Horii, Z., Saki, T., Tamura, Y., and Tanaka, K. (1961), *Chem. Pharm. Bull. 9*, 442.
Kaiser, B. L., and Kaiser, E. T. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 36.
Kaiser, E. T., and Carson, F. W. (1965), *Biochem. Biophys. Res. Commun. 18*, 457.
Kirschner, M. W., and Schachman, H. K. (1971), *Biochemistry 10*, 1900.
Latt, S. A., and Vallee, B. L. (1971), *Biochemistry 10*, 4253.
Martell, A. E., Ed. (1964), *Chem. Soc., Spec. Publ. No. 17*, 409.
Narahashi, Y., Shibuya, K., and Yanagita, M. (1968), *J. Biochem. (Tokyo) 64*, 427.
Narahashi, Y., and Yanagita, M. (1967), *J. Biochem. (Tokyo) 62*, 633.
Petra, P. H. (1970), *Methods Enzymol. 19*, 460.
Quiocho, F. A., Bethge, P. H., Lipscomb, W. N., Studebaker, J. F., Brown, R. D., and Koeing, S. H. (1971), *Cold Spring Harbor Symp. Quant. Biol. 35*, 561.
Richards, E. G., and Schachman, H. K. (1959), *J. Phys. Chem. 63*, 1578.
Riordan, J. F., Sokolovsky, M., and Vallee, B. L. (1967a), *Biochemistry 6*, 358.
Riordan, J. F., Sokolovsky, M., and Vallee, B. L. (1967b), *Biochemistry 6*, 3609.
Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 1460.
Schrecker, A. W. (1957), *J. Org. Chem. 22*, 33.
Simpson, R. T., Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 616.
Smith, E. L., Lumry, R., and Polglase, W. (1951), *J. Phys. Chem. 55*, 125.
Sokolovsky, M., Riordan, J. F., and Vallee, B. L. (1966), *Biochemistry 5*, 3582.
Streitz, T. A., Ludwig, M. L., Quiocho, F. A., and Lipscomb, W. N. (1967), *J. Biol. Chem. 242*, 4662.
Ukita, C., Tamemasa, O., and Motomatsu, H. (1951), *J. Pharm. Soc. 71*, 1044.
Vallee, B. L., Riordan, J. F., Bethune, J. L., Coombs, T. L., Auld, D. S., and Sokolovsky, M. (1968), *Biochemistry 7*, 3547.
Vallee, B. L., Rupley, J. A., Coombs, T. L., and Neurath, H. (1955), *J. Biol. Chem. 217*, 253.
Weast, R. C., Ed. (1967), Handbook of Tables for Organic Compound Identification, 3rd ed, Cleveland, Ohio, Chemical Rubber Publishing Co.
Webb, J. L. (1963), Enzyme and Metabolic Inhibitors, Vol. I, New York, N. Y., Academic Press.
Whitaker, J. R. (1966), *Biochem. Biophys. Res. Commun. 22*, 6.
Wolfenden, R. (1972), *Accounts Chem. Res. 5*, 10.
Yonetani, T. and Theorell, H. T. (1964), *Arch. Biochem. Biophys. 106*, 243.

Ultraviolet Difference Spectroscopic Studies of the Binding of Ligands to Rabbit Muscle Aldolase†

Alvin L. Crowder, III, Robert Barker,* and Charles A. Swenson

ABSTRACT: The binding of D-arabinitol 1,5-diphosphate, 1,5-pentanediol diphosphate, and inorganic phosphate to rabbit muscle aldolase (EC 4.1.2.7) has been investigated by ultraviolet difference spectroscopy. Binding of D-arabinitol 1,5-diphosphate, a potent inhibitor, causes a change in the environment of a tryptophan residue. The hydroxyl groups of the ligand are necessary for perturbation of the tryptophan residue. By ultraviolet difference spectroscopic studies of the binding of arabinitol 1,5-diphosphate in the presence of ethylene glycol the environmental change was shown to arise from a conformational adjustment in the enzyme which increases the exposure of tryptophan to solvent.

Binding of the competitive inhibitor D-arabinitol 1,5-diphosphate (Ara-P_2[1]) to rabbit muscle aldolase (EC 4.1.2.7) has been shown to produce changes in the environment of the tyrosyl and tryptophyl residues as measured by ultraviolet difference spectroscopy (Lehrer and Barker, 1971). Certain competitive inhibitors have been shown to quench the fluorescence of rabbit muscle aldolase (Rose and O'Connell, 1969). Similar results were obtained with rat muscle aldolase (Suh and Barker, 1971) and the quenching was shown to depend upon the presence and orientation of hydroxyl and/or carboxyl groups in the inhibitor. In addition the presence of hydroxyl groups can increase the binding constant by an order of magnitude as can the orientation of hydroxyl groups in polyhydroxylic derivatives (Hartman and Barker, 1965; Suh and Barker, 1971). In the present paper two aspects of ligand binding are examined by ultraviolet difference spectroscopy. First, the relationship between the quenching of fluorescence and the generation of a difference spectrum when a ligand is bound to aldolase was examined. Second, the cause of the spectral changes that occur on binding was sought using difference spectroscopy in the presence of the solvent perturbant ethylene glycol.

