Conformational Changes in Rabbit Muscle Aldolase. Kinetic Studies*

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ABSTRACT: The effects of temperature on the K_m, K_{equil}, and V_{max} for D-fructose 1,6-diphosphate and the K_I for D-arabinitol 1,5-diphosphate for rabbit muscle aldolase (EC 4.1.2.7) have been determined. Values for the thermodynamic parameters associated with binding, activation, and the overall reaction are reported. The enzyme undergoes a temperature-dependent transformation which is interpreted as demonstrating the existence of at least two forms of the enzyme which may have a $\Delta H^{\ddagger}$ of interconversion as low as 10 kcal mole^{-1}.

Temperature-dependent transitions in proteins have been described and interpreted in terms of multiple forms existing in solution. A number of enzymes have been shown to be subject to such transformations (Massey *et al.*, 1966; Kayne and Suelter, 1968). Studies on enzymes possessing such flexibility are complicated by its occurrence, particularly if they are carried out at or near the transition temperature. For example, allosteric effects might be observed in the region of a transition temperature if one form of the enzyme is stabilized by substrates. Such a stabilization has been shown to occur in the case of D-amino acid oxidase (Massey *et al.*, 1966). Temperature-dependent transitions could also influence the number and types of binding and catalytic sites in enzymes.

Rabbit muscle aldolase has been reported to undergo a temperature-dependent transition by Massey *et al.* (1966), but details of the study have not been published. Richards and Rutter (1961b) also stated that the Arrhenius plot for the enzyme was curved (concave downward) but did not interpret this in terms of the existence of multiple forms of the enzyme.

This study was undertaken to determine whether a transition could be observed, and if it was, whether it influenced binding as well as catalysis. In addition, the effect of temperature, and substrate and inhibitor binding on the spectral properties of the enzyme have been studied and will be published separately.

Materials and Methods

Rabbit muscle aldolase (EC 4.1.2.7) preparation I was prepared from frozen adult rabbit muscle by the methods of Taylor *et al.* (1948) and Swenson and Boyer (1957). The enzyme was recrystallized seven times from ammonium sulfate containing 3×10^{-3} M EDTA. Before storage, the enzyme was recrystallized once from an EDTA-free ammonium sulfate solution. The enzyme had a specific activity of 10.0–12.0 μmoles of FDP[1] cleaved per min per mg of protein at 25° as measured by a slight modification of the procedure of Richards and Rutter (1961a). The enzyme appeared homogeneous by sedimentation velocity in 0.1 M sodium chloride–0.1 M mercaptoethanol (pH 6.2) with a symmetrical peak having $s_{20,w}$ = 7.4 S. It contained less than 0.002% triose phosphate isomerase as assayed by the method described by Beisenherz (1955). Its molecular weight was assumed to be 156,000 (Castellino and Barker, 1968).

Preparation II was purchased from Boehringer und Soehne (lot 606750) as a crystalline suspension in 52% ammonium sulfate with a specific activity of 10.0–12.0 units at 25°; it showed a single symmetrical peak in the ultracentrifuge with $s_{20,w}$ = 7.80 S. The preparation contained less than 0.05% of triose phosphate isomerase activity.

The following rabbit muscle enzymes were purchased from Boehringer und Soehne as 1% suspensions in ammonium sulfate and used without further purification: triose phosphate isomerase, α-glycerophosphate dehydrogenase, and a mixture of the two.

Fructose 1,6-diphosphate tetracyclohexylammonium salt · $10H_2O$ purchased from Boehringer und Soehne and fructose 1,6-diphosphate (trisodium salt) · $7H_2O$ purchased from Wessex Biochemicals Ltd. were used without further purification.

NADH monohydrate, purchased from the Sigma Chemical Co., was used without further purification.

Dihydroxyacetone phosphate (disodium salt) was prepared from the dicyclohexylammonium salt of the dimethyl ketal (a gift from G. R. Gray) by the method of Ballou and Fischer (1956). It was stored at −5° in solution at pH 4.5.

Disodium DL-glyceraldehyde 3-phosphate was prepared from the diethyl acetal monobarium salt, purchased from Boehringer und Soehne, as described by Ballou and Fischer (1955).

D-Arabinitol 1,5-diphosphate tetracyclohexylammonium salt was prepared from D-arabinose by the method of Hartman

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[1] Abbreviations used are: FDP, fructose 1,6-diphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; NADH, β-dihydronicotinamide–adenine dinucleotide.

and Barker (1965). The cyclohexylammonium salt was used directly or converted into the sodium salt.

Specific Activity and Kinetic Measurements. Stock solutions prepared in 0.15 M glycylglycine buffer (pH 7.5) were as follows: 1.65×10^{-3} M NADH, 1.09×10^{-2} M FDP, 0.1 ml of the mixture of α-glycerophosphate dehydrogenase–triose phosphate isomerase (10 mg of protein/ml) diluted to 4.0 ml, and a dilution of aldolase containing from 0.05 to 0.1 mg of protein per ml. The protein concentration in the aldolase solution was determined by its optical density (at 280 mμ) using an $\epsilon_{280\ m\mu}^{0.1\%}$ value of 0.938 (Donovan, 1964).

