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Conformational Changes in Rabbit Muscle Aldolase. Ultraviolet Spectroscopic Studies*

G. M. Lehrer† and R. Barker‡

ABSTRACT: The binding of D-arabinitol 1,5-diphosphate (Ara-P_2) to rabbit muscle aldolase (EC 4.1.2.13) produces a spectral change which indicates that both tryptophyl and tyrosyl residues are affected. The titration curves are not hyperbolic indicating the possibility of cooperativity in the binding process or differences between sites. Tryptophan appears to be present in, or close to, the binding site. The temperature-induced difference spectrum of aldolase does not vary in intensity linearly with temperature over the range 0–50° supporting the view that the enzyme can undergo a temperature-induced conformational change. The temperature-induced difference spectra of a number of model compounds have been examined and shown to vary in intensity linearly with temperature. Ara-P_2 binding does not stabilize the enzyme against heat denaturation. Ara-P_2 binding does affect the difference spectrum produced by lowering the temperature indicating that binding may produce a similar conformation at all temperatures.

The effect of temperature on K_m and V_{max} for the cleavage of fructose 1,6-diphosphate (Fru-P_2[1]) by rabbit muscle aldolase and on K_I for the binding of D-arabinitol 1,5-diphosphate (Ara-P_2) by the enzyme has been described previously (Lehrer and Barker, 1970). Van't Hoff and Arrhenius plots of the binding and velocity data all showed curvature and were interpreted as indicating the existence of at least two active forms of the enzyme which would be present in equal amounts at approximately 28°, the transition temperature for their interconversion. It was not possible to determine the enthalpy of interconversion of the two forms of the enzyme, and appreciable quantities of both forms of the enzyme may be present over the temperature range of interest (4–45°).

The aldolase reaction can be thought of as consisting of three steps, binding, catalysis, and unbinding. The thermodynamics of the binding and catalysis steps were obtained, and it was found that the binding step was most strongly influenced by temperature. Below the transition temperature the thermodynamic parameters for binding were $\Delta G° = -7.2$ kcal mole^{-1}, $\Delta H° = 11.5$ kcal mole^{-1}, $\Delta S° = +63.8$ eu. Above the transition temperature these values were $\Delta G° = -7.4$ kcal mole^{-1}, $\Delta H° = -13.8$ kcal mole^{-1}, and $\Delta S° = -20.9$ eu.[2] Activation parameters were not so dramatically affected. These findings led to the proposal that the enzyme undergoes a conformational change as the temperature is varied, but that when the substrate is bound the same catalytically active form is induced (Scheme I). If a conformational change is involved it might be sufficiently large to result in the perturbation of tyrosine or tryptophan chromophores.

Alternatively, the observed changes in ΔH with temperatvre can be explained on the basis of a large and constant difference in heat capacity (ΔC_p) between the enzyme and the enzyme–substrate (or inhibitor) complex. In this case, ΔH will be zero at some temperature and will be of different sign above and below that temperature (Glasstone, 1946). This explanation does not require a conformational change in the protein at the temperature where ΔH is zero and the temperature perturbation of the enzyme would be linear (Bello, 1969).

In an attempt to distinguish between these possibilities, the temperature difference spectra of aldolase and of some model compounds were obtained, and the effect of binding Ara-P_2 on the temperature-induced difference spectrum of aldolase was determined. It is important to ascertain whether transitions in ΔH and ΔS for enzymatic processes are due to conformational changes or to heat capacity differences, since, in some cases, these changes are important to the function of the organism (Somero and Hotchachka, 1968)

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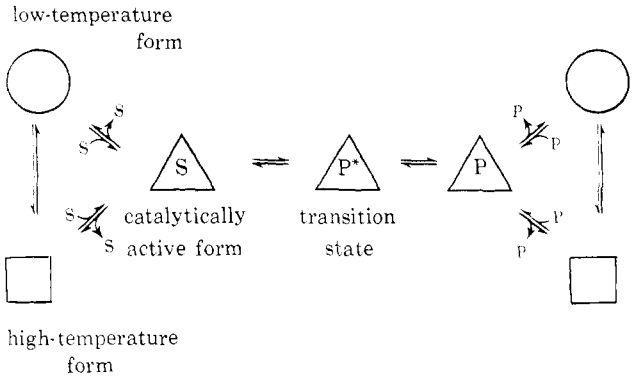
† Present address: Department of Medicine, Mt. Sinai Medical School, New York, N. Y. 10029.

‡ To whom correspondence should be addressed.

[1] Abbreviations used are: Fru-P_2 = D-fructose 1,6-diphosphate; Ara-P_2 = D-arabinitol 1,5-diphosphate.

[2] In the original publication errors were made in the signs for ΔS which are corrected here.

SCHEME I



and the mechanisms involved in producing them need to be established.

Materials and Methods

Enzymes, coenzymes, and substrates were described previously (Lehrer and Barker, 1970).

D-Arabinitol 1,5-diphosphate tetracyclohexylammonium salt (Ara-P_2) was prepared from D-arabinose by the method of Hartman and Barker (1965) and carefully purified. The cyclohexylammonium salt was used directly or converted into the sodium salt. Other chemicals were reagent grade and used without further purification.

Temperature Difference Spectra. Difference spectra were obtained on a Cary Model 15 recording spectrophotometer equipped with thermostatable cell jackets. The temperatures of the reference and of the sample compartments could be controlled independently through the use of separate circulating baths. Calibrated thermistors were used to record the temperature in the cuvets. The thermistors were inserted through pieces of plexiglas having the dimensions of the outer walls of the cuvet which were positioned so that when the base of the plexiglas rested in the cuvet top, the thermistor tip was in the center of the cuvet in the solution above the light path. A piece of surgical rubber tubing was then stretched over the cuvet and the plexiglas to provide a seal between them.

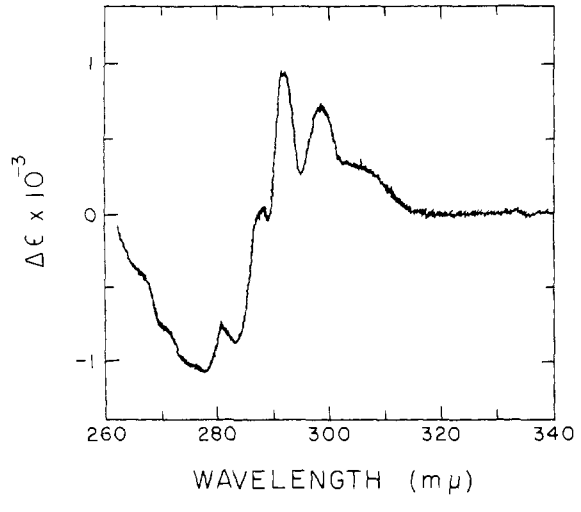


FIGURE 1: Difference spectrum of aldolase produced by a temperature difference of 18°; sample at 28.6°; reference at 10.6° (aldolase, 2.13 mg/ml of 0.15 M glycylglycine buffer, pH 7.4 at 25°).