Experimental Section

Materials. Rabbit muscle aldolase was prepared from adult New Zealand rabbits (6–8 lb) by the method of Taylor *et al.* (1948) as modified by Lehrer and Barker (1971).

† From the Department of Biochemistry, University of Iowa, Iowa City, Iowa 52240. *Received November 10, 1972.* Supported in part by a Public Health Service Grant (HD 02618) and a Public Health Service Research Career Development award to C. A. Swenson (GM 42,384) from the Institute of General Medical Sciences.

[1] Abbreviations used are: Ara-P_2, D-arabinitol 1,5-diphosphate; Pen-P_2, 1,5-pentanediol diphosphate; P_i, inorganic orthophosphate.

A mixture of triosephosphate isomerase and α-glycerolphosphate dehydrogenase isolated from rabbit muscle was purchased from Boehringer Mannheim Corporation as 1% suspensions in ammonium sulfate. This mixture was used without further purification.

D-Fructose 1,6-bisphosphate tetracyclohexylammonium salt·$10H_2O$ was purchased from Boehringer Mannheim Corporation and used without further purification.

β-Dihydronicotinamide adenine dinucleotide disodium salt, monohydrate, purchased from the Sigma Chemical Co., was used without further purification.

Ara-P_2 was prepared from D-arabinose by the method of Hartman and Barker (1965). Ara-P_2 was purified using ion exchange chromatography (Foote and Wold, 1963) and recrystallized as the tetracyclohexylammonium salt. The compound had no inorganic phosphate according to the method of Gomori (1942) as modified by Lehrer and Barker (1971) and gave one spot with paper chromatography in the solvent systems methanol–ammonia–water (7:2:1, v/v) or ethanol–ammonia–water (6:2:2, v/v) using the acid molybdate spray of Hanes and Isherwood (1949) for detection of phosphate esters.

The tetracyclohexylammonium salt of Pen-P_2 was prepared from 1,5-pentanediol obtained from Aldrich Chemical Co. using the method of Hartman and Barker (1965) and was purified and characterized in the manner described for Ara-P_2.

Sodium phosphate, monobasic, tris(hydroxylmethyl)aminomethane (Tris), 0.1 N sodium hydroxide, and ammonium molybdate were obtained from Fisher Scientific Co. Glycylglycine and Dowex 50 (H^+ form) were purchased from the Sigma Chemical Co. Ethylene glycol and Dowex 1-X8 (Cl^- form) were obtained from Baker Chemical Co. Elon and cyclohexylamine were purchased from Eastman Organic Chemicals. Sodium bisulfite and lithium chloride were purchased from Mallinckrodt Chemical Works. Diethylmalonic acid was purchased from Aldrich Chemical Corporation. All of these compounds were used without further purification.

Instruments. Hydrogen ion activities were monitored with a Corning Model 12 Research pH meter used with a Radiometer combination electrode, N. GK2322C.

A Gilford-Modified Beckman DU spectrophotometer equipped with a Photovolt recorder and thermostated with a Haake Model NBS circulating water bath was used for routine absorbance and kinetic experiments. A Cary Model 15 recording spectrophotometer with thermostated cell holders, maintained at a constant temperature by a Neslab Model RT3 circulating refrigerated water bath, was used for ultraviolet difference spectra.

Methods. The specific activities of aldolase preparations were determined using a modification (Lehrer and Barker, 1971) of the method of Richards and Rutter (1961). All preparations had specific activities of from 11 to 16 μmol of D-fructose 1,6-bisphosphate cleaved per minute per milligram of protein. Protein concentrations were determined using $E^{0.1\%}_{280\ nm} = 0.938$ (Donovan, 1964).

The tetracyclohexylammonium salts of Ara-P_2 and Pen-P_2 were converted to the acids by passing the salts through a Dowex 50 (H^+ form) column. The acids were titrated with sodium hydroxide to the second end point to determine pK_a values and the concentration of phosphate ester in the solution. The solutions were brought to pH 7.5 ± 0.1 at 25° with hydrochloric acid and diluted to an appropriate volume in volumetric flasks. A 1.0 M solution of inorganic P was prepared by dissolving 0.10 mol of monobasic sodium phosphate in 70 ml of water adjusting to pH 7.5 with sodium hydroxide, and bringing the volume to 100 ml. These stock solutions were diluted to concentrations utilized in the experiments with the appropriate buffers at pH 7.5 ± 0.05 at the appropriate temperature: 0.15 M glycylglycine, 0.02 M Tris, and 0.01 M diethylmalonate.

