Recently, it was reported that the activity of rabbit P450 1A2 is markedly increased at elevated sodium phosphate concentration. Here, the possible structural change of rabbit P450 1A2 accompanying the NaCl-induced increase in its enzyme activity is investigated by fluorescence spectroscopy, circular dichroism, and absorption spectroscopy. It was found that NaCl increased α-helix content and lowered β-sheet content of P450 1A2 in the presence as well as in the absence of a phospholipid. Intrinsic fluorescence emissions also increased with increasing NaCl concentration. The low spin iron configuration of P450 1A2 shifted toward the high spin configuration in response to the increased salt concentration. The effect of increased potassium phosphate concentration. Here, the possible structural change of P450 1A2 induced by increased NaCl concentration was found that the activity increase of rabbit P450 1A2 was associated with the conformational change accompanying the NaCl-inclusion. The relationship between conformational changes and activities of the P450 is discussed.

Conformational Change of Cytochrome P450 1A2 Induced by Sodium Chloride

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EXPERIMENTAL PROCEDURES

Chemicals—L-α-Dilauroyl-sn-glycero-3-phosphocholine (DLPC) and cumene hydroperoxide were from Sigma. 7-Ethoxyresorufin and resorufin were obtained from Molecular Probes, Inc. (Eugene, OR). Other chemicals were of the highest grade commercially available.

Enzyme Sources—P450 1A2 was purified from liver microsomes of 5,6-benzoflavone-treated rabbits as described (5). The P450 1A2 was electrophoretically homogeneous and had a specific P450 content of 17 nmol/mg protein. NADPH-P450 reductase was purified to an apparent homogeneity from phenobarbital-treated rabbits as described (6).

Assays—Two different approaches were taken to examine the effect of salt on the P450 1A2 activity. For the NADPH-P450 reductase-supported reactions, 0.5 nmol of P450 1A2, 0.6 nmol of NADPH-P450 reductase, 15 μg of DLPC, and a NADPH-generating system were mixed together. The volume of the reaction mixture was 0.5 ml with varying concentrations of potassium phosphate buffer (0–400 mM, pH 7.4) and NaCl (0–0.5 M). In all of these cases, the NADPH-generating system consisted of final concentrations at 10 μmol glucose 6-phosphate, 0.5 mm NADP+, and 2 μl of yeast glucose 6-phosphate dehydrogenase/ml. The reaction mixtures were incubated at 25 °C for 3 min, and reactions were initiated by adding 2 μl of 7-ethoxyresorufin (to 50 μM) or 7-ethoxyresorufin (to 5 μM) as the substrates.

When the reaction was supported by cumene hydroperoxide, each incubation contained 0.5 nmol of P450 1A2 and 15 μg of DLPC in addition to the same concentrations of the substrates, phosphate buffer, and NaCl (or KCl) as described above for the NADPH-P450 reductase-supported reactions. The reaction was initiated by the addition of 2 μl of cumene hydroperoxide (to 0.1 mM).

The reaction mixtures were incubated at 25 °C for 10 min and the products were estimated fluorometrically as described elsewhere for the assay of 7-ethoxyresorufin O-deethylation (7) and 7-ethoxyresorufin O-deethylation (8).

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Protein was assayed using a bicinchoninic acid procedure according to the manufacturer’s directions (Pierce). P450 concentrations were determined by Fe3+/CO versus Fe2+/CO difference spectroscopy (9).

Fluorescence Spectroscopy—All experiments were done in 80 mM potassium phosphate buffer at 25 °C. Fluorescence intensity was measured on a Shimadzu RF5301 PC spectrophotometer (Shimadzu Corp., Tokyo) in a thermostated cuvette. Protein concentration was 1 μM. The excitation wavelength was 295 nm, and the background light scattering was determined with identical solutions without the protein. The aggregation of the protein was followed by 90° light scattering at 450 nm.

