Effect of Alcohols on the Structure and Function of D-Amino-Acid Oxidase†

Hirobumi Ōhama, Nobuhiko Sugiura, Fumio Tanaka, and Kunio Yagi*

ABSTRACT: The absorption spectrum of D-amino-acid oxidase (D-amino-acid:oxygen oxidoreductase (deaminating), EC 1.4.3.3) was significantly perturbed by various alcohols; typical fine structures were observed in the visible absorption bands, accompanied by blue shifts of the peaks. Both fluorescence intensity and fluorescence polarization were increased upon the addition of alcohols, indicating that the coenzyme is not liberated from the apoenzyme but the hydrophobicity of the environment of the enzyme-bound flavin is increased. Upon the addition of alcohols, the circular dichroism of the enzyme was markedly modified in the visible and near-ultraviolet regions, while that of the apoenzyme in the near- and far-ultraviolet regions was scarcely modified, indicating a change in the interaction between the flavin coenzyme and protein. Both the apparent maximal velocity and the apparent Michaelis constant of the enzyme were increased by the addition of alcohols. The presence of alcohols tends to dissociate the dimer of this enzyme into the monomer, but the dissociation does not fully explain the increase in the maximal velocity of the enzyme by alcohols, because the increase in the maximal velocity caused by alcohols is larger than that expected from the dissociation. Since the rate of formation of the purple intermediate was decreased by alcohols in both the dimer and the monomer, the increase in the maximal velocity could be ascribed to an increase in the rate of dissociation of the enzyme–product complex. This increase could be ascribed to the protein conformational change, which is probably provoked by combination of alcohols with the enzyme at a locus other than that for substrate binding.

The appearance of fine structure in the 450-nm absorption band of D-amino-acid oxidase (D-amino-acid:oxygen oxidoreductase (deaminating), EC 1.4.3.3) upon forming a complex with benzoate was first noticed by Yagi and Ozawa (1962). This phenomenon was interpreted by Massey and Ganther (1965) to mean that the flavin chromophore is surrounded by a hydrophobic environment, on the basis of the data of Harbury et al. (1959) of the solvent effect on the flavin chromophore. The perturbation of absorption spectra of flavins by organic solvents has been amply discussed (Harbury et al., 1959; Koziol and Knobloch, 1965; Koziol, 1966, 1969; Kotaki et al., 1966, 1967; Yagi et al., 1967b, 1969; Palmer and Massey, 1968). Accordingly, it seemed worthwhile to investigate the effect on the enzyme of substances which reduce the dielectric constant of the medium, though the problem was suspected to be more complicated due to the interaction of alcohols with both the protein and FAD.[1] In fact, ethanol both induces a change in the absorption spectrum and enhances the catalytic activity of D-amino-acid oxidase (Ōhama et al., 1973; Shiga and Shiga, 1973). These remarkable phenomena were suggested to be caused by an interaction between the protein and ethanol, which induced a local change in the environment of the FAD binding site. In this paper, we consider the interrelationship between the modification of the catalytic activity and the change in the enzyme structure caused by the addition of alcohols.

Materials and Methods

D-Amino-acid oxidase was prepared from hog kidney according to the method of Yagi et al. (1967a, 1970b). The apoenzyme was obtained from the holoenzyme by the method of Massey and Curti (1966). Concentration of the holoenzyme was expressed in terms of the bound coenzyme, FAD. Protein concentration was measured by the biuret method (Gornall et al., 1949). Alcohols used in the present study are of spectroscopic grade.

Absorption and difference spectra were measured in a Beckman DK-2A spectrophotometer using a 10-mm quartz cell. A Yanaco SPS-1 stopped-flow spectrophotometer connected to a storage oscilloscope was used to follow the changes in absorbance at a fixed wavelength. Details of the method have been described elsewhere (Yagi et al., 1972c).

Fluorescence intensity and polarization were measured with a Shimadzu RF-502 spectrofluorophotometer according to the method described elsewhere (Yagi et al., 1975). Fluorescence polarization, P, was calculated from the following equation:

$$P = \frac{I_{\parallel} - I_{\perp}C}{I_{\parallel} + I_{\perp}C}$$

where $I_{\parallel}$ and $I_{\perp}$ are the measured fluorescence intensities parallel and perpendicular to polarized light and C is the instrumental correction factor, which was determined according to the method of Azumi and McGlynn (1962).

