# Analysis of the Reconstitution of Oligomeric Enzymes by Cross-Linking with Glutaraldehyde: Kinetics of Reassociation of Lactic Dehydrogenase†

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ABSTRACT: Cross-linking with glutaraldehyde with subsequent $NaDodSO_4$–polyacrylamide gel electrophoresis has been introduced as a convenient method for studying the association of oligomeric proteins [Hermann, R., Rudolph, R., & Jaenicke, R. (1979) *Nature (London) 277*, 243–245]. In the present paper, an improved version of this approach was applied to the analysis of the complex association behavior of the tetrameric lactic dehydrogenase from pig muscle. Monomers, dimers (as intermediates of reconstitution), and tetramers could be quantitatively determined during reconstitution. The initial fast formation of dimers from monomers does not reach completion; a certain amount of monomers remains during the whole reconstitution process. Monomers and dimers disappear parallel to the formation of tetramers. The reassociation behavior of lactic dehydrogenase is described by a kinetic mechanism comprising a dissociation–association equilibrium of monomers and dimers [characterized by an equilibrium constant $K = (3 \pm 1) \times 10^8$ L mol$^{-1}$] followed by the rate-limiting association of dimers to tetramers [described by a second-order rate constant $k = (3.15 \pm 0.15) \times 10^4$ L mol$^{-1}$ s$^{-1}$]. Tetramerization is found to strictly parallel reactivation.

Nascent polypeptide chains fold spontaneously to their "native" three-dimensional structure without the need for additional information beyond that contained in the specific amino acid sequence of a given protein and its aqueous environment.

In the case of stoichiometrically and geometrically well-defined oligomers, structure formation implies association steps which must be properly coordinated with the folding reactions (Jaenicke, 1978). Some association processes of a number of oligomeric enzymes have been determined from an analysis of the reactivation kinetics (Jaenicke, 1979; Jaenicke & Rudolph, 1980).

For lactic dehydrogenase (LDH)[1] from pig muscle, reactivation was found to obey simple second-order kinetics (Rudolph & Jaenicke, 1976). Various aspects of the reconstitution of this enzyme have been previously analyzed, e.g., the influence of coenzymes and temperature (Rudolph et al., 1977a), high pressure (Schade et al., 1980a,b), or the competition of aggregation and reactivation (Zettlmeissl et al., 1979). However, in these studies it could not be decided whether the rate-limiting association reaction observed during reactivation had to be attributed to dimer or tetramer formation. In the present investigation, chemical cross-linking and subsequent $NaDodSO_4$–polyacrylamide gel electrophoresis were used to determine the complete association mechanism of lactic dehydrogenase from porcine muscle.

As shown in a preliminary report (Hermann et al., 1979), glutaraldehyde as a bifunctional reagent (Richards & Knowles, 1968; Habeeb & Hiramoto, 1968; Monsan et al., 1975) was found to satisfy all necessary prerequisites for cross-linking during association. As will be demonstrated, the reagent allows fast fixation as well as the quantitative determination of intermediates of association even at very low enzyme concentrations, so that the population analysis is rendered possible under the conditions of optimum reconstitution.

## Materials and Methods

*Substances.* Lactic dehydrogenase from pig heart and skeletal muscle and NADH were purchased from Boehringer, Mannheim; dithioerythritol (DTE) was from Roth, Karlsruhe, and glutaraldehyde [purissimum, 25% (w/v) aqueous solution] from Fluka, Basel. Acrylamide, bis(acrylamide), ammonium persulfate, $NaDodSO_4$, *N,N,N′,N′*-tetramethylethylenediamine, and Serva-blue R 250 were from Serva, Heidelberg. All other reagents were of A-grade purity, obtained from Merck, Darmstadt. Quartz bidistilled water was used throughout.

*Standard buffer* was 0.1 mol/L sodium phosphate buffer, pH 7.6, containing 1 mmol/L EDTA. Stock solutions of the enzyme (~4–6 mg/mL) were prepared by repeated dialysis at 4 °C against standard buffer plus 1 mmol/L DTE.

