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Reversible Dissociation of Aldolase into Unfolded Subunits*

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Dissociation of rabbit muscle aldolase into three highly unfolded subunits has been observed at *p*H values of 2.9 and below. These subunits appear to be of equal or nearly equal molecular weight. Reversal studies demonstrated that the subunits would, *in vitro*, reassociate and refold in a rapid, spontaneous, and specific manner upon neutralization. The sedimentation coefficient, catalytic activity, and immunological reactivity of the reassociated enzyme appear to be identical to those of native protein. This establishment, *in vitro*, of an enzyme structure of high molecular weight (150,000) and complexity (three chains) provides strong support for the hypothesis that the amino acid sequence of proteins uniquely determines their chain configuration and the spatial relationship of subunits. The mechanism of the process appears to involve, in both directions, an intermediate form. The available data are best explained by the assumption that this intermediate is a trimer of unfolded subunits.

The mechanism of the formation of globular proteins, which seemingly possess exceedingly complex and specific three-dimensional configurations, is a central problem in biochemistry. Much information has been gained concerning the way in which the amino acid sequence is dictated and formed, but much less attention has been paid to the equally important question of how the molecular configuration is prescribed. Especially interesting in this respect are the multichain enzymes, in which not only the folding of individual chains but the assembly of a definite number of these chains (in a specific spatial relationship) into the biologically active unit must be explained.

An important glycolytic enzyme, aldolase, has been shown to be of the multichain type. This has been demonstrated both by the detection of three carboxyl end-groups (Kowalsky and Boyer, 1959) and by dissociation of the enzyme by a detergent (Ramel *et al.*, 1961). Since the native protein has been reported (Taylor and Lowry, 1956) to have a molecular weight of 149,000, dissociation and reassociation experiments should provide a critical test of the hypothesis that in the biosynthesis of proteins the folding and association are dictated by the amino acid sequence. Also, the splitting of such a large unit into subunits offers considerable advantage in its further study.

Early observations by Gralen (1939) suggested that the dissociation of aldolase into its constituent peptide chains might be accomplished in acidic solutions. The use of such a mild and easily reversed environmental condition seemed a promising line of approach.

A preliminary report of our results has been given (Deal and Van Holde, 1962). Concurrently, similar studies have been carried out by Stellwagen and Schachman (1962a,b).

Experimental

Reagents and Buffer Solutions.—Fructose-1,6-diphosphate was obtained from K and K Laboratories, DPNH from Pabst Laboratories, and α-glycerophosphate dehydrogenase (containing triose phosphate isomerase) from C. F. Boehringer and Soehne. All other chemicals were of the best available commercial preparations.

All of the buffer solutions which were used were prepared from distilled deionized water through which nitrogen gas had been bubbled. Buffer solutions contained 0.05 M NaCl and 0.002 M EDTA, in addition to the appropriate amounts of sodium citrate and citric acid.

Aldolase.—Crystalline rabbit muscle aldolase was prepared by the method of Taylor (1955), with the modifications suggested by Kowalsky and Boyer (1959). Protein concentrations were determined spectrophotometrically with use of Taylor's (1955) value $E^{0.1\%}_{1\text{ cm}} = 0.91$ at 280 mμ. Assays of enzymatic activity were made by the hydrazine test (Jagannathan *et al.*, 1956; Richards and Rutter, 1961) and a coupled enzyme assay (Racker, 1947; Richards and Rutter, 1961). For immunologic tests, the Ouchterlony (Kabat and Mayer, 1961) technique of double diffusion on agar plates was used.

Sedimentation and Electrophoresis.—A Spinco Model E ultracentrifuge, equipped with a phase plate schlieren diaphragm and RTIC unit, was employed. All sedimentation experiments utilized Kel-F or filled-Epon centerpieces. Sedimentation velocity experiments were at rotor speed settings of 52,640 rpm, while sedimentation equilibrium experiments were generally carried out at 12,590 rpm (for the subunits) or 7,447 rpm (for the native aldolase). In the sedimentation equilibrium studies, short liquid columns (about 1.6 mm) were used

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in a double-sector filled-Epon cell with Kel-F oil below the solution. In most cases, about 24 hours was judged sufficient for the attainment of equilibrium. Sedimentation coefficients were determined in the usual manner and converted to standard conditions of water and 20°. All photographic plates were measured with a comparator.

