pentane analog, the cyclohexane analog, and *threo*-γ-methyl-L-glutamate are, respectively, 100, 43, 29, and 27. It may also be relevant that the K_m value for ammonia with the cyclopentane analog is about 10% of that found with the cyclohexane analog. These calculations and considerations seem to provide an explanation for the more effective utilization of ammonia with the cyclopentane analog; however, it is evident that additional investigations are required for full understanding of the significance of the observed kinetic parameters. The calculations show that the more puckered the ring, the more nearly the cyclopentane analog approaches the extended conformation of L-glutamate and therefore the better it fits to the previously calculated enzyme binding sites. While it is clearly of importance that the conformation of *cis*-1-amino-1,3-dicarboxycyclopentane ultimately be determined by independent methods, the present studies suggest the possibility that enzymatic approaches may be of value in conformational analysis.

References

Allinger, N. L., Hirsh, J. A., Miller, M. A., Tyminski, I. J., and Van-Catledge, F. A. (1968), *J. Amer. Chem. Soc. 90*,1199.

Conly, J. C. (1953), *J. Amer. Chem. Soc. 75*, 1148.

Connors, T. A., and Ross, W. C. J. (1960), *J. Chem. Soc.*, 2119.

Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.

Gass, J. D. (1970), Doctoral Dissertation, Cornell University.

Gass, J. D., and Meister, A. (1970a), *Biochemistry 9*, 842.

Gass, J. D., and Meister, A. (1970b), *Biochemistry 9*, 1380.

Haresnape, J. N. (1953), *Chem. Ind. (London)*, 1091.

Kagan, H. M., Manning, L. R., and Meister, A. (1965), *Biochemistry 4*, 1063.

Kagan, H. M., and Meister, A. (1966a), *Biochemistry 5*, 725.

Kagan, H. M., and Meister, A. (1966b), *Biochemistry 5*, 2423.

Kay, F. W., and Perkins, W. H. (1906), *J. Chem. Soc. 89*, 1640.

Meister, A. (1968), *Advan. Enzymol. 31*, 183.

Nakanishi, K. (1962), Infrared Absorption Spectroscopy-Practical, San Francisco, Calif., Holden-Day, p 45.

Rowe, W. B., Ronzio, R. A., Wellner, V. P., and Meister, A. (1970), *Methods Enzymol. 17A*, 900.

Shemyakin, M. M., Shchukina, L. A., Vinogradova, E. I., Kolosov, M. N., Vdovina, R. G., Karapetyan, M. G., Rodionov, V. Ya., Ravdel, G. A., Shvetsov, Yu. B., Bamdas, E. M., Chaman, E. S., Ermolaev, K. M., and Semkin, E. P. (1957), *J. Gen. Chem. USSR 27*, 817.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Tiwari, R. D., and Sharma, J. P. (1970), The Determination of Carboxylic Functional Groups, New York, N. Y., Pergamon Press, p 77.

Wilt, J. W., Parsons, C. T., Schneider, C. A., Schultenover, D. G., and Wagner, W. J. (1968), *J. Org. Chem. 33*, 695.

In Vitro Assembly of Aldolase. Kinetics of Refolding, Subunit Reassociation, and Reactivation†

John W. Teipel‡

ABSTRACT: The kinetics of renaturation of aldolase, denatured in 6 M guanidine hydrochloride, were measured as a function of temperature, substrate (fructose 1,6-diphosphate), Gdn·HCl, and enzyme concentration. A comparative study was made of the *in vitro* rates of refolding, subunit reassociation, and reactivation. The major regain of secondary and tertiary structure, as determined by optical rotation, was complete within 30 sec of renaturation for temperatures between 0 and 25° and Gdn·HCl concentrations between 0 and 2 M. Dispersion curves for the renatured enzyme were nearly identical to that for native aldolase. The kinetics of subunit reassociation, as determined by Rayleigh light scattering, were biphasic—phase I an initial rapid association producing a mixture of monomers and dimers and phase II a further slow association leading to the formation of primarily tetramer. The rate of phase II reassociation increased with temperature and substrate concentration, decreased with Gdn·HCl concentration and was independent of initial enzyme concentration. Rates of regain of enzymatic activity closely paralleled phase II rates of reassociation under all renaturing conditions. Reactivation and reassociation kinetics were consistent with two or more rate-limiting sequential or parallel first-order reactions. Renaturation results are discussed in terms of an assembly mechanism for aldolase in which (1) dissociated and unfolded polypeptide chains both rapidly refold and partially reassociate to yield a mixture of structured, but inactive, monomers and dimers, (2) either or both of these species subsequently undergo a slow, first-order, minor conformational change, followed by (3) rapid association to tetramers with simultaneous regain of enzymatic activity.

Numerous studies have now indicated that many subunit proteins after complete disruption of their secondary, tertiary, and quaternary structure will spontaneously reassume a native or near native conformation simply upon removal of the denaturing conditions. In spite of these studies, however, still little is known about the mechanism, or sequence of events, leading from the dissociated and unordered polypeptide chain to the associated and uniquely structured subunit protein. A kinetic investigation of the *in vitro* renaturation of the subunit enzyme aldolase (fructose 1,6-di-

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phosphate:D-glyceraldehyde 3-phosphate lyase, EC 4.1.2.) from 6 M Gdn·HCl[1] was therefore undertaken in an effort to gain further insight into this assembly process.

The renaturation of a subunit protein, denatured in 6 M Gdn·HCl, may be conceptually divided into two processes: (1) refolding of the polypeptide chains, *i.e.*, regain of secondary and tertiary structure, and (2) reassociation of the polypeptide chains, *i.e.*, regain of quaternary structure. An earlier kinetic investigation of several subunit enzymes (Teipel and Koshland, 1971a,b) focused primarily on structural changes involved in the refolding process. This previous study indicated that the major regain of secondary and tertiary structure occurred rapidly (within 1 min) and that further minor conformational changes, leading to the return of enzymatic activity, occurred much more slowly (over several hours). The experiments reported here focus primarily upon the reassociation process, with the objective of determining the rate of regain of quaternary structure relative to the rates of regain of secondary and tertiary structure (fast and slow) studied earlier. This inquiry may thus be posed in the form of two questions. (1) Does major refolding of the polypeptide chains precede, accompany, or follow reassociation? (2) Does regain of enzymatic activity precede, accompany or follow reassociation? To answer these questions a comparative study was made of the kinetics of refolding, as determined by optical rotation, the kinetics of reassociation, as determined by Rayleigh light scattering, and the kinetics of reactivation, as determined by enzyme assay. From these investigations a mechanism for the renaturation of aldolase, based on kinetic observations, was formulated.