*Enzyme concentrations* were determined spectrophotometrically on the basis of $A^{0.1\%}_{280nm} = 1.40$ cm$^2$ mg$^{-1}$ (Jaenicke & Knof, 1968). Enzyme activity was determined in standard buffer plus 0.74 mmol/L pyruvate and 0.2 mmol/L NADH at 366 nm in a thermostated Shimadzu UV 110-02 spectrophotometer coupled to a Servogor recorder. The specific activity of native LDH from pig muscle at 25 °C was 580 ± 35 IU/mg.

*Acid dissociation* of the native enzymes was achieved by 15-min incubation at 20 °C after 1:10 dilution of the stock solution in 0.1 mol/L $H_3PO_4$ containing 1 mol/L $Na_2SO_4$. Under these conditions, the dissociated monomers form loose aggregates, as indicated by turbidity. These precipitated monomers redissolve immediately upon transfer to pH 7.6. Complete dissociation of the enzyme into monomers by the given procedure was proved by hybridization experiments of both isoenzymes from heart ($H_4$) and skeletal muscle ($M_4$). The rationale of this dissociation method, which gives yields of reactivation close to 100%, will be discussed under Results. *Reassociation* was initiated by 1:50 to 1:400 dilution in standard buffer at 20 °C.

*The kinetics of reactivation* were analyzed by taking aliquots at defined times, the optical assay being performed as de-

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[1] Abbreviations and symbols used: DTE, 1,4-dithioerythritol; EDTA, (ethylenedinitrilo)tetraacetic acid; *K*, equilibrium constant (2M ⇌ D); *k*, rate constant of tetramer formation; LDH, lactic dehydrogenase (EC 1.1.1.27); $H_4$ and $M_4$ refer to isoenzymes from heart and skeletal muscle, respectively; NADH, reduced nicotinamide adenine dinucleotide; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; T, D, and M, tetrameric, dimeric, and monomeric states of LDH.

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scribed. The *kinetics of reassociation* were determined by cross-linking with glutaraldehyde, separation of cross-linked particles of different molecular weight by $NaDodSO_4$–polyacrylamide gel electrophoresis, and quantification of the respective protein bands by densitometry.

*Cross-linking* of the reassociating enzyme was achieved by rapid mixing of 5- (10-) mL aliquots with 0.2- (0.4-) mL of glutaraldehyde (25% w/v). The cross-linking reaction was stopped after 2 min by adding a ~10-fold excess of solid $NaBH_4$, in this way reducing unreacted glutaraldehyde to the corresponding alcohol and stabilizing hydrolyzable imines by reduction to amines. After 20-min incubation, excess $NaBH_4$ was destroyed by lowering the pH to pH ~4 by addition of a small volume of concentrated $H_3PO_4$.

The cross-linking procedure was optimized with respect to glutaraldehyde concentration, and the rate of cross-linking was determined. Associates not covalently linked were dissociated by 10-min incubation at 100 °C in the presence of 1.7% (w/v) $NaDodSO_4$. Buffer salts and reactants were removed by exhaustive dialysis against 0.2% (w/v) $NaDodSO_4$ at room temperature. For increases in protein concentration, the solutions were lyophilized and redissolved in a small volume of water (0.2–1.0 mL) containing 50 mmol/L DTE. Samples were heated (2 min at 100 °C) in the presence of the SH reagent to avoid artifacts due to disulfide bridges.

*$NaDodSO_4$–polyacrylamide gel electrophoresis* was performed with standard buffer containing 0.1% (w/v) $NaDodSO_4$ as the reservoir buffer; the upper gel contained 5% total acrylamide concentration ($T$), 30% of which was bis(acrylamide) ($C$) (Hjertén & Mosbach, 1962) in standard buffer plus 0.1% (w/v) $NaDodSO_4$, and the running gel 6.5% $T$ and 3.2% $C$ in 0.4 mol/L sodium phosphate buffer, pH 7.6, plus 1 mmol/L EDTA and 0.2% (w/v) $NaDodSO_4$.

Electrophoresis was performed for ~7 h with 5–10 mA/tube in 10-cm tubes (i.d. 0.5 cm). Samples of 50–200 μL were applied; their density was increased by addition of sucrose. Staining and fixation of the protein bands were achieved by 24-h incubation in a 45:10:45 mixture of methanol, acetic acid, and water containing 0.2% (w/v) Serva-blue with subsequent destaining at 50 °C in a 25:10:65 mixture of methanol, acetic acid, and water. Scanning of the gels was performed at 560 nm in a Gilford 2400 S spectrophotometer equipped with a gel scanning device. The peak areas, as determined by planimetry, were found to be proportional to protein concentration over a wide range of protein concentrations (Hermann, 1979). Each run was calibrated with appropriate proteins of known molecular weight.