CD measurement was performed with a JASCO, Model J-20, spectropolarimeter. A 10-mm quartz cell was used for CD measurement in the visible wavelength region (330–550 nm) and the near-ultraviolet region (240–320 nm), and a 0.2-mm quartz cell for that in the far-ultraviolet region (190–250 nm). CD data were expressed in terms of molar ellipticity for the spectra in the visible wavelength region and in terms of mean residue ellipticity for those in the ultraviolet region. The mean residue weight for both the apoenzyme and holoenzyme was assumed to be 115, as previously reported (Yagi et al., 1967a; Sugiura et al., 1973). Samples of the enzyme were examined at the concentration of 1×10^{-4} M for the measurement of the visible CD spectrum and at the con-

† From Institute of Biochemistry, Faculty of Medicine, University of Nagoya, Nagoya 466, Japan. *Received December 18, 1975.*

[1] Abbreviations used are: FAD, flavin adenine dinucleotide; CD, circular dichroism.

TABLE I: Effects of Various Alcohols on Spectral Parameters of D-Amino-Acid Oxidase.[a]

Additives	Peak$_1$(ϵ)	Peak$_2$(ϵ)	$\Delta\epsilon_{492}$	$\Delta\epsilon_{387}$
FAD	450 nm (1.13×10^4)	375 nm (9.30×10^3)		
Enzyme	455 nm (1.13×10^4)	371 nm (9.73×10^3)		
FAD + methanol	450 nm (1.16×10^4)	375 nm (9.50×10^3)		
Enzyme + methanol	451 nm (1.13×10^4)	367 nm (9.35×10^3)	7.27×10^2	10.29×10^2
Enzyme + ethanol	445 nm (1.14×10^4)	363 nm (8.00×10^3)	13.66×10^2	21.29×10^2
Enzyme + 1-propanol	441 nm (1.26×10^4)		20.24×10^2	

[a] The enzyme (7.0×10^{-5} M) was dissolved in 0.017 M pyrophosphate buffer, pH 8.3, and absorption spectra were measured in the absence and presence of alcohols. Concentrations of alcohols were fixed at the molar fraction of 0.033. Peak$_1$ and peak$_2$ represent longer and shorter wavelength absorption bands, respectively. ϵ is molar extinction coefficient at wavelength of the absorption peak. $\Delta\epsilon_{492}$ and $\Delta\epsilon_{387}$ are difference extinction coefficients at 492 and 387 nm, at which peaks of difference absorption spectrum were observed.

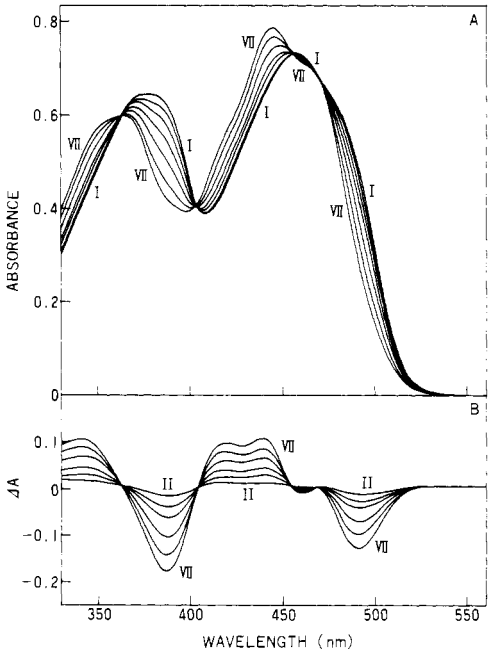

FIGURE 1: Change in the absorption spectrum of the holoenzyme upon addition of methanol. (I) Holoenzyme (6.5×10^{-5} M) dissolved in 0.017 M pyrophosphate buffer, pH 8.3; (II) I + methanol (2.5%); (III) I + methanol (5.0%); (IV) I + methanol (7.5%); (V) I + methanol (10.0%); (VI) I + methanol (12.5%); (VII) I + methanol (15.0%). (A) Absorption spectra; (B) difference spectra between curves from II to VII and curve I.

centration ranging from 0.5 to 1.5 mg/ml for the measurement of the near- and far-ultraviolet CD spectra. All spectroscopic measurements were made 10 min after mixing the enzyme solution with alcohol, since the change in the spectrum of the enzyme caused by alcohol takes a few minutes to reach its maximum.