Assays were performed in 1-cm path-length quartz cuvets. For specific activity measurements, the following volumes of stock solutions were added to each cuvet: 0.5 ml of NADH solution, 0.2 ml of FDP, 0.2 ml of the α-glycerophosphate dehydrogenase–triose phosphate isomerase mixture, and a volume of buffer such that the total volume after addition of aldolase would be 3.0 ml. The cuvets were placed in a Cary Model 15 recording spectrophotometer equipped with thermostatable cell jackets and temperature regulation was maintained by a Haake Model F or a Neslab Model RT3 circulating bath. The absorbance of the solution at 340 mμ was recorded, and the constancy of the reading verified for 2 min. The reaction was then initiated by addition with thorough mixing of 0.05 or 0.1 ml of the aldolase solution and the decrease in absorbance was recorded. A unit of aldolase solution was defined as the micromoles of FDP cleaved per minute per milligram of protein.

For K_m and V_{max} determinations, measurements were performed with the same stock solutions except that the stock FDP solution was $1.05–1.10 \times 10^{-3}$ M for temperatures up to 40° and $2.11–2.24 \times 10^{-3}$ M for temperatures above 40°. Aldolase stock solutions contained 0.01–0.26 mg of preparations I or II per ml of buffer. Two solutions of D-arabinitol 1,5-diphosphate cyclohexylammonium salt were used at each temperature—the stock solution and a 1:2 dilution of it in buffer. The concentration of the stock solution was $1.53–1.72 \times 10^{-5}$ M for temperatures below 40° and 2.77×10^{-5} M for temperatures above 40°. The buffer was adjusted to pH 7.5 at the temperature of the assays.

The assay mixture contained 0.5 ml of NADH, 0.2 ml of the α-glycerophosphate dehydrogenase–triose phosphate isomerase mixture, from 0.05 to 0.25 ml of aldolase solution (final amount of aldolase in assays: 2.9–26.0 μg), and buffer to 2.9 ml. The reaction was started by the addition of 0.1 ml of FDP. Five FDP concentrations were used: the stock solution and four dilutions of it in buffer (the dilutions were 1:3, 1:6, 1:9, and 1:11 or 1:12). The temperature within the cells was measured before and after the absorbance change using a calibrated thermistor (Yellow Springs Instruments 44003) positioned in the cuvet so as not to interfere with the light path. The solution was allowed to attain temperature equilibrium before the addition of substrate. The temperature was maintained within 0.2°. At each temperature experiments were performed to ensure that the auxiliary enzymes and reagents used in the assay system were not limiting.

The values of K_m and V_{max} as well as their respective standard deviations were obtained from a least-squares analysis of the data treated according to Lineweaver and Burk (1934). This treatment gives larger values for the standard deviation than the method of fitting to a rectangular hyperbola recommended by Wilkinson (1961). Programs for the Linc-8 computer for the determination of K_m, K_i, and V_{max} using both methods are available on request.

The inhibitor constant, K_i, was measured using the procedure described above except that 0.1 ml of D-arabinitol 1,5-diphosphate was added to the assay mixture prior to addition of FDP and only 2.8 ml of buffer was used. Since two D-arabinitol 1,5-diphosphate concentrations were used, two values of K_i were obtained at each temperature. The values of K_i were calculated from least-squares analyses of Lineweaver–Burk plots. The ranges of the inhibitor constants were calculated using the slope of the line for the inhibited reaction plus one standard deviation and the slope of the line for the normal reaction minus one standard deviation.

Equilibrium Measurements. A conical centrifuge tube containing 1.0 ml of aldolase (preparation I) at a concentration of about 1 mg/ml in 0.15 M glycylglycine buffer (pH 7.5) was kept at constant temperature by means of a water bath. To the aldolase was added 1 ml of FDP (trisodium salt) also in glycylglycine buffer, which had been kept at the same temperature. After 10-min incubation, 0.2 ml of a 20% trichloroacetic acid solution in water was added. The protein precipitate was centrifuged at 2000 rpm for 10 min and the concentrations of GAP and/or DHAP present in the supernatant fluid were determined. GAP and DHAP, but not FDP, were hydrolyzed by 20-min exposure to 1 N alkali to yield P_i (Baer and Fischer, 1943, 1956). The P_i was then measured by a slight modification of the method described by Gomori (1942). To aliquots of the samples was added 1.0 ml of the acid molybdate solution and 1.0 ml of elon. Water was then added to a total of 10.0 ml. The color of the phosphomolybdate complex was allowed to develop for at least 20 min, and the absorbance at 660 mμ was obtained by reading on a Gilford-modified Beckman DU spectrophotometer. The values obtained were corrected by subtracting the absorbance values of aliquots which had not undergone basic hydrolysis, as well as blank values obtained from FDP samples which had been subjected to alkaline hydrolysis. The concentration of alkali-labile phosphate was obtained from a standard curve.

The concentration of FDP at equilibrium was obtained by subtracting from the initial FDP concentration, one-half of the amount of P_i released by alkaline hydrolysis.

A second method of estimating the equilibrium concentrations of reactants utilized the oxidation of NADH by DHAP in the presence of α-glycerophosphate dehydrogenase followed by further oxidation of NADH by the addition of triosephosphate isomerase which catalyzes the reaction GAP $\rightleftarrows$ DHAP.

The solutions used were 1.65×10^{-3} M NADH in buffer, 0.1 ml of α-glycerophosphate dehydrogenase diluted to 1 ml with buffer and 0.1 ml of triose phosphate isomerase diluted to 10 ml with buffer.