Approximately 20 ml of the stock solution of the enzyme was placed in small diameter Visking dialysis tubing and dialyzed against 20–50 volumes of the appropriate buffer in a flow system for 10–24 hr. The dialyzed enzyme solution was clarified by centrifugation at 27,000*g* and the pH, concentration, and specific activity of the enzyme were determined.

Difference spectra were measured at 25° in cylindrical tandem cells (Herskovits and Laskowski, 1960) with 1.000-cm path lengths at constant dynode and sensitivity settings of 3.0 and 2.5, respectively; slit widths did not exceed 1.5 mm, which is equivalent to a spectral slit of below 3 nm. The sample compartments are numbered from 1 to 4. Compartments 1 and 2 are in the sample beam with compartment 1 being closest to the monochromater. Compartments 3 and 4 are in the reference beam with compartment 3 being closest to the monochromater. Compartments 1 and 3 always contained the protein sample and compartments 2 and 4 contained the reference solution which had the same composition as the protein solution except for the presence of the protein.

Perturbants were added and spectra were obtained in two different ways. In both cases, solutions were clarified by centrifugation at 27,000*g* for 10–15 min. With tightly bound ligands, 2.6 ml of protein solution (1.8–2.5 mg/ml) was added to compartments 1 and 3 and the same quantity of reference solution was placed in compartments 2 and 4. Tracings were taken from 350 to 240 nm with the potentiometers adjusted so that the deviations from the absorbance at the 350-nm reference position were less than 0.005 absorbance unit on the expanded scale (full scale–0.1 absorbance unit). Small aliquots of the ligand were added sequentially to compartments 1 and 4 and similar small aliquots of buffer were added to compartments 2 and 3. The ligand was added in quantities such that the mole ratio of ligand to enzyme was varied from approximately 0.3 to 300. After each addition of ligand, a spectrum was obtained. The absorbance *difference* between 294 and 298 nm ($A_{294} - A_{298}$) was used to evaluate the extent of ligand binding.

For solvent perturbation studies with ethylene glycol a set of gravimetrically calibrated 5-ml volumetric flasks was used to make up the solutions before transferring them to the tandem cells. To each flask was added an aliquot of protein from the dialyzed stock solution to give a final concentration of 1.5–2.0 mg/ml. An amount of ethylene glycol was added and the volume adjusted to 5 ml with distilled water. The transient high concentration of ethylene glycol never exceeded 60%. Since 50% ethylene glycol does not produce denaturation observable by difference spectroscopy it is assumed that no denaturation occurred during sample preparation. Percentages of ethylene glycol indicate the weight of ethylene glycol per unit volume. A base-line tracing from 350 to 240 nm was measured for the protein in the buffer only. The solutions in compartments 1 and 4 were removed, the compartments were cleaned, and new solutions with the appropriate ethylene glycol concentrations were added. Spectra were measured after the solutions had achieved temperature equilibrium and corrected by subtraction of the base-line absorbance. Throughout a series of experiments base-line tracings were measured to assure the accuracy of the procedure.

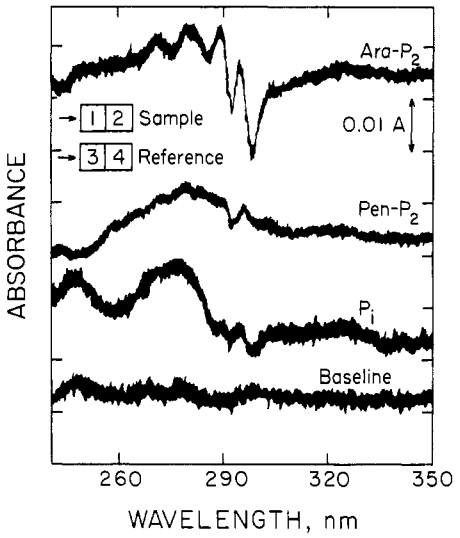

FIGURE 1: The difference spectra caused by the binding of saturating concentrations of Ara-P_2, Pen-P_2, and P_i to aldolase at 25°. Compartments 1-4 contained 0.01 M diethylmalonic acid, pH 7.5, and compartments 1 and 3 contained 1.31×10^{-5} M aldolase in the base-line spectrum. Compartments 1 and 4 were brought to 2.35×10^{-4} M Ara-P_2, 4.51×10^{-4} M Pen-P_2, and 2.72×10^{-2} M P_i for the ligand spectra. Dynode and sensitivity settings were 3.0 and 2.5, respectively, for all spectra.