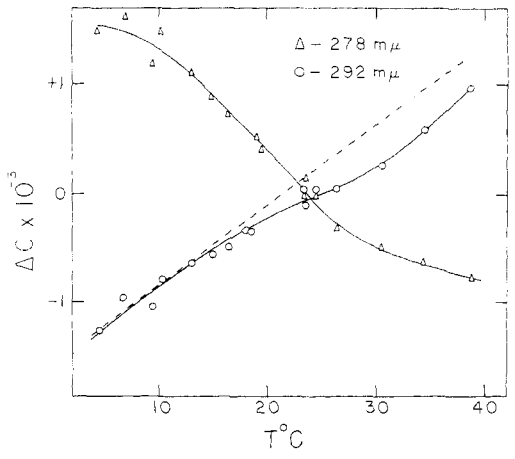


FIGURE 2: Effect of temperature on the extinction coefficient difference of aldolase [2.06 mg/ml (1.32×10^{-5} M) in 0.15 M glycylglycine buffer, pH 7.5]. Results expressed as the extinction coefficient difference between a reference temperature of 23.5 ± 0.3° and the particular temperature. Difference spectra were obtained at intervals as the temperature was decreased to 4.2 and then increased to 38.7°. Turbidity was present at 40.0°. The dashed straight line is the change expected from studies of the temperature perturbation of model compounds (see text).

Preparations of aldolase were dialyzed three times against at least 300 volumes of 0.15 M glycylglycine buffer, pH 7.5. Both protein solution and dialysate were passed through Millipore filters. Nitrogen gas was then bubbled through the dialysate for 10–15 min to remove oxygen present. This N_2-flushed buffer was used to dilute the aldolase to the desired concentration.

Solutions of amino acids, *N*-acetyl ethyl esters, and analogs were prepared in glycylglycine, stirred overnight, passed through Millipore filters, and N_2 flushed. Direct spectra were obtained and the concentrations were calculated from their

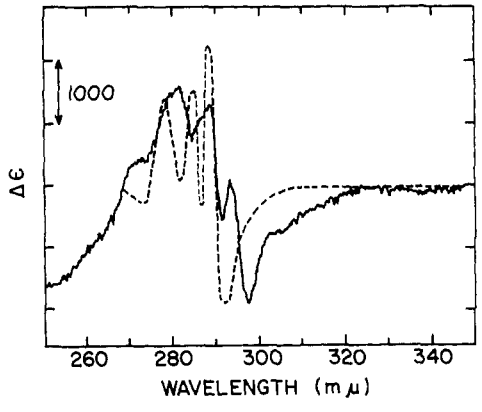


FIGURE 3: Comparison of Ara-P_2 induced difference spectrum of aldolase with a calculated curve. ——, observed difference spectrum produced by the presence of arabinitol diphosphate. (Aldolase = 0.97×10^{-5} M, Ara-P_2 = 5.38×10^{-5} M in 0.15 M glycylglycine, pH 7.5). -----, spectrum calculated from the solvent perturbation data of tyrosine and tryptophan in 20% ethylene glycol (Herskovits and Sorensen, 1968a,b) with the assumption that the protein fabric was the perturbant (Donovan, 1964). The calculated difference spectrum is that expected if a net of 1.2 tryptophan residues became exposed while a net of 5.8 tyrosine residues became buried when aldolase is saturated with inhibitor.

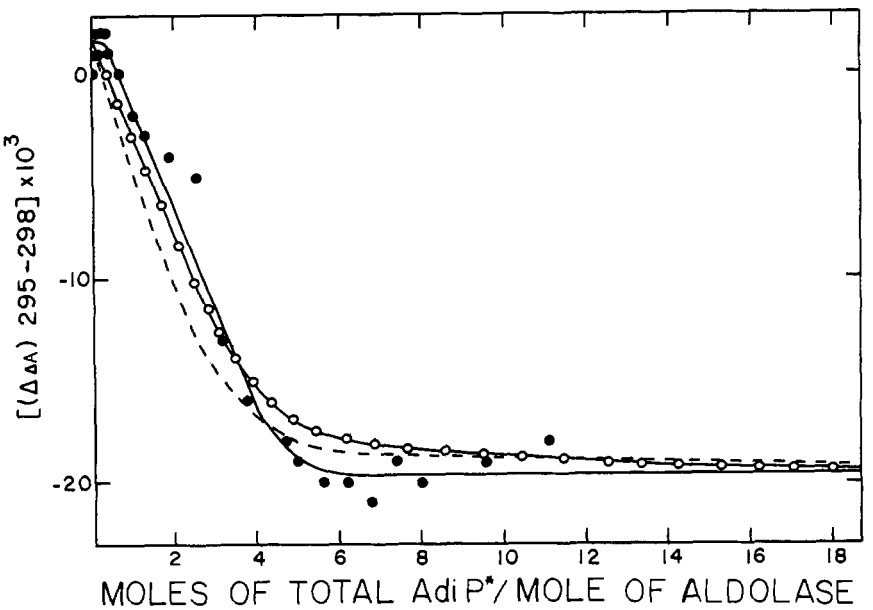


FIGURE 4: Effect of Ara-P_2 on the absorbance difference between 295 and 298 mμ of aldolase. The temperature was kept between 26.9 and 28.2°. The concentration of aldolase was 1.08×10^{-5} M before addition of Ara-P_2. -O-O-, observed; -----, theoretical curve calculated assuming a total of 3 equivalent binding sites per molecule and K_I 2.2×10^{-6} (Lehrer and Barker, 1970); -O-O-O-, theoretical curve assuming 4 equivalent binding sites per molecule and $K_I = 2.2 \times 10^{-6}$ M.

maximum absorbances using ϵ_{275} 1400 for tyrosine (Wetlaufer, 1962) and ϵ_{278} 5500 for tryptophan (Beaven and Holiday, 1952) or from weighed samples.