The assay mixture contained 0.5 ml of NADH, 0.1 ml of α-glycerophosphate dehydrogenase, and 2.3 ml of buffer. The reaction was initiated by the addition of a 0.1-ml aliquot of the sample under study. When the absorbance decrease had stopped, 0.02 ml of triosephosphate isomerase was added to the cuvet and the ensuing oxidation was recorded. The amount of DHAP was calculated using the relationship

$$\text{mmoles of DHAP/}\lambda = \frac{3.0\Delta A_{340} - \lambda A_1}{\epsilon}$$

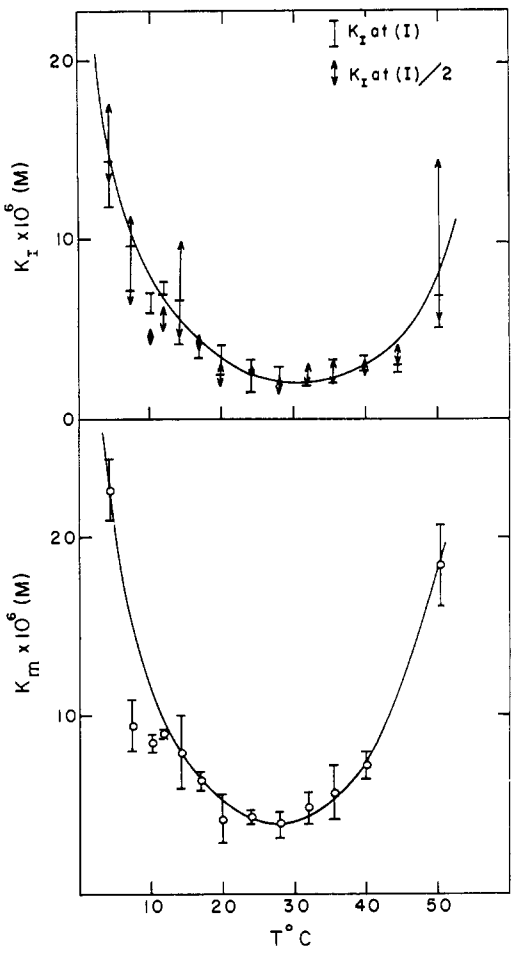

FIGURE 1: The effect of temperature on K_I for the aldolase–arabinitol diphosphate complex and on K_m for the aldolase-catalyzed cleavage FDP. Points were obtained by curve fitting as described in the text. Bars above and below the points indicate one standard deviation (see Methods).

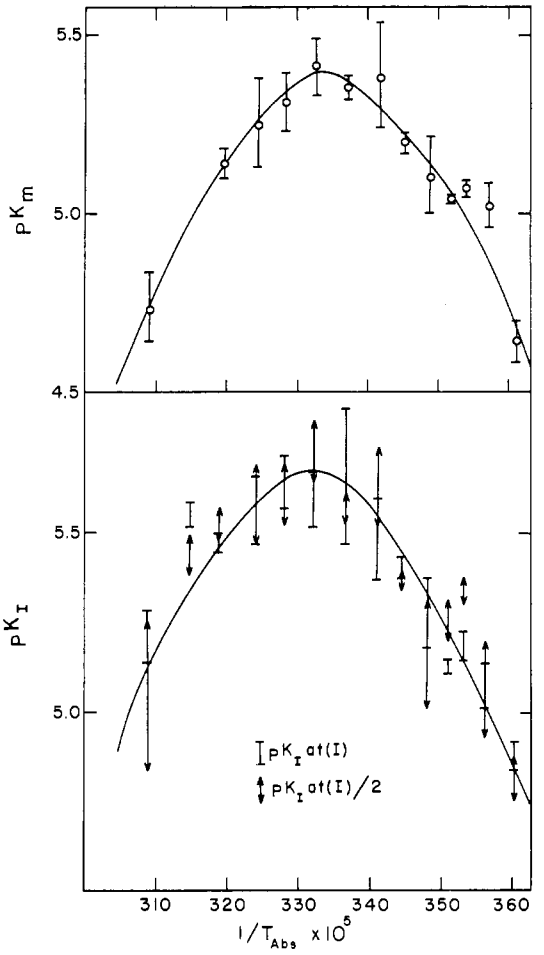

FIGURE 2: Plots of pK_m and pK_I *vs.* $1/T$ using the data presented in Figure 1. The bars above and below the pK values indicate one standard deviation. Enthalpy values estimated by least-squares analyses of the points below 20° and of those above 30° are presented in Table I.

where x represents the volume in milliliters of aliquot added to the assay mixture, ΔA_{340} is the change in absorbance at 340 mμ observed before addition of triose phosphate isomerase, A_i is the initial absorbance of the NADH in the assay mixture, and ϵ is the molar extinction coefficient of NADH (6.22×10^3).

The amount of GAP in the solution was calculated from the total amount of triose phosphate present minus the amount of DHAP. The total amount of triose phosphate was calculated using the relationship

$$\text{mmoles of triose phosphate}/x = \frac{3.02\Delta A_{340} - (x + 0.02)A_i}{\epsilon}$$

where ΔA_{340} represents the total change in absorbance (after addition of both enzymes), and the other terms remain as described above.

The FDP concentration at equilibrium was calculated as described above and the K_{equil} was obtained using the equation: K_{equil} = [DHAP][GAP]/[FDP].

Thermodynamic Parameters. The experimental energy of activation, $E^{\ddagger}$, in units of calories per mole was calculated from the slope of the line obtained by plotting log V_{max}/mg of protein (or log specific activity) against $1/T$ (slope = $-E^{\ddagger}/2.303R$). The slopes and their standard deviations were obtained from a least-squares analysis of the data. The enthalpy of activation, $\Delta H^{\ddagger}$, was calculated using the equation: $\Delta H^{\ddagger} = E^{\ddagger} - RT$, and the entropy of activation, $\Delta S^{\ddagger}$, using

$$\frac{\Delta S^{\ddagger}}{4.576} = \log k - 10.753 - \log T + \frac{E^{\ddagger}}{4.576T}$$

where k is the rate constant expressed in sec^{-1} and is equal to the product of V_{max}/mg of protein and the factor

$$\frac{1.56 \times 10^5 \text{ mg}}{\text{mmole}} \frac{1 \text{ min}}{60 \text{ sec}} \frac{10^{-3} \text{ mmole}}{\mu\text{mole}}$$

The free energy of activation $\Delta F^{\ddagger}$ was obtained using $\Delta F^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$.

