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Kinetics of Reactivation of Rabbit Muscle Aldolase after Denaturation and Dissociation in Various Solvent Media*

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Abstract. The denaturation of aldolase from rabbit muscle in various solvents leads to significant qualitative and quantitative differences with respect to the structural disintegration of the enzyme. The differences refer to the quaternary structure and to the conformation which is changed only slightly in $MgCl_2$ while in guanidine · HCl or urea at pH ~ 2 the molecule is close to the state of the random coil.

Using the enzymic activity as a quantitative measure for the refolding process, the reaction order and the rate constants of the processes of structure formation ($\vartheta_i \rightarrow N^*$) are found to be identical.

This observation suggests a common intermediate D in the process of renaturation after denaturation and dissociation in the different solvent media. D may be considered an intermediate state with a defined number of nucleation centers whose rapid formation is predetermined by the aminoacid sequence.

As taken from the first order kinetics in the given range of enzyme concentration, transconformation reactions are rate limiting in the obligatory pathway of refolding. At low enzyme concentrations second order steps gain importance which indicates that the enzymic activity is significantly modified by the formation of the native quaternary structure.

Key words: Aldolase – Denaturation – Kinetics – Refolding.

Introduction

The refolding of oligomeric enzymes after denaturation and dissociation under various conditions yields enzymically active products which are indistinguishable from the native molecules according to all available physico-chemical criteria (Anson, 1945; Jaenicke et al., 1975; Rudolph and Jaenicke, 1976; Engelhard et al., 1976). Concerning the formation of the three-dimensional structure in vivo this finding has been considered to prove the thermodynamic determination of protein folding (Anfinsen and Scheraga, 1975; Wetlaufer and Ristow, 1973). However, there might be different pathways in vivo and in vitro, because the chain may fold in vivo as it is

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synthesized on the ribosome, while in vitro the whole polypeptide chain is simultaneously involved in the process of refolding. Whether or not kinetic constraints are of importance in protein structure formation is not unambiguously established so far. If there exist path-limitations in the refolding from the completely unordered state, i.e. if the attainment of the free energy minimum shows different kinetics depending on the mode of denaturation, thermodynamic parameters cannot be sufficient to describe the process.

The following experiments are based on this consideration. Aldolase from rabbit muscle was transferred into the denatured state at acid pH (Rudolph et al., 1976, 1977) or by treatment with $MgCl_2$ (Hsu and Neet, 1973, 1975), urea (Stellwagen and Schachman, 1962), guanidine · HCl (Teipel, 1972, Chan et al., 1973) and guanidine · HCl at elevated temperature or acid pH. After having reached the final state of denaturation and dissociation comparative measurements of the rates of reactivation were performed. The rate constants for the reactivation reaction after denaturation and dissociation in the different denaturants were found to be indistinguishable within the limits of error.

Obviously there exists an intermediate D in the transition from the denatured state (ϑ) to the native state (N) which is transformed into N in rate determining steps[1]:

$$\vartheta \longrightarrow \mathrm{D} \overset{\text{slow}}{\longrightarrow} \mathrm{N}.$$

Materials and Methods

Fructose-1.6-bisphosphate aldolase from rabbit muscle, fructose-1.6-bisphosphate, NADH, and the mixture of glycerol-3-phosphate dehydrogenase and triose phosphate isomerase for the coupled assay were purchased from Boehringer (Mannheim), bovine serum albumin (BSA) from Serva (Heidelberg), guanidine · HCl and urea, both ultrapure from Schwarz-Mann (New York), dithiothreitol (DTT) from Calbiochem. (Lucerne), dithioerythritol (DTE) from Roth (Karlsruhe). Trypsin (Boehringer) was treated with L-(1-tosylamido-2-phenyl) ethyl-chlormethyl ketone (Serva) (Carpenter, 1967). All other reagents were A-grade substances from Merck (Darmstadt). Quartz bidistilled water was used throughout.