The rate of catalytic reaction of the enzyme was determined by measuring oxygen consumption with a Beckman Oxygen Sensor (Yagi et al., 1967a) in the absence or presence of alcohols. D-Alanine was used as substrate, and the concentration of the enzyme was 1×10^{-7} M. The reaction was checked by measuring the product, pyruvate (Friedemann, 1957).

Sedimentation measurements were performed in a Spinco, Model E, analytical ultracentrifuge with a standard 12-mm cell.

All experiments described here were performed in 0.017 M pyrophosphate buffer, pH 8.3, at 20 °C. When alcohols were added to the medium, the pH of the solution was readjusted before measurement.

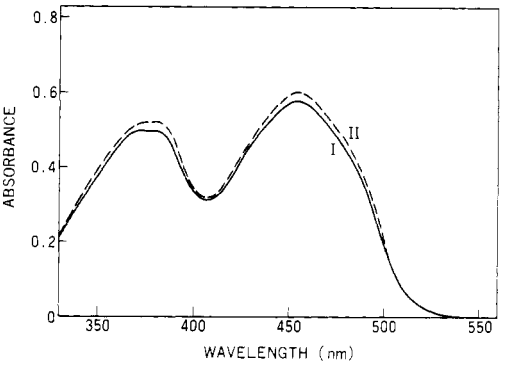

FIGURE 2: Effect of sucrose on the absorption spectrum of the enzyme. (I) Holoenzyme (5.1×10^{-5} M) dissolved in 0.017 M pyrophosphate buffer, pH 8.3; (II) I + sucrose (52.5%).

Results

Effects of Various Alcohols and Sucrose on the Visible Absorption Spectrum of the Holoenzyme. Figure 1 shows the change in the visible absorption spectrum of the enzyme upon the addition of methanol. Marked blue shifts of both absorption peaks and pronounced hyperchromism of the longer wavelength absorption band and hypochromism of the shorter wavelength band were observed. Isosbestic points appeared in the absorption spectra, indicating that an equilibrium occurs between the original enzyme species and the enzyme species induced by alcohols. Another characteristic modification of the spectrum was the appearance of typical resolution of both absorption bands. These phenomena were commonly observed with other alcohols. Table I summarizes the effect of various alcohols on the absorption spectrum of the holoenzyme at their concentration fixed at the molar fraction of 0.033 (corresponding to that of 10% ethanol). The characteristic modification of the spectrum became more prominent with an increasing carbon number of alcohol molecules. On the other hand, the spectrum of free FAD was not significantly modified upon the addition of methanol; only slight hyperchromism of both absorption bands was observable (Table I). This result indicates that the significant perturbation of the spectrum observed with the holoenzyme could be ascribed to the intervention of the apoenzyme, not to the direct interaction between alcohol and FAD itself.

A high concentration of sucrose is known to lower the dielectric property of water. Figure 2 shows the absorption spectrum of the holoenzyme in the presence of 52.5% sucrose, the dielectric constant of which is about 64.5 corresponding to that of 33% methanol or of 27% ethanol solution (Åkerlöf, 1932). Lowering the dielectric constant even less than that of

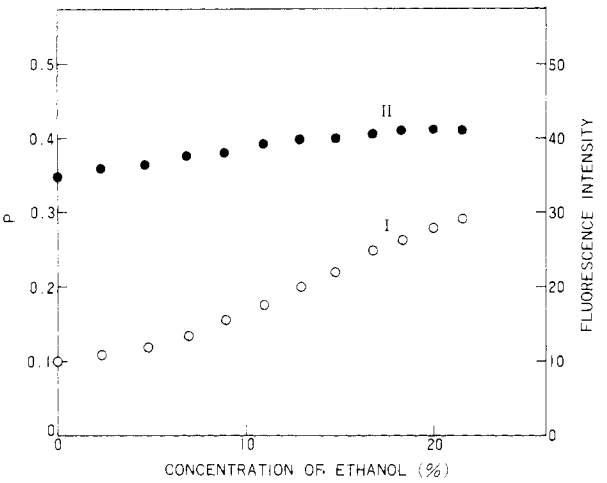

FIGURE 3: Effect of ethanol on fluorescence intensity and polarization of the holoenzyme. Final concentration of the enzyme was 5.1×10^{-5} M. (I) Fluorescence intensity; (II) fluorescence polarization. Excitation, 450 nm; emission, 530 nm.