*Polyacrylamide gel electrophoresis of the native isoenzymes* before and after hybridization was performed according to Davis (1964) with 0.1 mol/L Tris-HCl buffer, pH 8.6, as the reservoir buffer (circulated by a peristaltic pump to maintain a constant pH during electrophoresis) and 0.2 mol/L Tris-HCl, pH 8.6, as the gel buffer. The gels consisted of 6.5% $T$ and 3.2% $C$.

## Results

*Optimization of the Dissociation–Reassociation Process.* In a first series of experiments, denaturation conditions providing maximum recovery of enzyme activity were investigated. High yields of reconstitution are a necessary prerequisite for the analysis of association by cross-linking since large amounts of aggregates might perturb the particle distribution. It is known that the yield of reactivation of oligomeric enzymes is determined by the kinetic competition of folding and aggregation (Jaenicke & Rudolph, 1977; Zettlmeissl et al., 1979). One should, therefore, expect the yield of reactivation to be increased by stabilizing the enzyme in its dissociated state close to its native backbone structure. Addition of a stabilizing salt, e.g., $Na_2SO_4$, during dissociation increases the yield of reactivation (Table I). In the presence of 1 mol/L $Na_2SO_4$, the dissociated monomers form a precipitate which is instantaneously redissolved to monomers upon transfer to reconstitution conditions. The dissociation under the given conditions is proven by hybridization experiments; reconstitution after simultaneous dissociation of LDH-$H_4$ and LDH-$M_4$ gives the hybrid pattern expected for complete dissociation (Figure 1A). Reconstitution of separately dissociated isoenzymes by rapid mixing with standard buffer (using two coupled syringes) yields an identical hybrid pattern (Figure 1B). This strongly indicates that rapid dissociation–association under dissociating conditions of species other than monomers is not responsible for the observed hybridization. Slight deviations of the hybridization patterns from binomial distribution are caused by a strong dependency of hybridization on the reconstitution conditions (R. Hermann, unpublished results).

Table I: Yield of Reactivation of Porcine Lactate Dehydrogenase (LDH-$M_4$) after Acid Dissociation in the Presence of Varying Amounts of $Na_2SO_4$ [a]

| $[Na_2SO_4]$ (mol/L) | reactivation yield (%) |
|---|---|
| 1.0 | 83 |
| 0.8 | 65 |
| 0.6 | 46 |
| 0.4 | 40 |
| 0.1 | 18 |
| 0.0 | 18 |

[a] Reactivation in standard buffer after 15-min incubation at 20 °C in 0.1 mol/L $H_3PO_4$ in the presence of the given amounts of salt; $C_{LDH}$ = 5.8 μg/mL.

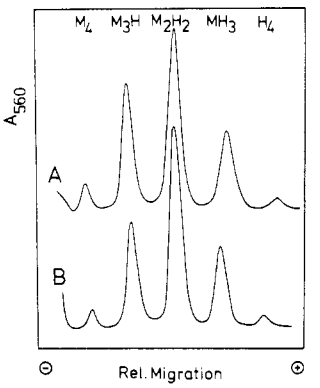


FIGURE 1: Determination of the state of dissociation of lactic dehydrogenase from pig muscle by hybridization with the heart isoenzyme. Dissociation by 15-min incubation at 20 °C in 0.1 mol/L $H_3PO_4$ plus 1.0 mol/L $Na_2SO_4$ and reconstitution by dilution in standard buffer at 20 °C after joint incubation of both isoenzymes in the dissociated state (A) or after rapid mixing of the dissociated isoenzymes immediately prior to reconstitution (B). Separation of the hybrids formed upon reconstitution by polyacrylamide gel electrophoresis.

*Optimization of the Cross-Linking Procedure.* Cross-linking by glutaraldehyde as a kinetic tool for the analysis of the association of oligomeric proteins has to satisfy three requirements: (1) intramolecular (intersubunit) cross-linking of associated particles must be quantitative; (2) intermolecular cross-linking of particles not in direct contact must be negligible; (3) intramolecular cross-linking must be fast compared to the association reaction under consideration.

