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Heme maintains catalytically active structure of cytochrome P-450

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Treatment of purified cytochrome P-450 LM2 and its liposome-bound form with hydrogen peroxide led to complete destruction of the P-450 heme. The apoenzyme thus produced could be reconstituted to catalytically active cytochrome P-450 by incubation with hemin, the reconstitution efficiency being 50% for the soluble enzyme and 80% for the liposome-bound enzyme. The removal of heme from the soluble hemoprotein resulted in a 3-fold decrease in the efficiency of its incorporation into sonicated liposomes. The contents of 5 secondary structure forms in the native, apo- and reconstituted holoenzymes were estimated from their circular dichroism spectra. It was thus found that the helix content increased from 34% to 60% upon removal of the heme from the native enzyme. We suggest that the increase in the helix content leads to a reduction of the incorporation efficiency into liposomal membranes.

Cytochrome P-450; Apoenzyme; Heme; Secondary structure; Hydrogen peroxide

1. INTRODUCTION

Cytochrome P-450 contains protoheme IX as prosthetic group, the main role of which is to produce an active oxygen species that is to be inserted into the substrate molecule. Removal of the heme from cytochrome P-450 leads to its inactivation. It is, however, unclear whether this removal affects the structure of the enzyme.

Sadano and Omura [1] have reported that the in vivo half life of the protein moiety of cytochrome P-450 is longer than that of the heme moiety, suggesting that during its life time cytochrome P-450 can change heme and the protein structure either remains unchanged or reversibly changes upon the loss of heme.

Reversible removal of heme has been reported for such hemoproteins as globins, *b*-type cytochromes, peroxidases and cytochrome P-450$_{cam}$ [2-8], but reversible removal of heme has not yet been successfully achieved for microsomal cytochromes P-450 such as LM2. In this paper, we report a method for reversible removal of heme from cytochrome P-450 LM2 and compare the structure and catalytic activity of the native, apo- and reconstituted holoenzymes.

2. MATERIALS AND METHODS

Cytochrome P-450 LM2 was purified from liver microsomes of phenobarbital-treated rabbits [9]. The peroxidase activity of the enzyme was assayed as described [10]. Cytochrome P-450 content was determined by the method of Omura and Sato [11].

Proteoliposomes were obtained by incubation of purified cytochrome P-450 LM2 with sonicated liposomes prepared from microsomal lipids as described [12]. The percentage of protein incorporation into the liposomes is referred to as 'incorporation efficiency'.

For determination of heme, a sample up to 0.1 ml was mixed with 2.9 ml of 1 N NaOH for 20 min with shaking. The heme concentration was estimated from absorbance at 420 nm using a millimolar extinction coefficient of 70 mM^{-1} cm^{-1}.

For preparation of apocytochrome P-450 LM2, 10 nmol of the hemoprotein in 0.5 ml of 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol, was incubated with 0.5 M hydrogen peroxide for 40 min at 20°C. The mixture was then diluted 50-fold with the buffer and concentrated on an Amicon cell using a PM-30 membrane. This procedure was repeated twice. The concentrated mixture was dialyzed for 4 h against 10 vols of the buffer containing 5000 units of catalase. All the above procedures were carried out under argon atmosphere.

To reconstitute cytochrome P-450 LM2, hemin solution was slowly added (for 20 min to a final concentration of 1 mM) to 1 ml of 0.1 M potassium phosphate buffer, pH 7.4, containing 10 nmol of apoenzyme and 20% glycerol. The mixture was continuously stirred at 20°C and then diluted 100-fold. The diluted solution was concentrated by using a PM-30 membrane. This procedure was repeated twice. To make sure that proper reconstitution had been achieved, the sample obtained was subjected to chromatography on an aminooctyl Sepharose 4B column. Cytochrome P-450 was then purified according to Imai et al. [9].

A new method was used for spectroscopic assay of protein content in the presence of detergent. Protein concentration, C (mg/ml), is given by the equation,

$$C = k(A_{\lambda_1} - A_{\lambda_2}),$$

where $k = 1/(E^{0.1\%}_{\lambda_1} - E^{0.1\%}_{\lambda_2})$. This value was an average obtained for 12 proteins whose extinction coefficients, $E^{0.1\%}_{\lambda}$, were evaluated by the method of van Iersel et al. [13]. A_{λ_i} is the absorbance of a protein in the presence of detergent at the wavelength of λ_i; λ_1 and λ_2 were chosen by minimization of the contribution of detergent absorption

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relative to that of protein [14] in the wavelength region of 205–225 nm. The proteins used were bovine serum albumin, human serum albumin, cytochrome *c* and myoblobin (all from Serva) and the detergent used was Emulgen 913 (from Kao-Atlas). A cell of 0.1 cm light path was used. Three equations were thus obtained for calculating *C*.

