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Comparative Experiments of Protein Denaturation for Studying Structural Difference of Homologous Membrane Proteins: P-450$_{scc}$ and P-450$_{11\beta}$

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The topological difference between two homologous membrane proteins, cytochromes P-450$_{scc}$ and P-450$_{11\beta}$ in the inner membrane of bovine adrenocortical mitochondria, was studied by denaturation experiments, using urea and 1-pentanol as denaturants. Both P-450s denatured at the urea concentration of about 2.5 M that is in the concentration range of the denaturation of soluble proteins. In contrast, P-450$_{11\beta}$ was denatured by 1-pentanol, which is an efficient denaturant of membrane proteins, at a much lower concentration than P-450$_{scc}$. These results suggested that the membrane domain is more strongly coupled with the soluble functional domain in P-450$_{11\beta}$ than P-450$_{scc}$. It was also indicated that the comparative experiments of protein denaturation by urea and alcohol provide a simple method to identify the structural difference of homologous membrane proteins.

KEYWORDS: P-450$_{scc}$, P-450$_{11\beta}$, membrane topology, denaturation experiments, structural model

1. Introduction

Eukaryotic cytochromes P-450 are intrinsic membrane proteins in endoplasmic reticula and mitochondria which perform a variety of important biological functions such as the biosynthesis of hormones. Because of the functional importance, the amino acid sequences[1)] and the structures of P-450s have been studied extensively. However, the tertiary structures that are essential for the understanding of the functions are known for only five soluble P-450s from bacteria and fungi,[2–6)] and the structural analyses by X ray-diffraction and NMR have not been successful for any eukaryotic P-450. Therefore, various other kinds of methods have been employed to obtain structural information of eukaryotic P-450s: limited proteolysis,[7–10)] chemical modification,[11,12)] site-directed mutagenesis,[13–15)] and theoretical modeling.[16–20)] The results of such investigations indicated that the structures of eucaryotic P-450s may not be classified into unique topology, although they have high sequence homology, particularly near the active site. For example, it is considered that many microsomal P-450s have a transmembrane helix near the N-terminal end,[9,10,12,13,16–20)] while long hydrophobic segments are not observed at the same position for many mitochondrial P-450s.[21)] Thus, a new question arises about the structure of P-450s: What is the difference between the structures of various eucaryotic P-450s, and how can we detect the difference by a simple method?

In order to answer these questions, we have carried out denaturation measurements of a pair of P-450s in adrenocortical mitochondria, P-450$_{scc}$ and P-450$_{11\beta}$ as a typical example. These P-450s are located in the same inner membrane of adrenocortical mitochondria and have similar functions of hydroxylation of steroids in which the proteins utilize molecular oxygen for the function.[22)] Furthermore, the sequences of P-450$_{scc}$ and P-450$_{11\beta}$ show homology of 30% as a whole and the local homology near the active site is as high as 60%. However, they seem to have different structures with respect to the interaction with the membrane. The membrane topology of P-450$_{scc}$ was shown to have a large part protruded to the water phase as judged from rotational diffusion measurements in proteoliposomes and limited proteolysis.[23,24)] In addition, purified P-450$_{scc}$ was water soluble, forming oligomers. On the other hand, the cytochrome P-450$_{11\beta}$ is highly hydrophobic and easily forms large aggregates during various experiments. The cytochrome P-450$_{scc}$ is easily transferred from submitochondrial particles to liposomes by 30 min. incubation, while such phenomenon does not occur for P-450$_{11\beta}$[25)] Therefore, these P-450s provide a good system for testing a new method to identify the structural difference between homologous membrane proteins.

In this study, we have comparatively measured the denaturation of P-450$_{scc}$ and P-450$_{11\beta}$ by urea and 1-pentanol. Urea is a highly hydrophilic molecule that is usually used for denaturation experiments of various soluble proteins, while alcohols are efficient denaturants of membrane proteins. The results indicated that the two P-450s denatured at the same concentration of urea, whereas their alcohol denaturation behaviors were very different. Namely, P-450$_{scc}$ was insensitive to alcohol, while P-450$_{11\beta}$ denatured at about 70 mM pentanol. Although we cannot exactly determine the topology of the P-450s only from the present denaturation experiments, it was prove that the comparative denaturation measurements are useful for identifying some topological differences between membrane proteins. In fact, the denaturation measurements of P-450$_{scc}$ and P-450$_{11\beta}$ suggested that the membrane domain is more strongly coupled to the soluble functional domain for P-450$_{11\beta}$ than P-450$_{scc}$.