Circular Dichroism Measurements—Circular dichroic spectra were recorded on a Jasco J700 spectropolarimeter (Japan Spectroscopic, Tokyo) at 25 °C in a thermostated cuvette. The calibration of the spectropolarimeter was performed using α-10-camphorsulfonic acid, which shows a molar ellipticity of 7800 degrees cm2/dmol at 290.5 nm in an aqueous solution. CD spectra of P450 1A2 were obtained using 1 μM protein in a 0.1-cm pathlength cell. Blanks (buffer with or without sodium chloride) were determined by Fe3+/CO versus Fe2+/CO difference spectroscopy (9).

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lipids) were routinely recorded and subtracted from the original spectra.  

**Absorption Spectroscopy**—Absorption spectra were recorded with a Perkin-Elmer Lambda 16 spectrophotometer (Norwalk, CT). All measurements were done at 25°C with 1 μM protein concentration in 80 mM potassium phosphate buffer with a 1-cm pathlength. Because of the high dilution (1 μM), spectral measurements on P450 1A2 were reliable only in the range of 360–480 nm.

Stock solutions of P450 1A2 and NADPH-P450 reductase contained 20% glycerol, but final glycerol concentrations in all experiments were kept below 0.2% as glycerol affects the NADPH-P450 reductase-mediated reactions (10) and the protein structure (4).

**RESULTS AND DISCUSSION**

**Effects of Salt on Catalytic Activities of P450 1A2**—In order to investigate the relationship between the electrolyte-induced conformational change and the enzyme activity, we extended the work by Schenkman et al. (3) by examining the P450 1A2-catalyzed reaction in the presence of various concentrations of potassium phosphate, NaCl, and KCl. The enzymatic activity of P450 1A2 was quantified by measuring its ability to catalyze the O-deethylation of 7-ethoxyresorufin and 7-ethoxycoumarin.

The activities toward both 7-ethoxyresorufin and 7-ethoxycoumarin, caused by cumene hydroperoxide in the absence of the reductase and NADPH, increased by 50–60% when the concentration of potassium phosphate was raised from 5 to 80 mM in the reconstituted system (Fig. 1, A and C). The addition of 0.05–0.3 mM NaCl to the P450 1A2 solution caused the gradual increase of activities (Fig. 1, B and D). This rather dramatic stimulatory effect continued beyond the concentration of 80 mM. When we used sodium phosphate instead of potassium phosphate, the effect of sodium phosphate was similar to that of NaCl (data not shown) as described previously (3).

Using the reconstituted system containing P450 1A2, NADPH reductase, and NaCl in addition to DLPC, it was observed that the activities increased about 5-fold when potassium phosphate concentrations were increased from 5 to 50 mM with 7-ethoxyresorufin as the substrate (Fig. 1A) and increased about 4-fold with 7-ethoxycoumarin as the substrate (Fig. 1C). With both substrates, the activities declined above the potassium phosphate concentration of 50 mM. As the salt concentration of the solution was increased beyond 50 mM, the activities declined. This effect is the same as that of sodium phosphate (3). The salt effect seems to be the result of influences on the interaction between P450 and NADPH-P450 reductase (11).

Catalytic activities were also observed in 10 mM HEPES buffer containing 0.05–0.5 mM NaCl or KCl. The stimulatory effect of salts was routinely observed and provided about 1.5- and 1.4-fold enhancement when 0.2 mM NaCl and 0.2 mM KCl, respectively, were added (data not shown). An apparent turnover number of 0.107 nmol of product produced per nmol of P450 1A2/min in 10 mM HEPES buffer was found.

Recently, it has been shown that some human P450-catalyzed reactions are also stimulated by high concentrations of salts (NaCl, KCl, or MgCl₂) (12). By increasing the ionic strength from 5–10 to 100–200 mM sodium phosphate or sodium chloride, it was observed that the rate of P450 reduction in microsomes and in reconstituted systems containing P450 1A2, P450 2B4, several rat P450 enzymes, and NADPH-P450 reductase was enhanced. This suggests that P450 has a higher affinity for the NADPH-P450 reductase at higher ionic strength (10, 11). These results have been interpreted to mean that the stimulatory effect of increased ionic strength is due to an enhancement of the interaction between P450 and NADPH-P450 reductase and is not due to a spin shift (10). Although the shift from low to high spin resulted in an increased fast phase reduction of P450 2B4 (13), no change was noted in the spin equilibrium in the reconstituted system of P450 2B4 and in microsomes at various NaCl or sodium phosphate concentrations (10).