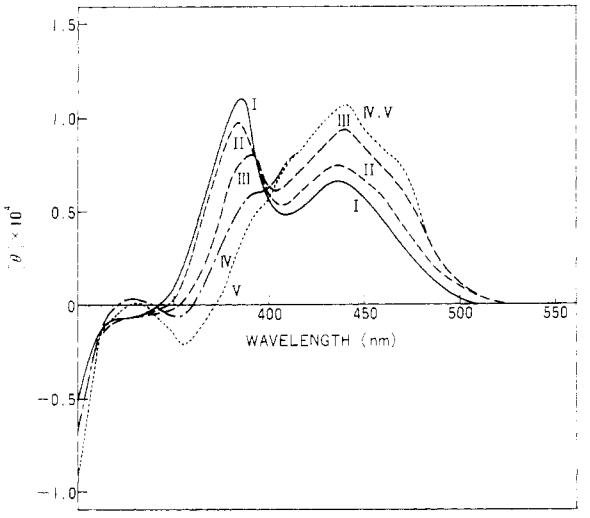

FIGURE 4: Effect of ethanol on the visible CD spectrum of the holoenzyme. (I) Holoenzyme (1.0×10^{-4} M) dissolved in 0.017 M pyrophosphate buffer, pH 8.3; (II) I + ethanol (5.0%); (III) I + ethanol (10.0%); (IV) I + ethanol (15.0%); (V) I + ethanol (20.0%). [θ], Molar ellipticity.

20% ethanol did not give any significant spectral change of the enzyme, except for a slight hyperchromism of both absorption bands.

Effects of Alcohol on Fluorescence of the Enzyme. Since the perturbation of the absorption spectrum mentioned above is considered to be caused by the change in the interaction between the apoenzyme and the coenzyme, the interaction was further studied fluorometrically. Figure 3 shows the effect of ethanol on the fluorescence intensity and polarization of the holoenzyme. It is obvious that the fluorescence intensity of the enzyme increased as the concentration of ethanol added increased. This indicates two possibilities: the increase in fluorescence efficiency of bound FAD or the release of FAD from the enzyme, since the fluorescence intensity of free FAD is higher than that of FAD bound to the apoenzyme. To discriminate between these two possibilities, fluorescence polarization data are useful. As is shown in Figure 3, fluorescence polarization was increased from 0.347 to 0.406 upon the addition of 20% ethanol. Considering that the polarization anisotropy obtained from the equation $(\frac{2}{3})(1/P - \frac{1}{3})^{-1}$ can be

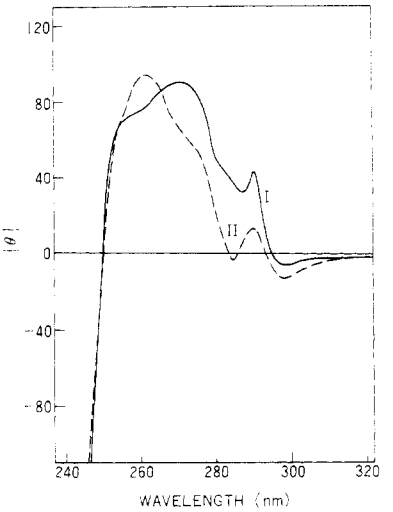

FIGURE 5: Effect of ethanol on the near-ultraviolet CD spectrum of the holoenzyme. (I) Holoenzyme (0.74 mg/ml) dissolved in 0.017 M pyrophosphate buffer, pH 8.3; (II) I + ethanol (10.0%). [θ], Mean residue ellipticity.

represented as an average of free (0.026) and bound FAD (0.400) weighed by respective fluorescence intensity (Weber, 1952; Yagi et. al., 1975), the above increase in fluorescence intensity should be ascribed to the increased fluorescence efficiency of bound FAD. This could be caused by the change in the environment surrounding FAD.

Effects of Various Alcohols on Circular Dichroism of the Enzyme. Figure 4 shows the change in the visible CD spectrum of the enzyme induced by ethanol. With increasing concentration of ethanol, ellipticity at the longer wavelength CD band increased significantly, the shorter wavelength band almost disappeared, and a minor, negative band at around 355 nm emerged. The resolution of the longer wavelength CD band was observed. This is a clear demonstration of the fine structure of the CD band of this enzyme that had been proposed by Shiga and Shiga (1973). These phenomena were also found with other monohydric alcohols, and the changes became more pronounced with the increase in carbon number of alcohol molecules, as observed in the changes of the absorption spectra. The resulting CD spectrum of the enzyme induced by the addition of 20% ethanol was quite different from that of free FAD reported by Miles and Urry (1968).