Stock solutions of the enzyme (c = 6–17 mg/ml) were prepared by repeated dialysis at 3° C against the following buffers in the presence of 1 mM EDTA and 0.1 mM DTE:
0.2 M potassium phosphate pH 7.6;
0.05 M triethanolamine buffer pH 7.6;
0.2 M tris (hydroxy-methyl) aminomethane buffer pH 7.6 (Tris).

In order to stabilize the enzyme against proteolytic attack the crystalline enzyme was solved in phosphate buffer containing 0.2 mM phenylmethyl-sulfonylfluoride (PMSF); after 30 min incubation at 20° C the afore-mentioned dialysis was started.

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[1] Abbreviations used are: ϑ, D, N, aldolase in its fully denatured, intermediate, and native state; BSA, bovine serum albumin; c, enzyme concentration; CD, circular dichroism; G · HCl, guanidine · HCl; ORD, optical rotatory dispersion; U, urea

Aldolase activity was determined according to Racker (Racker, 1974); the specific activity of the native enzyme was 10–12 IU/mg. In order to prevent reactivation of the denatured enzyme during the optical test the modified assay according to Chan et al. was applied (Chan et al., 1973). Reactivation was analyzed by taking aliquots at defined times.

The *enzyme concentration* was calculated from $A^{1\%}_{280\ nm} = 9.1\ cm^2 \cdot mg^{-1}$ (Baranowski and Niederland, 1949). Molar concentrations are based on the subunit molecular weight of 40,000 (Kawahara and Tanford, 1966; Sia and Horecker, 1968; Reisler and Eisenberg, 1969), molar ellipticities as usual are calculated for the tetramer of 160,000.

Denaturation and dissociation were achieved by incubating the native enzyme at 20° C for 10 min to 24 h in the respective denaturants. Except for the denaturation with $MgCl_2$ where equilibrium was reached only after several hours, no time dependence on the extent of denaturation could be detected.

For *reactivation* dilution in the different buffers plus 1 mM EDTA and 0.1–10 mM DTE was applied. The final concentration of the denaturant in the renaturation mixture was kept constant in all experiments by adding calculated volumes of the denaturant. Based on systematic experiments to define optimum solvent conditions 0.2 M Tris + 10 mM DTE was used for denaturation and 0.05 M Tris + 10 mM DTE + 0.5 mg/ml BSA for renaturation. To follow the kinetics samples were taken until the plateau of final reactivation was reached ($\geqq$ 24 h). The percentage of reactivation was then calculated relative to the activity of N and N*. Polypropylene vessels were applied throughout.

Conformational analysis made use of circular dichroism (Roussel-Jouan, Dichrographe II), fluorescence (Hitachi-Perkin Elmer, MPF 2A), and optical rotatory dispersion (Perkin Elmer, Pol 241, and Cary 60).

Sedimentation velocity and sedimentation equilibrium experiments were performed in an analytical ultracentrifuge (Beckman, Model E, with high-sensitivity photoelectric scanning system).

Results

The following comparative kinetic experiments refer to the reaction $\vartheta \rightarrow N^*$. Their significance depends on the degree of unfolding in the denatured state ϑ and on the fact that the product of renaturation N* is indistinguishable from the native enzyme N (Engelhard et al., 1976). Data describing the native and the acid dissociated state were summarized earlier (Jaenicke et al., 1975; Engelhard et al., 1976). As suggested from the close correlation of deactivation, denaturation, and dissociation in these experiments the enzymic activity may be considered a quantitative measure of the reversible reaction of denaturation–renaturation $N \rightleftharpoons D$. However, full deactivation does not necessarily correspond to complete unfolding, i.e. random coil formation. Instead the enzyme in various denaturants shows different amounts of residual secondary structure so that the denatured enzyme in the various media represents a whole spectrum of states of solvation and/or denaturation (ϑ_i) rather than one distinct and well-defined state ϑ. This is illustrated by the following spectral data.