The enthalpies of binding and of the overall reaction were obtained using the van't Hoff equation. The negative loga-

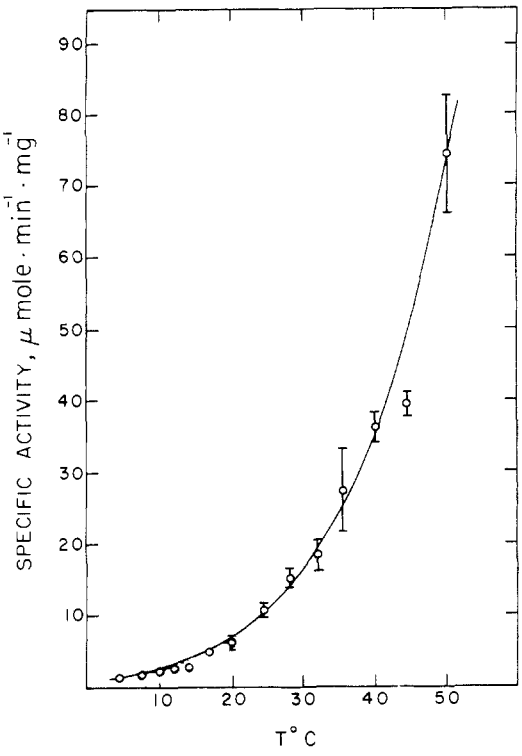

FIGURE 3: Effect of temperature on the specific activity of aldolase (preparation I): ○, values of the maximal velocity obtained by extrapolation; the bars above and below each circle indicate one standard deviation.

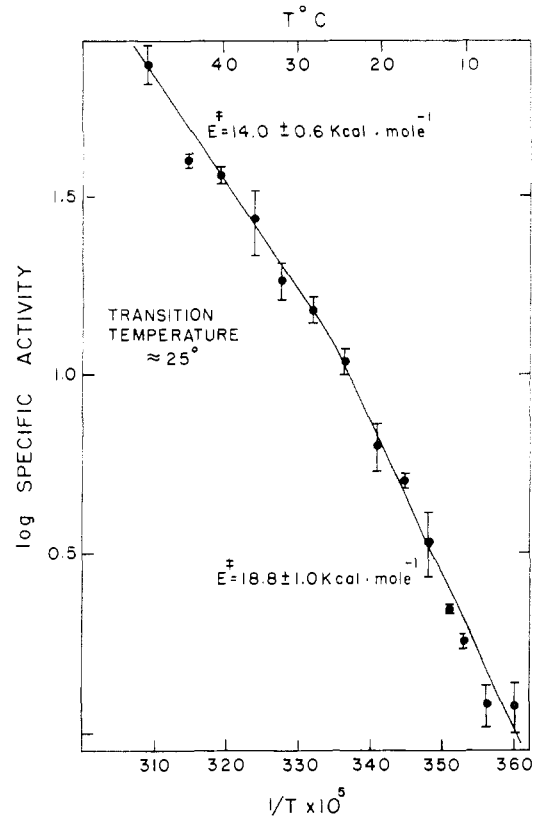

FIGURE 4: Arrhenius plots for the cleavage reaction of aldolase (preparation I). ● values obtained by extrapolation; the bars above and below each point indicate one standard deviation from the mean.

rithms of the dissociation constant (pK_m or pK_I) or of the equilibrium constant of the reaction (pK_{equil}) were plotted against $1/T$. The plots were not linear, and estimates of the heats of reaction below and above the critical temperature were obtained by least-squares analyses of the data points below 20° and of those above 30°, respectively.

The free energies were calculated from the equation $\Delta F = -RT \ln K$ using K_{equil}, K_m, or K_I. The entropies ΔS were obtained from $\Delta S = (\Delta H - \Delta F)/T$.

Temperature Stability. Nitrogen gas was bubbled for 10–15 min through a solution of 0.15 M glycylglycine buffer (pH 7.5) to remove oxygen. This buffer was used to prepare a stock solution of aldolase containing approximately 0.1 mg of protein/ml (0.6×10^{-6} M). In those experiments with inhibitor, the stock solution also contained 8.2×10^{-5} M D-arabinitol 1,5-diphosphate. An aliquot (0.3 ml) of this solution was pipetted into a small test tube and the tube was placed in a constant-temperature bath. The temperature was regulated with a Haake Model F circulating bath, and was measured with a National Bureau of Standards thermometer. The protein was incubated at each temperature for 20 min. Duplicate assays were performed at 25° using the assay system described above. The activity remaining after incubation was expressed as a percentage of the activity present before incubation.

Results

Effect of Temperature on the Kinetics and Equilibrium of the Reaction. The variations in the K_m for fructose diphosphate and K_I for arabinitol diphosphate are shown in Figure 1. The data are replotted in Figure 2 to obtain the enthalpy of binding. The effect of temperature on the maximum velocity of the reaction is shown in Figure 3. The data of Figure 3 are replotted in Figure 4 for the evaluation of the energy of activation. The data are best fitted by a curve which can be approximated by two straight lines intersecting at a "transition temperature."