2. Materials and Methods

2.1 Chemicals

Dithiothreitol (DTT), mannitol, EDTA, and 11-deoxycorticosterone (DOC) were purchased from Sigma (St. Louis, MO). 2-Methyl-1, 2-di-3-pyridyl-1-propanone (metyrapone) was purchased from Aldrich (Milwaukee, WI). All other chemicals were of the highest purity purchased from Wako Chemicals (Osaka). The DOC was dissolved in ethanol at a final concentration of about 20 μM. All other reagents were suspended in HEPES buffer containing 50 mM HEPES, 0.1 mM DTT, 320 mM mannitol, and 1 mM EDTA (pH 7.4).

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2.2 Preparation of enzymes

Submitochondrial particles (SMP) were prepared with French pressure methods from freshly prepared mitochondria of bovine adrenal cortex according to the method of Ohta *et al.*[26] The SMP were resuspended in the above-mentioned HEPES buffer (pH 7.4), and stored at $-80°C$.[27] The sample was reduced with sodium dithionite and bubbled for 30 s with CO. P-450_{scc} was selectively evaluated by the CO-difference spectrum in the presence of metyrapone which suppresses the CO binding to P-$450_{11\beta}$. The concentration of P-450_{scc} was about 250 nM and determined from the dithionite-reduced CO difference spectrum using the difference absorption coefficient $\Delta\varepsilon_{450-490} = 91\,mM^{-1}\,cm^{-1}$.[28] The concentration of P-$450_{11\beta}$ was also about 250 nm and measured by 11-deoxycorticosterone (DOC) induced spectrum with a maximal value at 390 nm and a minimal value at 420 nm, and the difference absorption coefficient was $\Delta\varepsilon_{390-420} = 69\,mM^{-1}\,cm^{-1}$.[29]

2.3 Denaturation experiments

We used urea and 1-pentanol for denaturation experiments. In the denaturation measurements of P-450_{scc}, the denaturant was added to an SMP suspension and stirred by a magnetic stirrer at room temperature for 1 h and 45 min. Metyrapone was added to the suspension 15 min. before the spectral measurements. The ratio of native P-450_{scc} was estimated from the dithionite-reduced CO difference absorption in the presence and in the absence of denaturants. In the measurements of P-$450_{11\beta}$, two cuvettes containing the same SMP suspension were prepared, and ethanol solution of DOC was added to one of the cuvettes and pure ethanol was added to another sample for reference. Then, the DOC induced spectra in the presence and in the absence of denaturants were measured for the estimation of the ratio of the native P-$450_{11\beta}$.

3. Results

We measured the denaturation behaviors of P-450_{scc} and P-$450_{11\beta}$ in bovine adrenocortical mitochondria by urea and 1-pentanol. The state of P-450_{scc} could be monitored by the CO difference absorption spectra in the presence of metyrapone. Figure 1(a) shows the CO difference absorption spectra of P-450_{scc} in the presence of urea in the concentration in the range between 0 and 4 M. The difference spectra showed a peak at 450 nm in the absence of urea, which indicates the native state of P-450_{scc}. A significant peak appeared at 420 nm above 2 M urea, and the spectral peak at 450 nm completely shifted to 420 nm at the concentration of 4 M urea. The change in the difference spectra indicated that P-450_{scc} was denatured by urea at the concentration of about 2.5 M. Figure 1(b) shows the DOC induced spectra of P-$450_{11\beta}$ at various urea concentrations. The DOC induced spectrum of P-$450_{11\beta}$ began to decrease above 2 M urea, until the peak completely disappeared at the urea concentration of 4 M.

