Kinetics of the Conformational Change and Subunit Dissociation of Aldolase

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The kinetics of acid denaturation of muscle aldolase were examined by stopped-flow fluorescence and stopped-flow light scattering measurements. The denaturation reaction was found to consist of three distinguishable steps——step I with fluorescence increase only, step II with both fluorescence increase and light scattering decrease, and step III with fluorescence decrease alone. Circular dichroism and sedimentation equilibrium measurements were also made at different pH's in the course of the acid denaturation. It was concluded that in the acid denaturation of this enzyme a rearrangement of the secondary or tertiary structures takes place rapidly (4 s^{-1} at 21.5 °C and at pH 3.4), and then the subunit dissociation occurs more slowly (0.04 s^{-1} at 21.5 °C and at pH 3.4), with further conformational change. The subunit dissociation was found to be caused by two H^+ per tetramer.

We have recently proposed a new method of characterizing the subunit-structure of a macromolecule.[1] The method consists of the use of a stopped-flow light scattering measurement in combination with a stopped-flow fluorescence measurement. By this combination, one can establish a sequence of *cause and effect* between an inter-subunit dissociation reaction and an intra-subunit conformation change in the macromolecule in question. The method was once applied to the subunit structure of concanavalin A and *Limulus* haemocyanin.[1] Practically the same method was followed by Tashiro *et al.*[2] with bovine liver glutamate dehydrogenase. In this paper, we examine the applicability of this method with a slightly more complex example, namely aldolase.

Rabbit muscle aldolase is an enzyme which splits fructose 1,6-diphosphate to yield glyceraldehyde 3-phosphate and dihydroxyacetone phosphate in the second stage of glycolysis.[3] The enzyme is tetramer with each subunit having a molecular weight of 40 000.[4] Kawahara and Tanford showed that the four-subunit structure of the enzyme is dissociated into monomers by guanidine hydrochloride.[5] The enzyme is also dissociated by urea or acid.[6,7] The subunit reassociation and reactivation of denatured aldolase were found to be slow processes (3 h at 0 °C), and the kinetics of these processes were once examined by Teipel.[8] The kinetics of unfolding and subunit dissociation of the enzyme, however, have not been examined so far.

Experimental

Aldolase. Rabbit muscle aldolase was purchased from Sigma as a crystalline suspension in 3.2 M† ammonium sulfate. After centrifugation of the suspension the enzyme solution was obtained by dialyzing it against 0.01 M sodium citrate buffer (pH 5.4) for a few days at 4 °C. Protein concentrations were determined spectrophotometrically using the value of $E^{1\%}_{1\,cm}$=9.1 at 280 nm.[9] Buffer systems used are: citric acid+sodium citrate buffer (in the pH 6—3 range) and sodium citrate+HCl buffer (in the pH 2—2.7 range). The enzyme solution was filtered everytime before the light scattering measurement through a 0.45 μm Millipore type HA filter.

Fluorescence Measurements. Fluorescence spectra were observed by a Hitachi Fluorescence Spectrophotometer model 650-10S with circulating water to maintain the temperature of the cell at 25 °C.

Circular Dichroism Measurements. Circular dichroism spectra were measured by a JASCO CD Spectrometer J 20 at room temperature. All the measurements were made with a 1 mm path length quartz cell.

Stopped-flow Measurements. The rate of fluorescence and light scattering changes (in intesity) were examined with a Union Giken stopped-flow spectrophotometer RA-401 in combinaton with a microcomputer RA-450 system.[10] We used a Hoya UV 33 cut filter (which allows the emitted light of wavelength longer than 330 nm to come into the detector) in the fluorescence measurements. For the light scattering measurements a 4 mW He-Ne laser was used. For introducing the laser beam into the cell window, an optical fiber was used. This makes it easier to guide the beam and to adjust the position and direction of the light path than a convensional mirror system. All scattering measurements were made at the angle of 90° to the incident beam, since the dimension of the protein molecule was small relative to the wavelength of the light.

Results

Fluorescence Spectra. We first observed the fluorescence spectra of rabbit muscle aldolase at several pH values and the results are shown in Fig. 1. As is seen in the figure, the emission maximum is shifted towards longer wavelength on going from pH 4.4 to pH 3.4. The pH dependence of the wavelength (λ_{max}) of the emission maximum and the intensity are shown in Fig. 2. From this figure it is suggested that the enzyme is denatured in acidic pH and the denaturation point is around pH 3.7. This value is in agreement with the previously proposed denaturation point.[6]

Circular Dichroism. The observed molecular ellipticity [θ] of this enzyme at 225 nm was plotted against pH as shown in Fig. 2 (c). In the acid denaturation a loss of gross secondary or tertiary structure is seen from the figure.