Correction for the effect of temperature on the viscosity of water was calculated from data from the *Handbook of Chemistry and Physics* (1958 edition). The viscosities of the solvents relative to water were determined from flow times in a Cannon-Ubbelohde No. 50 viscometer; densities were measured with a hydrometer. Correction was also made for the temperature dependence of the partial specific volume of aldolase; for 3° a value of 0.724 ml/g was estimated from the data of Taylor and Lowry (1956) and for 20° their value of 0.742 ml/g was used. The value assumed at 18°, 0.740, was interpolated.

Electrophoresis experiments were conducted in a Spinco Model H electrophoresis instrument with use of a standard 11-ml cell.

Results

Preliminary experiments in acetate buffered solutions in the *p*H range 4.4–4.0 indicated that rapid and complete dissociation of aldolase at low temperature could be achieved only at low *p*H. These studies also showed that low protein concentration seemed to minimize the production of large aggregates and suggested that exclusion of oxygen from the solutions increased the stability. Furthermore, it was found that, under proper conditions, reassociation of the aldolase could be achieved.

Accordingly, a series of experiments was planned, with the objective of studying the nature and stability of the products formed from aldolase in the *p*H range below 4.0, and the dependence of the reassociation process upon *p*H.

A freshly prepared three-times-crystallized aldolase sample was used in these experiments, all of which were conducted in the cold unless otherwise noted. The crystalline aldolase suspension in ammonium sulfate solution was first centrifuged and the precipitate was dissolved in 0.01 M citrate (Na) buffer at *p*H 5.4. The enzyme solution was then dialyzed for 48 hours. The dialyzed aldolase solution was diluted with solvent from a concentration of 0.23% to 0.15% before acidification.

Four parallel experiments were set up for acidification. To nine volumes of each aldolase solution was added one volume of a freshly prepared acid buffer so as to yield 0.14% aldolase solutions with the following buffer compositions: (1) 0.1 M HCl, 0.009 M citrate (Na), *p*H 1.10, (2) 0.109 M citrate (Na), *p*H 2.44, (3) 0.059 M citrate (Na), *p*H 2.92, and (4) 0.059 M citrate (Na), *p*H 3.33. Seventy-seven hours after acidification, one 2-ml portion of each solution was taken to 18° and held at this temperature for 8–9 days as a stability test, and another 2-ml aliquot of each was dialyzed back to *p*H 5.4 over a 60-hour period at 3°. The remainder of each acidified solution was used for immediate characterization.

Characterization of Subunits.—Native aldolase has a sedimentation coefficient of about 7.7 *S* at *p*H 5.4, and the molecular weight has been reported by Taylor and Lowry (1956) to be 149,000. As is shown in Table I, the sedimentation coefficients of about 1.9 *S* are observed at *p*H values below 3. Because of the low concentrations employed, these values, as well as that at *p*H 5.4, should be close to infinite dilution values.

Table I

Sedimentation Data Showing Effect of *p*H on Dissociation, Stability, and Reassociation of Aldolase

The relative amount of 7.7 *S* material was determined by measuring the area under the sedimenting peak with the comparator, correcting for radial dilution, and comparing with the corrected area of the original protein control. The *p*H 5.4 sample is a control. Further details are given in the text.

	Acidified $s_{20,w}$		Reassociated	
*p*H	3°	18°, 8–9 days	$s_{20,w}$ 3°	% Recovery
5.4	7.73	—	7.73	Control
3.33	3.63	17.6 and 2.4	7.74	58
2.92	1.93	2.16	7.76	50
2.44	1.76	2.00	7.65	55
1.10	1.92	2.05	7.79	58

Similarly, charge effects should be small; this is supported by the fact that the values of *S* do not vary much with *p*H below *p*H 2.9.