The CD spectrum in the near-ultraviolet region of the holoenzyme is shown in Figure 5. The perturbation in the near-ultraviolet CD spectrum by ethanol was also noticeable in the holoenzyme. The near- and far-ultraviolet CD spectra of the apoenzyme, however, were scarcely perturbed by ethanol, which were nearly identical with those reported elsewhere (Ōhama et al., 1973). These observations indicate that the molecular interaction between the apoenzyme and the coenzyme is influenced by alcohols and that the secondary structure of the enzyme was not modified by them at the concentration range, where the spectral perturbation in the visible wavelength region was observed.

Effects of Alcohols on the Catalytic Activity of the Enzyme. Figure 6 shows the double-reciprocal plots of the catalytic rate against the concentration of D-alanine in the absence and presence of ethanol. A linear correlation between e_0/v and $1/[S]$ was observed in the presence of ethanol. Both the apparent maximum velocity (V_{max}) and the apparent Michaelis constant (K_M) increased with increasing concentration of ethanol. The higher concentration of ethanol, however, resulted

TABLE II: Changes in Kinetic Parameters of the Enzyme upon the Addition of Various Alcohols.[a]

Organic Solvents	Molar Fractions of Solvents: 0.017 V_{max}	0.017 V/V_0	0.017 K_M(mM)	0.033 V_{max}	0.033 V/V_0	0.033 K_M(mM)
None	417		1.5	417		1.5
Methanol	448	1.08	2.4	521	1.25	4.0
Ethanol	541	1.30	5.3	645	1.55	8.3
1-Propanol	588	1.41	10.3	741	1.78	30.3
2-Propanol	508	1.22	5.9	571	1.37	13.0
1-Butanol	323[b]	0.78[b]	10.9[b]			
2-Butanol	625	1.50	17.5			
tert-Butyl alcohol	541	1.30	9.1	386[b]	0.93[b]	6.8[b]
tert-Amyl alcohol	690	1.66	28.6			

[a] Reaction mixture for the determination of the enzymatic activity contained the enzyme (1.0×10^{-7} M), FAD (2×10^{-5} M), and D-alanine (concentrations shown in Figure 6) in 0.017 M pyrophosphate buffer, pH 8.3. Reaction mixture was saturated with air. The increase in O_2 concentration of the medium upon the addition of alcohols (molar fraction of the solvents, 0.033) was minute. O_2 uptake due to the enzymatic reaction was measured polarographically. V_{max} and K_m values are apparent. V/V_0 represents ratio of values of V_{max} in the presence and absence of alcohols. [b] The enzyme seemed to be denatured partially as judged from the turbidity of the higher concentration of the holoenzyme solution in the presence of alcohols.

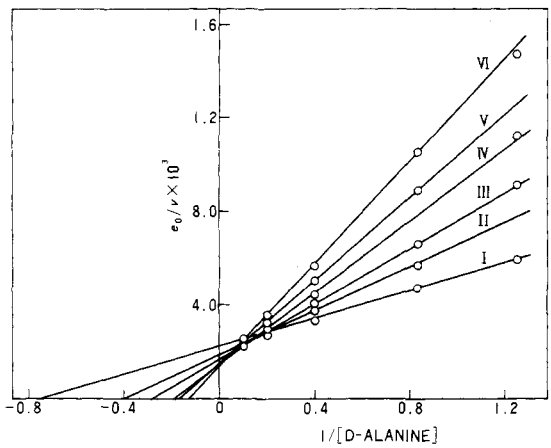

FIGURE 6: Effect of ethanol on the kinetic behavior of the enzyme. Reaction mixture contained the enzyme (1.0×10^{-7} M), FAD (2×10^{-5} M), and D-alanine (concentrations in the figure) in 0.017 M pyrophosphate buffer, pH 8.3. Reaction mixture was saturated with air. O_2 uptake due to the enzymatic reaction was measured polarographically. (I) In the absence of ethanol; (II) in the presence of ethanol (5.0%); (III) in the presence of ethanol (7.5%); (IV) in the presence of ethanol (10.0%); (V) in the presence of ethanol (12.5%); (VI) in the presence of ethanol (15.0%). The increase in O_2 concentration of the medium upon the addition of ethanol to 15% was less than 1%.

in denaturation of the enzyme, so that the elevation of catalytic rate was not observed at concentrations above 20%. The effects of various alcohols on kinetic parameters are summarized in Table II. Both V_{max} and K_M values increased with increasing carbon number of alcohols, but branched alcohols seem to be less effective than primary alcohols having the same carbon number.