For each oligomeric system, the cross-linking conditions have to be specifically optimized to satisfy the given requirements

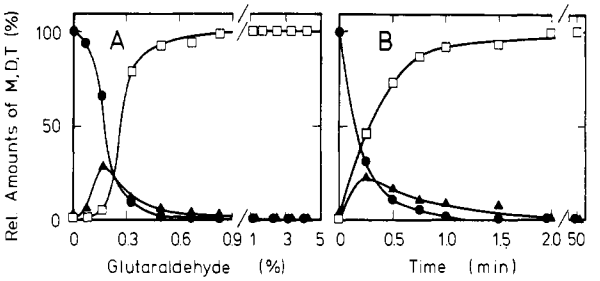


FIGURE 2: Cross-linking of native LDH from pig muscle by glutaraldehyde at $C_{LDH}$ = 10 μg/mL in standard buffer at 20 °C. The relative amount of monomers (●), dimers (▲), and tetramers (□) was determined by densitometry after $NaDodSO_4$–polyacrylamide gel electrophoresis. Minute amounts of octamers (resulting from intermolecular cross-linking at high glutaraldehyde concentrations) are included in the amount of tetramers. (A) Cross-linking of the native enzyme by 2-min incubation in the presence of increasing amounts of glutaraldehyde. (B) Cross-linking kinetics at a glutaraldehyde concentration of 0.8% (w/v).

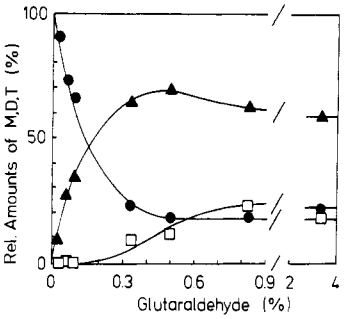


FIGURE 3: Influence of glutaraldehyde concentration on the particle distribution determined by cross-linking of reassociating LDH from pig muscle. Aliquots of the reconstituting enzyme were simultaneously cross-linked (2 min at 20 °C, $C_{LDH}$ = 5.5 μg/mL) by addition of varying amounts of glutaraldehyde 2 min after initiation of reconstitution. Symbols are the same as those given in Figure 2.

(e.g., by variation of the relative glutaraldehyde concentration, etc.). As a first approach, optimization was performed by cross-linking the native enzyme. Incomplete or excessive cross-linking of native tetrameric LDH can easily be determined by $NaDodSO_4$–polyacrylamide gel electrophoresis. Quantitative cross-linking of the tetramers (without dissociation products or higher aggregates) is achieved after 2-min incubation in the presence of ≥0.8% (w/v) glutaraldehyde (Figure 2A). In the presence of 0.8% (w/v) glutaraldehyde, the reaction is finished after 1.5 min (Figure 2B).

Quantitative cross-linking of the native enzyme does not necessarily imply that intermediates of association, which may be less stable than the native enzyme, are quantitatively cross-linked as well. The changes in polarity and dielectric constant of the buffer induced by the addition of glutaraldehyde may cause dissociation of the dimeric intermediate before cross-linking can occur. In the present experiments, this possible artifact was excluded by simultaneous cross-linking of the reassociating enzyme 2 min after initiation of its reconstitution with variable amounts of glutaraldehyde. The relative proportions of monomers, dimers, and tetramers observed in the reconstitution solution are not significantly changed at glutaraldehyde concentrations > 0.8% (w/v); at higher glutaraldehyde concentrations, a slight decrease in the amount of dimers was observed (Figure 3).

Optimum cross-linking conditions used in the subsequent kinetic analysis of the reconstituting enzyme are 2-min incubation at 20 °C in the presence of 1.0% (w/v) glutaraldehyde (cf. Figure 6). Quantitative cross-linking of native lactic dehydrogenase under these conditions is demonstrated in the