Figure 2 shows the fraction of native P-450s estimated from the CO difference spectra in the presence of metyrapone and the DOC induced spectra as a function of the urea concentration. When the urea was used as a denaturant, the fraction of both P-450s remained constant in the urea concentration range below 2 M. The concentration dependence for P-450_{scc} and P-$450_{11\beta}$ was very similar, indicating that the stability of the soluble domain is the same. Furthermore, the critical urea

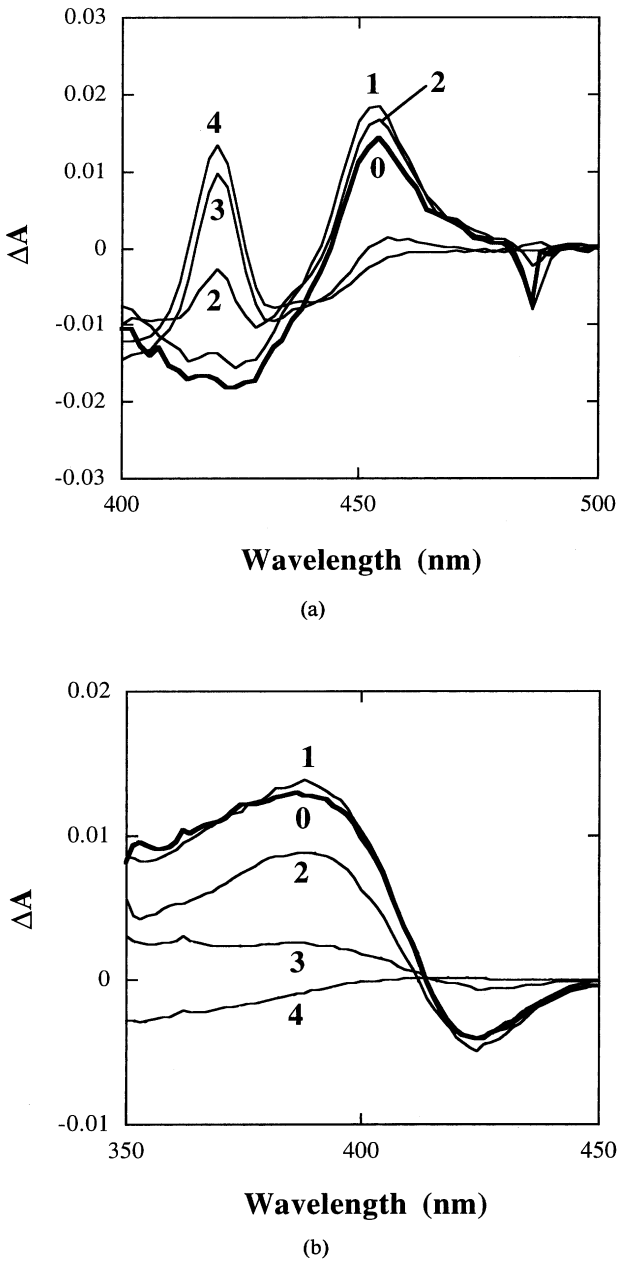

Fig. 1. The effect of urea on the characteristic spectra of P-450s in bovine adrenocortical mitochondrial P-450s: (a) the CO-difference spectra of submitochondrial particle suspensions in the presence of metyrapone which selectively show the state of P-450_{scc}; (b) the DOC-induced spectra which show the state of P-$450_{11\beta}$. The numbers in the diagram indicate the concentration of urea: 0, 0 M; 1, 1 M; 2, 2 M; 3, 3 M; 4, 4 M.

concentration for the denaturation is in the range of the denaturation of most soluble proteins. Because the difference spectra in our measurements monitor the native structure of the active site, the experimental results lead to the conclusion that the active site of both P-450s is located in the soluble domain.

The denaturation measurements were also carried out using 1-pentanol as a denaturant, between 0 and 100 mM. Figure 3(a) shows the CO difference absorption spectra in the presence of metyrapone. The results indicated that P-450_{scc} did not denature by 1-pentanol in this concentration range. However, the DOC induced spectrum of P-$450_{11\beta}$ decreased gradually by the increase of the pentanol concentration, and complete denaturation was attained at 70 mM (Fig. 3(b)).