Equal volumes of the solution under study in 1-cm quartz cuvets were placed in the sample and reference compartments of the spectrophotometer. The cell compartments were flushed with nitrogen throughout the experiment. Base-line difference spectra from 350 to 240 mμ—*i.e.*, spectra of the two solutions held at the same temperature—were obtained and subtracted from all subsequent spectra.

The temperature of the reference compartment was held constant (±0.8°) throughout the experiment (at a temperature between 24 and 30°) and that of the sample compartment was first lowered to a minimum value and then brought back to the initial temperature in approximately 5° increments. Duplicate spectra were obtained when the temperature had stabilized after each increment. When the temperature had reached the initial value, the base line was checked before examining the higher temperature range. The changes in absorbance were normalized to the 330–350-mμ region (Beaven and Holiday, 1952). The specific activity of the aldolase in the sample compartment was checked after the completion of each experiment, and the reversibility of the spectral effects was demonstrated.

In other experiments, continuous recordings of the change in absorbance with temperature at wavelengths of various maxima or minima in the difference spectra were obtained. The temperature of the reference compartment was held constant in a range from 25 to 30° throughout these experiments and that of the sample compartment was raised at approximately 1° min^{-1} from 4°. In the case of aldolase, the temperature and the change in absorbance were recorded until the protein formed a precipitate in the cuvet.

Ara-P_2 Difference Spectra. Aldolase was dialyzed against three changes of buffer. Both enzyme and dialysate were clarified by passage through Millipore filters. The desired concentration of protein was obtained by diluting the enzyme with N_2-flushed dialysate. Two 1-cm quartz cells were placed in tandem in jacketed cell holders in each of the Cary Model 15 compartments. The temperature was maintained constant by means of a circulating bath.

Identical volumes of the protein solution were placed in the front cells (with regard to the source of radiation) of both sample and reference compartments; the same volume of buffer was placed in the back cell of each compartment. After scanning the solutions from 350 to 240 mμ to obtain base-line difference spectra, identical volumes of the inhibitor were added to the protein solution of the sample compartment and to the buffer solution of the reference compartment. The same volumes of buffer were added to the protein solution of the reference compartment and to the buffer solution of the sample compartment (Herskovits, 1967). The solutions were then scanned from 350 to 240 mμ.

Results

Effect of Temperature on the Ultraviolet Difference Spectrum of Aldolase. Temperature has a pronounced effect on the ultraviolet spectrum of aldolase (Figure 1). The relationship between the extinction coefficient difference at both 292 and 278 mμ and temperature is not linear and has an inflection between 20 and 30° (Figure 2). The protein precipitates, and its specific activity decreases, when the temperature is maintained above 40° for a few minutes. To obtain data at higher temperatures, a continuous recording at 292 mμ was performed, and the temperature was increased from 4 to 55° during 40 min before any precipitation occurred. A second transition above 50° is then apparent which results in an increase in absorbance and is probably due to aggregation.

Effect of Binding of Ara-P_2 on the Ultraviolet Absorption of Aldolase. The effect of binding Ara-P_2 is shown in Figure 3. As the amount of inhibitor is increased, the difference spectrum increases up to a maximum. The "titration" curves shown in Figures 4 and 5 do not indicate simple association; at temperatures above, below, and in the transition range

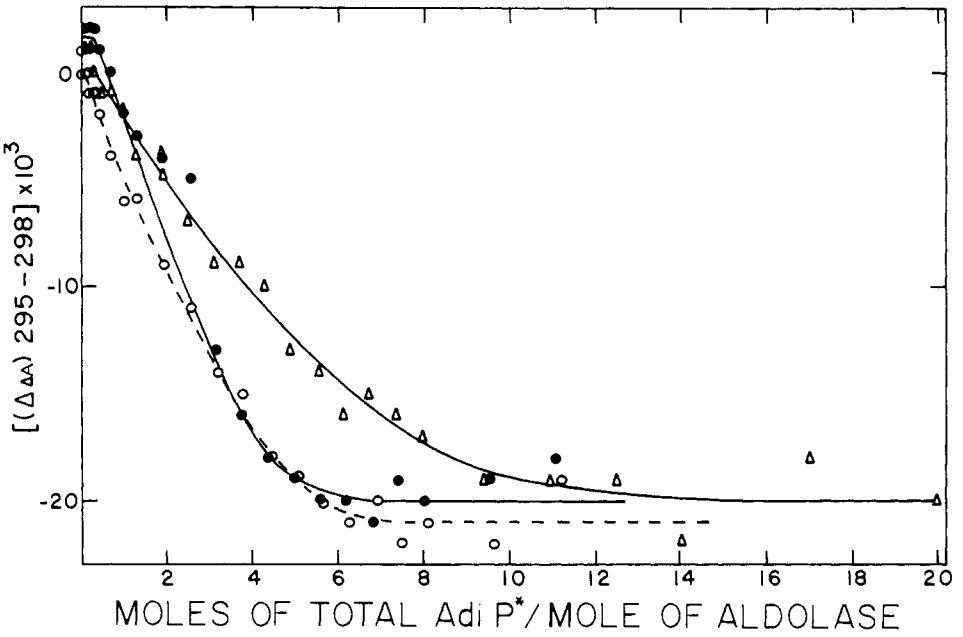


FIGURE 5: Effect of Ara-P_2 on the absorbance difference between 295 and 298 mμ of aldolase at various temperatures. -○--○-, temperature kept between 9.6 and 11.1°, aldolase concentration before addition of Ara-P_2 was 1.07×10^{-5} M; -●-●-●-, temperature kept between 26.9 and 28.2°, aldolase concentration before addition of AdiP was 1.08×10^{-5} M; -△-△-△-, temperature kept between 33.9 and 35.0°, concentration of aldolase before addition of Ara-P_2 was 1.09×10^{-5} M.

of aldolase, there appear to be at least two phases in the binding which have Hill coefficients of approximately 1.6 below half-saturation and approximately 3.8 above half-saturation. The spectrum expected if a net of 1.3 tryptophan residues became buried while a net of 5.8 tyrosine residues became exposed (Donovan, 1964, Herskovits and Sorensen, 1968a,b) is compared in Figure 3 to the difference spectrum obtained by concentrations of Ara-P_2 sufficient to saturate the enzyme. At saturation, the enzyme had 3.3 ± 0.3 binding sites as determined by equilibrium dialysis using Ara-P_2-*l*-^{14}C and membranes having a molecular weight retention of 70,000 (Sartorius S-11536).