The effect of temperature on the equilibrium constant is shown in Figure 5, and the data are replotted in Figure 6 to obtain the enthalpy of the overall reaction. Similar results were obtained using the alkali-labile phosphate procedure described in Materials and Methods. The thermodynamic parameters of the catalytic process are listed in Table I. The enzyme was stable over the range 0–45° for the period of all experiments reported. D-Arabinitol 1,5-diphosphate at the levels tested had no effect on the stability of the enzyme.

Discussion

The data presented in Figures 1 and 2 suggest that the K_m of fructose diphosphate approximates the true dissociation constant for the enzyme–substrate complex. Its behavior parallels that of the dissociation constant, K_I, for arabinitol diphosphate, a potent inhibitor of the aldolase reaction (Hartman and Barker, 1965). Taking into consideration experimental error, K_m is of the same magnitude and reaches a minimum value at the same temperature as K_I. The enthal-

TABLE I: Thermodynamic Parameters for the Aldolase-Catalyzed Cleavage Reaction: FDP $\rightleftarrows$ DHAP + GAP.

		Calculated Values		
		Just below the Transition	Just above the Transition	Lit. Values and Sources
ΔF (cal mole^{-1})	Binding	−7,200 (20°)	−7,400 (31.9°)	
	Activation	15,400 (20°)	15,500 (31.9°)	15,000[a]
	Overall	6,500 (21.1°)	6,400 (30°)	5,820 (28°)[b]
				5,590 (38°)[b]
				5,340 (48°)[b]
				5,590 (37°)[c]
ΔH (cal mole^{-1})	Binding	11,500	−13,800	19,400 above 28°)[a,d]
	Activation	18,200 (20°)	13,400 (31.9°)	14,000 below 28°)[a,d]
	Overall	14,300	10,600	13,130[b]
				14,000[e]
ΔS (eu)	Binding	−63.8 (20°)	20.9 (31.9°)	
	Activation	9.2 (20°)	−7.3 (31.9°)	−5.5[a,d]
	Overall	26.5 (21.1°)	13.8 (30°)	24[f]
$E^{\ddagger}$ (cal mole^{-1})	Activation			20,000 above 28°[a,d]
				14,600 below 28°[a,d]
		18,800	14,000	16,000[g]
		22,500[i]	15,000[i]	15,000[h]

[a] Richards and Rutter (1961b). [b] Herbert *et al.* (1940). [c] Lehninger *et al.* (1955). [d] Value calculated from Richards and Rutter (1961b). [e] Meyerhof and Lohman (1935). [f] Rutter (1961). [g] Drechsler *et al.* (1959). [h] Beisenherz *et al.* (1953). [i] Values obtained using the hydrazine assay (Jagannathan *et al.*, 1956) and saturating substrate concentrations.

pies of binding for both compounds are also of the same magnitude and vary in the same manner with temperature. Therefore, it appears that the events occurring on binding of fructose diphosphate and of arabinitol diphosphate to the enzyme are similar.

The results presented in Figure 4 show that, for aldolase, a transition occurs at approximately 25° which affects V_{max} and produces a form of the enzyme which can lower the activation energy for the reaction. Massey *et al.* (1966) reported a value of 16°, and we obtained a transition at 18° when V_{max} was determined using 2×10^{-3} M FDP to saturate the enzyme. However, the energies of activation calculated from these data were the same as those calculated from V_{max} obtained by extrapolation. There is no indication of substrate inhibition. The discrepancy between the two transition temperatures (18 and 25°) could indicate that the substrate (or one of the products formed in the assay system) stabilizes the high-temperature form of the enzyme, thereby lowering the temperature of the transition. The presence of substrate or products should, however, have no effect on the values of the energies of activation of the two forms of the enzyme.

When V_{max} was measured using saturating substrate concentrations and the hydrazine assay system (Jagannathan *et al.*, 1956), a transition in the range 22–28° was observed. Since the products of this reaction are not the same as those in the coupled enzyme system, the probability of substrate stabilization is decreased. Activation energies calculated from the hydrazine data are approximately 22,500 cal mole^{-1} below the transition temperature and 16,000 cal mole^{-1} above it. These values are in very good agreement with the values that can be calculated from the data of Richards and Rutter (1961b) presented in Table I.

The free energy of binding is positive and approximately the same for all temperatures (Table I). By itself, this could indicate that the binding process is not affected by tempera-

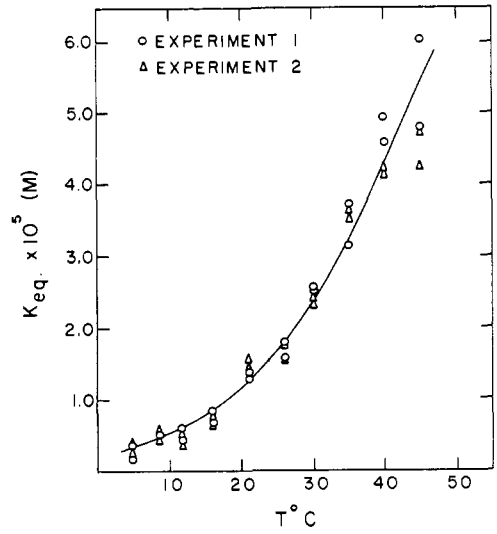

FIGURE 5: Effect of temperature on the equilibrium constant of the overall cleavage reaction catalyzed by aldolase (preparation I). Both sets of data were obtained using the α-glycerophosphate–triose isomerase assay described under Materials and Methods.