$C = 0.639(A_{208} - A_{224})$; $C = 0.846(A_{210} - A_{224})$; $C = 1.113(A_{212} - A_{224})$

We tested the validity of these equations using the four above-mentioned proteins in the concentration range of 0.1–0.3 mg/ml in the presence of 0.005–0.01% Emulgen 913. The mean magnitude of error of measurement in 34 experiments was 7%. Specific contents of cytochrome P-450 determined by this technique are about 30% higher than those estimated by the method of Lowry et al. [15] using bovine serum albumin as a standard.

The contents of 5 secondary structure forms were calculated from circular dichroism spectra of freshly dissolved proteins by the method of Bolotina et al. [16]. Preparations were dissolved in 5 mM potassium phosphate buffer, pH 7.4, in the presence of 5×10^{-4}% Emulgen 913.

3. RESULTS AND DISCUSSION

The main criterion in choosing a procedure for preparation of apocytochrome P-450 is the possibility of subsequent reconstitution of the native enzyme. Several methods are available for removal of heme from hemoproteins including cytochrome P-450$_{cam}$. Most of them involve strong acidification, exposure to organic solvents, and addition of heme acceptors [2–8]. However, none of these methods allowed us to obtain apocytochrome P-450 whose incubation with hemin could restore the properties of the native enzyme. The main reason for the failure seems to be the instability of the enzyme structure at pH values lower than 3.5. The technique described by Ingelman-Sundberg et al. [17], who used apomyoglobin as a heme acceptor, proved to be ineffective as well, because the removal of heme by this method was incomplete. Therefore, we decided to use an entirely different method involving the destruction of heme in situ by the action of hydrogen peroxide. Incubation of purified cytochrome P-450 LM2 with hydrogen peroxide for 40 min led to complete destruction of the heme. As shown in table 1, the addition of hemin to the apoenzyme thus prepared resulted in partial restoration of the native properties of cytochrome P-450. The reconstituted enzyme, when reduced and mixed with CO, showed an absorption peak at 420 nm in addition to the characteristic absorption peak at 450 nm due to the ferrous carbonyl complex. The contents of adsorbed hemin and cytochrome P-420 in the preparation could not be accurately determined because of their unknown spectral properties under the given conditions.

Upon reconstitution *p*-nitroanisol *O*-demethylation activity was restored completely when determined on the basis of cytochrome P-450 content (table 1). However, the activity on the basis of protein content was only 50% of that of the native enzyme. Since neither hemin nor cytochrome P-420 can catalyze *p*-nitroanisol *O*-demethylation [18], it can be concluded that only 50% of the apoenzyme was reconverted to cytochrome P-450. When purified from the reconstitution mixture, the cytochrome showed the same activity with the native enzyme even on the basis of protein content. The incomplete reconversion of the apoenzyme can be accounted for by the hydrogen peroxide induced damage of the protein in addition to the instability of the protein.

To elucidate the effect of heme destruction on the properties of the protein, we studied the ability of the apoenzyme to be incorporated into the phospholipid bilayer of liposomes. It is known that purified cyto-

Table 1

Properties of native, apo- and reconstituted holocytochrome P-450 LM2

Preparation	Molar ratio		*p*-Nitroanisol *O*-demethylation activity		Efficiency of incorporation into liposomes (%)
	heme / protein*	P-420 + hemin / protein	nmol product $\times 10^{-3}$/s / nmol P-450	nmol product $\times 10^{-3}$/s / nmol holo- + apo-P-450	
Native P-450	0.9	0	60	60	27
Apo-P-450	0	0	0	0	7
Reconstituted P-450	0.8	0.25	60	30	16
P-450 isolated after reconstitution	0.95	0	60	60	25
Liposome-bound native P-450	1.0	0	200	200	-
Liposome-bound apo-P-450	0	0	0	0	-
Liposome-bound reconstituted P-450	15	-	-	160	-
Reconstituted P-450 isolated from proteoliposomes	0.9	0.1	60	55	-

* Protein determination was carried out according to Lowry et al. [15].

chrome P-450 LM2 can be incorporated into liposomes with an efficiency of 25–30% at temperatures higher than the phase transition point [12]. Under the same conditions the incorporation efficiency of the apoenzyme did not exceed 7% (table 1), suggesting that a structural change had occurred in the enzyme molecule upon the heme loss.