Experimental Procedure

Reagents. Rabbit muscle aldolase was purchased from Boehringer Mannheim as a crystalline suspension in 2 M ammonium sulfate. The specific activity of the enzyme was approximately 10 units/mg when assayed according to the procedure of Penhoet *et al.* (1969).

Fructose 1,6-diphosphate and a mixture of glycerol-1-phosphate dehydrogenase:triosephosphate isomerase was purchased from Boehringer Mannheim. DPNH was obtained from Sigma and Gdn·HCl (Ultra Pure) from Mann Research Laboratories.

Denaturation. Aldolase was denatured by incubation of the enzyme in a solution of 6 M Gdn·HCl–0.05 M Tris-Cl–0.1 M 2-mercaptoethanol–1 mM EDTA (pH 7.5) for 20–24 hr at 0°.

Renaturation. Aldolase was renatured by a rapid 100:1 dilution of the above enzyme–Gdn·HCl solution into a physiological buffer. Except where noted elsewhere, this buffer was composed of 0.1 M KCl, 0.05 M Tris-Cl, 0.01 M 2-mercaptoethanol, and 1 mM EDTA at pH 7.5 and will be defined as the standard renaturation buffer or SRB. The temperature of renaturation varied between 0 and 25° (see Results for details).

Enzyme Assay. The activity of native and renatured aldolase was measured spectrophotometrically on a Beckman ACTA III recording spectrophotometer equipped with a Lauda constant-temperature circulating bath. Aldolase activity was measured by a linked enzyme assay originally described by Racker (1947). A small aliquot of enzyme was added to a 1-ml solution of 0.05 M Tris-Cl–2 mM fructose 1,6-diphosphate–0.25 mM DPNH–0.1 mM EDTA (pH 7.5), containing approximately 25 μg of a mixture of glycerol-1-phosphate dehydrogenase:triosephosphate isomerase. The oxidation of DPNH was monitored at 340 mμ. All aldolase concentrations were determined spectrophotometrically using $E^{1\%}_{280\,m\mu} = 9.1$ (Kabashi *et al.*, 1966).

Light-scattering measurements were conducted with unpolarized light at 436 mμ using a Brice-Phoenix Model 2000 light-scattering photometer equipped with a Varian Model G-4000 dual-channel recorder. The instrument was absolutely calibrated with an opal glass reference standard (Brice *et al.*, 1950). In addition, measurement of Rayleigh's ratio for benzene, a widely used calibration standard, yielded $R_{90} = 47.9 \times 10^{-6}$ cm^{-1} in close agreement with accepted values for this parameter (Kratohvil *et al.*, 1962). All scattering measurements were made at an angle of 90° to the incident beam, since the dimensions of the macromolecular solutes were small relative to the wavelength of light. Molecular weights were calculated from scattering intensities as initially described by Brice *et al.* (1950) and later slightly modified by Tomimatsu and Palmer (1959, 1963).

The partial specific refractive indices of aldolase at constant chemical potential of the diffusible components $(dn/dc)_\mu$ (Casassa and Eisenberg, 1964) were employed for all molecular weight determinations. Values of $(dn/dc)_\mu$ were measured with a Brice-Phoenix differential refractometer as described by Brice and Halwer (1951).

Scattering measurements were made with a cylindrical cell (75 × 26 mm i.d., purchased from Phoenix Precision Instruments) possesing flat entrance and exit faces. The temperature of the cell was regulated by means of a triple-walled brass cylindrical jacket, a modified version of an earlier jacket described by Trementozzi (1957). Water of the desired temperature was circulated through the inner shell, and, for temperatures less than 10°, dry nitrogen was flushed through the outer shell to prevent condensation. A small magnetic stirrer was built into the base plate directly beneath the scattering cell and controlled by a rheostat outside the scattering compartment.

Rates of reassociation, as measured by light scattering, were routinely determined by the following procedure. Approximately 0.15 ml of denatured aldolase was added to 15 ml of stirring renaturation buffer in the scattering cell *via* a length of polyethylene tubing leading from outside the scattering compartment. Both enzyme and buffer were previously filtered through a 0.22 μ Millipore type HA filter using only plastic fittings. Initial turbidity measurements were made within 30 sec of dilution and subsequently monitored as a function of time.

Sedimentation velocity measurements were made with a Spinco Model E analytical ultracentrifuge equipped with a high-intensity ultraviolet light source, monochromator, photoelectric scanning system, and temperature control unit. Velocities were measured at 280 mμ using the scanner optics and at a speed of 60,000 rpm. Sedimentation velocity values were corrected to water at 20° by the usual procedure.

Optical rotation measurements were conducted on a Durrum Jasco Model ORD/UV-5 recording spectropolarimeter. All measurements were made at 0° with a 1-cm path-length, water-jacketed cell.

Results

Light Scattering. Aldolase, a tetrameric enzyme, is dissociated by 6 M Gdn·HCl into its individual polypeptide chains (Kawahara and Tanford, 1966; Castellino and Barker, 1968). Renaturation from 6 M Gdn·HCl will therefore

[1] Abbreviations used are: Gdn·HCl, guanidine hydrochloride; SRB, standard renaturation buffer (see Experimental Section for details).