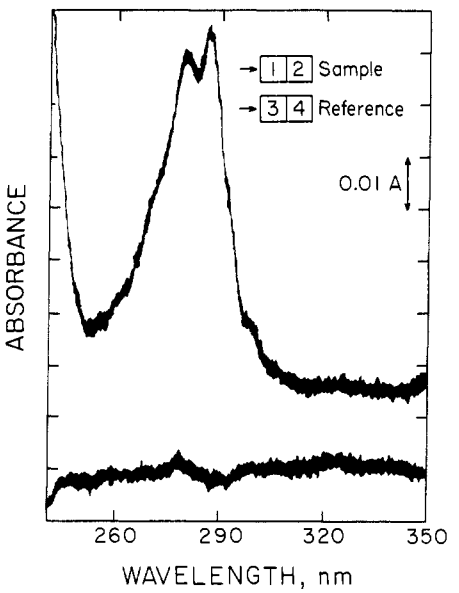

FIGURE 3: The difference spectrum caused by the effect of 50% (w/v) ethylene glycol on aldolase. Compartments 1–5 contained 0.01 M diethylmalonic acid, pH 7.5, and compartments 1 and 3 contained 1.42×10^{-5} M aldolase for the base-line spectrum. The solution in compartment 1 was replaced with 1.42×10^{-5} M aldolase and 50% ethylene glycol in 0.01 M diethylmalonic acid pH 7.5, and the solution in compartment 4 was replaced with 50% ethylene glycol in 0.01 M diethylmalonic acid, pH 7.5, for the ethylene glycol spectrum. Spectra were measured at a dynode of 3.0 and a sensitivity of 2.5.

Results

In Figure 1, representative difference spectra caused by the binding of saturating levels of Ara-P_2, Pen-P_2, and P_i to aldolase at 25° are shown. The Ara-P_2 spectrum has minima at 298, 292, 284, and 275 nm and maxima at 287, 279, and 272 nm. The difference spectrum of Pen-P_2 does not have a minimum at 298 nm, but does have one at 292 nm. There are also maxima at 295 and 280 nm although none are as pronounced as those observed with Ara-P_2. There are minima at 298 and 292 nm and maxima at 294 and 275 nm in the difference spectrum produced by P_i.

A titration curve produced by the binding of increasing amounts of P_i to aldolase is shown in Figure 2. The error bars represent the average experimental error of ±0.0005 Å. This error was estimated from more than 50 difference spectra obtained under a variety of conditions. The method of estimation probably exaggerates the error statement. Donovan (1969b) estimated the instrumental error to be approximately ±0.0002 Å. The data can be fitted to a rectangular hyperbola which gives a value of $3.0 \times 10^{-3} \pm 0.6 \times 10^{-3}$ M for the dissociation constants of the enzyme–P_2 complex. Changes in absorption were too small to permit accurate titration for Pen-P_2.

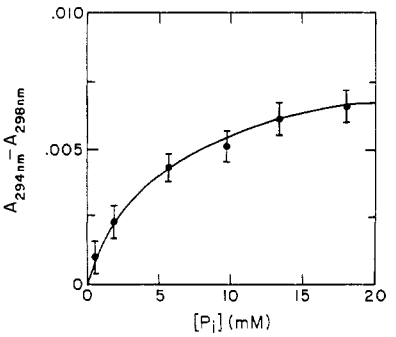

FIGURE 2: A difference spectral titration curve of the binding of P_i to aldolase at 25°; aldolase concentration = 1.54×10^{-5} M. The error bars represent the average experimental error of ±0.0005 Å.

In Figure 3, the difference spectrum developed by aldolase in the presence of 50% ethylene glycol is presented. There are maxima at 287 and 279 nm and shoulders at 293, 298, and 305 nm. These peaks and shoulders were observed in all difference spectra obtained generated by 20–50% ethylene glycol. Additional shoulders are present below 279 nm. The spectrum is presented for comparison with the binding difference spectra presented in Figure 1.

The difference in absorbance ($A_{294} - A_{298}$), produced by the binding of Ara-P_2 to aldolase in buffer and in 35% ethylene glycol, is plotted in Figure 4. The equivalence point was not changed with different concentrations of ethylene glycol, but the absorbance changes at saturating levels of Ara-P_2 became smaller as ethylene glycol concentrations were increased. The dependence of the absorbance change on ethylene glycol concentration is shown in Figure 5.

In Figure 6, the change in absorbance at three wavelengths, 279, 287, and 298 nm, is plotted *vs.* the concentration of ethylene glycol which produced the difference spectrum. The lines in Figure 6 show a least-squares fit of the data.