We therefore compared the structures of native cytochrome P-450, the apoenzyme and the reconstituted hemoprotein by analyzing their circular dichroism spectra. Table 2 shows the contents of 5 secondary structure forms in the native, apo- and reconstituted holoenzymes. The contents of different secondary structures vary depending on the technique used for protein assay, a fact that explains different α-helix contents reported by different authors for cytochrome P-450 LM2 [19, 20]. If a cytochrome P-450 preparation is contaminated by the apoenzyme, the absolute ellipticity will be overestimated when cytochrome P-450 content is determined by the method of Omura and Sato [11]. The secondary structure of the native protein calculated in this way is identical with that reported by Shimizu et al. [20] who determined protein content by the same technique. Protein determination by the method of Lowry et al. [15] also results in overestimation of the ellipticity. When protein content was assayed by this method, no β-turns could be detected in the apoenzyme (table 2), a finding which is hardly likely for a globular protein. The α-helix content calculated on the basis of the protein assay procedure proposed in this study is identical with that reported by Chiang and Coon [19]. This is probably due to the fact that the latter authors used the Lowry method corrected for cytochrome P-450. In any case, the results shown in table 2 indicate that the loss of heme is accompanied by an increase in α-helix content. This could be due to a structural change in the hydrophobic core of the molecule, which facilitates the formation of new helical regions [21]. The structure of proteins analogously changes upon the addition of detergents [20,22].

Liposome-bound apocytochrome P-450 LM2 was prepared by treating the liposome-bound hemoprotein with hydrogen peroxide. Unlike the case for the free apoenzyme, as much as 80% of the liposome-bound apoenzyme could be reconverted to the holoenzyme, as can be seen in table 1 (compare *p*-nitroanisol *O*-demethylation activity of the reconstituted enzyme based on protein content with that of the native liposome-bound enzyme based on cytochrome P-450 content). Since a 15-fold excess of hemin was used in this reconstitution, the absorption spectrum of the reconstituted cytochrome P-450 in the ferrous-CO complex was distorted. However, when the reconstituted hemoprotein was solubilized from the proteoliposomes and partially purified by aminooctyl Sepharose 4B column chromatography [9], it was identical with the native enzyme in its spectral and catalytic properties.

Table 2

Determination of secondary structures of cytochrome P-450 LM2

Preparation	Protein assay	$f\alpha$	$f\beta_a$	$f\beta_p$	$f\beta_t$	f_c	δ**
Native P-450	1	41	5	2	13	39	3.9
	2	34	2	6	13	44	2.5
	3	71	8	0	14	7	9.1
Control***	1	41	5	3	13	38	3.1
	2	32	1	8	13	46	2.6
Apo-P-450	1	75	5	0	0	20	13.0
	2	60	12	0	16	12	7.8
Reconstituted P-450	1	38	6	3	13	40	2.5
	2	33	4	6	13	44	1.8

Molar fractions of amino acid residues in α-helices ($f\alpha$), antiparallel β-sheets ($f\beta_a$), parallel β-sheets ($f\beta_p$), β-turns ($f\beta_t$) and random coil regions (f_c) in %.

* 1, According to Lowry et al. [15]; 2, method proposed in this study (see section 2); 3, according to Omura and Sato [11].

** Standard deviation between a CD spectrum calculated from reference spectrum and experimental spectrum.

$$\delta = \left\{ \sum_{\lambda} ([\theta]_{\lambda.\mathrm{calc}} - [\theta]_{\lambda.\mathrm{exper}})^2 \Big/ \sum_{\lambda} [\theta]_{\lambda.\mathrm{exper}} \right\}^{1/2} \times 100\%$$

*** Enzyme subjected to the same procedures except for the addition of H_2O_2.

In conclusion, the removal of heme from cytochrome P-450 LM2 results in a reversible change in its structure. The apoenzyme thus prepared can be reconverted to the native enzyme by the addition of hemin. The liposome-bound apoenzyme can be reconstituted more readily than its free counterpart, probably because the membrane-bound protein is more stable in structure.

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