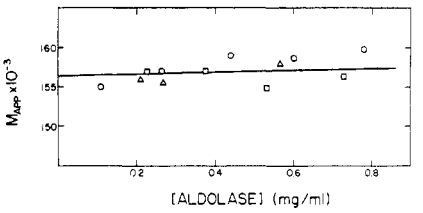

FIGURE 1: Apparent weight-average molecular weight (M_{app}) of aldolase as a function of enzyme concentration. Values of M_{app} for native aldolase were determined by light scattering in the following buffers: 0.1 M potassium phosphate, pH 7.0, 25° (○); 0.05 M Tris-Cl, 0.1 M KCl, 0.01 M 2-mercaptoethanol, and 1 mM EDTA, pH 7.5, 25° (□); same as (□) except at 0° (△).

be accompanied by a fourfold increase in molecular weight, assuming that the renatured species is also a tetramer. Light scattering has been employed to measure the rate of this increase in molecular weight, and hence the rate of reassociation, upon renaturation.

In Figure 1 is shown the apparent molecular weight, M_{app}, of native aldolase in several different buffers at 25 and 0° measured as a function of enzyme concentration. The weight-average molecular weight, $\bar{M}_w$, obtained by extrapolation to zero enzyme concentration was 156,300 ± 1000, a value in good agreement with other experimental determinations for native aldolase (Kawahara and Tanford, 1966; Castellino and Barker, 1968; Sine and Hass, 1969). It may be observed that for the enzyme concentration range examined (*i.e.*, concentrations <0.8 mg/ml) the change in apparent molecular weight with concentration lies within experimental error of $\bar{M}_w$. Thus for the renaturation experiments described below, which were conducted at enzyme concentrations, buffer concentrations, pH and temperatures similar to those described in Figure 1, only apparent molecular weights were measured and these values assumed equal to $\bar{M}_w$.

In Figure 2 is shown the increase in molecular weight with time for the renaturation of aldolase at three different concentrations of enzyme. In each case the rate of increase in molecular weight was followed for approximately 2 hr at 0°, after which the temperature of the renaturation solution was quickly raised to 25° and further changes in molecular weight monitored at this temperature. For the lowest concentration of aldolase (0.07 mg/ml) the following kinetic profile is observed. There is a rapid reassociation (within 30 sec) at 0° of the monomeric enzyme ($M \simeq 39{,}000$) to a multisubunit specie(s) ($\bar{M}_w \simeq 60{,}000$). This rapid phase of reassociation is followed by a much slower increase in molecular weight,

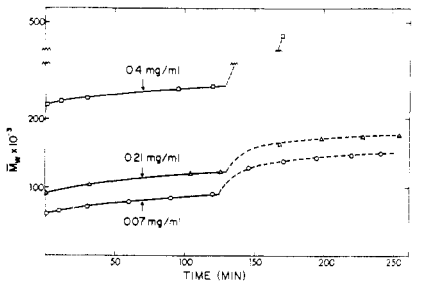

FIGURE 2: Rate of reassociation of aldolase as a function of enzyme concentration. Aldolase was renatured by dilution into SRB to the final concentrations indicated. Weight-average molecular weight ($\bar{M}_w$) was monitored as a function of time at 0° (solid curves) and 25° (dashed curves).

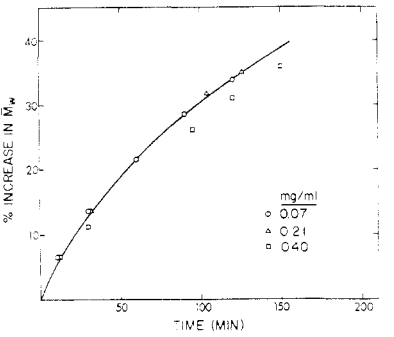

FIGURE 3: Rate of reassociation of aldolase as a function of enzyme concentration. Data taken from Figure 2 at 0° are plotted as a per cent increase in weight-average molecular weight with time. Rates of reassociation were measured at the enzyme concentrations indicated.

reaching a value of approximately 90,000 after 2 hr at 0°. An increase in the temperature of the renaturation medium to 25° raises this slow rate of reassociation severalfold, and the enzyme, after 2 additional hr, approaches a final molecular weight of 156,000 consistent with that of native tetrameric aldolase. This renaturation profile for aldolase at 0.07 mg/ml was reproducible over five separate experiments and at enzyme concentrations as low as 0.04 mg/ml (limit of light-scattering precision).

It may be observed from Figure 2 that renaturation at higher concentrations of enzyme (0.21 and 0.40 mg per ml, respectively) had two effects on reassociation. First, a substantial increase in the value of $\bar{M}_w$ measured at 30 sec was seen with increasing enzyme concentration. Secondly, at the highest aldolase concentration tested (0.40 mg/ml), an increase in the temperature of renaturation to 25° precipitated a further large increase in $\bar{M}_w$ not seen for the lower enzyme concentrations at this temperature. Both increases were attributed to nonspecific aggregation of the enzyme. Despite these general effects of enzyme concentration on renaturation, however, the rate of increase in $\bar{M}_w$ with time at 0° *after the first 30 sec* was the same for all three concentrations of enzyme. This result may be seen more clearly in Figure 3 where the per cent increase in $\bar{M}_w$ after 30 sec is plotted as a function of time for the three concentrations of aldolase. It may be observed that the rate of increase in $\bar{M}_w$ at 0° is the same over a more than fivefold range of initial enzyme concentration.

A study of the effect of the *initial* temperature of the renaturation medium on reassociation led to the following observations. First, there was a substantial increase in molecular weight values measured at 30 sec for temperatures greater than 0°. Initial values of $\bar{M}_w$ for aldolase diluted in SRB at 10° (0.2 mg/ml) were, for example, more than twice those determined for renaturation in SRB at 0°. Renaturation of the enzyme in SRB at 20° (0.2 mg/ml) resulted in an immediate and pronounced visible turbidity. Secondly, rates of the slow phase of reassociation increased appreciably with increasing temperature.

The effect of substrate on the kinetics of aldolase reassociation was minimal. The presence of 1 mM fructose 1,6-diphosphate in the renaturation medium increased only slightly the rate of reassociation of aldolase (0.08 mg/ml) at either 0 or 10°. No effect of substrate on reassociation was observed at higher enzyme concentrations.