Sedimentation Equilibrium. Dissociation of aldolase into dimer and monomer has next been examined at acidic pH. According to the results of sedimen-

† 1M=1 mol dm^{-3}.

tation equilibrium measurements (see Table 1) aldolase is considered to be tetramer at pH 5.4, a mixture of dimer and monomer at pH 3.5, and monomer at pH 2.7.

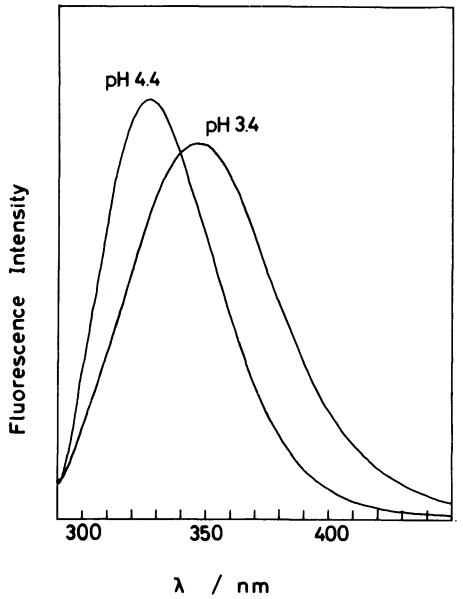

Fig. 1. Fluorescence spectra of aldolase (0.13 mg ml^{-1}), excited at 280 nm. Solvents, 0.1 M citrate buffer. Temperature, 25 °C.

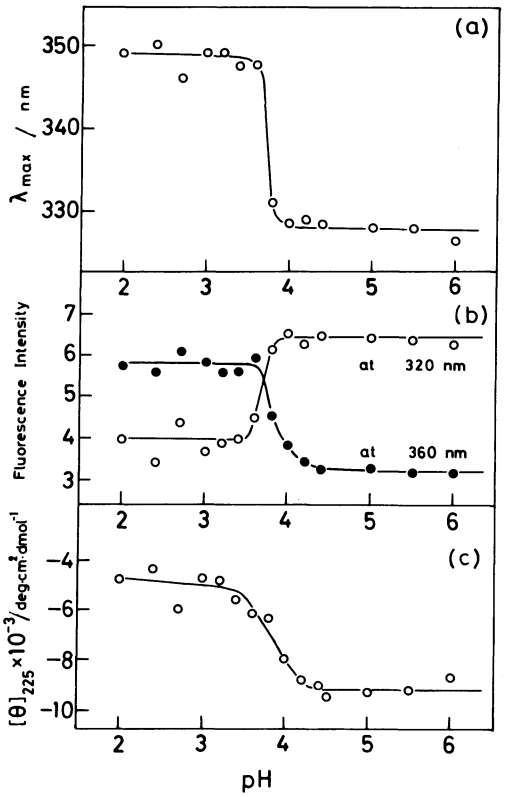

Fig. 2. Effects of pH on (a) the fluorescence peak position, (b) fluorescence intensities, (c) circular dichroism of aldolase. For the fluorescence measurements, the protein concentration was 0.13 mg ml^{-1}, and for the circular dichroism measurements 1.3 mg ml^{-1}. For both measurements, the solvents were 0.1 M citrate buffer. Temperature, 25 °C.

This conclusion is consistent with the previous results.[4]

Kinetic Measurements. After a rapid change of pH from 5.4 to 3.4 of aldolase solution, time-dependent fluorescence and light scattering measurements were made. As may be seen in Fig. 3 (a) and (b), the fluorescence increase (excited at 280 nm and observed all the

Table 1. Apparent molecular weights of aldolase determined by sedimentation equilibrium measurements[a] at different pH's

pH 5.4	174 000± 3 600
pH 3.5	59 800—87 200[b]
pH 2.7	50 300± 400

a) All the experiments were made with aldolase solutions of 0.44 mg ml^{-1} in 0.1 M sodium citrate buffer. b) At pH 3.5, log c *versus* r^2 relation (where c is the concentration and r the distance from the axis of rotation) was not found to be linear, showing that the molecules are not homogeneous.