Effect of Ara-P_2 on the Temperature-Induced Difference Spectrum of Aldolase. The Ara-P_2 induced difference spectrum, the temperature-induced difference spectrum, and the effect of the addition of Ara-P_2 on the temperature-induced difference spectra are shown in Figures 6 and 7. The difference between the Ara-P_2 spectrum and the spectrum produced by perturbation with both the inhibitor and temperature is also plotted in curve C of Figure 6 and curve D of Figure 7 and compared to the temperature-induced spectra.

Effect of Ara-P_2 on the Temperature Stability of Aldolase. Samples were incubated at various temperatures for 20 min in glycylglycine buffer and adjusted to pH 7.5 at the temperature of incubation and then assayed for activity at 25°. The enzyme is stable up to 40° and loses 50% of its activity at 52°.

At concentration levels sufficient to saturate the enzyme (2×10^{-4} M) Ara-P_2 has no effect on the temperature stability of the enzyme (2×10^{-7} M).

Effect of Temperature on the Ultraviolet Absorption of Model Compounds. The ultraviolet spectra of tryptophan, tyrosine, *N*-acetyl tryptophan ethyl ester, *N*-acetyl tyrosine ethyl ester, indole, and phenol in aqueous solution show a marked dependence on temperature. Typical difference spectra due to a temperature difference of 27° are shown in Figure 8. The temperature-induced difference spectra of indole in water and acetonitrile are very similar; however, those of phenol while similar in shape are reversed (Figure 8). The spectra produced by the amino acids and the *N*-acetyl ethyl esters in aqueous solutions are essentially the same in shape and magnitude but are shifted to longer wavelengths (2–5 mμ). In all cases the intensity changes in the difference spectra were linear with respect to temperature as shown in Figure 13 for indole and phenol. The values of $\Delta\Delta\epsilon$ per degree for the compounds tested in water, acetonitrile, and heptane are given in Table I.

Discussion

Although in the following discussion only two conformations of aldolase will be considered, it should be remembered that the data can also be interpreted in terms of many conformations of the enzyme.

Effect of Temperature on Aldolase. The temperature-induced difference spectrum shown in Figure 1 has maxima at 298 and 292 mμ, a shoulder at 305 mμ, and minima at 285, 278, and 273 mμ. None of the model compounds had a distinct absorption maximum at or near 298 mμ although the 292-mμ maximum in the temperature-induced difference spectrum of tryptophan showed strong tailing toward longer wavelengths.

A trough at 298 mμ in the denaturation difference spectra of several proteins has been interpreted as due to spectral broadening which occurs when chromophores enter an aqueous environment (Keyes *et al.*, 1969). On the other hand, Ananthanarayanan and Bigelow (1969) have interpreted absorptions in the 300-mμ region as due to a minor absorption band of the tryptophyl residue which is sensitive to changes in the electrostatic environment. According to these authors a positive value for $\Delta\epsilon_{300}$ indicates that the process involves an increase in negative charge, a decrease in positive charge, or both in the region of the chromophore. Probably exposed groups only would be involved. However, the magnitude

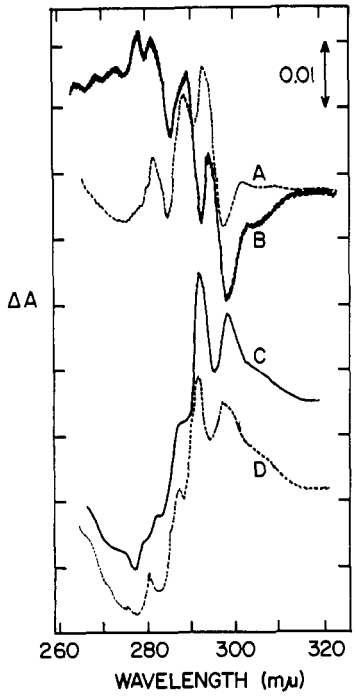


FIGURE 6: The effect of Ara-P_2 on the high temperature induced difference spectrum of aldolase (1.37×10^{-5} M). (A) Sample at 34° and 1×10^{-3} M with respect to Ara-P_2. Reference at 11°. (B) Sample and reference at 12°, sample 1×10^{-3} M with respect to Ara-P_2. (C) Calculated curve A − B. (D) Sample at 34°, reference at 11°.

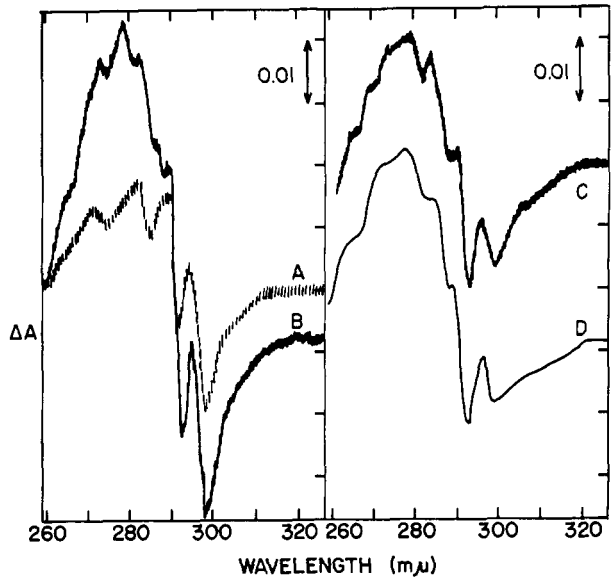


FIGURE 7: The effect of Ara-P_2 on the low temperature induced difference spectrum of aldolase (1.42×10^{-5} M). (A) Sample and reference at 12°, sample 1×10^{-3} M with respect to Ara-P_2. (B) Sample at 12°, reference at 36°, sample 1×10^{-3} M with respect to Ara-P_2. (C) Sample at 12° reference at 36°. (D) Calculated curve B − A.

of $\Delta\epsilon_{298}$ produced by a 37° temperature difference with aldolase which has 12 tryptophan residues per molecule is surprisingly large. It is as large as that produced by the complete denaturation of lysozyme which has six tryptophyl residues per molecule (Foss, 1961). If the effect is due entirely to a change in the electrostatic environment of the two exposed tryptophyl chromophores (Herskovits and Sorensen, 1968b; Donovan, 1969a), then they must be very close to the charged groups responsible. There are no absorptions in the model compounds corresponding to the strong shoulder in the aldolase spectrum at 305 mμ. In the enzyme some of the tryptophyl residues may be in an extremely nonpolar environment causing them to absorb at longer wavelengths and the 305-mμ absorption increase with temperature might be due to changes in their electrostatic environment.