Reactivation. In Figure 4, the per cent reactivation of aldolase with time is compared to the per cent increase in

$\bar{M}_w$ with time under three different renaturing conditions. These conditions were, respectively, renaturation in SRB at 0°, renaturation in SRB at 10° and renaturation in SRB plus 0.25 M Gdn·HCl at 0°. In each case the solutions were warmed to 25° after approximately 2 hr. In spite of the appreciably different kinetic profiles it may be observed that the kinetics of reactivation closely parallel those of reassociation. This close correspondence suggests that (1) the slow changes in $\bar{M}_w$ with time as measured by light scattering are not a result of nonspecific aggregation, but reflect an association process directly related to the assembly of native enzyme, and (2) reassociation and regain of biological activity are closely linked kinetic processes. In all three experiments the specific activity of the renatured enzyme after four hours was equal to or greater than that of the native enzyme.

Reactivation in the presence of 1 mM fructose 1,6-diphosphate also closely paralleled reassociation behavior in the presence of substrate, *i.e.*, substrate slightly increased rates of reactivation at low concentrations of enzyme, but had no effect at higher enzyme levels. No regain of activity was observed for renaturation in the presence of Gdn·HCl $\geq$1.0 M.

Reactivation of aldolase as a function of initial enzyme concentration is shown in Figure 5. Denatured aldolase was diluted into SRB at 0° to enzyme concentrations of 0.07, 0.17, and 0.30 mg per ml, respectively. It may be observed that the rates of reactivation are the same over the fourfold range of enzyme concentration tested. The solid curve passing through the experimental points was generated from the integrated rate equation derived for either (1) two sequential first-order reactions, *i.e.*

$$A \xrightarrow{k_1} B \xrightarrow{k_1'} C$$

or (2) two parallel first-order reactions, *i.e.*

$$A \xrightarrow{k_1} C$$

$$B \xrightarrow{k_1'} C$$

Both mechanisms yield the solid curve in Figure 5 given the proper choice of the magnitude of the first-order rate constants k_1 and k_1' and approximately equal values of A and B. The lower dashed curve shows the rate of reactivation predicted by second-order kinetics for an initial concentration fourfold less than that employed to generate the upper solid theoretical curve. Clearly the difference in reactivation rates predicted by second-order kinetics is outside the range of experimental error for the activities actually measured. The results of Figure 5 are thus indicative of a first-order process, since rates of reactivation were independent of concentration and are at least consistent with the sequential or parallel first-order mechanisms described above. Rates of reactivation conformed to the values predicted by the integrated rate equations up to 85% reactivation, reached after 20 hr at 0°.

Comparison of the rates of reactivation as a function of initial enzyme concentration in Figure 5 with rates of increase in $\bar{M}_w$ as a function of enzyme concentration previously described in Figure 3 shows that the two sets of data are virtually superimposable. The solid curve in Figure 3 was in fact generated by the same rate equation used to fit the reactivation data of Figure 5. This correspondence reaffirms the earlier conclusion that reactivation and reassociation are kinetically closely linked.

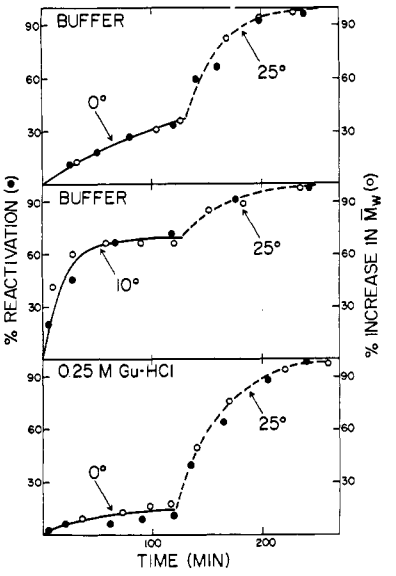

FIGURE 4: Comparison of rates of reactivation with rates of reassociation for aldolase. Per cent reactivation (●) and per cent increase in $\bar{M}_w$ (○) were measured as a function of time in three different renaturation mediums: SRB initially at 0° (top figure), SRB initially at 10° (middle figure), and SRB containing 0.25 M Gdn·HCl initially at 0° (bottom figure). Rates of reactivation and reassociation were measured at the initial temperature of renaturation (solid curves) and at 25° (dashed curves).

Sedimentation Velocity. The relatively slow rate of reassociation of aldolase permitted an investigation of the distribution of macromolecular species during renaturation by sedimentation velocity analysis. Denatured aldolase was diluted into SRB at 0 or 10° and centrifugation begun within 20 min of the initiation of renaturation. In Figure 6A the sedimentation velocity pattern of aldolase renatured at 0° is compared to that of native aldolase at 0° and in Figure 6B aldolase renatured at 10° is compared to that of the native enzyme at 10°. In each case native aldolase gave a single symmetrical boundary with an $s_{20,w}$ = 7.5 S. This $s_{20,w}$ is slightly lower than some earlier values reported for the native enzyme (Deal *et al.*, 1963; Kawahara and Tanford, 1966), but is in good agreement with other more recently determined

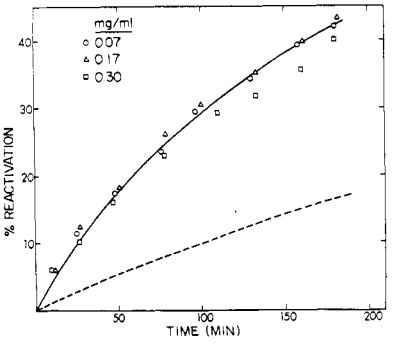

FIGURE 5: Rate of reactivation of aldolase as a function of enzyme concentration. Aldolase was renatured by dilution into SRB at 0° to final enzyme concentrations indicated. The solid curve was generated from integrated rate equations for sequential first-order or parallel first-order mechanisms (see text). The dashed curve is the theoretical curve predicted by second-order kinetics for a fourfold decrease in initial concentration relative to that employed to generate the solid curve.