Discussion

The experiments reported here were designed to explore two aspects of the binding of ligands to aldolase. First, the ultraviolet difference spectra produced on binding three ligands to aldolase were measured to determine if the perturbation of the tryptophyl residues found by this method parallel the fluorescence quenching by the same compounds (Suh and Barker, 1971; Rose and O'Connell, 1969). The second facet of the problem was to determine whether the spectral changes caused

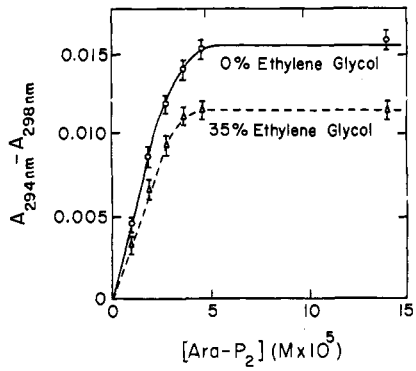

FIGURE 4: The difference in absorbance, $A_{294} - A_{298}$, produced by binding of Ara-P_2 to aldolase *vs.* the concentration of ligand in aqueous buffer and in 35% ethylene glycol; aldolase = 1.34×10^{-5} M. The error bars represent the average experimental error of 0.0005 Å.

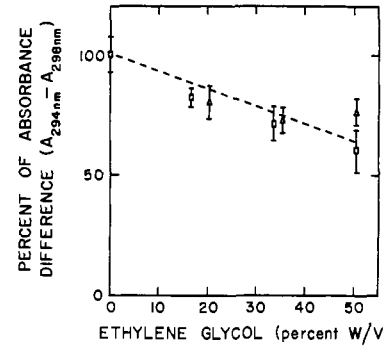

FIGURE 5: The absorbance change produced by the binding of a saturating concentration of Ara-P_2 to aldolase at different concentrations of ethylene glycol plotted as a percentage of the absorbance change produced in buffer *vs.* the percentage of ethylene glycol. The error bars represent the standard deviation from the mean. Data from two sets of experiments are presented.

by binding Ara-P_2 to aldolase are due to a conformational change of the protein or to a proximity effect of the hydroxyl groups of the ligand to a tryptophyl chromophore.

The difference spectrum caused by the binding of Ara-P_2 to aldolase (Figure 1) has a sharp minimum at 298 nm, which probably is due to the polarization blue shift of a tryptophyl residue that has entered a more polar environment, a region of different charge density, or a region of decreased refractive index (Yanari and Bovey, 1960; Donovan, 1969b). It can be assumed that the maximum at 292 nm of *N*-acetyltryptophyl ethyl ester, observed by Herskovits and Sorensen (1968a) in 20% aqueous (v/v) ethylene glycol, which has a greater refractive index than water, has the same basis as the minimum at 298 nm caused by the binding of the ligand to aldolase. This assumption is supported by the observations of Yanari and Bovey (1960) who have shown that the spectrum of indole is shifted to longer wavelengths with increasing refractive index of the solvent. These shifts are 5.4 nm in carbon tetrachloride which has a refractive index of 1.473. The refractive index of the protein interior is usually much higher than this (1.60–1.62; Yanari and Bovey, 1960) so that the wavelength shifts produced by the transfer of indole into the protein interior may be larger than those produced by transfer from water to carbon tetrachloride. If, in addition, it is assumed that the perturbing power of the protein interior is 6.3 times that of 20% ethylene glycol (Donovan, 1969a), it can be estimated that the absorbance change at 298 nm is caused by the exposure of approximately 0.4 mol of tryptophyl residues to solvent or to an environment having the same refractive index as the solvent when 1 mol of ligand binds (Lehrer and Barker, 1971). In this study, with several preparations of enzyme, qualitatively similar but quantitatively different difference spectra were obtained. These variations are sufficient to produce values for the numbers of chromophores changing environments on binding that differ by as much as 25%. These variations do not apply, however, to calculations of the stoichiometry of binding based on difference spectroscopy.

Most of the experiments reported here were carried out using diethyl malonate as a buffer. Approximately 90% of the active sites of the enzyme are occupied by this buffer anion prior to the addition of a specifically bound perturbant. On this basis the difference spectra presented in Figure 1 reflect the difference between the protein with the active site occupied by buffer anion and with the active site occupied by the specific ligand. Difference spectra are qualitatively and quantitatively similar in diethylmalonate, Tris, and glycylglycine buffers.

The difference spectrum caused by the binding of Pen-P_2 to aldolase (Figure 1) does not have a minimum at 298 nm, indicating that the hydroxyl groups of Ara-P_2 are necessary to produce the change in environment of the tryptophyl residue. On the other hand P_i gives a difference spectrum which has a very small minimum at 298 nm. This may be due to a small change in the environment of a tryptophyl residue caused by the hydroxyl group of P_i which could mimic the effect of the hydroxyl group of Ara-P_2 or by a slightly different charge effect for P_i as compared to Ara-P_2 (Andrews and Forster, 1972). These findings agree with the fluorescence quenching experiments of Suh and Barker (1971) who showed that the hydroxyl groups of polyhydric alcohol diphosphates, such as Ara-P_2, are necessary for maximal quenching and that P_i is more effective than Pen-P_2 in quenching the fluorescence of rat muscle aldolase. Both ultraviolet spectral and fluorescence changes would be produced by a tryptophyl residue entering an environment of lower refractive index.

