solvent concentrations (20 to 30% methanol or 10 to 30% ethanol) the greater increase in accessibility is correlated with P-420 formation and probably represents conformational change even though Trp_{121} apparently remains in a largely hydrophobic environment as indicated by the fluorescence peak remaining at 335 nm.

DISCUSSION

The peak position in the fluorescence spectrum of tryptophan is generally dependent on the dielectric constant (ε) of the medium, being at 355 nm in aqueous solution (ε = 78) and shifting to shorter wavelength with a decrease in the constant; for example, 310-330 nm in butyl ether (ε = 4), p-dioxane (ε = 2.2), or n-hexane (ε = 1.9) (16,17). Accordingly, the dielectric constant of the environment of a Trp residue can be estimated from the peak position using a dielectric scale. From the emission maximum of 335 nm found for P-450 isozyme 2, the dielectric constant of the environment of the Trp residue was estimated to be less than 15. The quenching and solvent perturbation experiments presented in this paper indicate that this residue is partly exposed to the solvent. Thus, the estimated dielectric constant may be that near the "exterior" of the protein. The dielectric constants previously reported are 34-48 for the region of Glu_{35} and Asp_{52} and about 40 for the region of His_{15} of hen egg-white lysozyme (18,19), and less than 49 for the interacting region between subtilisin BPN' and Streptomyces subtilisin inhibitor (20). Since the dielectric constant in the interior of proteins is believed to be very low, such as 1-5 (21,22), the value found in our work for the environment of P-450 Trp_{121} supports the view that this residue exists in the boundary area between the surface and interior of the protein.

Our findings suggest that Trp_{121} is in an environment that is partly entered by methanol and ethanol, with accessibilities greater than those estimated for iodide and cesium ions. In the latter case, the results are probably influenced by electrostatic interaction as well as the size of these hydrated ions. The solvent perturbation is not affected by the electrostatic field on the protein and may therefore give a more reliable estimate of solvent accessibility. Lehrer (10) reported that the K_Q value of tryptophyl residues for iodide ion quenching depends on the environment; for example, K_Q is 12 for N-acetyltryptophanamide, 22 for a copolymer containing 97% lysine and 3%

tryptophan, and 0.3 for a copolymer containing 99% glutamate and 1% tryptophan. The low K_Q values obtained with iodide and cesium ions, with hydrated diameters of 3.0 and 2.5 Å, respectively (23), suggest that collisions of these ions with Trp_{121} are highly influenced by charged groups on the cytochrome. It should be noted that, according to the tentative topological model proposed on the basis of the amino acid sequence of this cytochrome (4), Trp_{121} is located among charged groups exterior to the membrane bilayer on the cytosolic side.

P-450 isozyme 2 is known to be largely aggregated in the hexameric to octameric state in aqueous solution (24). If the distance between Trp_{121} of apoprotein and the heme(s) of the neighboring protein(s) in the aggregate is within the Förster's critical distance (30 to 40 Å (25,26)), the fluorescence of the tryptophyl residue should be quenched almost completely even in the mixed aggregates of holo- and apoproteins. The fact that F_{335} is quenched in a linear and almost stoichiometric manner with respect to the increase in specific content suggests that the fluorescence of Trp_{121} is quenched by a single heme, presumably in the same protein molecule. The most reasonable estimate for the distance between Trp_{121} and the heme in the aggregated form is less than 40 Å for the intramolecular case and greater than 40 Å for the intermolecular case.

Whether the tryptophyl residue facilitates electron transfer from NADPH via the reductase flavins to the heme of P-450 isozyme 2 remains to be established. Recently, Schwarze et al. (27) estimated the distance between the NH_2-terminal methionine of this cytochrome and its heme group to be 26.1 Å by measuring the energy transfer from the fluorescein isothiocyanate-modified methionine to the heme. They concluded that this distance is too large to be surmounted by a thermally activated tunneling mechanism, and that the NH_2-terminus does not participate in the electron transfer pathway from reductase to the heme.

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REFERENCES

1. White, R.E., and Coon, M.J. (1980) Annu. Rev. Biochem. 49, 315-356.
2. Black, S.D., and Coon, M.J. (1985) in Cytochrome P-450: Structure, Mechanism, and Biochemistry (Ortiz de Montellano, P.R., Ed) Plenum Press, New York, in press.
3. Haugen, D.A., and Coon, M.J. (1976) J. Biol. Chem. 251, 7929-7939.
4. Tarr, G.E., Black, S.D., Fujita, V.S., and Coon, M.J. (1983) Proc. Natl. Acad. Sci. USA 80, 6552-6556.
5. Udenfriend, S. (1962) Fluorescence Assay in Biology and Medicine, Academic Press, New York.
6. Weber, G., and Teale, F.J.W. (1959) Faraday Discuss. Chem. Soc. 27, 134-141.

7. Chiang, Y.-L., and Coon, M.J. (1979) Arch. Biochem. Biophys. 195, 178-187.
8. Warburg, O. (1932) Angew. Chem. 45, 1-6.
9. Bücher, T., and Kaspers, J. (1947) Biochim. Biophys. Acta 1, 21-34.
10. Lehrer, S.S. (1971) Biochemistry 10, 3254-3263.
11. Ricchelli, F., Rossi, E., Salvato, B., Jori, G., Bannister, J.V., and Bannister, W.H. (1983) in Oxy Radicals and Their Scavenger Systems. Vol. 1. Molecular Aspects (Cohen, G. and Greenwald, R.A., Eds), pp. 320-323, Elsevier, New York.
12. Cuatrecasas, P., Edelhoch, H., and Anfinsen, C.B. (1967) Proc. Natl. Acad. Sci. USA 58, 2043-2050.
13. Maddaiah, V.T., Collipp, P.J., Sharma, R.K., Chen, S.Y., and Thomas, J. (1972) Biochim. Biophys. Acta 263, 133-138.
14. Handbook of Chemistry and Physics, 56th Edition (1975) Weast, R.C., Editor, CRC Press, Cleveland, p. E56.
15. Herskovits, T.T., and Sorensen, M. (1968) Biochemistry 7, 2533-2542.
16. Teale, F.W.J. (1960) Biochem. J. 76, 381-388.
17. Cowgill, R.W. (1967) Biochim. Biophys. Acta 133, 6-18.
18. Kangawa, K., Matsuo, H., and Narita, K. (1976) Seibutsubutsuri 16, 145-153.
19. Kuramitsu, S., Ikeda, K., and Hamaguchi, K. (1977) J. Biochem. (Tokyo) 82, 585-597.
20. Inouye, K., Tonomura, B., Hiromi, K., Fujiwara, K., and Tsuru, D. (1979) J. Biochem. (Tokyo) 85, 1127-1134.
21. Brant, D.A. (1972) Annu. Rev. Biophys. Bioeng. 1, 369-408.
22. Schulz, G.E., and Schirmer, R.H. (1978), Principles of Protein Structure, p. 30, Springer-Verlag, Berlin.
23. Kielland, J. (1937) J. Am. Chem. Soc. 59, 1675-1678.
24. French, J.S., Guengerich, F.P., and Coon, M.J. (1980) J. Biol. Chem. 255, 4112-4119.
25. Stryer, L. (1960) Radiation Res. Suppl. 2, 432-451.
26. Förster, T. (1960) Radiation Res. Suppl. 2, 326-339.
27. Schwarze, W., Bernhardt, R., Jänig, G.-R., and Ruckpaul, K. (1983) Biochem. Biophys. Res. Commun. 113, 353-360.