The temperature-induced absorption increase at 292 mμ could be due to tryptophyl residues entering a less polar or more negative environment (Donovan, 1969b). Alternatively, they may simply reflect the increase in absorbance due to changing the temperature. In Figure 8A the difference spectra of indole in water and acetonitrile are shown. These spectra have not been corrected for volume increases due to increasing the temperature, neither has the spectrum of aldolase shown in Figure 1. However, both sets of data were obtained using solutions of approximately the same total absorbance in the region of interest and approximate comparisons can be made between the uncorrected spectra. For indole, $\Delta\epsilon_{291}$ per degree Celsius in water is approximately +6; in acetonitrile $\Delta\epsilon_{292}$ per degree Celsius is approximately +2. Assuming that these two solvents are adequate models for the interior and exterior of the protein and that there are two external and ten internal tryptophyl residues (Donovan, 1969a), then the $\Delta\epsilon_{292}$ per degree Celsius for aldolase should be +32, that observed is +67. Acetonitrile may not be a very good model for the interior of the protein, and this analysis does not take into account changes in the electrostatic environment. It does demonstrate, however, that a large part of the spectral change at 292 mμ is due to thermal perturbation of tryptophyl residues.

Changes in absorption at shorter wavelengths could be due to changes in the environment of both tryptophyl and tyrosyl residues. For example, the difference spectrum of tryptophan produced by increasing the pH shows strong negative bands at 276 and 263 mμ (Donovan *et al.*, 1961). The difference spectrum of *N*-acetyl tryptophan ethyl ester

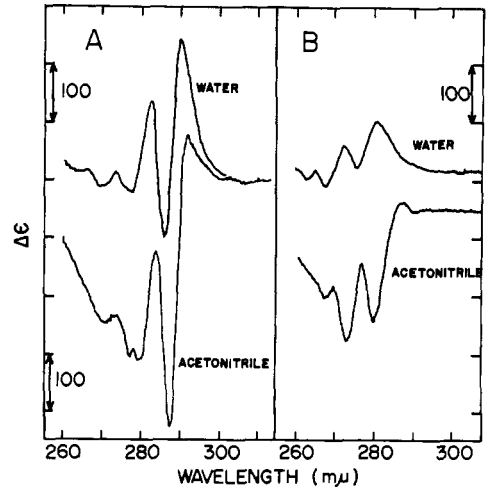


FIGURE 8: The temperature difference spectra of model compounds in acetonitrile and water: A, indole; B, phenol, approximately 1×10^{-4} M solutions with the sample at 40.4° and the reference at 13.4°. Spectra are not corrected for the change in volume of samples with temperature. Values for $\Delta\Delta\epsilon$ at maxima and minima are given in Table I.

TABLE I: Absorption Maxima and Minima, Extinction Coefficients, Difference Spectra Maxima, and the Change in Absorbance per Degree.[a]

DL-Tryptophan Water		*N*-Acetyl-L-tryptophan Ethyl Ester				Indole					
		Water		Acetonitrile		Water		Acetonitrile		Heptane	
$m\mu^b$	ϵ ($\times 10^{-3}$)	$m\mu^b$	ϵ ($\times 10^{-3}$)	$m\mu^b$	ϵ ($\times 10^{-3}$)	$m\mu^b$	ϵ ($\times 10^{-3}$)	$m\mu^b$	ϵ ($\times 10^{-3}$)	$m\mu^b$	ϵ ($\times 10^{-3}$)
288	3.76	288	4.62	290	6.90	286	3.90	287.5	4.64	288	3.58
285.5 (min)	3.64	286 (min)	4.52	287 (min)	6.51	284 (min)	3.75	285 (min)	3.84	285.5 (min)	1.23
279.5	4.47	280	5.57	281.5 (s)	8.25	277	5.21	280 (s)	5.82	279.5	4.62
273 (s)	4.30	273	5.21	279	8.30	270	5.34	277	5.95	277.5 (s)	4.40
243 (min)	2.10	246 (min)	2.36	276 (min)	8.02	237 (min)	1.87			275.5 (min)	3.87
				274	8.07			271	6.36	272 (s)	5.92
				243 (min)	2.53			237	1.80	267	5.76
										264 (min)	5.44
										261.5	5.59
										253 (s)	4.03
										238 (min)	1.52
$m\mu^c$	$\Delta\epsilon$ deg^{-1}	$m\mu^c$	$\Delta\epsilon$ deg^{-1}	$m\mu^c$	$\Delta\epsilon$ deg^{-1}	$m\mu^c$	$\Delta\epsilon$ deg^{-1}	$m\mu^c$	$\Delta\epsilon$ deg^{-1}	$m\mu^c$	$\Delta\epsilon$ deg^{-1}
292.5	+4.4	293	+3.5	289.5	−15.8	290	+5.9	292	+1.8	290.5	+5.2
288	−5.1	288	−6.4	286 (min)	−11.3	286	−6.3	287	−15.4	288	−22.0
284.5 (min)	−0.4	284.5 (min)	−1.6	280	−15.8	282.5	+0.9	284 (min)	−5.5	286 (min)	0.0
278	−4.0	278	−8.1	275 (min)	−11.7	227.5	−4.8	280	−12.2	282	−15.7
274.5 (min)	−2.69	274 (min)	−6.8	272	−13.0	273 (min)	−3.1	275 (min)	−11.2	280.5	−17.0
270	−3.6	268	−8.1			270	−4.2	270	−10.0	276 (min)	−10.0
										268	−15.5
										264 (min)	−10.0
										262	−13.0
DL-Tyrosine Water		*N*-Acetyl-L-tyrosine Ethyl Ester				Phenol					
		Water		Acetonitrile		Water		Acetonitrile		Heptane	
$m\mu^b$	ϵ ($\times 10^{-2}$)	$m\mu^b$	ϵ ($\times 10^{-2}$)	$m\mu^b$	ϵ ($\times 10^{-2}$)	$m\mu^b$	ϵ ($\times 10^{-2}$)	$m\mu^b$	ϵ ($\times 10^{-2}$)	$m\mu^b$	ϵ ($\times 10^{-2}$)
282 (s)	11.7	282 (s)	12.1	285	13.0	275 (s)	11.4	278.5	15.0	277.8	25.4
276	13.9	275.5	14.3	282 (min)	12.8	269.5	13.8	276 (min)	12.5	275.6 (min)	10.1
		264 (s)	11.3	277	15.4	264 (s)	11.2	272	18.2	272.4	25.0
245	2.4	247 (min)	01.8	270 (s)	10.8	247 (min)	0.46	267 (s)	13.6	268.5 (min)	14.9
				247 (min)	1.3			237 (min)	0.57	268	15.1
										267 (min)	13.6
										265	16.9
										262 (s)	11.8
										259.6	9.8