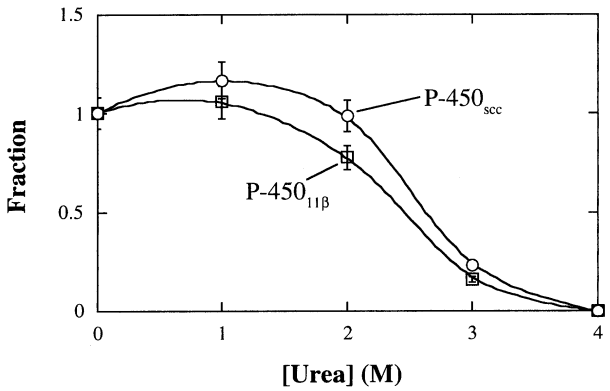

Fig. 2. The urea concentration dependence of native P-450s in bovine adrenocortical mitochondria: ◯, P-450$_{scc}$; □, P-450$_{11\beta}$. The ordinate is the fraction of native species of P-450s, which was estimated from the ratio of the signal in the presence of urea to the corresponding value without urea. Error bars represent the scattering of data.

As shown in Fig. 4, the denaturation behaviors in the case of 1-pentanol were quite different between the two kinds of P-450s: P-450$_{scc}$ remained native even at the pentanol concentration of 100 mM, whereas the native P-450$_{11\beta}$ monotonically decreased by the addition of 1-pentanol and the concentration of half denaturation was about 30 mM (Fig. 4).

4. Discussion

It is well known that most soluble proteins denature by guanidine hydrochloride or urea in the concentration range between 1 and 4 M. However, membrane proteins with a small water protruded part, such as bacteriorhodopsin, are stable against those denaturants. Bacteriorhodopsin did not denature by guanidine hydrochloride even at 8 M.[30] This fact indicates that urea does not significantly affect the interactions in the hydrophobic region of the membrane and that the denaturation of membrane proteins by urea indicates the existence of a considerably large soluble domain. In this work, we have monitored the state of the functional site of heme. Therefore, the influence of urea means that the functional site is in or near the soluble domain. Since both P-450$_{scc}$ and P-450$_{11\beta}$ denatured by urea at the same concentration of about 2.5 M, it should be reasonable to assume that both P-450s have considerably large soluble domains, in which their active sites are located.

It is also known that alcohol is an effective denaturant of the structure of a transmembrane domain. The tertiary structure of bacteriorhodopsin and other membrane proteins was denatured by 1-pentanol of about 50 mM.[31–33] When we compared the denaturation behaviors of a typical membrane protein, bacteriorhodopsin,[33] to those of the mitochondrial P-450s, the alcohol denaturation of P-450$_{11\beta}$ was very similar to that of bacteriorhodopsin, while there was no alcohol effect on the structure of P-450$_{scc}$.

The difference in the alcohol denaturation behaviors between P-450$_{scc}$ and P-450$_{11b}$ should provide information on the topology of the membrane proteins but the mechanisms of the difference may not be elucidated solely by the present experiment. However, the fact that alcohol does not destroy the secondary structure of a membrane protein but effectively denatured the tertiary structure leads to a plausible explanation of the significant difference in the alcohol denaturation