To determine the step which is accelerated by alcohols in the overall reaction, the effect of alcohols on the formation of the purple intermediate was examined. First, the formation of the purple intermediate was examined by mixing anaerobically the enzyme (1.8×10^{-4} M) with D-alanine (5×10^{-2} M), lithium pyruvate (1×10^{-1} M), and ammonium sulfate (5×10^{-2} M) in the presence of 10% ethanol; a typical spectrum of the purple intermediate complex (Yagi et al., 1967c) was observed, which was identical with that observed in the absence of ethanol. Then, the effect of alcohols on the rate of formation of the purple intermediate was studied. By the stopped-flow method, the absorbance changes at 550 nm upon the anaerobic mixing of the enzyme with D-alanine in the absence and presence of 10% ethanol were traced. As can be seen in Figure 7, they are obviously biphasic. The rapid and slow reaction components are ascribed to the dimer and monomer of this enzyme, respectively, as reported previously (Yagi et al., 1972c). In the absence of ethanol, the amounts of the dimer and monomer were calculated from Figure 7A by semilogarithmic plots to be 91% and 9%, respectively. This result is in good agreement with our previous one (Yagi et al., 1972c). In the presence of ethanol, the amounts were calculated from Figure 7B to be 72% and 28%, respectively. These results indicate that the monomer–dimer equilibrium of this enzyme (Yagi and Ohishi, 1972a; Sugiura et al., 1973) is shifted towards the monomer in the presence of ethanol. From Figure 7, the reaction rates of both the dimer and monomer in the absence of ethanol were calculated to be 85 and 14 sec^{-1}, respectively, and those of the dimer and monomer in the presence of 10% ethanol to be 44 and 7 sec^{-1}, respectively.

Effect of the Addition of Benzoate on the Spectral Change of the Enzyme Induced by Alcohol. Benzoate is known to form a complex with the enzyme in competition with the substrate and to exhibit a characteristic change in the absorption spectrum of the enzyme (Yagi and Ozawa, 1962). Figure 8 shows the effect of benzoate on the spectrum of the enzyme in the presence of 20% methanol. The absorption spectrum obtained in the presence of methanol was changed to that of the enzyme–benzoate complex formed in the absence of methanol. The same result was observed in the CD spectrum.

Effect of Ethanol on Sedimentation Pattern of the Enzyme. The effect of ethanol on the monomer–dimer equilibrium of this enzyme was studied by ultracentrifugation. In the presence of 10% ethanol, a slight asymmetry of the sedimentation pattern of the enzyme was observed, and the value of $s_{20,w}$ was slightly decreased as compared with that obtained in the absence of the cosolvent (Table III). By increasing the concentration of ethanol to 15%, $s_{20,w}$ was significantly decreased, indicating that the monomeric species of the enzyme increased.

Discussion

The addition of alcohols to the enzyme solution resulted in the resolution of the visible absorption bands of the enzyme