Two mechanisms for the perturbation of the tryptophyl residue are represented in Figure 7. In A, a tryptophyl residue is buried in the protein fabric and binding Ara-P_2 to the active site moves it into contact with the solvent by a conformational

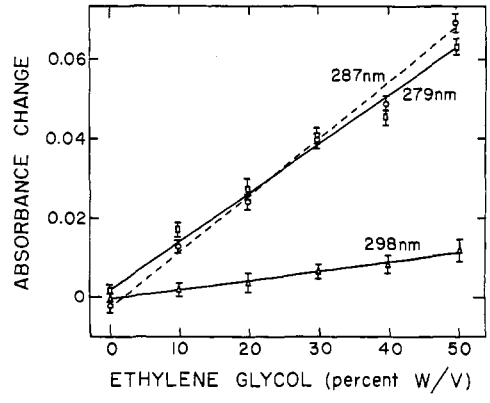

FIGURE 6: A plot of absorbance change at 298, 287, and 279 nm in the difference spectrum of aldolase in the presence of ethylene glycol *vs.* the concentration of ethylene glycol. The error bars represent the average error of two determinations for 287 and 279 nm and four determinations for 298 nm.

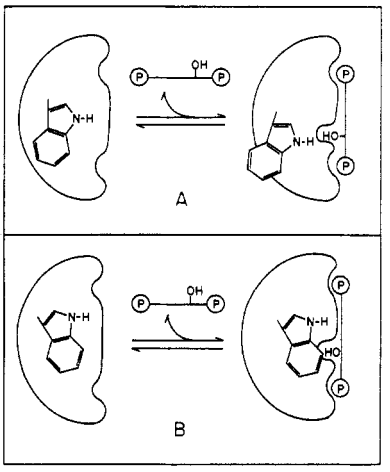

FIGURE 7: A schematic representation of two hypothetical causes for the perturbation of the tryptophyl residue caused by the binding of Ara-P_2 to aldolase: (A) a conformational change exposes the tryptophyl residue to the solvent; (B) a hydroxyl group of the ligand perturbs the tryptophyl residue directly.

change. In the second mechanism a tryptophyl residue buried in the protein fabric, as in B, is brought into contact with a hydroxyl group of Ara-P_2 when this ligand is bound. In both cases, the tryptophyl residue in question would experience a more hydrophilic environment.

To determine which of the mechanisms causes the environmental change of the tryptophyl residue, perturbation of the protein by ligand binding to the active site was studied in combination with solvent perturbation of the protein (Villanueva and Herskovits, 1971). The diagrams in Figure 7 can be used to understand the effect of the solvent perturbant, ethylene glycol, on the protein for the two cases. In the first case, the tryptophyl residue, which is exposed to the solvent upon binding of Ara-P_2 to the enzyme, will be affected by the presence of ethylene glycol. Thus, the difference spectrum produced by binding Ara-P_2 would be smaller in the presence of ethylene glycol since the tryptophyl residue would experience a smaller change in environment on entering a solution of ethylene glycol in water than it would experience on entering water. In the second case, the tryptophyl residue, which is buried in the active site and perturbed by the ligand, will not be affected by the presence of ethylene glycol. Thus, the difference spectrum produced by the binding of Ara-P_2 to aldolase will be unaffected by ethylene glycol if it is due to a proximity effect. Before these hypotheses can be tested it must be shown that neither the dissociation constant nor the stoichiometry of ligand binding is affected by the presence of ethylene glycol. The titration curves presented in Figure 4 show typical data demonstrating that neither of these parameters is changed significantly by ethylene glycol. It is also necessary to show that ethylene glycol does not denature the protein in the range of concentrations used. In Figure 6, the changes in absorption at several wavelengths are shown to be linearly related to ethylene glycol concentration. Since solvent denaturation should be a cooperative phenomenon, changes in conformation due to solvent effects should produce higher order relationships between perturbant concentration and absorbance. The changes observed are probably due entirely to the simple perturbation by the solvent of the exposed chromophores (Herskovits and Laskowski, 1962).