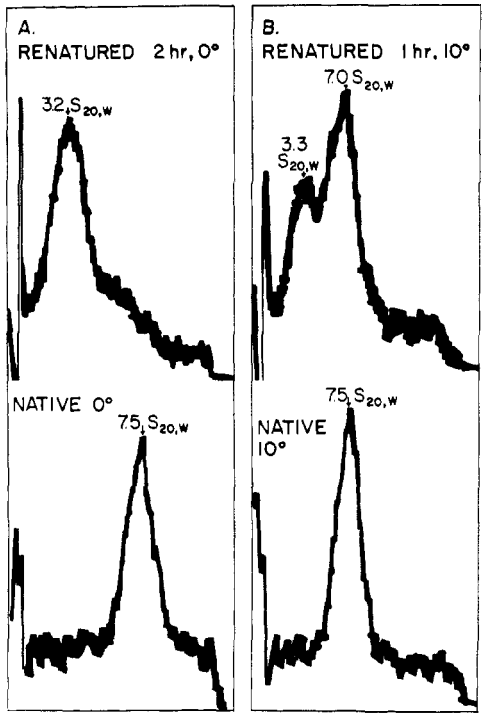

FIGURE 6: Sedimentation velocity patterns for native and renatured aldolase. Scanner traces of the first derivative of concentration *vs.* distance (*i.e.*, concentration gradient *vs.* distance) are presented. Sedimentation is from left to right. In part A, aldolase renatured at 0° for 2 hr (upper half) is compared to native aldolase (lower half). Both sedimentation velocity patterns were recorded after centrifugation at 60,000 rpm for 95 min at 0°. In part B, aldolase renatured at 10° for 1 hr (upper half) is compared with native aldolase (lower half). Both sedimentation velocity patterns were recorded after centrifugation at 60,000 rpm for 50 min at 10°. Enzyme concentrations ranged from 0.15 to 0.2 mg per ml.

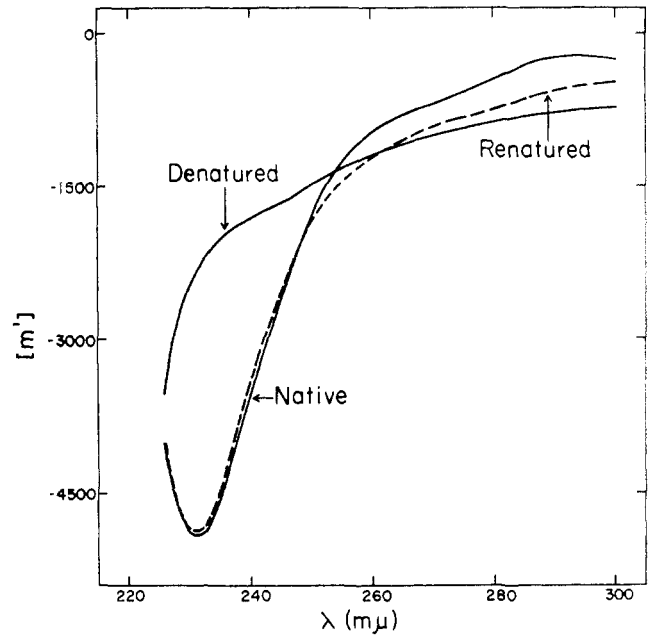

FIGURE 7: Optical rotatory dispersion curve for native, denatured and renatured aldolase. Native spectrum corresponds to native aldolase in 0.01 M Tris-Cl, 0.1 M KCl, 1 mM 2-mercaptoethanol, and 0.1 mM EDTA, pH 7.5 at 0°; renatured spectrum corresponds to renatured aldolase in same buffer at 0°; denatured spectrum corresponds to renatured aldolase *in same buffer containing* 6.0 M Gdn·HCl at 0°. Final enzyme concentrations ranged from 0.15 to 0.20 mg per ml.

coefficients (Wassarman and Kaplan, 1968; Reisler and Eisenberg, 1969).

Analysis of the sedimentation velocity patterns of the renaturing enzyme was made with the aid of relationships derived by Van Holde (1962) and Belford and Belford (1962) for systems with approximately equal association and sedimentation rates. Thus, in Figure 6A the major peak ($s_{20,w}$ = 3.2 S) was attributed to unassociated enzyme and the minor faster sedimenting shoulder ($s_{20,w} \simeq 7$ S) to enzyme primarily associated during the sedimentation process itself. In Figure 6B the first minor peak ($s_{20,w}$ = 3.3 S) was attributed to unassociated enzyme and the major peak ($s_{20,w}$ = 7.0 S) to enzyme primarily associated before the initiation of sedimentation. The faster sedimenting shoulder ($s_{20,w} \simeq 13$ S) probably represents a nonspecific aggregate and is consistent with the high $\bar{M}_w$ values measured by light scattering for renaturation at 10°.

A close correlation was observed between the per cent of 7 S or associated species, as determined by sedimentation velocity analysis, and per cent reactivation. For aldolase renatured at 0°, both reassociation (Figure 6A) and reactivation (Figure 4) had proceeded approximately 30% after 2 hr, and for aldolase renatured at 10°, both reassociation (Figure 6B) and reactivation (Figure 4) proceeded approximately 65% after 1 hr.

Two further observations concerning the sedimentation velocity analyses of renaturing aldolase were made. First, centrifugation of the enzyme renatured at 0° beyond 2 hr revealed a slower sedimenting shoulder on the trailing edge of the 3.2S peak. The 3.2S peak, therefore, appears to consist of at least two separate components which are not in rapid equilibrium. Secondly, sedimentation velocity analysis of aldolase renatured at 0° for 2 hr and at 25° for 2 additional hr (*i.e.*, aldolase nearly 100% reactivated) revealed a single symmetrical boundary with an $s_{20,w}$ = 7.4 S.