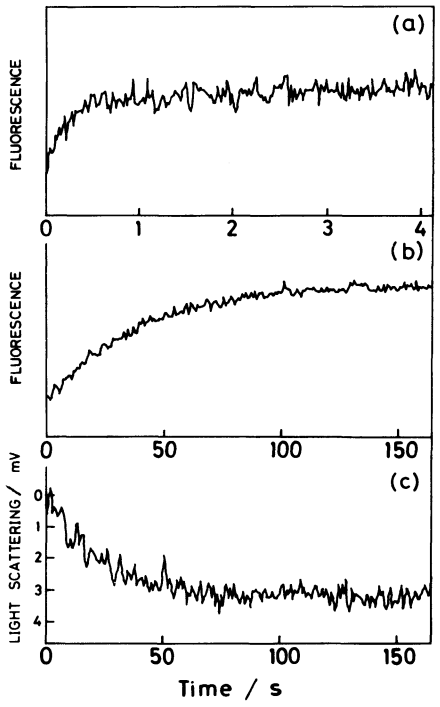

Fig. 3. The time dependence of the increase in the fluorescence intensity (a) and (b) and of decrease in the light scattering intensity (c), of aldolase solution on pH=5.4→3.4 jump. In each experiment, aldolase solution of 3.2 mg ml^{-1} in 0.01 M citrate buffer (pH 5.4) was rapidly mixed with an equal volume of 0.2 M citrate buffer (pH 3.4) at time zero with a stopped-flow device. The final pH was found to be 3.4 and the final concentration of aldolase was 1.6 mg ml^{-1}. Temperature, 21.5 °C. The fluorescence was excited at 280 nm and observed with a cut-filter of 330 nm. The light scattering was observed at 632.8 nm. The ordinate scale (read in mV) is arbitrary but should be taken as equal to that of Fig. 4(c), because all the experimental conditions were the same for these two recordings.

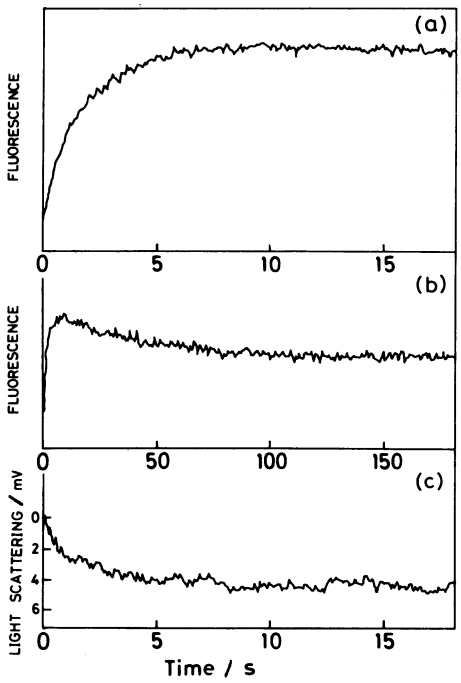

Fig. 4. The time dependent changes in fluorescence intensity ((a) and (b)) and in the light scattering intensity (c) of aldolase solution on pH=5.4→2.7 jump. In each experiment, aldolase solution of 3.2 mg ml^{-1} in 0.01 M citrate buffer (pH 5.4) was rapidly mixed with an equal volume of 0.2 M citrate buffer (pH 2.7) at time zero with the stopped-flow device. The final pH was found to be 2.7 and the final concentration of aldolase was 1.6 mg ml^{-1}. Temperature, 21.5 °C. The fluorescence was excited at 280 nm and observed with a cut-filter of 330 nm. The light scattering was observed at 632.8 nm. The ordinate scale (read in mV) is arbitrary but should be taken as equal to that of Fig. 3(c), because all the experimental conditions were the same for these two recordings.

emitted light of wavelength longer than 330 nm) takes place in two steps. In both steps the fluorescence intensity was found to increase as nearly first-order process with rate constants of 4.0 and 0.03 s^{-1} at 21.5 °C. In the course of these processes, however, the light scattering intensity (at 632.8 nm) was found to decrease in only one step. Its rate constant is nearly equal to that of the second step of the fluorescence increase (step II). Similar experiments were made with different final pH's (to which pH jumps were made from pH 5.4). An example (pH=5.4→2.7 jump) is shown in Fig. 4. The rate constants of the light scattering and fluorescence change were both found to be dependent on the final pH of the solution. As may be seen in Fig. 5, the rate constant of the second-step reaction (step II) is nearly proportional to square of the H^+ concentration, $[H^+]^2$. The rate constant of the first-step (step I) would probably be also proportional to $[H^+]^2$, but it could not be confirmed because the reaction was found too fast to be observed for pH≦3.2 (see Fig. 5). At pH lower than 3.2, on the other hand, even slower reaction (let us call it step III) came out, which could be monitored by a very slow fluorescence decrease. This is seen in Fig. 4 (b). Its rate

