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Oxidation-reduction Potential of Soluble and Membrane-bound Rabbit Liver Microsomal Cytochrome P-450 LM2

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The redox midpoint potentials of rabbit liver microsomal cytochromes P-450 and of soluble and membrane-bound rabbit liver microsomal cytochrome P-450 LM2 were determined using EPR-spectroscopy and absorption difference spectrometry with NADPH or dithionite as reductants. Using EPR, a redox midpoint potential of −0.36 V was obtained both for the low spin and the high spin components of microsomal cytochrome P-450. Spectrophotometrical determinations yielded very similar values: −0.37 V and −0.34 V for the low and high spin signals, respectively. Soluble cytochrome P-450 LM2 had a midpoint potential of −0.32 V. This redox potential was not significantly affected by incorporation of the protein into an artificial membrane structure or, furthermore, by the presence of cytochrome b_5 the same membrane.

The redox midpoint potentials of cytochromes P-450 are surprisingly low. Both the bacterial cytochrome P-450cam,[1] the hepatic cytochromes P-450[2,3] and the mitochondrial cytochrome P-450scc[4] have midpoint redox potentials around −0.30 V. In the liver microsomal hydroxylase system cytochrome P-450 receives electrons either from NADPH-cytochrome P-450 reductase, having midpoint potentials related to the various reduced forms between −0.11 and −0.36 V[5] or from cytochrome b_5[6,7] with a midpoint potential of about 0 V.[8] As a consequence of the non-functionally related redox potentials between the different proteins in the hydroxylase system, suggestions have been made that cytochrome P-450 exerts altered redox properties when it interacts with cytochrome b_5 in the native membrane.[8] In view of our results indicating altered properties of cytochrome P-450-catalyzed hydroxylation reactions upon incorporation of purified components of the hydroxylase system into a membrane structure,[9] it was considered of interest to evaluate whether the redox properties of cytochrome P-450 LM2 were affected by the presence of a neighbouring membrane or by cytochrome b_5 incorporated into the membrane.

The results indicate that the redox midpoint potential of cytochrome P-450 LM2 is unaffected by the membrane or by cytochrome b_5, when measurements are performed either by EPR-spectroscopy or absorption difference spectrometry using NADPH or dithionite as reductants.

MATERIALS AND METHODS

Microsomal phospholipids were prepared according to Bligh and Dyer.[10] Egg yolk phosphatidylcholine, type III E, was purchased from Sigma. Electrophoretically homogeneous cytochrome P-450 LM2 was prepared from phenobarbital-treated rabbits as described previously[11]. The method, based on that described by Haugen and Coon,[12] yielded preparations with specific contents of 12.5–14.2 nmol/mg protein. Cytochrome b_5 was purified as described elsewhere.[7]

Unilamellar phospholipid vesicles containing cytochrome P-450 LM2 were prepared in the following way. Ten mg of egg yolk phosphatidylcholine or microsomal phospholipids in chlo-

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roform was taken to absolute dryness under nitrogen. The residue was suspended in 0.5 ml of 50 mM potassium phosphate buffer, pH 7.4, containing 20 % glycerol and sonicated at 22 °C under nitrogen until clarity, using an MSE sonifier. One ml of cytochrome P-450 LM2 (13–15 μM) was then added and the solution was incubated for 20 min at 37 °C. This procedure resulted in complete incorporation of the cytochrome into the vesicles as was evident from Sepharose 4 B chromatography of the preparations (*cf.* Ingelman-Sundberg *et al.*[11]).

Oxidation-reduction potential measurements were carried out with EPR at low temperature, essentially as described previously.[13] The following modifications were made. Antraquinone 2-sulfonate was added to the gas scrubbing bottles and the second bottle contained 0.2 g sodium dithionite per 100 ml of 10 mM potassium hydroxide. The samples were withdrawn by overpressure through the gas outlet by a bent glass tube leading into a quartz EPR tube. The following redox mediators in a final concentration of 50 mM were used: phenosafranine T, benzyl viologen and methylviologen. In negative direction the potentials were adjusted by a solution containing 0.1 M sodium dithionite and 20 mM potassium hydroxide. The EPR spectra were recorded with a Varian V-5402 spectrometer as described before.[14]

Oxidation-reduction potential measurements were also carried out at 20 °C using a dye equilibrium technique. The samples in butyl rubber sealed glass spectrophotometer cuvettes were reduced anaerobically under stirring by a flow of argon for 1 h. The argon was cleaned by passage over heated BASF catalyst R 3–11 followed by passage over a gas scrubbing bottle containing water to restore the moisture to the gas. Each sample contained one of the dyestuffs phenosafranine, safranine T or benzylviologen. To vary the degree of oxidation, small amounts of sodium dithionite, NADPH or potassium ferricyanide were added to the sample. The difference light absorption spectra were recorded by an Aminco DW-2 UV-VIS spectrophotometer. The potentials of the solutions were then calculted by the observed light absorption using

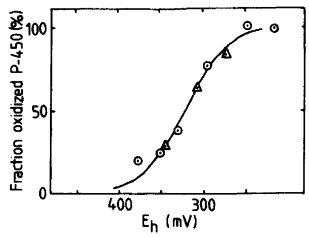

Fig. 1. Potentiometric titration of soluble cytochrome P-450 LM2. To samples with 11 μM cytochrome P-450 LM2 in 0.1 M potassium phosphate buffer, pH 7,0, containing mediators (*cf.* Methods) and 20 % glycerol, 0.1 M sodium dithionite in 20 mM potassium hydroxide was added to adjust the potential. The samples were subsequently frozen and analyzed by EPR as described under Methods. ▽ indicates the results obtained during reoxidation of the hemoprotein.