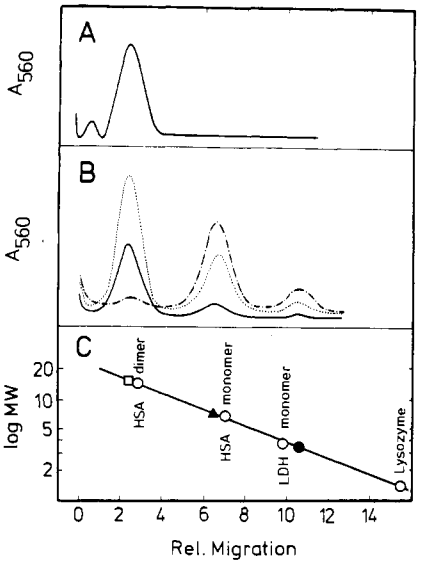


FIGURE 4: Band pattern and molecular weight determination of the products obtained by cross-linking of native and reconstituting LDH from pig muscle by $NaDodSO_4$–polyacrylamide gel electrophoresis. Cross-linking at $C_{LDH}$ = 2.8 μg/mL was performed at 20 °C by 2-min incubation in the presence of 1.0% (w/v) glutaraldehyde. (A) Cross-linking of native enzyme. (B) Cross-linking of reconstituting enzyme 30 s (–·), 25 min (···), and 24 h (—) after initiation of reconstitution. (C) Determination of the molecular weight of the cross-linking products by comparison with proteins of known molecular weight. For the cross-linking products of LDH observed in (A) and (B), the following molecular weights were estimated: tetramers, 150 000 ± 15 000; dimers, 70 000 ± 7000; monomers, 35 000 ± 3500. Symbols are the same as those given in Figure 2.

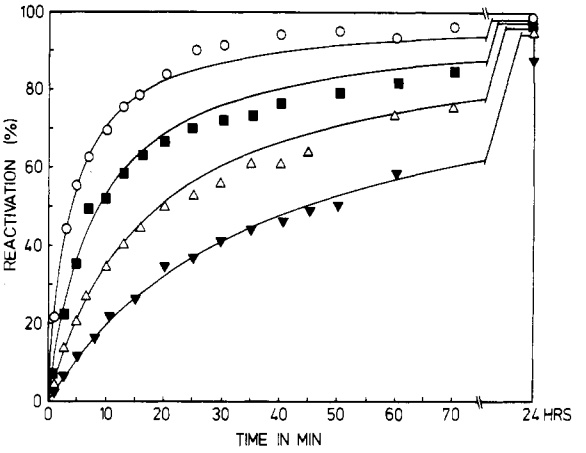


FIGURE 5: Kinetics of reactivation of LDH from pig muscle after acid dissociation. Reactivation at 20 °C by dilution in standard buffer after 15-min incubation (20 °C) in 0.1 mol/L $H_3PO_4$ in the presence of 1.0 mol/L $Na_2SO_4$ at $C_{LDH}$ = 0.56 mg/mL. Reactivation was performed at the following enzyme concentrations (μg/mL): 1.39 (▼), 2.78 (△), 5.55 (■), and 11.1 (○). Full lines are calculated for tetramer formation according to eq 6.

densitogram shown in Figure 4A. Cross-linking of reconstituting enzyme after various times of reconstitution reveals three bands on $NaDodSO_4$–polyacrylamide gels which are identified as monomers, dimers, and tetramers (Figure 4B,C). Trimers are not detectable.

*Kinetic Analysis of Reactivation and Reassociation.* The combined use of fast cross-linking with glutaraldehyde and subsequent $NaDodSO_4$–polyacrylamide gel electrophoresis allows the quantitative determination of the kinetics of association of oligomeric enzymes (Hermann et al., 1979). The parallel determination of the reactivation kinetics is expected to reveal whether intermediates of association possess catalytic activity.