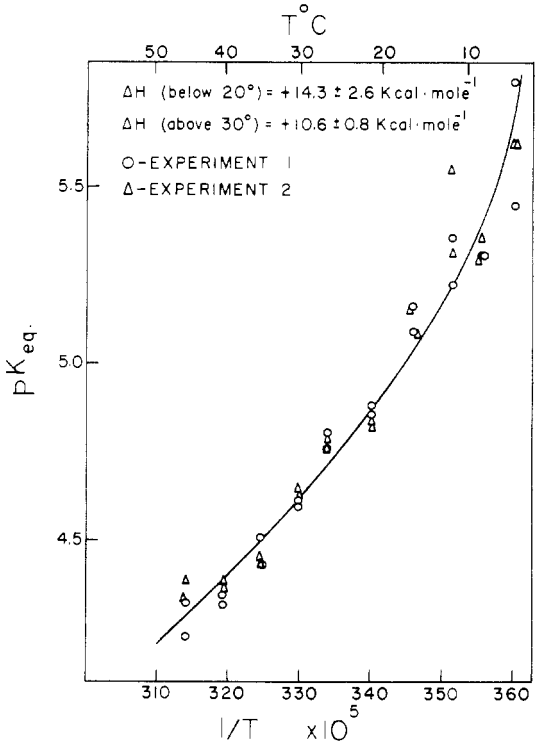

FIGURE 6: Plot of pK_{equil} *vs.* $1/T$ using the data presented in Figure 5.

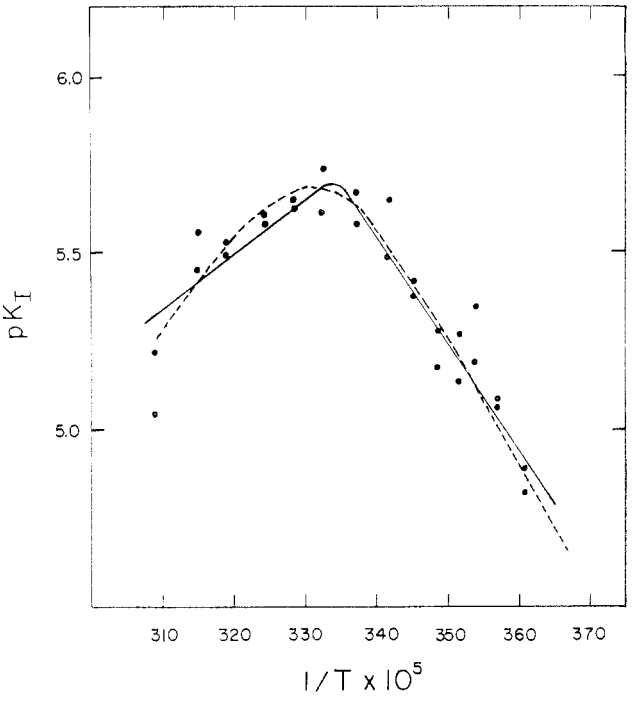

FIGURE 7: Calculated variations in pK_I *vs.* $1/T$ assuming different values of $\Delta H^{\ddagger}$ for the transition between two forms of rabbit muscle aldolase. (—) $\Delta H^{\ddagger}$ = 1000 kcal/mole, critical temperature = 29.9°. ΔH_b above the critical temperature = −13.88 kcal/mole, ΔH_b below the critical temperature = 6.85 kcal/mole. (---) $\Delta H^{\ddagger}$ = 10 kcal/mole, critical temperature = 29.6°, ΔH_b above the critical temperature = −13.88 kcal/mole, ΔH_b below the critical temperature = 11.15 kcal/mole. ● values for pK_I.

ture. However, an examination of the enthalpies shows that the binding is endothermic at low temperatures and exothermic at high temperatures. Massey (1953) has described changes from positive to negative enthalpies of binding of the inhibitors *tran*-saconitate, succinate, and malonate to fumarate hydratase. The large negative entropy observed at temperatures above the transition could be due to tightening of the protein structure upon binding. Such a change has been ascribed to various factors among which are the "freezing" of water structure (Klotz, 1958), hydrogen-bond formation (Yanari and Bovey, 1960), hydrophobic bond formation (Tanford, 1962; Klotz and Franzen, 1962; Brandts, 1964a,b), and electrostatic interactions. These large changes in the thermodynamics of binding clearly indicate the occurrence of a change in the properties of the enzyme.

Smaller changes with temperature in the thermodynamics of the catalytic process are observed in the enthalpy and entropy terms (Table I). The values of the thermodynamic parameters obtained are in good agreement with those available in the literature (Table I).

The curvature in the Arrhenius plots (Figure 4) as well as in the plots of pK_m and pK_I *vs.* $1/T$ (Figure 2) can be attributed to the existence of at least two forms of the enzyme, each being able to bind the substrate or inhibitor, but having different affinity constants and different energies of activation. Such a model has been discussed by Massey *et al.* (1966) for D-amino acid oxidase. It assumes that two forms of the enzyme are in equilibrium, that they exist in equal amounts at the transition temperature, and that increasing the temperature favors the formation of the high-temperature form and *vice versa*.

Other explanations can be offered for the curvature in the Arrhenius plots (Dixon and Webb, 1965). Phase changes in the solvent could produce discontinuities of the kind observed. However, at present no clear evidence that such phase changes occur in water has been advanced. The reaction has been shown to consist of a series of discrete steps, and at 35°, the release of dihydroxyacetone phosphate has been shown to be rate limiting (Rose *et al.*, 1965). If rate constants for steps in the cleavage have different temperature coefficients, then it is possible to obtain discontinuities in Arrhenius plots as different rate constants become dominant. However, the observance of a discontinuity in the plot of K_I *vs.* T (Figure 1) at a temperature close to the discontinuity in the Arrhenius plot is best explained by assuming that both are due to the same fundamental change in the enzyme. Thus, if the break in the Arrhenius plot is due to a change in the rate-limiting step, then the latter change is produced by a change in the protein which also affects binding.