Characterization of the Denatured State

Figure 1 shows the denaturation effects of denaturants on aldolase using various spectral parameters. The concentration range of the denaturant where deactivation (denaturation) and reactivation (renaturation) occur does not coincide under the given experimental conditions (Fig. 2); in general the transition $N \rightarrow \vartheta$ takes place at much higher concentrations of the denaturant as compared to the transition $\vartheta \rightarrow N^*$. Longer times of incubation are essential only in the case of the $MgCl_2$-dependent dissociation where the midpoint of the $N \rightarrow \vartheta$ transition changes from 4.2 M to 3.3 M after 2 and 26 h, respectively. The residual helicities in the different denaturing media and the results of the respective sedimentation analyses are summarized in

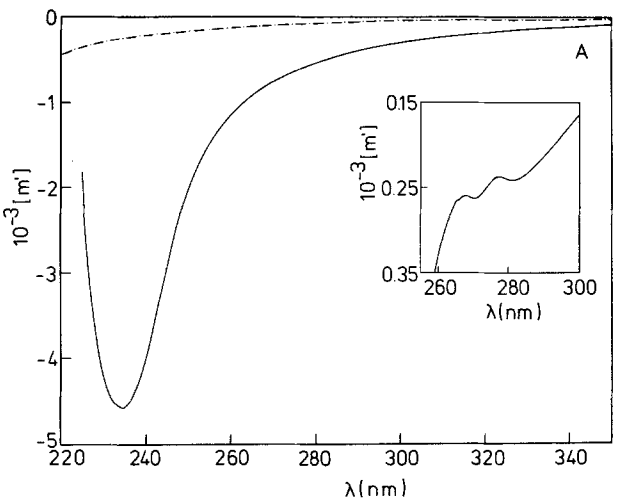

Fig. 1A

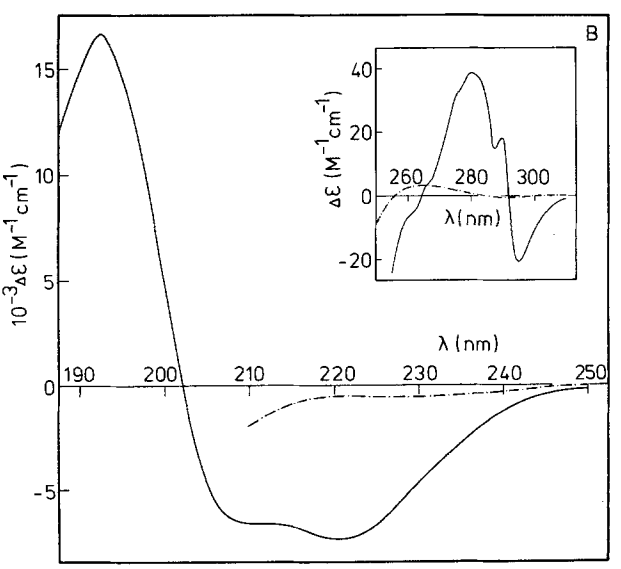

Fig. 1B

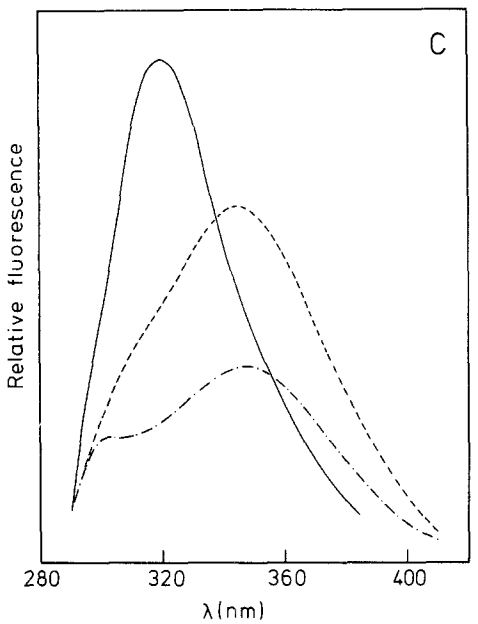

Fig. 1C

Fig. 1A–C. Spectral properties of native and denatured aldolase. **A** Optical rotatory dispersion in 0.2 M Tris buffer pH 7.6, 1 mM EDTA, 0.1 mM DTE; c = 0.25 mg/ml, pathlength 1 cm, 25° C. (——) native; (– · – · –) denatured in 7.2 M G · HCl for 1 h at 20° C. Insert: Near UV ORD at c = 3.8 mg/ml, pathlength 2 cm. **B** Far UV circular dichroism in 0.2 M Tris buffer pH 7.6, 1 mM EDTA, 0.1 mM DTE; c = 0.3–1 mg/ml, pathlength 0.1 mm, 25° C. (——) native; (– · – · –) denatured in 7.2 M G · HCl for 0.5 h at 20° C. Insert: Near UV CD, pathlength 1 cm. **C** Protein fluorescence in 0.2 M triethanolamine (TEA) or Tris buffer pH 7.6, 1 mM EDTA, 0.1 mM DTT; c = 0.35 mg/ml, 25° C, λ_{exc} = 275 nm. (——) native in TEA buffer; (– · – · –) denatured in TEA buffer plus 7.0 M G · HCl; (– – –) denatured in Tris buffer plus 7.2 M urea

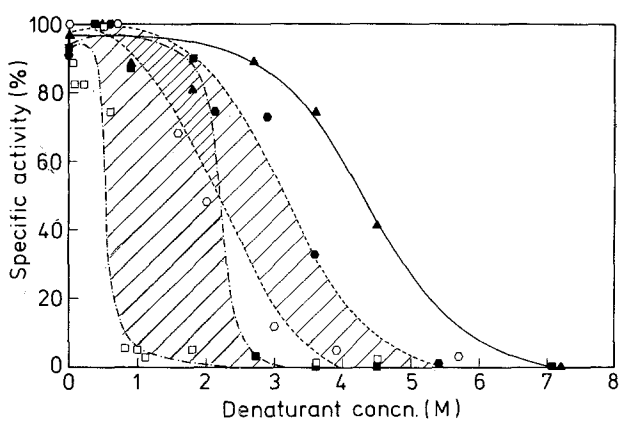

Fig. 2. Deactivation and reactivation of aldolase as a function of denaturant concentration in 0.2 M Tris buffer pH 7.6, 1 mM EDTA, 0.1 mM DTE. Deactivation at 20° C; reactivation at 8° C. Filled symbols: Deactivation of native aldolase, open symbols: Reactivation of deactivated aldolase. –▲– Deactivation (1 h) in urea at c = 0.12 mg/ml. - -●- - Deactivation (26 h) in $MgCl_2$ at c = 1.2 mg/ml. - -○- - Reactivation (22 h) in $MgCl_2$ at c = 0.063 mg/ml after deactivation (26 h) in 5.6 M $MgCl_2$. — · ■ · — Deactivation (3 h) in G · HCl at c = 1.2 mg/ml. — · □ · — Reactivation (24 h) in G · HCl at c = 0.02–0.06 mg/ml after deactivation (1 h) in 7.2 M G · HCl. Hatched areas indicate hysteresis (see text)

Table 1. Effect of various denaturants on the particle weight and conformation of rabbit muscle aldolase, 20° C[a]

Solvent	pH	$M_w \cdot 10^{-3}$	$s^0_{20,w}$ (S)	f_H (%)[b]
0.05 M Tris/HCl	7.6	160	7.90	34 ± 4
0.2 M glycine/H_3PO_4	2.3	40	2.0	17 (19)[c]
3.2 M $MgCl_2$	7.6	42–73[d]		~ 40
5.4 M $MgCl_2$	7.6	≦ 75	6	~ 25
7.2 M U	2–8	42 (42)[c]	(1.6)[c]	~ 6 (8)[c]
7.2 M G·HCl (20 and 95° C)	2–8	40		0