Optical Rotation. An earlier investigation of the renaturation kinetics of six different enzymes, including aldolase, revealed that the regain of gross secondary and tertiary structure was complete within 1 min of the initiation of renaturation (Teipel and Koshland, 1971b). These previous studies were all conducted at 25°. Since many of the reassociation and reactivation measurements described here were conducted at 0°, some in the presence of Gdn·HCl, it was also of interest to determine the rate of refolding of aldolase under these conditions.

The rate of refolding (*i.e.*, the rate of regain of secondary and tertiary structure) of aldolase was estimated by monitoring the change in optical rotation of the renaturing enzyme at 232 mμ with time. Aldolase, denatured in 6 M Gdn·HCl, was renatured by a rapid 100:1 dilution into a buffer of 0.01 M Tris-Cl, 0.1 M KCl, 1 mM 2-mercaptoethanol, and 0.1 mM EDTA, pH 7.5 at 0°. The first mean residue rotation, [*m'*], measured approximately 30 sec after the initiation of renaturation, was −4860 (deg cm²)/dmole, a value close to that measured for the native enzyme at 232 mμ. This rotation remained constant for three hours, at which time the experiment was terminated. The rotatory dispersion spectra between 225 and 300 mμ for the renatured enzyme is compared with the dispersion curves at 0° for native aldolase and denatured aldolase in Figure 7. Several observations may be made concerning these spectra. First, the denatured enzyme appears to possess little ordered secondary or tertiary structure as judged

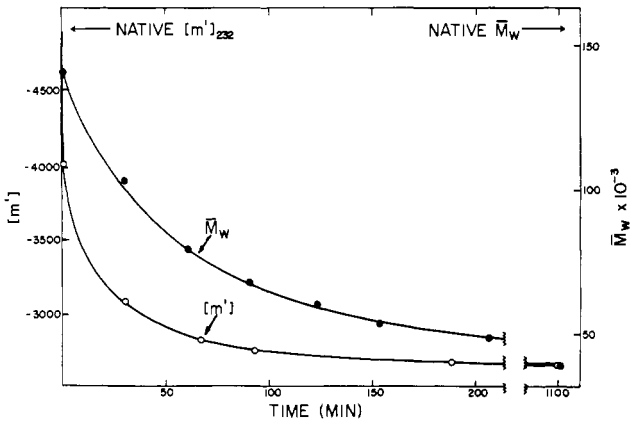

FIGURE 8: Comparison of the rates of unfolding and disassociation for aldolase. Native aldolase was diluted into SRB containing 2.0 M Gdn·HCl at 0° and the decrease in $[m']$ at 232 (○) and $\bar{M}_w$ (●) measured with time.

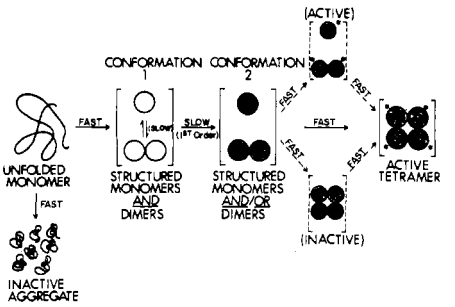

FIGURE 9: Schematic representation of the proposed renaturation mechanism for aldolase. See text for details.

by its plain dispersion curve. The native enzyme, on the other hand, contains a considerable degree of secondary structure, primarily in the form of α helix, as indicated by the pronounced trough in its dispersion curve at 232 mμ. The dispersion curve for the renatured enzyme is seen to be nearly identical with that of the native enzyme below 250 mμ, suggesting that the polypeptide backbone structure and particularly the α-helical regions of the two conformations are very similar. Consequently, the observation that $[m']_{232}$ for renatured aldolase was, within 30 sec, nearly identical with that of the native enzyme indicates a rapid and nearly complete regain of this backbone structure upon renaturation. These results are the same as those found for renaturation at 25°, and indicate that the rate of refolding, although probably temperature dependent, is still not reduced at 0° to a level discernible on the time scale of these experiments. Comparison of the native and renatured dispersion spectra between 250 and 300 mμ reveals tertiary structural differences between the two conformations within the local environment of certain aromatic residues. The small magnitude of the optical rotations at these wavelengths, however, precluded a kinetic investigation of structural changes in these regions.

In a further attempt to reduce the rate of refolding of aldolase, the enzyme was renatured at 0° in the presence of 2.0 M Gdn·HCl. Tanford *et al.* (1966) observed, for example, that the rate of renaturation of lysozyme was lowered in the presence of Gdn·HCl. The change in optical rotation at 232 mμ for aldolase, however, was once again complete within 30 sec of the initiation of renaturation. The kinetics of renaturation in the presence of Gdn·HCl greater than 2.0 M were not feasible because of the prohibitively small difference in optical rotation between the renatured and denatured enzyme.

Denaturation. Aldolase was denatured by diluting 100:1 native enzyme into SRB containing 2 M Gdn·HCl at 0°. The rate of unfolding, as measured by the decrease in optical rotation at 232 mμ, was compared with the rate of dissociation, as measured by the decrease in $\bar{M}_w$ determined by light scattering. The results of these experiments are shown in Figure 8. It may be observed that $[m']_{232}$ decreased faster than $\bar{M}_w$. The final molecular weight, measured approximately 19 hr after the start of denaturation, was 39,000, consistent with that expected for the monomeric subunit. This final value of $\bar{M}_w$ was the same as that measured for the renaturation of aldolase in 2 M Gdn·HCl. The final value of $[m']_{232}$, which remained constant over a period of 12 hr, was -2660 (deg cm^2) dmole. This value is considerably lower than $[m']_{232} = -3150$ measured for renaturation of the enzyme in 2 M Gdn·HCl, and indicates that one (or both) of the two aldolase conformations is not at equilibrium. The faster decrease in $[m']$ relative to $\bar{M}_w$ was primarily due to the large change in optical rotation within the first minute of denaturation. Given that the change in $[m']$ is an accurate reflection of changes in secondary and tertiary structure, the results of Figure 8 indicate that aldolase becomes partially unfolded before disassociating. This order is the converse of that which occurs upon renaturation in buffer, where refolding is essentially complete before reassociation begins. These alternate mechanisms do not, of course, violate the principle of microscopic reversibility since denaturation and renaturation were conducted under different thermodynamic conditions (*i.e.*, denaturation in 2 M Gdn·HCl; renaturation in 0 M Gdn·HCl.