Weight-average and *z*-average molecular weights of the material at *p*H 2.92 were calculated from a sedimentation equilibrium experiment (Fig. 1) by the methods described by Van Holde and Baldwin (1958); the values were M_w = 51,100, M_z = 52,000. The approach to sedimentation equilibrium has been used in the manner described by Sophianopoulos *et al.*[1] (1962) to calculate a value of 4.0×10^{-7} cm² sec.$^{-1}$ for the diffusion coefficient ($D_{20,w}$). This, together with the sedimentation coefficient of 1.93 *S*, yields $M_{s,D}$ = 46,000, in fair agreement with the sedimentation equilibrium results.

These data suggest that aldolase dissociates into three subunits of equal or nearly equal size and that dissociation is rapid and essentially complete below *p*H 3. Further support for the idea that the subunits are similar is provided by an electrophoresis experiment at *p*H 2.44 in 0.109 M citrate buffer. No resolution of components was seen under these conditions (Fig. 1). However, a small difference in net charge might be difficult to detect, since the molecules presumably have a large average net charge at this *p*H.

The values of the sedimentation and diffusion coefficients each correspond to a frictional ratio of about 2.3, assuming a molecular weight of 50,000. This result would be most simply interpreted as indicating the subunits to be highly unfolded, for an explanation based on the idea of "rod-like" subunits requires[2] an axial ratio for the subunits of about 28:1 if a prolate model is assumed.[3] Such subunits seem hard to reconcile with a "parent" molecule of axial ratio about 6.

Since the sedimentation coefficient is nearly the same at all *p*H values below 3.0, it would seem that the gross

[1] The equation for the calculation of *D* is misprinted in that paper. The correct equation is:

$$\left[\left(\frac{\partial c}{\partial r}\right)_{(eq)} - \left(\frac{\partial c}{\partial r}\right)_{(i)}\right] = \text{const} - \frac{\pi^2 D}{(r_b - r_a)^2}$$
$$\left[1 + \frac{(r_b - r_a)^2}{\pi^2}\left(\frac{\omega^2 M(1 - \bar{v}\rho)}{2RT}\right)\left(\frac{r_b + r_a}{2}\right)^2\right]$$
$$\left[1 + \frac{3}{\pi^2}\left(\frac{r_b - r_a}{r_b + r_a}\right)^2\right] \cdot t$$

[2] Neglecting water of hydration effects.

[3] An oblate model requires the completely unrealistic value of 45:1.