mμ[c]	Δε deg^{-1}	mμ[c]	Δε deg^{-1}	mμ[c]	Δε deg^{-1}	mμ[c]	Δε deg^{-1}	mμ[c]	Δε deg^{-1}	mμ[c]	Δε deg^{-1}
287	+2.3	287	+2.6	291	+0.85	281	+2.4	285	+0.92	281.5	+1.6
282.5	−0.19	283	−0.18	285	−3.5	277	−0.37	280	−5.1	278.5	−11.2
278	+0.75	279	+0.36	282 (min)	−1.5	274	+0.84	276.5 (min)	−1.7	276	+0.8
274.5	−0.57	275	−1.1	278	−3.5	269	−1.5	273	−5.5	274.8	−0.3
						266 (min)	−0.65	269 (min)	−2.7	274	0.0
						263	−1.1	266.2	−3.3	21.5	−8.1
										269.5	−1.4
										268.5	−2.4

[a] Solutions were from 1×10^{-3} to 1×10^{-4} M with respect to solute. Absorption spectra were obtained at 25° and difference spectra over a range of at least 25°. [b] Wavelengths of absorption maxima and minima; (min) = a minimum, (s) = a shoulder. [c] Wavelength of inflection in the temperature difference spectrum; + indicates an increase in absorbance with increasing temperature, − a decrease.

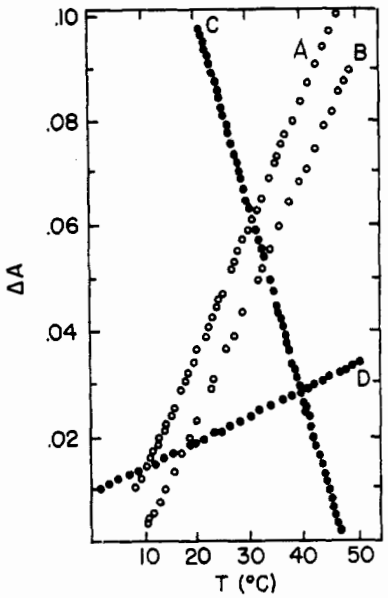


FIGURE 9: The change in absorbance with temperature of phenol and indole in water and acetonitrile: (A) phenol (9.65×10^{-4} M) in 0.15 M glycylglycine buffer, pH 7.5 at 281 mμ; (B) indole (3.7×10^{-4} M) in 0.15 M glycylglycine buffer, pH 7.5 at 290 mμ; (C) phenol (7.5×10^{-4} M) in acetonitrile at 280 mμ; (D) indole (2.5×10^{-4} M) in acetonitrile at 292 mμ.

produced by 20% dimethyl sulfoxide shows a similar negative Δε (Herskovits and Sorensen, 1968a). The temperature-induced difference spectra of indole and phenol in acetonitrile have a strong negative Δε below 290 mμ as shown in Figure 8. Thus, the effect of temperature on the 46 phenolic chromophores of aldolase, 28 of which are buried and 18 of which are in contact with the solvent (Donovan, 1969a), can account adequately for the effect observed; but an increase in negative charge close to a tryptophanyl residue could also contribute to the absorbance change in the 260–290-mμ region.

The observation that the temperature difference spectrum of aldolase is not linear (Figure 2), whereas those of the model compounds are linear (Table I, Figure 9, and Bello, 1969) is of particular interest since it probably indicates that a conformational change is occurring in the enzyme. In Figure 2 the change in absorbance at 292 mμ is shown and compared with the change that would be expected if thermal perturbation of the chromophores in the protein was similar to that observed for the model systems. The spectral change is completely reversible up to 45°, but above that temperature precipitation can occur rapidly.

The deviation of the spectral change at 292 mμ from linearity could be due to a structural change resulting in tryptophyl residues entering a more aqueous environment or to their entering a more positive environment. However, the effect is small and there is no indication in the spectrum of an abrupt conformational change such as would be expected if a transition with a large ΔH was occurring. The spectral change is more consistent with a transition having a small ΔH but does not preclude the occurrence of a more dramatic change which does not result in a large perturbation of the tryptophyl or tyrosyl residues.

Changes at the wavelengths of other maxima or minima in the difference spectrum are similar to those observed at 292 mμ in that they are not linear (Figure 2).

Effect of Ara-P_2 Binding on Aldolase. A potent competitive inhibitor was used in this study rather than the substrate since the latter can permanently modify the enzyme (Woodfin, 1967) and since the proportion of substrate and products varies with temperature and concentration. The interaction of 1,3-dihydroxy-2-propanone phosphate with aldolase produces an increase in absorbance in the region of 240 mμ which is attributed to Schiff base formation; however, Fru-P_2 and glyceraldehyde 3-phosphate produce no change in this region (Topper *et al.*, 1957; Mehler and Bloom, 1963). In addition, Adelman *et al.* (1968) have proposed that conformational changes are induced by binding a variety of substrate analogs which protect aldolase from heat denaturation and digestion by carboxypeptidase. Rose and O'Connell (1968) have shown that a number of substrates and inhibitors protect aldolase from trypsin digestion and quench the fluorescence of the enzyme; they also confirmed the increase in absorbance at 240 mμ observed with dihydroxyacetone phosphate.

The difference spectrum produced by a saturating level of Ara-P_2 is shown in Figure 3. Strong minima are present at 298 and 292 mμ and maxima at 289, 282, and 272 mμ. The Ara-P_2 induced difference spectrum is almost the inverse of that produced by an increase in temperature; they are compared in Figure 6; spectrum B and C, respectively. Clearly, tryptophyl chromophores are perturbed when Ara-P_2 is bound, and the perturbation could be due to either an increase in positive charge or to an increase in the polarity of their environment (Keyes *et al.*, 1969; Ananthanarayanan and Bigelow, 1969). If the tryptophyl residues involved were close to, or in, the active site, the reverse effect might be expected since binding involves a highly negatively charged species, Ara-P_2. The hydroxyl groups on the inhibitor might be brought close to a chromophore, however, increasing the polarity of its environment and producing the effect observed at 298 mμ. This possibility has been tested by examining the difference spectra produced by saturating amounts of a number of α,ω-alkanediol diphosphates. In all cases much smaller perturbations were observed (A. L. Crowder and R. Barker, unpublished observations). On this basis it appears possible that the Ara-P_2 induced spectrum may involve direct perturbation of tryptophyl residues in the active site. An increase in the polarity of the medium in the region of tryptophyl residues would also be expected to quench the fluorescence of the protein. Rose and O'Connell (1968) observed that 1,4-butanediol diphosphate gave 7% quenching. We have observed that Ara-P_2 quenches much more efficiently than does pentanediol 1,5-diphosphate supporting the interpretation that the hydroxyls of Ara-P_2 are important in the perturbation of tryptophyl residues (B. Suh and R. Barker, unpublished observations).