When the concentration of ethylene glycol in the solvent produces a milieu equivalent to that of the interior of the protein the binding of Ara-P_2 should produce no observable difference spectrum. Donovan (1969a) has shown that 126% (w/v) ethylene glycol would be equivalent to the interior of the protein. Thus, if the decrease in the Ara-P_2 difference spectrum produced by ethylene glycol concentration in the 0–50% range is extrapolated to obtain the decrease that would be produced if 126% ethylene glycol was used the proportion of the Ara-P_2 binding difference spectrum due to the movement of protein chromophores into the solvent can be estimated. Extrapolation of the data of Figure 5 to 126% ethylene glycol using a least-squares analysis would decrease the binding difference spectrum by 86 ± 10% indicating that the same proportion of the binding difference spectrum is due to movement of chromophore to the solvent. Thus, the majority of the difference spectrum produced by binding 3.3 mol of Ara-P_2 to aldolase (Castellino and Barker, 1966) which results in the exposure of 1.4 ± 0.2 tryptophyl residues to solvent can be attributed principally to a conformational change involving 1.2 ± 0.2 mol of tryptophyl residue. It is interesting to note that the Ara-P_2 binding exposes approximately the same number of additional tryptophyl residues as are exposed in the native enzyme. Herskovits and Sorenson (1968b) and Donovan (1969a) found that 2.0–2.5 tryptophyl residues are exposed to solvent in the native enzyme. We find that 1.4 ± 0.2 residues are exposed by Ara-P_2 binding. These values were obtained in slightly different ways from the experimental data. Herskovits and Sorenson (1968a) used 20% ethylene glycol and curve fitting; in this study we have used 50% ethylene glycol and peak intensities. Coincidentally, using the latter procedure we find that 1.4 ± 0.2 tryptophyl residues are in contact with solvent in the *native* enzyme.

In summary, the amount of spectral change produced by Ara-P_2 binding that can be ascribed to a proximity effect of alcohol groups in the ligand appears to be quite small and within experimental error may be zero. The small difference spectra observed with Pen-P_2 and P_i which are qualitatively different from those observed with Ara-P_2 may be a reflection of such proximity effects. Thus, the binding of hydroxyl containing ligands results in a conformational change that is not produced when nonhydroxylic ligands are bound. This conformational change is probably small and may involve a fractional increase in the exposure of a tryptophyl residue or residues to the solvent.

References

Andrews, L. J., and Foster, L. S. (1972), *Biochemistry 11*, 1875.
Castellino, F. J., and Barker, R. (1966), *Biochem. Biophys. Res. Commun. 23*, 182.
Donovan, J. W. (1964), *Biochemistry 3*, 67.
Donovan, J. W. (1969a), *J. Biol. Chem. 244*, 1961.
Donovan, J. W. (1969b), *in* Physical Principles and Techniques of Protein Chemistry, Part A, Leach, S. J., Ed., New York, N. Y., Academic Press, p 158.
Foote, C. D., and Wold, F. (1963), *Biochemistry 2*, 1254.
Gomori, G. (1942), *J. Lab. Clin. Med. 27*, 955.
Hanes, C. S., and Isherwood, R. A. (1949), *Nature (London) 164*, 1107.
Hartman, F. C., and Barker, R. (1965), *Biochemistry 4*, 1068.
Herskovits, T. T., and Laskowski, M. (1960), *J. Biol. Chem. 235*, PC56.
Herskovits, T. T., and Laskowski, M. (1962), *J. Biol. Chem. 237*, 2481.
Herskovits, T. T., and Sorensen, M. (1968a), *Biochemistry 7*, 2523.

Herskovits, T. T., and Sorensen, M. (1968b), *Biochemistry 7*, 2533.
Lehrer, R. M., and Barker, R. (1971), *Biochemistry 10*, 1705.
Richards, O. C., and Rutter, W. J. (1961), *J. Biol. Chem. 236*, 3185.
Rose, I. A., and O'Connell, E. L. (1969), *J. Biol. Chem. 244*, 126.
Suh, B., and Barker, R. (1971), *J. Biol. Chem. 246*, 7041.
Taylor, J. F., Green, A. A., and Cori, G. T. (1948), *J. Biol. Chem. 173*, 591.
Villanueva, G. G., and Herskovits, T. T. (1971), *Biochemistry 10*, 4589.
Yanari, S., and Bovey, F. A. (1960), *J. Biol. Chem. 235*, 2818.