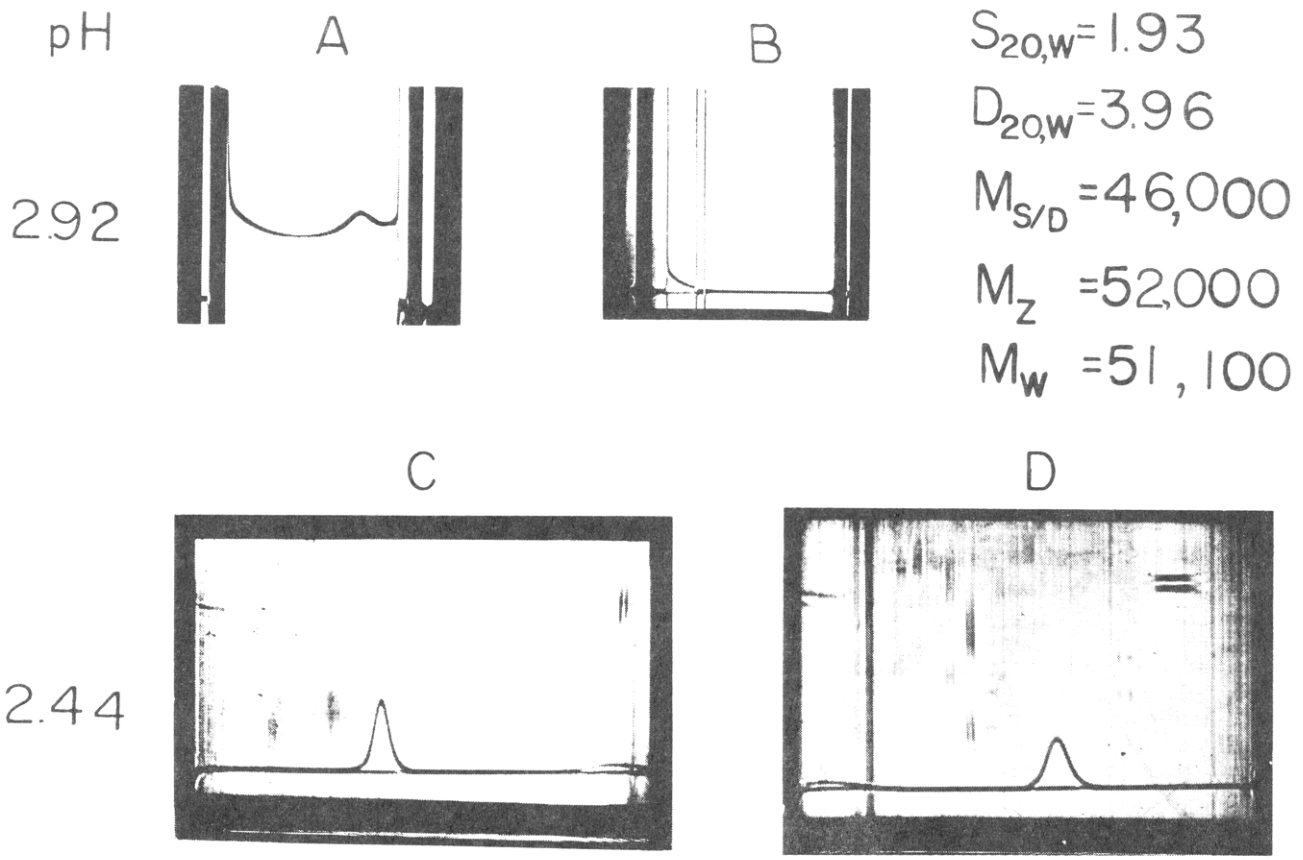

FIG. 1.—Characterization of aldolase subunits. (*A*) Sedimentation velocity at *p*H 2.92 (see text), 52,640 rpm, 50°, bar angle, 3°. (*B*) Sedimentation equilibrium at *p*H 2.92, 12,590 rpm, 70°, bar angle, 3°. (*C*), (*D*) Ascending and descending electrophoretic patterns, respectively, at *p*H 2.44. The experiment had proceeded for 8 hours.

configuration does not change further at low *p*H. Furthermore, no appreciable change was observed in the sedimentation coefficient under these conditions over a period of 4 days at 3°; even at 18° for 8–9 days only a slight increase is observed (Column 3, Table I). Thus, the subunits produced at low *p*H appear to be stable.

Behavior at pH 3.33 and Above.—The behavior at *p*H 3.33 is more complex. The product formed initially shows a single boundary sedimenting with a rate (3.63 *S*) intermediate to that of the native enzyme and the subunits. Sedimentation equilibrium experiments showed this material to have a weight-average molecular weight of 138,000. The substance was somewhat heterogeneous; the graph of $(1/r)(dn_c/dr)$ *vs.* n_c (see Van Holde and Baldwin, 1958) consisted of two straight line portions; one of these (the major component) corresponded to a value of M = 153,000, the other to a value of M = 108,000.

This product also differs in stability from the materials obtained below *p*H 3. After 9 days at 18°, two components were observed; a slowly sedimenting material, possibly subunits, of $s_{20,w}$ = 2.4 *S* and a considerable amount of aggregated material ($s_{20,w}$ = 17.6 *S*).

In order to test further the effect of *p*H and time of standing upon the amount of 3.7 *S* species produced and its stability, experiments were performed at *p*H 3.40, 3.46, and 3.77. At *p*H 3.40 almost all the protein is in the 3.7 *S* peak, whereas at *p*H 3.77 most of the protein is found in the 7.7 *S* peak. Furthermore, there is little change in the relative amounts of 3.7 *S* and 7.7 *S* material with time at 3°.

An experimental series also was conducted at *p*H 3.40 with a much higher protein concentration (7.2 mg/ml) in an effort to test further whether the step from 7.7 *S* to 3.7 *S* represents an unfolding without significant dissociation or whether it corresponds to dissociation into folded subunits. If this were dissociation, the equilibrium should shift toward the 7.7 *S* species at high concentrations of protein, whereas an unfolding step should show no such change. In fact, this shift did not occur, suggesting that this is a process of unfolding without dissociation.