In view of these considerations, it is most logical to conclude that the discontinuities observed are due to the existence of at least two forms of rabbit muscle aldolase that exists in equilibrium with each other. These two forms have different affinities for FDP and D-arabinitol diphosphate and different catalytic capabilities.

Massey *et al.* (1966) showed that the existence of two forms of an enzyme which can undergo a temperature-dependent transformation give discontinuities in Arrhenius plots which are more or less abrupt, depending on the $\Delta H^{\ddagger}$ of the trans-

formation. We have calculated values for the $\Delta H^{\ddagger}$ of the temperature-dependent aldolase transformation by assuming a value for $\Delta H^{\ddagger}$ and calculating the proportions of the two forms of the enzyme at various temperatures. Values were then assigned to the ΔH for binding to each form using the data for the effect of temperature on K_I well above and well below the transition temperature. From the values of ΔH_b, values for K_I over the temperature range of interest were calculated and used to compute values for the apparent K_I. The process was repeated using different values for $\Delta H^{\ddagger}$ until a satisfactory fit of the experimentally determined values to the theoretical curve was obtained (Figure 7).

A similar approach was utilized to calculate $\Delta H^{\ddagger}$ by fitting to the data on V_{max}. Fitting to the V_{max} and K_I data can be achieved equally well using values of 10 or 1000 kcal per mole for the conversion. If the larger value is more nearly correct then both forms of the enzyme are present over a very narrow range of temperature close to the transition temperature. If the $\Delta H^{\ddagger}$ for the transition is 10 kcal/mole then appreciable amounts of both forms are present over the whole range studied.

Susor *et al.* (1969) have demonstrated that rabbit muscle aldolase can be separated into five electrophoretically distinct bands each having a high specific activity. This finding clearly demonstrates that the two-state model utilized in this discussion is an oversimplification. However, the data presented here does indicate that there is a temperature-dependent change in the binding and catalytic properties of the enzyme. Whether each of the electrophoretically distinct forms has similar properties or whether the observed change is due to different forms dominating at different temperatures remains to be established.

Some of the effect of temperature on the binding reaction must be due to changes in the ionization and solvation of the substrate or inhibitor. That the substrate and products are affected differently is shown by the changes in ΔF, ΔH, and ΔS for the overall reaction (Table I). The differences in these parameters on either side of the transition are similar to those for ΔF, ΔH, and ΔS of the activation process ($\Delta\Delta F$ = 100 cal mole^{-1}; $\Delta\Delta H$ = 4800 cal mole^{-1}, $\Delta\Delta S$ = 16.5 eu) but quite different to those for ΔF, ΔH, and ΔS of binding ($\Delta\Delta F$ = 200 cal mole^{-1}, $\Delta\Delta H$ = 25,300 cal mole^{-1}, $\Delta\Delta S$ = 84.2 eu). Now ΔH of activation represents a difference between the enthalpy content of the enzyme–substrate complex and that of the transition state; both involve the enzyme. However, ΔH of the overall process represents a difference between the enthalpy content of the substrate and that of the products and does not involve the enzyme. ΔF and ΔS values can be similarly defined. Because of this, it may be most reasonable to assume that the similarities in the effect of temperature on the thermodynamics of the overall process and the activation process are fortuitous. However, it is possible that the substrate molecule in ES resembles free substrate and that in the transition state resembles products; at least in terms of thermodynamic properties. The thermodynamic parameters of the overall process and of activation should then be determined in part by the properties of the substrate and products. If the enzyme had the same thermodynamic properties when combined with the substrate over the temperature range studied, then the values of $\Delta\Delta F$, $\Delta\Delta H$, and $\Delta\Delta S$ for activation and the overall process might be identical. These thoughts lead to the conjecture that the temperature-dependent transition involves only the free enzyme. The conformational change is overcome by binding the substrate and the catalytic process involving the enzyme–substrate complex and the transition state are affected in a normal way by temperature.