There is ample evidence that rabbit muscle aldolase has at least three binding sites for substrate analogs and that the sites are equivalent (Castellino and Barker, 1966; Ginsburg and Mehler, 1966). On this basis each mole of Ara-P_2 bound produces a change in ϵ_{292} of -160 ± 25 absorbance units, equivalent to the transfer of approximately 0.1[3] of an indole chromophore from a hydrophobic environment to water (Donovan, 1969b). Transfer to the environment provided by the hydroxyl groups of Ara-P_2 probably would produce a smaller molar perturbation, and more than 0.1 of an indole group may be involved in the change.

Spectral changes at shorter wavelengths (260–290 mμ) could be due to either tryptophan or tyrosine residues. The indole chromophore, perturbed so as to produce a minimum at 292 mμ, would give rise to maxima at lower wavelengths so that the direction of the change in the 260–290-mμ region is consistent with the transfer of an indole residue to a more positive environment (Donovan *et al.*, 1961). The shapes of both the Ara-P_2 induced difference spectrum and the temperature-induced difference spectrum of aldolase bear a strong resemblance to the difference spectrum obtained when tryptophan at pH 11.0 is compared to tryptophan at pH 1.3 (Donovan *et al.*, 1961) except that all absorptions in aldolase are shifted to longer wavelengths by from 6 to 30 mμ. Although a shift of 6 mμ is possible, a shift of 30 mμ is not, making it unlikely that the perturbations observed in the 260–290-mμ region are entirely due to tryptophyl residues. The observed changes would be produced if tyrosyl residues enter less polar or more negatively charged environments. Attempts were made to fit the observed difference spectra by summing the spectra produced by solvent perturbation using the data of Herskovits and Sorensen (1968a,b); an example is given in Figure 3. The fit is not very good as shown in the figure, but can be vastly improved by shifting the calculated spectrum approximately 6 mμ toward longer wavelengths, that is by equating the 298-mμ absorption of aldolase with the 292-mμ absorption of tryptophan. The calculated spectrum is that expected if 0.4 tryptophyl residues enter a more polar or a more positively charged environment and if 1.93 tyrosyl residues experienced the opposite change when one molecule of Ara-P_2 is bound to aldolase. These effects could be produced by the hydroxyl groups of the inhibitor perturbing a tryptophyl residue and its negative charges perturbing tyrosyl residues without any dramatic changes in conformation.

The discrepancy between the number of groups calculated as being perturbed when the 298-mμ rather than the 292-mμ absorption in the aldolase difference spectrum is considered to be due to the transition which produces the 292-mμ absorption in model compounds points to the need for better understanding of the relationship between observations on model compounds and on proteins.

Previous studies have indicated that at least three molecules of inhibitor bind to aldolase with approximately the same value for K_I (Castellino and Barker, 1966; Ginsburg and Mehler, 1966). The enzyme used in the present study had 3.3 ± 0.3 binding sites for Ara-P_2 at 5×10^{-4} M inhibitor. If no cooperativity is present in binding to aldolase, then the change in absorbance with increasing amounts of Ara-P_2 would be expected to follow a normal titration curve. This is not the case as can be seen from the data in Figure 4. At $27.5 \pm 0.7°$ the addition of approximately one molar equivalent of Ara-P_2 produces a much smaller change in absorbance than would be predicted on the assumption that all sites are equivalent. When the change in the difference spectrum is plotted according to Hill (1936), two phases can be seen in the binding; at lower concentrations the Hill coefficient is 1.6 and at higher concentration it is 3.8, clearly showing that cooperativity exists in the development of the difference spectrum. Aldolase does not show cooperativity in its kinetics or in its binding, and we cannot reconcile the observed spectral changes with these facts.

The binding of Ara-P_2 to aldolase produces similar difference spectra at 10, 27, and 34°; the magnitude of the change is

[3] One-tenth of an indole residue implies that the perturbation involves only a portion of the chromophore, or that the change in environment is only one-tenth as effective as the transfer from the interior of the protein to water.

also similar as shown in Figure 5. Two phases are apparent in the binding at all temperatures as shown by plotting the data according to Hill (1936); however, the greatest divergence from a simple titration is shown by the spectral changes at 27°, in the region of the conformational change implied by kinetic studies. A protein undergoing a temperature-dependent conformational change should show cooperativity in binding a ligand in the temperature range of the conformational change providing that binding stabilizes one conformer. On the other hand, the finding that a protein shows cooperativity in binding a ligand over a wide range of temperatures could be due to the presence of varying amounts of different conformers which have different ligand-induced difference spectra. The fact that the binding difference spectra of aldolase with Ara-P_2 at low and high temperatures are very similar argues against this possibility, and it is more probable that the phenomenon involves a change in the enzyme conformation caused by the binding of the first molecule of Ara-P_2. The same results would be obtained if the first binding site was structurally different from the others; however, this situation would be expected to result in a difference in K_I which has not been observed.

Effect of Ara-P_2 Binding of the Temperature-Induced Difference Spectrum of Aldolase. As pointed out above, the difference spectra produced by Ara-P_2 and a temperature increase are similar but of opposite sign.

The hypothesis implicit in Scheme I requires that the enzyme be converted by binding substrate (or inhibitor) to a form which is, or is capable of becoming, the catalytically active form. Thus, the addition of Ara-P_2 to either form would produce a common form. A temperature-induced conformational change can be inferred from the deviation from linearity observed in the absorbance increase as shown in Figure 2, and is supported by the coincidental change in ΔH of binding reported previously (Lehrer and Barker, 1970). Assuming that the deviation in absorption increase reflects a conformational change and that the catalytically active form always has the same conformation, it should be possible to observe an effect of Ara-P_2 binding on the temperature difference spectrum. The spectra should not be strictly additive as would be the case if binding and temperature-perturbation were entirely independent. When the high-temperature form of the enzyme is further preturbed by the addition of Ara-P_2, the two effects are almost perfectly additive, as shown in Figure 6. Spectrum C, which was obtained by subtracting B (the Ara-P_2 induced spectrum) from A (the combined temperature and Ara-P_2 spectrum), is very similar to spectrum D which was produced by temperature alone. The match is not perfect; the calculated spectrum is shifted slightly to longer wavelengths, but the peak areas are relatively close to those of the experimental spectrum. However, when a similar experiment is carried out perturbing the low-temperature form with Ara-P_2 and comparing it to the low-temperature form, the two effects are not additive as shown in Figure 7.