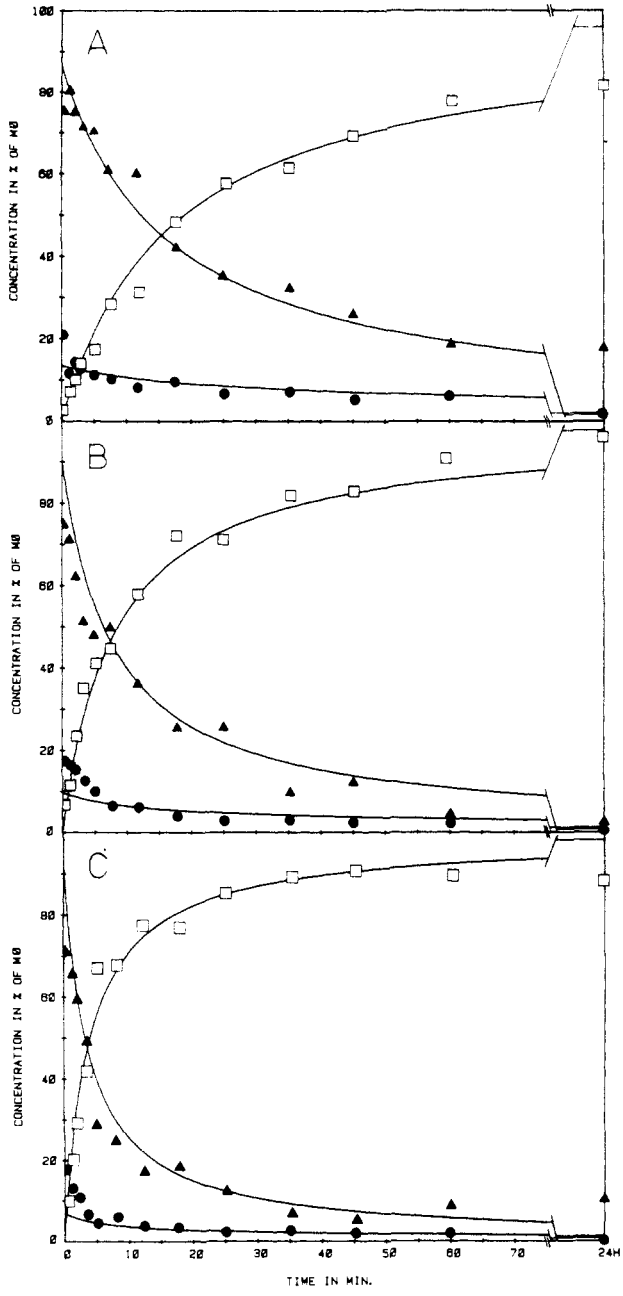


FIGURE 6: Kinetics of reassociation of LDH from pig muscle as determined by cross-linking with glutaraldehyde and subsequent $NaDodSO_4$–polyacrylamide gel electrophoresis. Reconstitution procedure as in Figure 5, cross-linking as described in Figure 4, and symbols as given in Figure 2. Reassociation was determined at the following enzyme concentrations (μg/mL): 2.78 (A), 5.55 (B), and 11.1 (C). The full lines are calculated for a tetramer (T), dimer (D), and monomer (M) ratio by using eq 3, 4, and 6 with $K = 3 \times 10^8$ L mol$^{-1}$ and $k = 3.15 \times 10^4$ L mol$^{-1}$ s$^{-1}$.

The rate of the presently determined reactivation kinetics of LDH displays the same concentration dependence which was previously described by a simple second-order reaction [Figure 5; for comparison, see Rudolph & Jaenicke (1976)].

Reassociation, as determined by the cross-linking and $NaDodSO_4$–polyacrylamide gel electrophoresis method, shows a complex association pattern (Figure 6). Qualitative inspection of the kinetic traces proves tetramerization to parallel reactivation, with an identical concentration dependence of the rate in both cases. Since the reaction starts with the homogeneous monomer, it is obvious that 70–80% of these monomers form dimers in a fast reaction before tetramerization takes place. Dimerization does not go to completion since a certain amount of monomers is observed over the whole