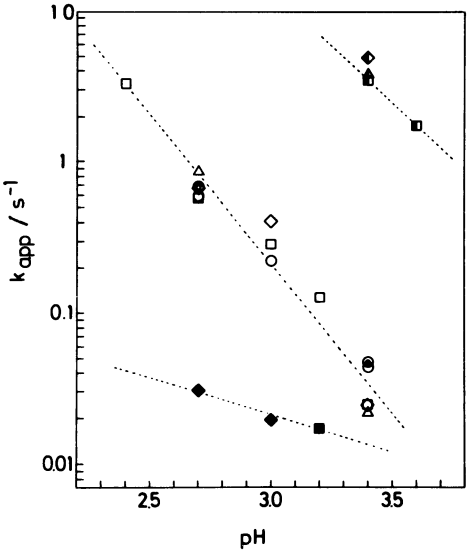

Fig. 5. Observed rate constants of the fluorescence and light scattering changes plotted against the pH values to which the pH jumps (from pH=5.4) were made at 21.5 °C.
▲ △ Fluorescence changes observed with aldolase concentration 6.2 mg/ml. ◐ ◇ ◆ Fluorescence changes, at 1.6 mg ml^{-1}. ◧ □ ■ Fluorescence changes, at 0.67 mg ml^{-1}. ◎ Light scattering changes, at 6.2 mg ml^{-1}. ○ Light scattering changes, at 1.6 mg ml^{-1}.

constants are plotted in Fig. 5 with black squares. The kinetic measurements were also done at different aldolase concentrations, 0.67—6.2 mg/ml. The rate constants of both fluorescence and light scattering changes, however, were found to be independent of the protein concentration (see Fig. 5).

Discussion

Present experimental results indicate that the acid denaturation of rabbit muscle aldolase consists of three distinguishable steps. Step I is a fast reaction in which fluorescence increases but no change in the light scattering takes place. Step II is a slow reaction in which a fluorescence increase and a light scattering lowering take place simultaneously. Step III is a further slower reaction in which fluorescence decreases and no light scattering change occurs. An interpretation of this set of steps is schematically represented in Fig. 6.

Apparently, the subunit dissociation takes place in step II. The rate constant of this dissociation reaction is about 0.04 s^{-1} at pH 3.4 but 0.6 s^{-1} at pH 2.7. The amplitude of the light scattering change is greater at pH 2.7 than that at pH 3.4 (see Figs. 3(c) and 4(c)). These facts may be interpreted by taking into account the finding (by sedimentation equilibrium measurements) that at pH 3.4 aldolase tetramer dissociates only into dimer-monomer mixture, whereas at pH 2.7 completely into monomer (Table 1). The rate of light scattering decrease was found to be independent of the protein concentration. This fact is explained if we assume (i) that tetramer←dimer back reaction is very slow (≈10^{-3} s^{-1}),[8] and (ii) that dimer⇄monomer equilibrium takes place very fast (>1 s^{-1}). In order to explain

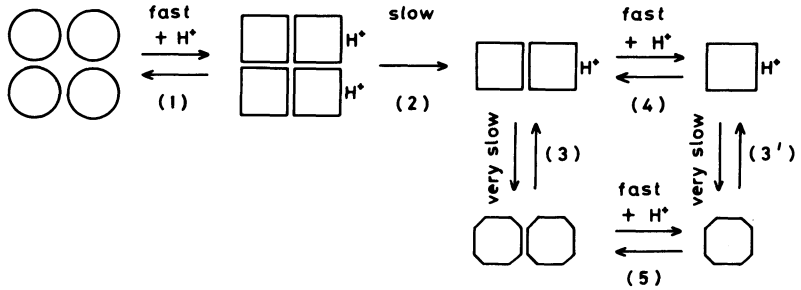

Fig. 6. Schematic representation of the proposed mechanism of acid denaturation of aldolase. We speculate that the observed step I corresponds to the process (1), the observed step II to the processes (2)+(4), and the observed step III to the processes (3)+(3') or (3)+(3')+(5).

the fact that this rate is proportional to $[H^+]^2$ we have to assume, in addition, (iii) that every tetramer has two sites of H^+ binding, which are essential to cause the tetramer→dimer dissociation. Because the step II fluorescence increase takes place simultaneously with the molecular weight change, it is likely (iv) that this fluorescence change is caused merely by a new exposure of some tyrosine or tryptophan groups located on the dimer surface, rather than an intra-dimer or intra-monomer conformation change.

The fluorescence change of step I (fast) must be attributed to an intra-tetramer, intra-dimer, or intra-monomer conformational change, because it is not accompanied by any molecular weight change. This fast conformational change seems to prepare for a two-site H^+ binding to cause the subunit dissociation. The fluorescence change of step III must be ascribed to an intra-monomer (intra-subunit) conformational change. This seems to be a final adjustment to reach a stable conformation of the monomer (or dimer) in acidic pH.

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Note added in proof. It is speculated that the two sites of proton binding, which are essential to cause the tetramer →dimer dissociation, are Asp 358 of the two β-subunits. In the α-subunits, this is replaced by Asn358 (This is the only difference in the amino acid sequences of α and β subunits; C. Y. Lai, *Arch. Biochem. Biophys.*, **166**, 358 (1975)), and cannot be protonated. Therefore, it is understandable that the effective protonation sites are only two, instead of four, per tetramer.