Reassociation.—As shown in Figure 2 and Table I, dialysis of the acidified aldolase solution back to *p*H 5.4 produced in every case a substance showing a single sedimenting boundary, with sedimentation coefficient identical to that of the native enzyme. The boundary spreading was slight, suggesting homogeneity; however, the corrected area under the schlieren curve corresponded in each case to only about 55% of the original protein. Spectrophotometric measurements indicated that the total protein concentration was unchanged, and a sedimentation equilibrium experiment showed the presence of some very high molecular weight material. Thus, the conclusion is drawn that the remaining 45% of the aldolase is in the form of large aggregates, apparently with a wide distribution of sizes.

While the above results indicate that at least the gross molecular configuration was regained, it seemed desirable to employ more sensitive tests of configurational specificity. Accordingly, the catalytic and immunologic activities of the neutralized samples were studied. The recovery of catalytic activity was approximately 55% in each case. This compares well with the per cent recovery (Table I) of protein as 7.7 *S* material and suggests that the specific activity of reassociated aldolase is comparable to that of the native

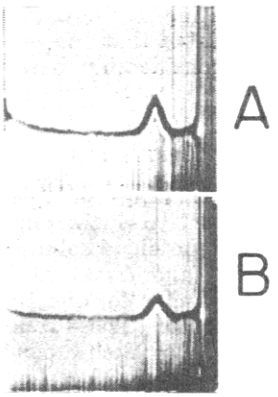

FIG. 2.—Typical sedimentation velocity diagrams for (*A*) native aldolase and (*B*) aldolase which had been dissociated (in this case, at *p*H 1.1) and reassociated by dialysis to *p*H 5.4 (see text). In both cases the *p*H was 5.4, the rotor speed 52,640 rpm, the temperature 3°, and the bar angle 50°. The pictures were taken 43 minutes after speed was attained.

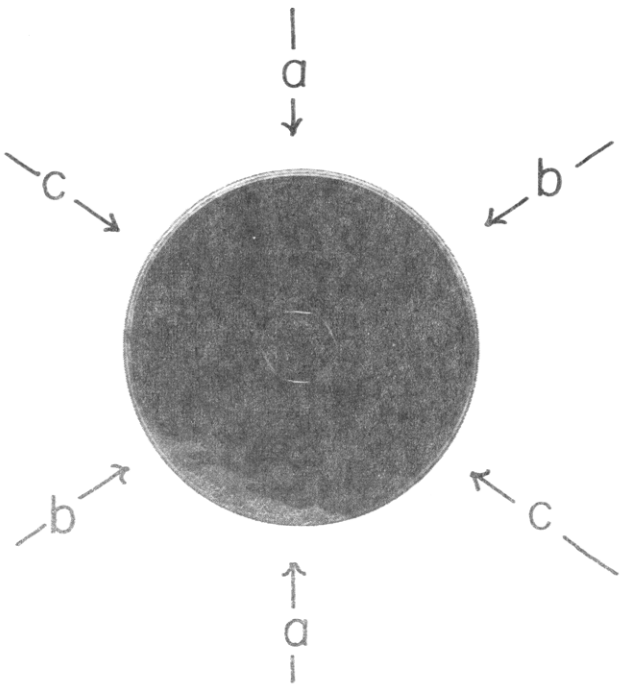

FIG. 3.—Photograph of agar plate from a double diffusion experiment showing immunologic identity of reassociated and native aldolase. Antialdolase serum was placed in the center well, and native aldolase solution at *p*H 7.5 in the wells labeled (*a*) and (*c*). The concentration of aldolase in (*c*) was half that in (*a*). In well (*b*) was placed aldolase solution, concentration nominally the same as (*a*), which had been acid-dissociated and reassociated by neutralization.

enzyme. Furthermore, reassociated and native aldolase react identically with antialdolase serum in double diffusion experiments (Fig. 3).