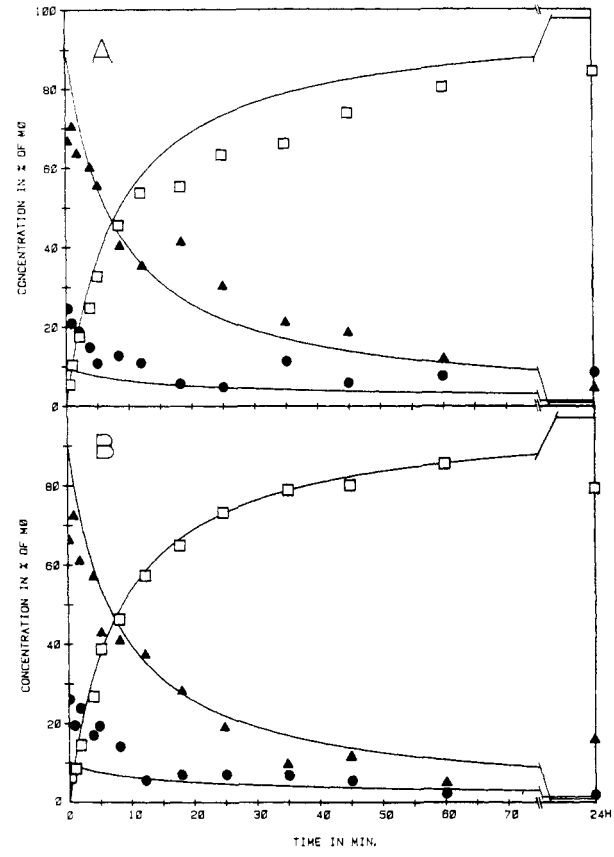


FIGURE 7: Determination of the kinetics of reassociation of LDH from pig muscle by cross-linking with various concentrations of glutaraldehyde. Reconstitution at $C_{LDH} = 5.55$ μg/mL as described in Figure 5 (symbols as given in Figure 2); cross-linking as described in Figure 3, except for the following variations in glutaraldehyde concentration in percent (w/v): 0.49 (A) and 1.85 (B). The full lines are the same as those in Figure 6B.

reassociation process. This complex kinetic behavior has two possible explanations: first, the cross-linking of the dimeric intermediates could simply be incomplete; second, the dimers and monomers could be in a fast dissociation–association equilibrium during reconstitution. As mentioned, the first alternative can be excluded on the basis of the previous optimization experiments. Additional evidence comes from association kinetics determined in the presence of various concentrations of glutaraldehyde (Figure 7). Since the monomer–dimer ratio is not significantly affected by a variation of the glutaraldehyde concentration, incomplete cross-linking of dimers can clearly be excluded.[2] The fact that hybridization studies of the reassociation reaction yield the same monomer–dimer ratio points in the same direction (R. Hermann, unpublished results). When the second alternative is considered, the most simple kinetic mechanism would consist of a fast association–dissociation equilibrium of the monomers and dimers, followed by their rate-determining association to tetramers, according to

$$4M \overset{K}{\rightleftarrows} 2D \overset{k}{\longrightarrow} T \qquad (1)$$

In this scheme, M, D, and T represent monomers, dimers, and tetramers, respectively; $K$ symbolizes the equilibrium constant of the monomer–dimer equilibrium and $k$ the rate-limiting

[2] At higher glutaraldehyde concentrations, slight perturbations in the monomer–dimer ratio are observed, without detectable effects on the fixation of the tetramers.

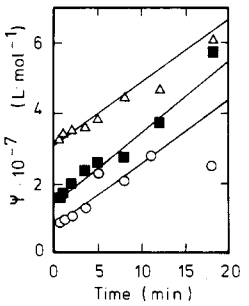


FIGURE 8: Determination of the association constant $k$, as defined in the kinetic model (eq 1), by linearization of the tetramerization data given in Figure 6 according to eq 6. Symbols △, ■, and ○ refer to tetramer formation depicted in parts A, B, and C, respectively, of Figure 6.

association of the dimers to native tetramers. For this model, the rate expression for the formation of [T] is

$$\frac{d[T]}{dt} = \frac{k}{2}[D]^2 \tag{2}$$

Combining the equilibrium constant

$$K = [D]/[M]^2 \tag{3}$$

with the equation providing mass conservation

$$[M]_0 = [M] + 2[D] + 4[T] \tag{4}$$

the reaction rate can be rewritten as

$$\frac{d[T]}{dt} = \frac{k}{2}\left(\sqrt{\frac{1}{2}[M]_0 - \frac{1}{16K} - 2[T]} - \frac{1}{4\sqrt{K}}\right)^4 \tag{5}$$

After separation of variables, integration of eq 5 (making use of the fact that [T] = 0 for $t$ = 0) gives

$$\psi = kt + C \tag{6}$$

with

$$\psi = \frac{3\sqrt{\frac{1}{2}[M]_0 + \frac{1}{16K} - 2[T]} - \frac{1}{4\sqrt{K}}}{3\left(\sqrt{\frac{1}{2}[M]_0 + \frac{1}{16K} - 2[T]} - \frac{1}{4\sqrt{K}}\right)^3} \tag{7}$$

and

$$C = \frac{3\sqrt{\frac{1}{2}[M]_0 + \frac{1}{16K}} - \frac{1}{4\sqrt{K}}}{3\left(\sqrt{\frac{1}{2}[M]_0 + \frac{1}{16K}} - \frac{1}{4\sqrt{K}}\right)^3} \tag{8}$$

Since $C$ is a constant, the rate constant for the tetramer formation can be determined according to eq 6 from the slope of a $\psi$ vs. $t$ plot.