[a] In solvents where denaturation depends on the time of incubation final values after long incubation are given. 0.2 M Tris/Hcl or phosphate buffer were used as solvents

[b] Helix content f_H estimated according to Doty-Yang, Moffitt-Yang, Greenfield-Fasman and Chen-Yang-Martinez; to correct for the refractive index of the various solvents the optical rotation was expressed as mean residue rotation. Mean residue weight: 113

[c] cf. Stellwagen and Schachman (1962)

[d] Concentration dependent dissociation $(M_w)_{min} = 42\,400 \pm 1\,000$; irreversible denaturation may interfere with the equilibration

Table 1. Under all conditions, except high $MgCl_2$-concentrations, sedimentation velocity and sedimentation equilibrium prove the enzyme to represent the homogeneous tetramer or monomer. In the presence of $MgCl_2$ nonlinear profiles in the ln c vs r^2 plot indicate a concentration dependent dissociation equilibrium including the dimer and monomer. As in the case of the acid dissociation (Engelhard et al., 1976) activity and structural parameters show closely parallel profiles. At low concentrations of the denaturants a slight increase in the specific activity is observed which is parallelled by respective changes of the spectral properties. With increasing concentration of the denaturant inflection points in the sigmoid transition curves are observed. They may indicate intermediates with well defined specific activity. Similar profiles of the conformational parameters suggest regions of ordered structure in the otherwise disordered enzyme molecule; another explanation can be derived from slow aggregation of the enzyme at moderate concentrations of the denaturant which leads to the observed hysteresis of the denaturation–renaturation profiles. The superposition of dissociation and formation of aggregates again leads to biphasic curves.

At high concentrations of the denaturant the totally inactive enzyme is characterized by limiting lower values of the conformational parameters. As shown in Table 1 these values differ for the various denaturants which corroborates the idea that different denatured states ϑ_i are formed instead of one single state ϑ. To illustrate this the estimates of the "helicity" at maximum concentrations of guanidine · HCl, urea, and $MgCl_2$ may be compared; the respective figures are ~ 0, ~ 6, and ~ 25% whereas acid denaturation at pH 2 gives ~ 17% residual "helicity". Similarly the amplitude of the fluorescence emission maximum in guanidine · HCl and urea is decreased to a different extent, 39% and 70%. On the other hand the various experimental methods obviously reflect different structural transitions. As an example the near-UV CD bands and the enzymic activity vanish at relatively low concentrations of the denaturant where the relative fluorescence and $\Delta\varepsilon_{220}$ have not yet reached their final plateau value (Fig. 3).