Characterization of the Reassociation Process.—When dissociated aldolase in acidic solutions was added directly to an assay medium at neutral *p*H, at 25°, the initial activity was almost negligible and increased rapidly with time (Fig. 4). This phenomenon appeared in both the hydrazine and the coupled enzymatic assay. In contrast, assays with native enzyme were characteristically linear from zero time (Fig. 4). The recovery of activity, as measured by the time dependence of the slope of graphs such as Figure 4, was found to fit a first-order rate law, with a half-life of 2 minutes

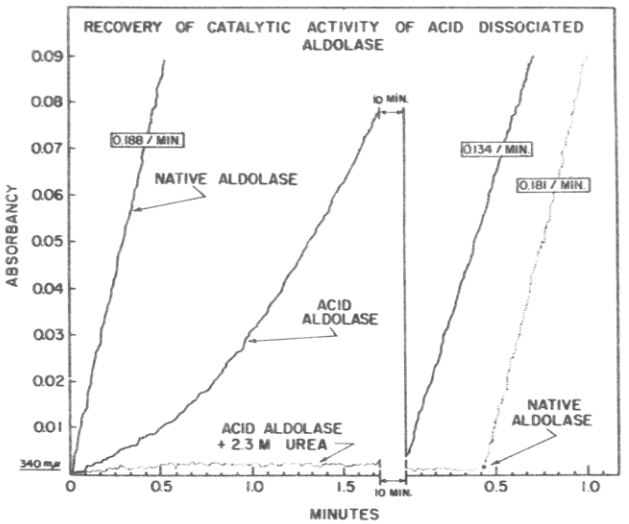

FIG. 4.—Time-dependent recovery of catalytic activity upon neutralization of aldolase subunits and the effect of 2.3 M urea on this recovery. Data obtained with a Cary spectrophotometer, using the α-glycerophosphate dehydrogenase assay. Three experiments are shown. The line on the left is an assay of native aldolase. The next two curves show the regain of catalytic activity when subunits at low *p*H are diluted into an assay medium at *p*H 7.5; the dotted horizontal line is an identical assay except for the presence of 2.3 M urea, and the line on the right was obtained when native aldolase was then added to this mixture.

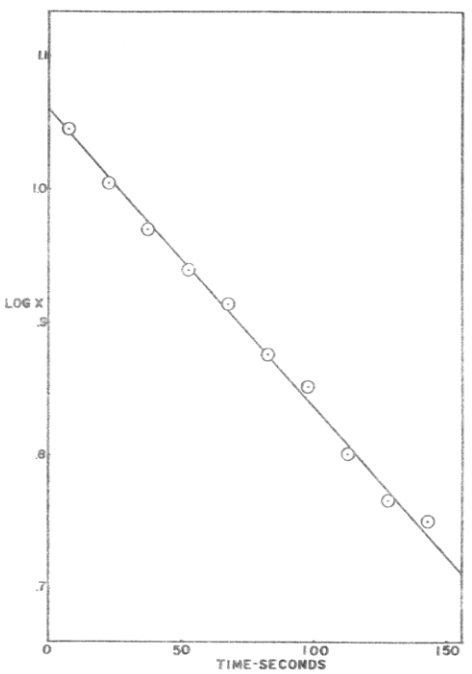

FIG. 5.—A graph showing that recovery of enzymatic activity follows first-order kinetics. The quantity x is the difference between the final activity and the activity at time t and is presumed to be proportional to the fractional recovery at a given time. Activity is measured as the slope of the OD *vs.* time curve (as in Fig. 4), expressed in arbitrary units. The data were taken from a coupled enzyme assay in which 5 μl of 0.14% aldolase (*p*H 2.92) were diluted into 3 ml of assay medium at *p*H 7.5, containing 0.1 M glycylglycine.

(Fig. 5). This indicates that a unimolecular process may be the rate-limiting step in the over-all recovery of the native state at these concentrations of enzyme.

If an acid solution of aldolase subunits is diluted into assay medium containing 2.3 M urea, no activity is

regained, as is shown by the horizontal line in Figure 4. Preliminary sedimentation experiments provided further evidence that the presence of 2.3 M urea prevents complete resumption of the native structure. However, this urea concentration is not sufficient to destroy the catalytic activity of native aldolase, as can be seen from the immediate appearance of activity when native enzyme is added to the above solution.

Since a solution containing 2.3 M urea does not destroy the structure of the native enzyme but does prevent the re-formation of the native structure by the subunits, an assay in 2.3 M urea provides a test for the presence of residual native enzyme in a dissociation solution. The absence of such activity in the solution tested (Fig. 4) indicates that there is essentially no residual native enzyme in the solution.