Discussion

The results of the experiments described above will be discussed in terms of the general renaturation mechanism for aldolase depicted in Figure 9. The structural species schematically illustrated are not intended to represent all intermediate conformations formed during the course of renaturation. Only those intermediates implicated by direct experimental observation have been included. The following major conclusions may be drawn with respect to the assembly mechanism proposed in Figure 9.

(1) Unfolded monomers refold rapidly upon renaturation. Regain of gross secondary and tertiary structure, as measured by optical rotation, was complete within 30 sec for all renaturing environments tested. Under conditions leading to high levels of reactivation, the secondary structure, especially α helix, regained within this interval was nearly identical with that observed for the native enzyme.

(2) Within the first 30 sec of renaturation two modes of reassociation occur: (a) an extensive, nonspecific association leading to the formation of high molecular weight aggregates, and (b) a specific, partial association leading to a mixture of structured monomers and dimers in conformation 1. Evidence for rapid nonspecific aggregation is derived from light scattering studies which indicate large initial $\bar{M}_w$ values after renaturation at elevated temperatures or enzyme concentrations. Sedimentation velocity analysis of the 10° renatured enzyme also indicates the presence of such aggregates in the form of 13S sedimenting species. These aggregates were judged inactive since no regain in enzymatic activity accompanied the rapid reassociation process.

The rapid specific mode of reassociation produces a mix-

ture of structured dimers and monomers in conformation 1. Several observations suggest that both dimeric and monomeric species are present within the first 30 sec of renaturation. For aldolase renatured at 0° and enzyme concentrations <0.1 mg/ml (*i.e.*, optimum renaturation conditions) the initial $\bar{M}_w$ recorded was ~60,000. This molecular weight is consistent with an approximately equal weight percent mixture of monomers ($M \simeq 39{,}000$) and dimers ($M \simeq 78{,}000$). For renaturation at higher temperatures and enzyme concentrations initial values of $\bar{M}_w$ may be misleading due to the formation of high molecular weight aggregates. In such cases, however, the further slow increase in molecular weight ($\Delta\bar{M}_w$) leading to tetramer consistently fell within the range $\Delta\bar{M} \simeq 80{,}000$ to $\Delta\bar{M}_w \simeq 100{,}000$, indicating an initial distribution of monomer and dimer.

Sedimentation velocity studies of renaturing aldolase also indicated the initial formation of both monomeric and dimeric enzyme. The relationship discerned between the sedimentation patterns and percent reactivation has suggested that the slower sedimenting boundaries represent the less associated inactive species formed during renaturation. Thus, in terms of the renaturation mechanism in Figure 9, the species of conformation 1 give rise to the 3.2S peak for renaturation at 0°, and the 3.3S peak for renaturation at 10°. Assuming that the sedimentation coefficient of aldolase varies as $M^{2/3}$, $s_{20,w}$ (dimer) $\simeq$ 4.7 S and $s_{20,w}$ (monomer) $\simeq$ 3.0 S. The intermediate $s_{20,w}$ values observed for the conformation 1 species is thus consistent with the presence of both monomers and dimers.

Two observations suggest that the equilibrium between monomer and dimer in conformation 1 is not rapid. First, for a rapid equilibrium the kinetics of association to form tetramer would be greater than first order. However, no dependence of the rate of association on initial enzyme concentration is observed. Secondly, as noted in the Results, sedimentation of the 0° renatured enzyme eventually revealed a shoulder on the trailing edge of the 3.2S peak, presumably attributable to monomer alone. For a rapid equilibrium between monomer and dimer the sedimentation boundary would have remained symmetrical.

(3) There is a slow first-order conversion of monomers *and* dimers in conformation 1 to monomers and/or dimers in conformation 2. The slow association of monomeric and dimeric enzyme to a higher molecular weight species was independent of initial enzyme concentration and hence judged to obey first-order kinetics. In order to explain the apparent first-order dependence of the association reaction (which must involve at least one second- or higher order step) it was necessary to postulate a reaction scheme consisting of a rate-limiting first-order isomerization reaction followed by a rapid second- or higher order association reaction. The conversion of conformation 1 species to conformation 2 species is the postulated slow first-order reaction.

As described in the Results, the kinetics of reassociation could be explained by either two sequential first-order reactions or two parallel first-order reactions. In terms of the structural species in Figure 9, both sequential and parallel pathways for the conversion of monomers and dimers in conformation 1 to monomers and/or dimers in conformation 2 may be easily envisioned. The renaturation scheme has been generalized to include either of these pathways.

(4) Enzyme in conformation 2 rapidly both associates to tetramer and regains enzymatic activity. Evidence that the associated species is tetramer is twofold: (a) aldolase renatured at 0° and enzyme concentrations <0.1 mg/ml gave a final weight-average molecular weight (~150,000) close to that determined for the tetrameric enzyme (156,000) and (b) sedimentation velocity analysis of fully reactivated enzyme revealed a single symmetrical boundary with an $s_{20,w}$ = 7.4 S. For aldolase reactivated at 0 and 10°, sedimentation velocity analysis revealed a faster sedimenting boundary with $s_{20,w} \simeq 7$ S. There is no obvious explanation for these slightly low $s_{20,w}$ values, except that perhaps under these renaturing conditions, the tetramer may be in rapid equilibrium with a lower molecular weight species.