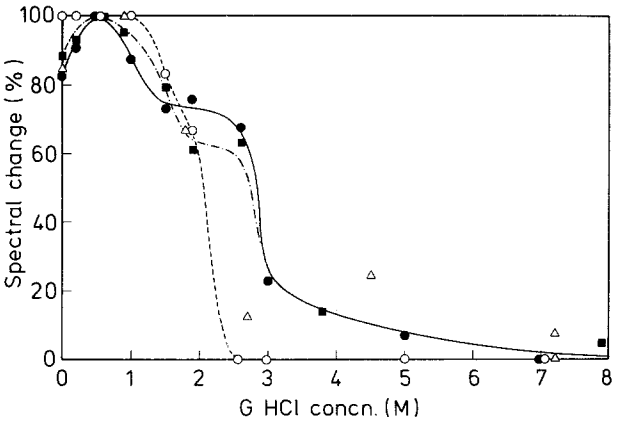

Fig. 3. Denaturation of aldolase as a function of G·HCl concentration, monitored by various spectral properties. 0.2 M Tris buffer pH 7.6, 1 mM EDTA, 0.1 mM DTE. –●– Fluorescence intensity at λ_{max} (25° C): λ_{exc} = 275 nm; c = 0.35 mg/ml, 24 h incubation at 20° C. - - ○ - - Maximum of fluorescence emission (25° C): λ_{exc} = 275 nm, c = 0.35 mg/ml, 24 h incubation at 20° C. –■– Circular dichroism (25° C): λ = 220 nm. c = 0.35–0.9 mg/ml, 0.5 h incubation at 20° C, pathlength 0.1 mm. △ λ_c calculated on the basis of the simple Drude equation. c = 1.62 mg/ml, 3 h incubation at 20° C, pathlength 1 cm. Percentage calculated for maximum spectral changes ≡ 100%

Characterization of the Renatured State

The yield of renaturation of aldolase after treatment with various denaturants depends on the conditions of denaturation and renaturation. Table 2 summarizes representative data which prove a high percentage of reactivation to occur even after drastic denaturation. Low enzyme concentration (c < 1 μg/ml) and high temperature cause irreversible loss of enzymic activity. For long term kinetic experiments optimum renaturation conditions in terms of the reproducibility and the yield of reactivation were found to be 0.05 M Tris/HCl + 10 mM DTE + 0.5 mg/ml BSA, 0° C. Applying these conditions to the enzyme after denaturation in different solvent media leads to a product of renaturation N* which shows the enzymic, hydrodynamic, and spectral properties of the native enzyme (N = N*) (Stellwagen and Schachman, 1962, Engelhard et al., 1976).

Kinetics of Reactivation

The kinetics of reactivation and renaturation after acid dissociation have been extensively investigated in previous experiments (Rudolph et al., 1976, 1977). In the present study the reactivation rates after denaturation to various states ϑ_i were compared. The reactivation kinetics of the enzyme in the given concentration range can be described by two consecutive, irreversible uni-bimolecular reactions (Rudolph et al., 1977). Since the unimolecular step produces an intermediate with partial activity the reaction order (calculated from the initial slopes of the kinetic traces) is expected to be only slightly greater than one. As shown by the double logarithmic plot in Figure 4 the evaluation of the reaction order yields *one* linear relationship

Table 2. Yield of reactivation of aldolase after denaturation in various denaturants. Renaturation at 20° C after dilution (1 : 50) with TEA buffer pH 7.6. To prevent reactivation in the tests trypsin was applied in the assay mixture (Chan et al., 1973). Reactivation was determined after up to 48 h

Denaturation				Reactivation	
Solvent	pH	Incubation (h)	T °C	c_p(µg/ml)	Yield (%)
7 M G·HCl/TEA	7.6	0.5	20	0.82	32
7.6 M G·HCl/TEA	7.6	0.5	20	6.9	47
	6.1	0.5	20	6.9	41
	4.7	0.5	20	6.9	65
	3.1	0.5	20	6.9	76
	2.2	0.5	20	6.9	81
	1.3	0.5	20	6.9	80
1.1–7 M G·HCl/P	7.6	0.5	20	17.9	90 ± 2
7.2 M G·HCl/P	7.6	2.0	52	25.0	99
7.2 M G·HCl/P	7.6	2.0	70	11.0	0
7.2 M G·HCl/P + N_2	7.6	2.0	95	23.0	20
7.2 M U	7.6	2.0	20	1–21	85
5.4 M $MgCl_2$/Tris	7.6	24	20	12.0	91
1 M gly/H_3PO_4	2.3	2.0	20	1–40	95

G · HCl, guanidine · HCl; U, urea; TEA, triethanolamine; P, phosphate; Tris, tris(hydroxy-methyl) aminomethane; gly/H_3PO_4, glycine/H_3PO_4 + 1 mM EDTA + 1 mM DTT; + N_2, solvent saturated with nitrogen