The recovery of enzyme activity on neutralization may be as high as 75% under favorable conditions. Optimal total recoveries were obtained at *p*H values from 6.0 to 7.5 and were independent of the concentration from 4 to 100 μg enzyme per ml. The presence of the substrate FDP did not alter the final recovery and in higher concentrations decreased the rate of recovery. The total recovery appeared to be dependent upon the quality of the aldolase preparation. With identical protocols being used, recoveries of 35–75% were obtained with different enzyme preparations. Addition of EDTA to the reaction mixture frequently slightly enhanced the recovery. Inclusion of mercaptoethanol or rigorous exclusion of oxygen were not markedly beneficial. Many of the critical factors determining the relative proportion of side-reactions leading to inactive molecules remain to be defined. The specificity of these side-reactions is indicated by the fact that addition of a thousand-fold excess of another protein (bovine serum albumin) had no effect on activity recovery.

Behavior of Carboxypeptidase-Digested Aldolase.—In view of the loss of catalytic activity which parallels the loss of three terminal tyrosine residues upon treatment of aldolase with carboxypeptidase (Drechsler *et al.*, 1959), it seemed possible that such modification would alter the process of dissociation and/or reassociation. Preliminary ultracentrifugal experiments show that carboxypeptidase-treated aldolase can be dissociated and reassociated in the same way as native enzyme. The *C*-terminal tyrosines do not seem to play a major role in this process.

An Intermediate Stage in the Reassociation Process.—A study at *p*H 4.6 yielded information about the rate and mechanism of reassociation. An acidified aldolase sample (0.06 M citrate, *p*H 2.92) was neutralized to *p*H 4.6 by dialysis against 0.01 M citrate at *p*H 5.4 over a period of 5 hours. Two sedimentation velocity experiments were then carried out at 3° on aliquots of this solution. Immediately after dialysis, a single peak with $s_{20,w}$ of 3.7 *S* was observed. Thirty hours later two peaks with sedimentation coefficients of 3.8 *S* and 8.0 *S* were found.

These experiments suggest that the reassociation process occurs in two steps: first, the relatively fast formation of 3.7 *S* material, and, second, a rate-limiting conversion of this material to the native enzyme. A substance of 3.6 *S* is also found on acidification of native aldolase to *p*H 3.33. Thus, it appears that this intermediate is an obligatory stage in both the dissociation and reassociation processes.

In order to test the effect of a higher *p*H on the rate of reassociation, acidified aldolase at a concentration of 1.4 mg/ml was neutralized to *p*H 6.5 by direct addition of base. Immediate ultracentrifugal analysis indicated the presence of material with sedimentation coefficient approximating that of native enzyme. This experiment demonstrated that the reaction to produce native enzyme was considerably faster at *p*H 6.5 than at *p*H 4.6.

Discussion

The experimental findings of this study indicate that in acid solution native aldolase undergoes dissociation into three highly unfolded subunits. On neutralization, these reunite and refold to form a molecule which is extremely similar to, and probably identical with, the native enzyme. These results are in agreement with those given by Stellwagen and Schachman (1962a,b). Our conclusions are supported by the following data: First, at low *p*H there is extensive loss of the specific and compact structure of native aldolase, as indicated by (*a*) a decrease in the molecular weight from 150,000 to 50,000 accompanied by change in the frictional ratio from 1.3 to 2.3, (*b*) a decrease in the value of the optical rotatory dispersion constant from 278 mμ to 220 mμ (Jirgensons, 1961), (*c*) a pronounced increase in the intrinsic viscosity from 0.057 dl/g to 0.227 dl/g (Jirgensons, 1961). Second, on neutralization of acidified aldolase the molecule assumes the original gross structure, as judged by sedimentation data. Moreover, since the reactivity of reassociated aldolase with aldolase antibody is identical with that of the original enzyme, and since the catalytic activity is recovered (in a time-dependent fashion), it seems likely that the detailed configuration of the native enzyme is restored. Since this recovery of activity can be prevented, even under assay conditions, by urea in concentrations which do not inhibit native aldolase, this catalytic activity cannot be due to a residual amount of native enzyme which was unchanged by the dissociating conditions.