**The NaCl-induced Conformational Changes of P450 1A2 in Aqueous Solution**—It was reported that the absorption spectrum of P450 1A2 is sensitive to its own concentration and to the ionic strength of the solution (14). At high protein concentration or at low potassium phosphate concentration, an appreciable light scattering due to aggregation was observed. Fig. 2 shows that, when the concentration of P450 1A2 was 1 μM and the concentration of potassium phosphate buffer (pH 7.4) was 80 mM or beyond, the spectrum was almost entirely originated from the absorption by the oxidized P450 1A2 without light scattering due to aggregation. Therefore, all the spectroscopic studies were performed under these conditions. It was reported that seven to eight P450 1A2 monomers associate with high affinity to form a basic oligomeric structure even at this low protein concentration (15). It has been shown, however, that P450 1A2 activity is not very dependent upon the state of aggregation of the enzyme (16). Glycerol, usually added to P450 solution to stabilize the protein, was omitted here to avoid its
The effect of NaCl on the secondary structure of the P450 1A2 was studied by CD in the far UV region. Fig. 3A shows the CD spectra of P450 1A2 in 80 mM potassium phosphate buffer (pH 7.4) in the presence of varying concentrations of NaCl. The CD spectra were curve-fitted by the least squares method into the reference spectra obtained from five proteins: myoglobin, lysozyme, ribonuclease A, papain, and lactate dehydrogenase (17). Analysis of the CD spectrum for the P450 1A2 in the absence of NaCl yielded 30% α-helix, 26% β-sheet, 23% β-turns, and 21% random structure (Table I). With an increasing concentration of NaCl, the α-helix content increased while those of β-sheet, β-turns, and random structure decreased. Fig. 3A shows that the CD spectra, measured in the NaCl concentration ranging from 0 to 0.3 M, pass through an isodichroic point at 202 nm. The presence of the isodichroic point observed for P450 1A2 indicates that the β-structure and random structure are in dynamic equilibrium with the α-helix structures. We did not observe any change in the CD spectrum when the concentration of NaCl was increased beyond 0.3 M up to 0.5 M. However, the spectra of P450 1A2 obtained at the concentration of potassium phosphate buffer of less than 80 mM did not agree well with reference spectrum (17) (data not shown) possibly due to light scattering as shown by Haugen and Coon (14). It is thought that the P450 1A2 forms heterogeneous aggregates at low salt concentrations with an apparent molecular weight larger than the basic oligomeric structure of P450 1A2. Measurements at high dilution (1 μM) prevented us from observing the CD spectrum of the P450 1A2 in the near UV range. The secondary structures of salt-free and salt-induced P450 do not agree with values predicted from the method by Tretiakov et al. (18).

The intrinsic fluorescence of P450 1A2 reflects mainly the individual environments of its intrinsic probes, which are spaced evenly throughout its sequence (19). The intrinsic fluorescence intensity of P450 1A2 at \( \lambda_{\text{max}} = 330 \text{ nm} \) obtained in the presence of 0–0.3 M NaCl is shown in Fig. 4. The fluorescence intensity increased with increasing NaCl concentration, but there was no change in the \( \lambda_{\text{max}} \). This indicates that the change brought about by NaCl reduces the quenching of the intrinsic fluorescence in the P450 1A2, but otherwise the overall environment of the intrinsic fluorophore appears to remain unchanged. We did not observe any further change in fluorescence intensity when the concentration of NaCl was higher (data not shown).

The Conformational Change of P450 1A2 by NaCl in the presence of 0–0.3 M NaCl is shown in Fig. 4. The fluorescence intensity of P450 1A2 at \( \lambda_{\text{max}} = 330 \text{ nm} \) was studied by CD in the far UV region. The presence of the intrinsic fluorophore appears to remain unchanged. We did not observe any further change in fluorescence intensity when the concentration of NaCl was higher (data not shown).