That association to tetramer must be rapid is inferred from the observed apparent first-order kinetics of reassociation as discussed above. That regain of activity is also rapid stems from the observation that the rate of reactivation closely parallels the rate of association. The finding that the concentration of the faster and slower sedimenting species seen in sedimentation velocity analysis corresponded with the concentration of active and inactive enzyme respectively is also consistent with this idea. In Figure 9 two alternate pathways by which monomers and/or dimers in conformation 2 may proceed to active tetramers are illustrated. In one, regain of activity precedes association, while in the other association procedes reactivation. The two pathways cannot be discriminated by the kinetic experiments described here. It should be noted that for the activation before reassociation mechanism, the monomers and/or dimers of conformation *2* could equally well be considered enzymatically active. Studies of the binding of aldolase to an insoluble matrix by Chan (1970), in fact, suggest that a covalently bound monomeric species is active. Certain kinetic evidence is also consistent with this idea.[2]

The most significant observations pertaining to the renaturation scheme in Figure 9 are that (1) polypeptide chain refolding occurs rapidly, (2) reassociation to tetramer is a slow, first-order reaction, and (3) reactivation closely parallels reassociation to tetramer. Less significant is the observation that a mixture of both monomeric and dimeric species appears as an intermediate during renaturation. Although considerable evidence points to the presence of monomers and dimers under the renaturation conditions employed in these experiments, it is not certain that a distribution of both species represents a necessary intermediate in all cases. The finding that the proportion of monomers and dimers changed appreciably with renaturation conditions, while the other major characteristics of the renaturation process, enumerated above, remained relatively constant, attests to the variable and somewhat uncritical nature of this distribution with respect to the overall renaturation mechanism. It is, therefore, quite plausible that a more specific renaturation process, *e.g.*, assembly of aldolase *in vivo*, might involve rapid refolding to only monomeric species with subsequent slow association to tetramer. Alternatively, dimers may represent the true intermediate subunit species with the structural monomers observed here being an artifact of *in vitro* renaturation.

Deal and coworkers (1963) have previously investigated the *in vitro* renaturation of acid denatured aldolase. Certain features of the renaturation mechanism suggested by these authors, *i.e.*, rapid association to an intermediate species followed by a slow, first-order conversion to active enzyme, are very similar to those proposed here for renaturation from Gdn·HCl. In the former studies the intermediate species was thought to resemble closely a component formed at pH 3.3 with a weight-average molecular weight of 138,000.

[2] William Chan, private communication.

In a more recent study of the renaturation of acid-denatured aldolase by Blatti (1969), however, somewhat different observations were reported. A molecular weight of only 80,000 (consistent with dimer) was measured for a subunit species formed at pH 3.35. Also, reassociation of this component to active enzyme was reported to follow second-order kinetics. Regardless of the mechanism of renaturation of acid-denatured aldolase, however, it is unlikely that renaturation of the enzyme from Gdn·HCl will proceed *via* an identical mechanism.

Finally, it should be emphasized that the kinetic studies reported here were conducted under essentially irreversible conditions, *i.e.*, rate of renaturation $\gg$ rate of denaturation. Other investigators (Tanford *et al.*, 1966; Ikai and Tanford, 1971; Shen and Hermans, 1972) have studied the kinetics of protein conformation change under reversible conditions, and consequently have been able to determine both equilibrium constants and forward and reverse kinetic constants. Clearly, the latter approach has advantages in terms of a more complete description of the particular conformational changes under investigation. However, solvent conditions leading to equilibrium constants close to unity, as measured by structural probes such as optical rotation, ultraviolet absorbance, viscometry, etc., generally also result in complete loss of catalytic activity. Conversely, solvent conditions favoring irreversible renaturation usually lead to substantial catalytic reactivation. Since some of the more interesting structural changes accompanying renaturation are those affecting the active site region, regeneration of a biologically active enzyme, albeit under irreversible conditions, has been favored in this and other renaturation studies.

References

Belford, G. G., and Belford, R. L. (1962), *J. Chem. Phys. 37*, 1926.

Blatti, S. P. (1969), *Diss. Abstr. B 29*, 3656.

Brice, B. A., and Halwer, M. (1951), *J. Opt. Soc. Amer. 41*, 1033.

Brice, B. A., Halwer, M., and Speiser, R. (1950), *J. Opt. Soc. Amer. 40*, 768.

Casassa, E. F., and Eisenberg, H. (1964), *Advan. Protein Chem. 19*, 287.

Castellino, F. J., and Barker, R. (1968), *Biochemistry 7*, 2207.

Chan, W. W.-C. (1970), *Biochem. Biophys. Res. Commun. 41*, 1198.

Deal, W. C., Rutter, W. J., and Van Holde, K. E. (1963), *Biochemistry 2*, 246.

Ikai, A., and Tanford, C. (1971), *Nature (London) 230*, 100.

Kabashi, K., Lai, C. Y., and Horecker, B. L. (1966), *Arch. Biochem. Biophys. 117*, 437.

Kawahara, K., and Tanford, C. (1966), *Biochemistry 5*, 1578.

Kratohvil, J. P., Dezelic, G. J., Kerker, M., and Matijevic, E. (1962), *J. Polymer Sci. 57*, 59.

Penhoet, E. E., Kochman, M., and Rutter, W. J. (1969), *Biochemistry 8*, 4391.

Racker, E. (1947), *J. Biol. Chem. 167*, 843.

Reisler, E., and Eisenberg, H. (1969), *Biochemistry 8*, 4572.

Shen, L. L. and Hermans, J., Jr. (1972), *Biochemistry 11*, 1836.

Sine, H. E., and Hass, L. F. (1969), *J. Biol. Chem. 244*, 430.

Tanford, C., Pain, R., and Otchin, N. (1966), *J. Mol. Biol. 15*, 489.

Teipel, J. W., and Koshland, D. E., Jr. (1971a), *Biochemistry 10*, 792.

Teipel, J. W., and Koshland, D. E., Jr. (1971b), *Biochemistry 10*, 798.

Tomimatsu, Y., and Palmer, K. J. (1959), *J. Polym. Sci. 35*, 549.

Tomimatsu, Y., and Palmer, K. J. (1963), *J. Phys. Chem. 67*, 1720.

Trementozzi, Q. A. (1957), *J. Polym. Sci. 23*, 887.

Van Holde, K. E. (1962), *J. Chem. Phys. 37*, 1962.

Wassarman, P. M., and Kaplan, N. O. (1968), *J. Biol. Chem. 243*, 720.