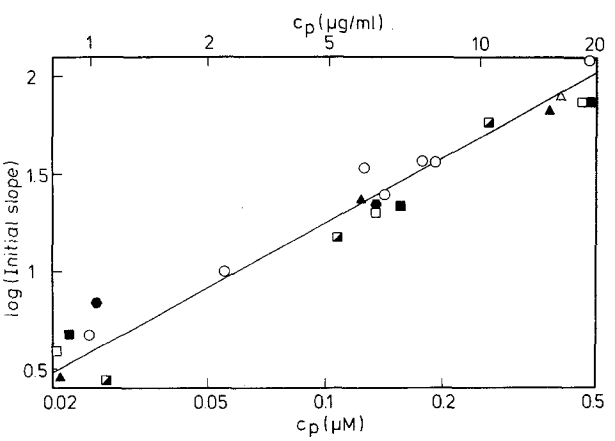

Fig. 4. Determination of the reaction order of the reactivation of aldolase after 0.5 h incubation in various denaturants. c = 1.1 mg/ml. pH 2.3: 1 M glycine/H_3PO_4, 1 mM EDTA, 10 mM DTE; pH 7.6: 0.2 M Tris buffer, 1 mM EDTA, 10 mM DTE. ○ pH 2.3, 20° C. ■ 7.2 M G·HCl pH 7.6, 20° C. □ 7.2 M G·HCl pH 2.3, 20° C. ◪ 7.2 M G·HCl pH 7.6, 95° C. ● 5.4 M $MgCl_2$ pH 7.6, 20° C. ▲ 7.2 M urea pH 7.6, 20° C. △ 7.2 M urea pH 2.3, 20° C. Reactivation in 0.05 M Tris buffer pH 7.6 plus 1 mM EDTA, 10 mM DTE, 0.5 mg/ml BSA at 0–8° C. Final values determined after up to 24 h of reactivation. Reaction order in the given concentration range: $n = 1.1$

Table 3. Range of apparent first order rate constants for the reactivation of aldolase after denaturation and dissociation in various solvent media (see text)

Denaturant	c (Renaturation) [μg/ml]	$k \cdot 10^4$ [s^{-1}]
pH 2.3	1	1.68–0.98
	5	1.97–1.36
	21	2.09–1.46
7.2 M G·HCl	1	1.68–1.03
	5	1.33–1.22
	21	1.55–1.36
7.2 M G·HCl, pH 2.3	1	1.45–0.69
	5	1.42–1.31
	21	1.49–1.21
7.2 M U	1	1.44–1.06
	5	1.72–1.17
	21	1.89–1.47
5.4 M $MgCl_2$	1	1.79–0.43
	5	2.57–2.32
	21	5.60–2.63

with $n > 1$ for the different tansitions $\vartheta_i \rightarrow N^*$. Similarly the different kinetics of renaturation are characterized by one single rate constant; i.e. the overall reactivation can be described in first approximation by a single first order reaction. From this we may conclude that the first order rate constants are an appropriate criterion to compare the overall reactivation rates after denaturation in various solvent media. Because of the deviations from first order ln (100-% A_{sp}) vs t is non-linear; therefore, the limiting slopes at < 40 min and > 50 min were used for comparison. Ranges of the apparent first order rate constants are summarized in Table 3. There is good agreement among the first order rate constants for the various reactions $\vartheta_i \rightarrow N^*$ which proves again that the different kinetics of renaturation are characterized by a single set of kinetic parameters. Enzyme concentration and denaturation conditions do not show a significant influence.

Discussion

The experimental results of the present study show that a variety of denaturing conditions leads to a reversible loss of enzymic activity without complete destruction of the three-dimensional structure. This holds especially for the $MgCl_2$-dependent dissociation which leads to an inactive form of the dimer and monomer containing significant residual secondary structure. Since independent kinetic evidence proves the subunits of aldolase to be partially active (Rudolph et al., 1977), the conformation of the molecule must be changed drastically. From this we conclude that deacti-

vation, denaturation, and dissociation do not lead to identical levels of structural and functional disintegration.