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secondary structure. The addition of glycerol (to 5–20% final concentration) to P450 1A2 with DLPC induced a higher degree of α-helix and decreased β-sheet contents (Table I).

When DLPC was present, the intrinsic fluorescence intensity of the P450 1A2 increased by about 13% as compared with that of the protein in the absence of DLPC. The intensity gradually increased by about 12% when the salt concentration in the protein solution was increased to 0.3 M NaCl (Fig. 4); further increased by about 12% when the salt concentration in the P450 1A2 increased by about 13% as compared with that in the absence (●) or presence (○) of DLPC. Excitation was made at 295 nm. All values are means of duplicate experiments.

When DLPC was present, the intrinsic fluorescence intensity of the P450 1A2 increased by about 13% as compared with that of the protein in the absence of DLPC. The intensity gradually increased by about 12% when the salt concentration in the protein solution was increased to 0.3 M NaCl (Fig. 4); further increased by about 12% when the salt concentration had no effect (data not shown). Regardless of the presence or absence of DLPC, it is likely that the increased salt concentration converts the conformation of P450 1A2 into a form where at least a part of the intrinsic probes is exposed to more hydrophobic environments as compared with that of the NaCl-free condition. These results suggest that the stimulation of catalytic activity by DLPC involves the increased secondary structure as well as increased interaction between P450 and NADPH-P450 reductase (21).

The P450 1A2 exists as a mixture of high and low spin iron forms with 2 peaks at 395 and 414 nm. Fig. 5 gives the absorption spectra of P450 1A2 complexed with DLPC in the presence of several concentrations of NaCl, and they are compared with the spectrum obtained in the absence of NaCl. As the concentration of NaCl increased, the absorption spectra shifted toward the high spin state in the spin equilibrium.

Recently, it has been shown that some typical P450 substrates, inhibitors, or cytochrome b5 can cause a shift in the spin equilibrium of P450 1A2 toward high spin (23, 24). It has been reported also that several P450 substrates or alcohols influence the conversion of rabbit and rat P450 1A2 from a high to low spin iron configuration (25–27). Interestingly, the effect of salt on P450 1A2 is very similar to the salt effect on bacterial P450cam (CYP101). That effect involves the configurational change toward the high spin state (28). In contrast, no change was noted in the spin equilibrium in the reconstituted system of P450 2B4 and in microsomes at various NaCl or sodium phosphate concentrations (10). It has been reported that the spin state was not correlated to catalytic activity of the rat P450s in reconstituted systems and that not all P450s exhibited a relationship between spin state and reduction potential (26).

**Conclusions**—This investigation established that the conformation of P450 1A2 was highly dependent on the concentration of salt. NaCl increased the α-helix content and lowered β-sheet content of P450 1A2. The presence of DLPC magnified this effect. Intrinsic fluorescence emissions also increased with an increasing concentration of NaCl. The low spin iron configuration of P450 1A2 shifted toward the high spin configuration in response to the increased NaCl concentration. These salt-induced conformations coincided with higher activity of P450 1A2-catalyzed reactions. It was not possible to decide whether the high α-helix content of P450 1A2 induced by salt was the cause of the increased catalytic activity or it was simply parallel NaCl-induced phenomena without direct connection. Since the ionic strength in vivo was about 0.15, the conformation of P450 1A2 induced by salt may have been actually the physiologically active form, which had high affinity for the NADPH-P450 reductase. The stimulation of the activity by increasing the ionic strength was consistent with the conformational change of P450 1A2 in addition to the stimulated interaction between P450 and NADPH-P450 reductase. Although some P450-catalyzed reactions were stimulated with an increased salt concentration (10–12), the physiological significance of this phenomenon is unclear yet.

P450 is a superfamily of proteins consisting of a number of families. Since P450 enzymes have considerable differences in their amino acid sequence (29, 30) and an effect observed with one of them would not necessarily apply to other microsomal P450 forms, the salt effect on the conformation could be an unique property of the P450 1A2. Analysis of structures of more individual P450s and their interactions with NADPH-P450 reductase in three dimensions is needed to elucidate the contributions of P450 structures to catalytic mechanisms.

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