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References

Baer, E., and Fischer, H. O. L. (1943), *J. Biol. Chem. 150*, 223.
Baer, E., and Fischer, H. O. L. (1956), *J. Am. Chem. Soc. 78*, 1659.
Ballou, C. E., and Fischer, H. O. L. (1955), *J. Am. Chem. Soc. 77*, 3329.
Ballou, C. E., and Fischer, H. O. L. (1956), *J. Am. Chem. Soc. 78*, 1659.
Beisenherz, G. (1955), *Methods Enzymol. 1*, 387.
Beisenherz, G., *et al.* (1953), *Z. Naturfor. 8B*, 555.
Brandts, J. F. (1964a), *J. Am. Chem. Soc. 86*, 4291.
Brandts, J. F. (1964b), *J. Am. Chem. Soc. 86*, 4302.
Castellino, F. J., and Barker, R. (1968), *Biochemistry 7*, 2207.
Dixon, M., and Webb, E. C. (1965), Enzymes, 2nd ed, New York, N. Y., Academic, pp 158ff.
Donovan, J. W. (1964), *Biochemistry 3*, 67.
Drechsler, E. R., Boyer, P. D., and Kowalsky, A. G. (1959), *J. Biol. Chem. 234*, 2627.
Gomori, G. (1942), *J. Lab. Clin. Med. 27*, 955.
Hartman, F. C., and Barker, R. (1965), *Biochemistry 4*, 1068.
Herbert, D., Gordon, H., Subrahmanyan, V., and Green, D. E. (1940), *Biochem. J. 34*, 1108.
Jagannathan, V., Singh, K., and Damodaran, M. (1956), *Biochem. J. 63*, 94.
Kayne, F. J., and Suelter, C. H. (1968), *Biochemistry 7*, 1678.
Klotz, I. M. (1958), *Science 128*, 815.
Klotz, I. M., and Franzen, J. S. (1962), *J. Am. Chem. Soc. 84*, 3461.
Lehninger, A. L., Sice, J., and Jensen, E. V. (1955), *Biochim. Biophys. Acta 17*, 285.
Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.
Massey, V. (1953), *Biochem. J. 55*, 172.
Massey, V., Curti, B., and Ganther, H. (1966), *J. Biol. Chem. 241*, 2347.
Meyerhof, O., and Lohman, K. (1935), *Biochem. Z. 275*, 430.
Richards, O. C., and Rutter, W. J. (1961a), *J. Biol. Chem. 236*, 3177.
Richards, O. C., and Rutter, W. J. (1961b), *J. Biol. Chem. 236*, 3185.
Rose, I. A., O'Connell, E. L., and Mehler, A. H. (1965), *J. Biol. Chem. 240*, 1758.
Rutter, W. J. (1961), *Enzymes 5*, 341.
Susor, W. A., Kochman, M., and Rutter, W. J. (1969), *Science 165*, 1260.
Swenson, A. D., and Boyer, P. D. (1957), *J. Am. Chem. Soc. 79*, 2174.
Tanford, C. (1962), *J. Am. Chem. Soc. 84*, 4240.

Taylor, J. F., Green, A. A., and Cori, G. T. (1948), *J. Biol. Chem. 173*, 591.

Wilkinson, G. N. (1961), *Biochem. J. 80*, 324.

Yanari, S., and Bovey, F. A. (1960), *J. Biol. Chem. 235*, 2818.

Characterization of a Fluorescent Complex between Auramine O and Horse Liver Alcohol Dehydrogenase*

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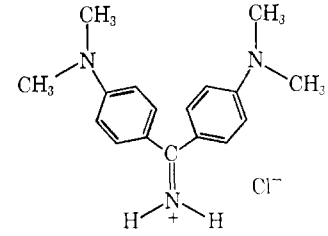
ABSTRACT: Auramine O exhibits an intense fluorescence in the presence of horse liver alcohol dehydrogenase but not with sixteen other proteins tested. The interaction between dye and protein has been studied by absorption and fluorescence spectroscopy, inhibition kinetics, and equilibrium dialysis. The results suggest two equal and independent dye binding sites with an association constant of 1.0×10^5 M^{-1}. The dye binding sites are distinct from the coenzyme and substrate binding sites as illustrated by the noncompetitive nature of the inhibition by auramine O. In the presence of oxidized nicotinamide-adenine dinucleotide (NAD^+), the number of dye binding sites is unchanged, the dye–protein association constant increases, and the dye fluorescence is quenched. Auramine O is displaced from the protein by addition of NAD^+ plus pyrazole. Energy transfer from intrinsic tryptophan to bound auramine O is observed, but no evidence has been obtained for transfer between dye molecules.

The binding of fluorescent dyes to specific sites on horse liver alcohol dehydrogenase has been under investigation in this laboratory with the aim of gaining insight into the nature of the active site and the function of the enzyme. Rose bengal and several *N*-arylaminonaphthalenesulfonates exhibit marked increases in fluorescence yield upon binding to liver alcohol dehydrogenase (Brand *et al.*, 1967; Conrad, 1968). Evidence was obtained that these dyes interact at the coenzyme binding regions of the protein.

It is of interest to note that the above dyes are anions. It is well documented that several buffer anions inhibit the catalytic activity of liver alcohol dehydrogenase (Sund and Theorell, 1963) and the binding of coenzymes (Li *et al.*, 1963). The binding of the fluorescent dyes could be due to their anionic character and/or their aromatic character. The fluorescence characteristics of the bound *N*-arylaminonaphthalenesulfonate dyes indicate that the binding sites are nonpolar regions on the enzyme (Turner and Brand, 1968).

This report describes the interaction of liver alcohol dehydrogenase with the *cationic* diphenylmethane dye, auramine O.

Auramine O does not fluoresce in water, ethanol, hexane, benzene, or dioxane. Intense visible fluorescence is observed in viscous solutions or when the dye is adsorbed to polyanions



such as DNA (Oster and Nishijima, 1964). The experiments to be described in this paper characterize a specific fluorescent complex between auramine O and liver alcohol dehydrogenase.

Materials and Instrumentation

Crystalline horse liver alcohol dehydrogenase, (EC 1.1.1.1) was obtained from C. F. Boehringer, Mannheim, West Germany, and dialyzed against 0.1 M sodium phosphate (pH 7.4) as previously described (Brand *et al.*, 1967). The molar extinction coefficient of liver alcohol dehydrogenase at 280 mμ was determined by the method of Sund and Theorell (1963). It was found to be within 2% of 3.54×10^4, the value reported by these authors. Other enzymes were obtained from the following sources: yeast alcohol dehydrogenase, bovine serum albumin, and urease from the Sigma Chemical Co., St. Louis, Mo.; beef liver glutamic dehydrogenase, rabbit muscle glycerol 1-phosphate dehydrogenase, rabbit muscle enolase, rabbit muscle aldolase, rabbit muscle triose phosphate isomerase, and α-chymotrypsin from C. F. Boehringer, Mannheim, West Germany; and rabbit muscle lactic dehydrogenase, egg-white lysozyme,

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