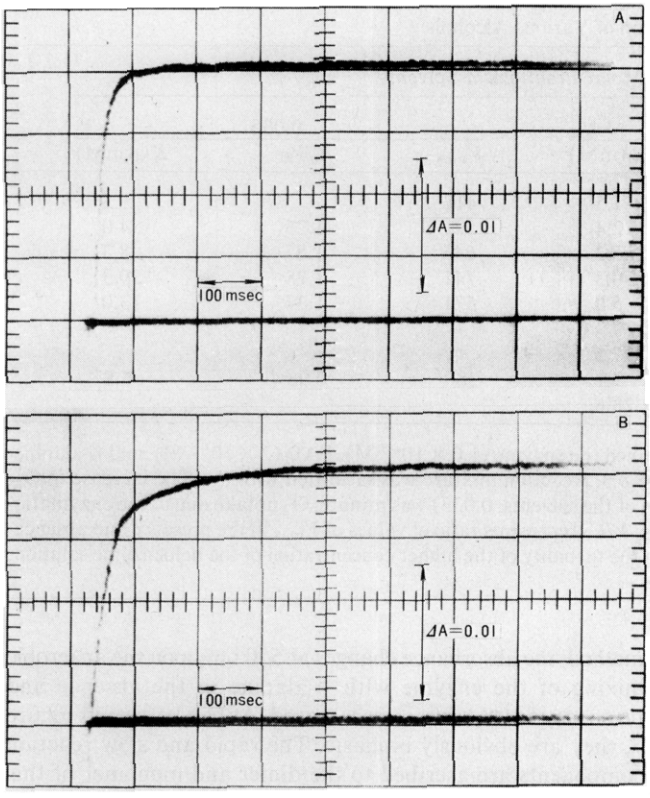

FIGURE 7: Effect of ethanol on the reaction of the enzyme with D-alanine under anaerobic conditions. Equal volumes of the anaerobic solutions of the enzyme (8.0×10^{-5} M) and D-alanine (5×10^{-2} M) were mixed in the absence and presence of ethanol (10.0%) and the transmittance change at 550 nm was followed (light path, 10 mm). The solutions were made anaerobic by bubbling argon gas followed by consuming a trace of oxygen by addition of glucose oxidase (1×10^{-7} M) and glucose (5×10^{-3} M). (A) In the absence of ethanol; (B) in th presence of ethanol (10.0%). Transmittance was converted to absorbance.

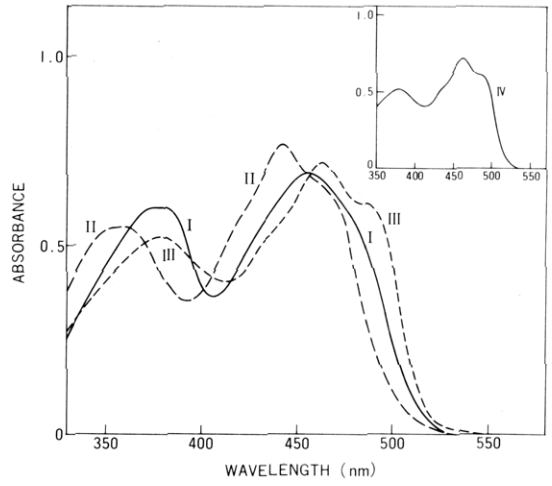

FIGURE 8: Formation of enzyme–benzoate complex in the presence of methanol. The enzyme–benzoate complex formation was observed by spectral change of the holoenzyme upon the addition of powdered sodium benzoate into a cuvette. (I) Holoenzyme (6.2×10^{-5} M) dissolved in 0.017 M pyrophosphate buffer, pH 8.3; (II) I + methanol (20.0%); (III) II + sodium benzoate (ca. 1×10^{-2} M); (IV) I + sodium benzoate (ca. 1×10^{-2} M).

TABLE III: Effect of Ethanol on $s_{20,w}$ of the Holoenzyme.[a]

Solvent System	$s_{20,w}$
0.017 M pyrophosphate, pH 8.3	7.7
0.017 M pyrophosphate + ethanol (10.0%)[b]	6.7
0.017 M pyrophosphate + ethanol (15.0%)[b]	4.1

[a] Concentration of the enzyme was 5 mg/ml. [b] Concentrations of ethanol were final ones.

accompanied with the blue shifts of their peaks, in agreement with our previous result (Ōhama et al., 1973) as well as with that of Shiga and Shiga (1973). The modified absorption spectrum observed in the present study coincides with the spectra of flavins dissolved in apolar media (Harbury et al., 1959; Koziol and Knobloch, 1965; Koziol, 1966, 1969; Kotaki et al., 1966, 1967; Yagi et al., 1967b, 1969; Palmer and Massey, 1968). Accordingly, the presently observed perturbation of the absorption spectrum of the enzyme provoked by alcohols could be interpreted to mean that the flavin chromophore of the enzyme is surrounded by a hydrophobic environment. This interpretation is supported by the fluorometric data; upon the addition of alcohols, the fluorescence intensity and polarization degree of the flavin moiety of the enzyme were increased, indicating that the flavin was not liberated from the enzyme but the fluorescence efficiency of bound FAD increased. On the other hand, a high concentration of sucrose did not perturb the absorption spectrum of the enzyme. This suggests that the effects of alcohols on the enzyme were not simply due to their action in decreasing the dielectric constant of the medium.