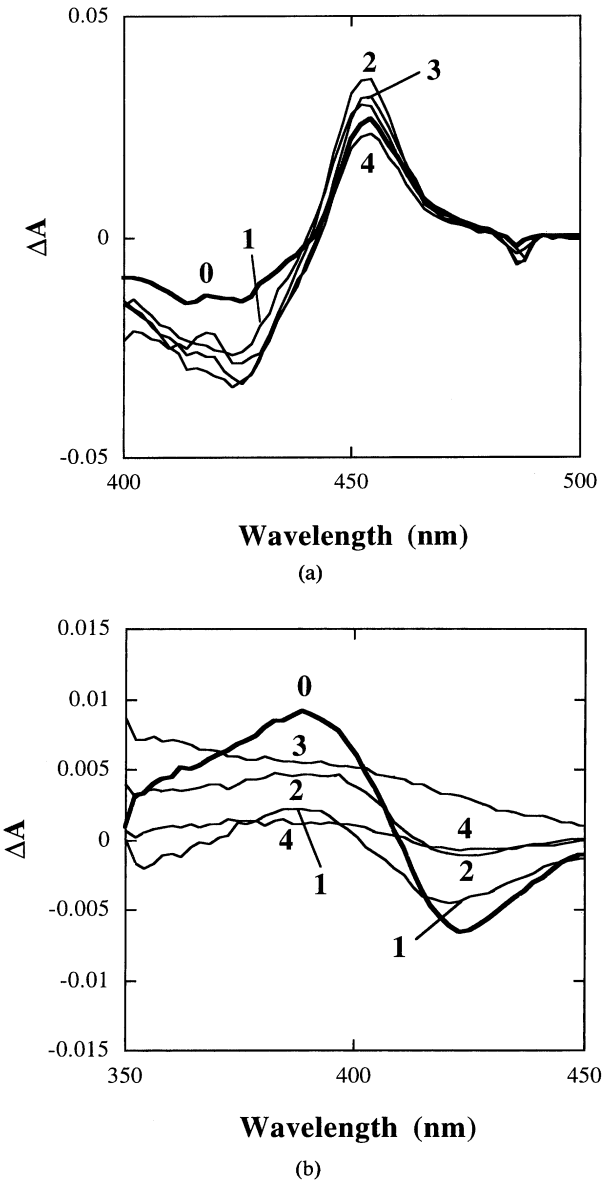

Fig. 3. The effect of 1-pentanol on the characteristic spectra of P-450s in bovine mitochondrial P-450s: (a) the CO-difference spectra of submitochondrial particle suspensions in the presence of metyrapone which selectively show the state of P-450$_{scc}$; (b) the DOC-induced spectra which selectively show the state of P-450$_{11\beta}$. The numbers in the diagram indicate the concentration of 1-pentanol: 0, 0 mM; 1, 25 mM; 2, 50 mM; 3, 70 mM; 4, 100 mM.

behaviors. If the membrane domain is comprised of a single transmembrane helix which only anchors a soluble functional domain, alcohol may not destroy the structure of such a membrane domain because there is not interhelix binding to be destroyed by the attack of alcohol molecules.[33] This consideration seems consistent with the fact that P-450$_{scc}$ is a membrane protein but is rather hydrophilic[23–25] and does not denature in the presence of alcohol. On the other hand, if a membrane protein has some tertiary structure, it may be destroyed by the addition of alcohol as in the case of the alcohol denaturation of bacteriorhodopsin. Then, the membrane protein has to become more hydrophobic as a whole than a protein with a single transmembrane helix. P-450$_{11\beta}$ exhibits characteristics similar to this kind of membrane protein.

Finally, the methodological aspect of this work should be

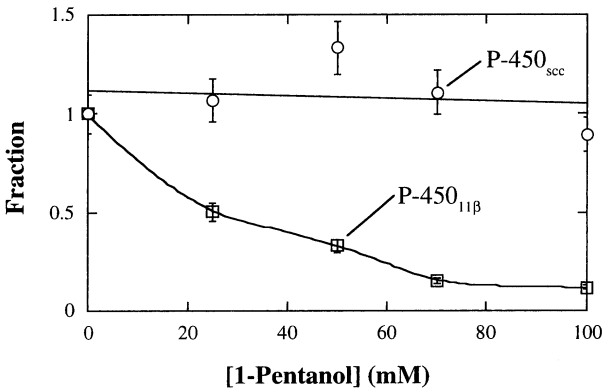

Fig. 4. The 1-pentanol concentration dependence of native P-450s in bovine adrenocortical mitochondria: ◯, P-450$_{scc}$; □, P-450$_{11\beta}$. The ordinate is the fraction of native species of P-450s, which was estimated from the ratio of the signal in the presence of 1-pentanol to the corresponding value without 1-pentanol. Error bars represent the scattering of data.

pointed out. In this work, the difference between P-450$_{scc}$ and P-450$_{11\beta}$ could be shown by very simple experiments of the comparative denaturation, and its usefulness in the identification of the difference between two membrane proteins is apparent. However, more basic investigation is necessary for determining the topology and the fraction of membrane domains by the comparative denaturation experiments. Recently, molecular structures at atomic resolution have been reported for several membrane proteins, and the denaturation experiments of those proteins will provide a more detailed correlation between the structural characteristics of membrane proteins and the effects of denaturing reagents.

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