In no case has complete recovery of native protein been achieved; nonetheless, the fact that a large fraction of the molecules can return to a highly specific configuration must be explained. The enormous number of forms and configurations potentially available to such a large and complex molecule argues that highly specific interactions between side-chains must be invoked to direct the formation of the specific configuration.

In particular, these results show that there is no need to postulate a "folding template" in the biosynthesis of aldolase. Specification of the amino acid sequence, together with the provision of appropriate *p*H and salt concentration, seem to be all that is required for both the association and folding processes. Furthermore, re-formation of enzymatically active protein has been observed to occur rapidly, as would be required of a physiologically significant process.

These results can be considered to extend to a higher level of molecular weight and complexity the observations which have been made by White (1961) concerning the spontaneous refolding of ribonuclease and by Levinthal *et al.* (1962) concerning the refolding and reassociation of alkaline phosphatase. A principal difference is the much more rapid formation of native aldolase as compared to ribonuclease and alkaline phosphatase, despite the greater complexity of the aldolase molecule. The difference might be explained by the requirement of a specific enzyme for the *rapid* formation of disulfide bonds, which are present in both ribonuclease and alkaline phosphatase but not in aldolase.

Mechanism.—While many models might be constructed to describe the sequence of steps in these processes, two obvious possibilities will be discussed.

These may be represented as shown in (*A*) and (*B*),

$$(A)\ N \rightleftharpoons N^* \rightleftharpoons 3S^*$$

$$(B)\ N \rightleftharpoons 3S \rightleftharpoons 3S^*$$

where the asterisk indicates unfolded species, and N and S refer to a trimer and monomer subunit respectively. To be acceptable, a mechanism must be consistent with the following observations:

1. A material with sedimentation coefficient about 3.8 S is observed either upon acidification to *p*H 3.3 or upon raising of the *p*H from 2.9 to 4.6. In the latter case this substance was formed immediately; 30 hours later both 3.8 S and 7.7 S material were found.
2. The regeneration of enzymatic activity upon dilution of acid aldolase (*p*H 2.9) into neutral (*p*H 7.5) assay medium follows first-order kinetics.
3. The material at *p*H 3.3 has been shown by sedimentation equilibrium to consist mostly of particles of molecular weight about 150,000, although a small amount of lower molecular weight material was present.

Model *A*, which postulates an unfolded trimer, N^*, as the intermediate form, is consistent with all observations. Model *B* is hard to reconcile with either the molecular weight data or the kinetic data. The possible argument that the 3.8 S boundary corresponds to an equilibrium mixture of 1.9 S and 7.7 S material is inconsistent with the subsequent observation of both 3.8 S and 7.7 S boundaries in the same experiment.

Mechanism *B* requires that the 3.8 S material be a folded monomer. If this were the case, the slow conversion of 3.8 S material to 7.7 S material at *p*H 4.6 would indicate the recombination step to be rate limiting, at least at the concentration of 1.4 mg ml. However, the kinetic data at assay concentrations show the rate-limiting step to be first order. Therefore, to fit Model *B* to the data would require the assumption that the rate-limiting step at high concentration (at *p*H 4.6) is a multimolecular process, while that at low concentration (at *p*H 7.5) is unimolecular. While not impossible, because of the difference in *p*H, such a change in kinetics seems most unlikely.

In conclusion, while the data do not rule out other possible mechanisms, Model *A* is strongly favored over Model *B*. In addition, it appears that any mechanism proposed must account for a species having a sedimentation coefficient of 3.8 S and a molecular weight close to that of the native protein.

Acknowledgments

Appreciation is expressed to Dr. Rhoda Blostein, whose assistance made possible the immunologic experiment, and to Mrs. C. K. Rhodes, who assisted in preliminary ultracentrifugal studies.

Added in Proof

We have recently had the privilege of examining a manuscript by E. W. Westhead, L. Butler, and P. D. Boyer, entitled "Binding Sites, Reactivation, and Possible —S—S— Groups of Rabbit Muscle Aldolase." These authors have also observed inactivation of aldolase at low *p*H, and reactivation upon neutralization. Under optimum conditions they have been able to obtain as high as 90% recovery of activity.

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