Mechanisms of Formation and Equilibria of 4a and 5 Adducts of an Isoalloxazine. Reaction of 10-(2′,6′-Dimethylphenyl)-3-methylisoalloxazine-6,8-disulfonate with Sulfite in Aqueous Media†

Thomas C. Bruice,* Laszlo Hevesi,‡ and Seiji Shinkai‡

ABSTRACT: The reaction of 10-(2′,6′-dimethylphenyl)-3-methylisoalloxazine-6,8-disulfonate (I) in aqueous sulfite–bisulfite buffers (30°, μ = 2.0) yields an equilibrium mixture of I plus 4a- and 5-sulfite adducts (**4a**, **5**$^-$, and **5H** of eq 1). From the pH dependence of the relative concentration of **4a** and the kinetically apparent acid dissociation constant of **5H** (*i.e.*, **5H** $\rightarrow$ **5**$^-$ with pK_{a_5}) the pH and buffer independent equilibrium constant (K_e = [**5H**]/[**4a**]) has been calculated to be 4.12×10^{-2}. Therefore, at 30° in aqueous solution, the 4a adduct is thermodynamically favored over the neutral 5 adduct. From studies of the dependence of the equilibrium constants K_x = [**4a**]/[I] and K_y = [**5**$^-$]/[I] upon pH and total sulfite buffer concentration ([S]$_T$) it was determined that K_x was proportional to $[HSO_3^-]^{1.0}$ and K_y proportional to $[SO_3^{2-}]^{1.0}$. These dependencies of K_x and K_y upon concentrations of buffer species establish that the forward reactions from I to **4a** and **5**$^-$ have in their rate expressions the terms $[HSO_3^-]$ and $[SO_3^{2-}]$, respectively, in excess over the retrograde reactions of **4a** $\rightarrow$ I and **5**$^-$ $\rightarrow$ I. The kinetics for approach to equilibrium in the conversion of I to products (**4a**, **5**$^-$, and **5H**) were studied under the pseudo-first-order conditions of [buffer] $\gg$ [I$_0$]. The pseudo-first-order rate constants (k_{obsd}) were found to be dependent upon three terms (eq 14); the first contained the product $[HSO_3^-][SO_3^{2-}]$, the second $[SO_3^{2-}]$, and the third was independent of buffer species but dependent upon the mole fraction of a reactant of pK_{app} (pK_{a_5}) assignable to dissociation of **5H** $\rightarrow$ **5**$^-$. With the knowledge of the dependence of the equilibrium ratios [**4a**]/[I] and [**5**$^-$]/[I] upon $[HSO_3^-]$ and $[SO_3^{2-}]$, the rate terms were assignable (Scheme I) to: general acid (by HSO_3^-) catalysis of nucleophilic attack of SO_3^{2-} upon I to yield **4a** and by microscopic reversibility general base (by SO_3^{2-}) catalysis of conversion of **4a** $\rightarrow$ I and unassisted nucleophilic attack of SO_3^{2-} upon I to yield **5**$^-$ with spontaneous conversion of **5**$^-$ $\rightarrow$ I. An alternate scheme that would satisfy both the thermodynamic and kinetic findings would be that of Scheme II. Here I is converted to **5H** *via* general acid (by HSO_3^-) catalyzed attack of SO_3^{2-} and to **5**$^-$ by unassisted attack of SO_3^{2-}, **4a** arising from rearrangement of **5H**. Arguments are presented which favor the mechanism of Scheme I.

This study deals with the reaction of SO_3^{2-} with 10-(2′,6′-dimethylphenyl)-3-methylisoalloxazine-6,8-disulfonate (I) to yield both 5 (**5**$^-$ + **5H**) and 4a (**4a**) adducts (eq 1). In the previous paper (Hevesi and Bruice, 1973) it was established that **4a** was the predominant product, at neutrality, in the reaction of I with SO_3^{2-}. This result may be compared to the reaction of eq 2 (Hevesi and Bruice, 1973) which typifies the normal mode of addition of SO_3^{2-} to flavines and flavinium salts (Müller and Massey, 1970). Inspection of the structures of I and II suggests that the formation of **4a** from I is due to steric and possibly electrostatic hindrance of approach of SO_3^{2-} to the N-5 of I. Also possible is the initial formation of a steric and electrostatically destabilized 5 adduct followed by migration of the SO_3^{2-} moiety to the 4a position (eq 3).

The object of this investigation has been to determine the difference in the free energies of **5H** and **4a** and to establish the mechanisms of their formation from I. Though the mechanisms of oxidation–reduction reactions involving flavines have been speculated to proceed *via* covalent adducts (Hamilton, 1971; Hemmerich, 1972), the only nucleophilic additions at the 5 position involve sulfite (Müller and Massey, 1970; Bruice *et al.*, 1971; Hevesi and Bruice, 1973) and phosphines (Müller, 1972), while the only 4a adduct arising *via* nucleophilic addition (dark) is **4a** (Hevesi and Bruice, 1973).

Experimental Section

Kinetic studies were carried out at the λ_{max} (435 nm) of I as previously described (Hevesi and Bruice, 1973). The concentration range of total sulfite buffer ([S]$_T$, where [S]$_T$ =

† From the Department of Chemistry, University of California, Santa Barbara, California 93106. *Received December 11, 1972.* This work was supported by grants from the National Institutes of Health and the National Science Foundation.

‡ Postdoctoral Fellows at the University of California.