All the above results indicate that alcohols combine with the enzyme to cause the conformational change of the apoenzyme resulting in the provocation of hydrophobic environment surrounding the flavin. The CD spectra in the near-ultraviolet and visible regions further indicate that alcohol combines with the enzyme and modifies the molecular interaction of flavin with the protein moiety.

The fact that the modification of the absorption and CD spectra was increased when the carbon number of alcohols was increased indicates that the binding between alcohol and the apoenzyme is hydrophobic in nature.

The effects of alcohols on the spectroscopic properties of the enzyme presented above are similar to the case of the enzyme–benzoate complex (Yagi and Ozawa, 1962) or the enzyme–straight chain fatty acid complex (Yagi et al., 1970a). They equally combine with the apoenzyme. However, apart from benzoate or straight chain fatty acid, alcohol combines with the enzyme at a locus other than that of the substrate binding. This is verified by the fact that alcohols neither inhibit the enzyme in competition with the substrate nor disturb the formation of the catalytic intermediate.

In interpreting further the change provoked in the interaction between the apoenzyme and coenzyme upon the addition of alcohols, the results obtained by Kotaki et al. (1970) seem suggestive. The vibrational structure in the visible absorption spectrum of flavin in apolar media was broadened and shifted towards longer wavelength by the formation of hydrogen bonding. These changes were accompanied by a decrease in the fluorescence efficiency. Kotaki et al. assumed from these phenomena that the flavin chromophore in the enzyme is in a hydrophobic environment but that it partly interacts with protein residue(s) or water molecule(s) via hydrogen bonding. If this assumption is valid, the appearance of fine structure in the absorption spectra and fluorescence characteristics in the presence of alcohols stated above can be ascribed to the elimination of hydrogen bonding(s) between the isoalloxazine moiety and amino acids residue(s) of the protein or water molecule(s).

Another important finding in the present study is the increase in the maximum velocity of this enzyme combined with alcohols, which also appeared in the earlier work by Tan and Lovrien (1972) with other enzymes. In this regard, dissociation of the dimer into the monomer should be firstly examined, since the monomer has been found to possess larger V_{max} than the dimer (Yagi et al., 1972b, 1973; Shiga and Shiga 1972). The presence of 10% methanol accelerates the dissociation of the dimer into the monomer to some extent at a high enzyme concentration, and the dissociation would be further accelerated if the enzyme concentration is low. However, even the complete dissociation of the dimer into the monomer cannot explain fully the effect of alcohols to increase V_{max} of the enzyme, since the presently observed effect of alcohols to increase V_{max} of the enzyme is larger than that expected from the dissociation of the dimer into the monomer.

The elevation of the maximum velocity of the enzyme by alcohols should be explained in terms of the acceleration of some step(s) of the reaction sequence. Under aerobic conditions, the reaction mechanism of the enzyme is expressed as follows:

$$E_{ox} + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E_{ox} \cdot S \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E' \cdot S'$$

$$E' \cdot S' + O_2 \xrightarrow{k_{+3}} E_{ox} \cdot P + H_2O_2$$

$$E_{ox} \cdot P \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} E_{ox} + P$$

where E_{ox} and $E' \cdot S'$ represent the oxidized form of the enzyme and the purple intermediate, respectively. In the presence of excess oxygen, V_{max} can be expressed by $k_{+2}k_{+4}/(k_{+2} + k_{+4})$ (Palmer and Massey, 1968), and the increase of k_{+2} and/or k_{+4} leads to an increase in the overall reaction rate of the enzyme. In the presence of ethanol, the rate of the purple complex formation, k_{+2}, was found to decrease both in the dimer and in the monomer. Therefore, the elevation of the maximum velocity of the enzyme by alcohols observed in the present study would be achieved by the increase in the rate of dissociation of the enzyme–product complex, k_{+4}.

It is clear that both K_M and the dissociation constant of the enzyme–product complex are increased by alcohols. The increase in both parameters should be ascribed to such a conformational change of the apoenzyme as to decrease the affinity of the enzyme for both substrate and product.

Although it is obvious that the presently observed characteristic change in hydrophobicity surrounding the coenzyme and the elevation of V_{max} are both due to the conformational change of the apoenzyme upon complex formation with alcohols, further investigation should show whether the hydrophobicity surrounding the coenzyme directly influences the catalytic activity of the enzyme.

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