Spectrum B of Figure 7 was produced when Ara-P_2 was added to the sample which gave spectrum C; it should be the summation of A and C if the effects of binding and temperature are additive. That the effects are not additive is shown by comparing spectrum D with C; spectrum D was obtained by subtracting A (the Ara-P_2 induced spectrum) from B (the combined Ara-P_2 and low-temperature spectrum) and should be identical with C (the low-temperature spectrum). The major differences are that the 298- and 292-mμ peaks are much smaller than in the temperature-induced spectrum (C), and the peaks below 290 mμ are much larger than expected. It should be noted that correction of the spectra in Figure 7 for the dilution due to the volume change produced by temperature differences is unnecessary. Both spectrum B and spectrum C would be corrected by the same amount, and spectrum D would show the same increase; however, the differences between C and D would be the same as those shown.

The lack of addivity in the perturbation of the enzyme with low temperature and inhibitor indicates that addition of inhibitor decreases the differences for trypotophyl residues between the high- and low-temperature forms. The differences for tyrosyl residues are increased. It is possible, but not necessary, that the tryptophyl residues which are responsible for most of the perturbation caused by temperature changes are the same ones perturbed by Ara-P_2 and that the presence of the inhibitor stabilizes the enzyme in the high-temperature form at the active site. The whole enzyme cannot be similarly affected, and binding inhibitor at the active site may enhance the effect of temperature on other parts of the protein.

It has previously been reported (Adelman *et al.*, 1968) that aldolase is protected from heat inactivation by compounds, such as Ara-P_2, which protect the enzyme against carboxypeptidase digestion; however, in our hands no protection by Ara-P_2 was observed.

The data presented here support the view that the variation in ΔH of binding of Fru-P_2 and Ara-P_2 to rabbit muscle aldolase with temperature is due to a temperature-dependent conformational change in the enzyme rather than to a large constant value for ΔC_p between the enzyme and the enzyme–substrate complex. Binding of inhibitor also produces a spectral change which may be due in part to local effects of the charged and polar groups of the bound molecule and in part to conformational changes.

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A Kinetic Analysis of the Reaction of Lysozyme with Oligosaccharides from Bacterial Cell Walls*

David M. Chipman

ABSTRACT: The kinetic behavior of lysozyme-catalyzed reactions of cell-wall oligosaccharides is analyzed. The model considers the various ways in which any oligomer can associate with the enzyme, and assumes that the association constant for any mode depends only on which subsites of the enzyme site are filled. Rates of cleavage of bound substrate to form a glycosyl enzyme intermediate and rates of hydrolysis or transfer of the intermediate to an acceptor are assumed to be the same for any productively bound substrate. The model can be solved by numerical integration with a digital computer, and has been fit to experimental data by a least-squares procedure. Using the parameters so obtained, further reactions can be satisfactorily modelled. The general behavior of the reactions and the significance of the values of the parameters are discussed. Nonproductive binding is seen to be of major importance in the reactions of small oligomers, which are hydrolyzed chiefly *via* pathways in which they react first as transglycosylation acceptors. Nonproductive binding is also seen to have implications for the mechanism of lysozyme action. The model used can be extended to deal with other endocatenases having transferase activity.

The kinetic behavior of lysozyme-catalyzed hydrolyses of oligosaccharides is quite complex, and has a number of peculiar features (Maksimov *et al.*, 1965; Chipman *et al.*, 1968). Reactions of certain oligosaccharides show the pronounced induction periods typical of autocatalytic reactions, and slow down markedly before the oligosaccharide mixture has reached its expected equilibrium composition (which should correspond to nearly complete hydrolysis). During the course of these reactions, oligomers of many different chain lengths appear in the reaction mixtures, and the enzyme synthesizes significant amounts of saccharides of higher degree of polymerization than the starting material by transglycosylation.

Because of these complications, very few true kinetic studies of the action of lysozyme on oligosaccharides have been carried out. Many studies aimed at the elucidation of the molecular mechanism of lysozyme action have utilized poor synthetic substrates (such as phenyl glycosides of mono- or disaccharides) in an attempt to simplify kinetic analyses (Osawa and Nakazawa, 1966; Lowe and Sheppard, 1968; Dahlquist *et al.*, 1968; Raftery and Rand-Meir, 1968; Tsai *et al.*, 1969). We feel that this is an unfortunate situation, as there may be more going on in some of the apparently simple reactions than meets the eye. In addition, we believe the complexities of the lysozyme reactions are intimately related to one of the most fascinating aspects of the molecular mechanism of lysozyme action, the role of "substrate distortion," or "strain." The complex reactions thus have an inherent information content which is unavailable from "simple" reactions.

Chipman *et al.* (1968) carried out a detailed study of the lysozyme-catalyzed reactions of bacterial cell wall oligosaccharides, $(GlcNAc\text{-}MurNAc)_n$,[1] and proposed a simple kinetic mechanism for the enzymic reaction which qualitatively explained all of the observations cited above. According to this mechanism (to be described below in detail), saccharides less than five pyranose units long are very poor substrates because they associate with the enzyme chiefly in nonproductive modes. Since larger oligosaccharides, which can be formed by transglycosylation, are much better substrates, the major reaction of "small" substrates is as acceptors for transglycosylation. Unfortunately, the kinetic mechanism proposed cannot be solved in closed form, and it was not tested mathematically when the above work was reported in 1968.

In order to test the adequacy of the proposed mechanism, and to provide the required tools for the analysis of reactions catalyzed by lysozyme, we have developed a mathematical model for such reactions, and computer programs for the numerical calculation of the time course of lysozyme-catalyzed reactions of oligosaccharides. We have also written a program for obtaining the kinetic parameters giving the best fit to

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[1] Abbreviations used: GlcNAc, *N*-acetyl-D-glucosamine; MurNAc, *N*-acetylmuramic acid. All oligosaccharides referred to are linked β-(1 → 4), with the reducing terminus written to the right.