High concentrations of guanidine · HCl cause full denaturation and dissociation. Whether the denatured state represents the random coil cannot be answered. It should be mentioned, however, that extreme conditions of denaturation, e.g. long incubation in guanidine · HCl at elevated temperature, lead to a considerable decrease of reactivation or to irreversible loss of activity. The effect may either be caused by chemical modifications or by the elimination of nucleation centers originating from the folding of the nascent chain in vivo.

Denaturation at high concentrations of urea (7.2 M) leads to results comparable to those in the presence of > 4 M guanidine · HCl.

In the case of acid denaturation coincident profiles of deactivation, denaturation and dissociation have been observed (Engelhard et al., 1976). Obviously protonation results in a strongly cooperative breakdown of part of the native structure which is accompanied by full deactivation.

In summary the different denaturants give rise to specific final states of denaturation characterized by different levels of "helicity".

In this respect the present experiments confirm earlier results of Kugimiya and Bigelow (1973), Tiffany and Krim (1973), Snape et al. (1974), and Tsong (1975). On the other hand renaturation and reactivation lead back to the enzyme in its native state (Engelhard et al., 1976).

The kinetics of reactivation deviate slightly from first order as a result of incomplete separation of uni- and bimolecular elementary processes (Rudolph et al., 1976). The different denaturing solvents do not affect the reactivation characteristics significantly. In all experiments full catalytic function of the enzyme requires the association of the (partially active) subunits. Regarding the rate constants k_t of the reactions $\vartheta_i \xrightarrow{k_t} \mathrm{N}^*$ no significant differences were detectable. This result is of importance in connection with the mechanism of refolding; it proves that the native tetramer is formed from the different states ϑ_i via a common intermediate D which is then transformed into the fully active molecule by identical rate determining steps. Independent pathways starting from different levels of residual secondary structure would yield different kinetic constants. The fact that the opposite holds does not necessarily imply one sequential pathway

$$\mathrm{n}\vartheta_1 \rightarrow \mathrm{n}\vartheta_2 \rightarrow \mathrm{n}\vartheta_3 \rightarrow \cdots \rightarrow \mathrm{nD} \underset{\text{slow}}{\xrightarrow{k_t}} \mathrm{N}^* \tag{1}$$

with k_t as rate constants of the rate determining steps. Instead

$$\left.\begin{matrix} \mathrm{n}\vartheta_1 \\ \mathrm{n}\vartheta_2 \\ \mathrm{n}\vartheta_3 \end{matrix}\right\rangle \mathrm{nD} \underset{\text{slow}}{\xrightarrow{k_t}} \mathrm{N}^* \tag{2}$$

seems to be an adequate mechanism to describe the observed data.

Since the kinetics of the various transitions $\vartheta_i \rightarrow \mathrm{N}^*$ do not show significant differences, the residual structural elements cannot be decisive for the kinetics of refolding. D represents a common intermediate indistinguishable regarding the number and type of nucleation centers.

This finding is in agreement with the postulate that short range interactions between next neighbours in the polypeptide chain are responsible for the nucleation in the refolding process. Evidently these interactions are not influenced by the different states of denaturation.

The apparent path-independence does not prove the acquisition of the native three-dimensional structure to be thermodynamically determined, because the various modes of denaturation represent different states rather than one single "denatured state". Therefore, the free energy of the renaturation reaction is expected to be different for the different transitions. The alternative obligatory pathways of reactivation (Eqs. 1 and 2) contain association steps which become obvious from the change from first to second order at very low enzyme concentrations (Rudolph et al., 1976). Whether the formation of the dimer or tetramer is responsible for the acquisition of full enzymic activity cannot be decided on the basis of the present experiments.

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