Table 1. Redox midpoint potentials of cytochrome P-450. Midpoint potentials were determined as described under Methods in liver microsomes isolated from phenobarbital-treated rabbits or in purified cytochrome P-450 LM2-preparations. When indicated, an equimolar amount of purified rabbit liver cytochrome b_5 was introduced into the vesicles together with P-450 LM2.

Sample	Assay Method	Reductant	$E_{m,7}$ (V)
Microsomes	EPR g=2.4	dithionite	–0.36
Microsomes	EPR g=8	dithionite	–0.36
P-450 LM2	EPR g=2.25	dithionite	–0.32
P-450 LM2 in vesicles	EPR g=2.25	dithionite	–0.32
P-450 LM2 in vesicles containing cytochrome b_5	EPR g=2.25	dithionite	–0.31
Microsomes	ΔA 465 nm	dithionite	–0.37
Microsomes	ΔA 648 nm	dithionite	–0.34
P-450 LM2 in vesicles	ΔA 455 nm	dithionite	–0.33
Microsomes	ΔA 465 nm	NADPH	–0.36
Microsomes	ΔA 648 nm	NADPH	–0.34

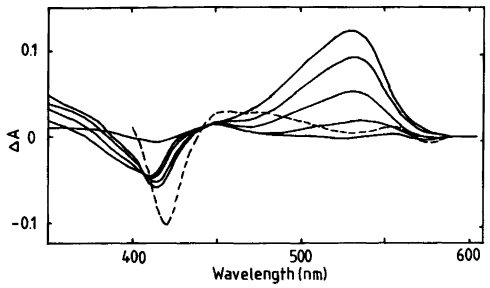

Fig. 2. Redox titration of membrane-bound cytochrome P-450 LM2 in the presence of safranine T. The cytochrome was incorporated into vesicles prepared from microsomal phospholipids as described under Methods. Safranine T was added to a final concentration of 2.7 μM and the solution was diluted with 50 mM potassium phosphate buffer, pH 7.4, containing 20 % glycerol to give a final concentration of the cytochrome of 3 μM. The potential was adjusted in negative and positive directions by small additions of 50 mM sodium dithionite in 10 mM potassium hydroxide and 25 mM potassium ferricyanide, respectively. A fully reduced sample is shown by the dotted line. The system potentials were measured at 530 nm from the fraction of safranine T oxidized, whereas the degree of cytochrome P-450 reduction was calculated farom the absorbance difference 455−419 nm.

the Nernst equation. At pH 7.4, the midpoint potentials of −265 mV and −298 mV were used for phenosafranine and safranine T, respectively.[15]

RESULTS

Redoxmidpoint potentials detected by EPR. The midpoint potentials of cytochrome P-450 were determined by EPR-spectroscopy at low temperatures in frozen anaerobic samples of rabbit liver microsomes or of purified cytochrome P-450 LM2 either in soluble (Fig. 1) or membrane-bound state. In microsomes, also the high spin EPR-signal at g=8.0 was detected, whereas with the purified protein, only the low spin signal was taken into consideration.

From the results presented in Table 1, it is evident that the midpoint potentials obtained were relatively similar and not dependent upon the presence of a surrounding membrane. A value of about − 0.34 V was obtained both for the high and low spin forms of cytochrome P-450 in microsomes and for the purified P-450 LM2 preparations in both the soluble and membrane bound state. The presence of an equimolar amount of cytochrome b_5 in the membranes did not affect the redox potential of cytochrome P-450 LM2.

Redoxmidpoint potentials detected by adsorption spectra. Midpoint potentials of cytochrome P-450 were also determined by a dye equilibrium technique at 20 °C. Difference absorption spectra of cytochrome P-450 LM2 in the presence of safranine T at different oxidation-reduction states are shown in Fig. 2. Even with this technique low midpoint potentials around −0.34 V were obtained for all cytochrome P-450 preparations and, furthermore, also for both the low and high spin forms of the protein, as detected by absorption difference at 465 and 648 nm, respectively, using sodium dithionite as reductant (*cf.* Table 1).

In microsomal samples NADPH was also used as reductant. However, in these cases the redox midpoint potentials of microsomal P-450 were not significantly different from those obtained when using dithionite either (*cf.* Table 1).

DISCUSSION

The results presented indicate that the membrane has little influence upon the redox properties of cytochrome P-450 LM2, when measurements were performed either by EPR at low temperatures or by absorption difference spectra at room temperature using phenosafranine, safranine T or benzylviologen as mediators. The results obtained using microsomal samples agree well with previous redox midpoint potentials of hepatic cytochrome P-450 determined by Waterman and Mason [16], and soluble cytochrome P-450 LM2 presented by Guengerich *et al.*[3] In experiments where we used NADPH as reductant, still a very low midpoint potential was obtained indicating that in this way an indirect reduction of P-450 *via* NADPH-cytochrome P-450 reductase, does not affect the midpoint potential obtained when measuring the formation of ferrous cytochrome P-450.

Recent results have more or less established the participation of cytochrome b_5 in the liver

microsomal hydroxylase system, where it most probably donates the second electron to cytochrome P-450.[6–8,18,19] The presence of cytochrome b_5 in the membrane did not affect the redox properties of P-450 LM2. A midpoint potential of cytochrome b_5 in these reconstituted membranes of about 0 V confirms the marked difference in redox properties between the two microsomal hemoproteins. It is therefore tempting to speculate about altered and more similar values of these midpoint potentials in the functionally working microsomal hydroxylase system. The results obtained by Werringloer and Kawano,[8] using cytochrome b-5 as an endogenous microsomal mediator, suggest that this is the case although an overestimation of the midpoint potential of P-450 seems plausible due to the measurement of the ferrous-carbonyl complex (including measurement of two equilibria) instead of ferrous P-450. Our recent results [19] indicate that cytochrome b_5, NADPH-cytochrome P-450 reductase and cytochrome P-450 form a functionally active ternary complex in the membrane. It thus seems likely that the formation of this complex in an intact membrane structure is a prerequisite for obtaining the native redox properties of the enzymes.

REFERENCES

1. Sligar, S. *Biochemistry 15* (1976) 5399.
2. Cinti, D., Sligar, S., Gibson, G. and Schenkman, J. *Biochemistry 18* (1979) 36.
3. Guengerich, F. P., Ballou, D. P. and Coon, M. J. *J. Biol. Chem. 250* (1975) 7405.
4. Light, D. R. and Orme-Johnson, N. R. *in Microsomes, Drug Oxidations and Chemical Carcinogenesis, Proc. of the Fourth International Symposium on Microsomes and Drug Oxidations,* Academic Press, New York, (1980) p. 67.
5. Vermilion, J. L. and Coon, M. J. *J. Biol. Chem. 253* (1978) 2694.
6. Estabrook, R. W., Hildebrandt, A. Baron, J., Netter, K. J., and Leibman, K. *Biochem. Biophys. Res. Commun. 42* (1971) 132.
7. Ingelman-Sundberg, M., and Johansson, I., *Biochem. Biophys. Res. Commun. 97* (1980) 582.
8. Werringloer, J. and Kawano, S. In Coon, M. J., Conney A. H., Estabrook, R. W., Gillette, J. R., Gelboin, H. V. and O'Brien, P. J. Eds., *Microsomes, Drug Oxidations and Chemical Carcinogenesis.* Academic Press, New York 1980 p. 469.
9. Ingelman-Sundberg, M. and Johansson, I. *Biochemistry 19* (1980) 4004.
10. Bligh, E. G. and Dyer, W. J. *Can. J. Biochem. Physiol. 37* (1959) 911.
11. Ingelman-Sundberg, M. and Glaumann, H. *Biochim. Biophys. Acta 599* (1980) 417.
12. Haugen, D. A. and Coon, M. J. *J. Biol. Chem. 251* (1976) 7929.
13. Bäckström, D., Lorusso, M., Andersson, K. and Ehrenberg, A. *Biochim. Biophys. Acta 502* (1978) 276.
14. Grasdalen, H., Bäckström, D., Eriksson, L. E. G., Ehrenberg, A., Moldeus, P., von Bahr, C. and Orrenius, S. *FEBS lett. 60* (1975) 294.
15. Stiehler, R. D., Chen, T.-T. and Clark, W. M. *J. Am. Chem. Soc. 55* (1933) 891.
16. Waterman, M. R. and Mason, H. S. *Arch. Biochem. Biophys. 150* (1972) 57.
17. Ingelman-Sundberg, M., Glaumann, H. and Johansson, I. In Coon, M. J., Conney, A. H., Estabrook, R. W., Gillette, J. R., Gelboin, H. V. and O'Brien, P. J. Eds., *Microsomes, Drug Oxidations and Chemical Carcinogenesis* Academic Press, New York 1980 p. 513.
18. Sugiyama, T., Miki, N. and Yamano, T. *Biochem. Biophys. Res. Commun. 90* (1979) 715.
19. Ingelman-Sundberg, M., Edvardsson, A.-L. and Johansson, I. In Sato, R. and Kato, R. Eds., *Microsomes, Drug Oxidations and Drug Toxicity* Japan Scientific Societies Press, Tokyo 1981 p. 187.

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