The determination of $k$ from the tetramerization data yields $k = (3.15 \pm 0.15) \times 10^4$ L mol$^{-1}$ s$^{-1}$ (Figure 8). According to eq 3, the equilibrium constant $K$ can be calculated from the observed concentrations of M and D during the whole process of reconstitution. Determination of $K$ from the monomer–dimer ratio at the reactivation half-time yields $K = (3 \pm 1) \times 10^8$ L mol$^{-1}$.

With the constants $k$ and $K$, the kinetics of tetramerization can be calculated by using eq 6; correspondingly, the amount of M and D can be determined from eq 3 and 4. The distribution of monomers, dimers, and tetramers, computed according to the kinetic model (eq 1), is given by the full lines in Figures 6 and 7. Comparison of the calculated relaxation curves with the experimental data shows excellent agreement, thus corroborating the proposed kinetic model. Reactivation shows a perfect agreement with the curves calculated for tetramer formation (cf. Figure 5). Since reactivation is found to parallel tetramer formation, the dimeric intermediates as well as the folded monomers cannot be enzymatically active to any appreciable extent.

## Discussion

Chemical cross-linking has become a ubiquitous tool in protein chemistry, its application including protein topography (Fasold et al., 1971; Wold, 1972), conformational transitions (Enns & Chan, 1978; Klemes & Citri, 1980), stoichiometry of subunits in oligomeric proteins (Davies & Stark, 1970) and their spatial arrangement (Bickle et al., 1972; Hucho et al., 1975; Hajdu et al., 1976; Friedrich et al., 1979), and fixation of proteins in the crystalline state (Quiocho & Richards, 1964), among others. In a previous paper, chemical cross-linking by glutaraldehyde was introduced as a new approach in studying the association kinetics of oligomeric systems (Hermann et al., 1979). In the present study, this method has been improved and applied to the association of lactic dehydrogenase from porcine skeletal muscle after acid dissociation.

Lactic dehydrogenase was chosen as a model system since this enzyme has been thoroughly studied with respect to its amino acid sequence (Eventoff et al., 1977) and backbone and quaternary structure (Holbrook et al., 1975) as well as its reactivation behavior (Anderson & Weber, 1966; Levitzki, 1972; Jaenicke, 1978; Jaenicke & Rudolph, 1980).

The enzyme is composed of four subunits in tetrahedral array. The intersubunit contact areas show strong pairwise interactions, suggesting a "dimer of dimers" structure (Holbrook et al., 1975). Previous reactivation studies using the porcine isoenzymes aimed at the kinetic analysis of the reconstitution with special emphasis on the effects of temperature, pressure, coenzymes, and the state of dissociation (Rudolph & Jaenicke, 1976; Rudolph et al., 1977a,b; Schade et al., 1980a,b; Jaenicke et al., 1981). Association was found to be rate limiting in the process of reactivation. Whether this association (which is obligatory for enzymatic activity) belongs to the dimerization of monomers or dimers could not be unequivocally decided.

Because of the aggregation reaction competing with reconstitution, reassociation of LDH cannot be determined by conventional methods such as laser light scattering (Zettlmeissl et al., 1979). The present cross-linking method is far less sensitive to perturbations by aggregation.

Optimum recovery of activity was obtained by acid dissociation in the presence of 1 mol/L $Na_2SO_4$. Complete dissociation of the enzyme (LDH-$M_4$) under these conditions was demonstrated by hybridization (with LDH-$H_4$). Addition of the stabilizing salt is supposed to protect elements of native backbone structure within the acid-dissociated monomers. That this holds true may be deduced from the fact that under the given conditions the yield of reconstitution is close to 100%. Extensive unfolding, e.g., by denaturation and dissociation in guanidine hydrochloride, leads to complications in the reconstitution characteristics due to additional slow folding reactions of the isolated chains with concomitant reduction of the yield of reactivation (G. Zettlmeissl, unpublished results).

Since native LDH does not show concentration-dependent dissociation at concentrations as low as 0.1 μg/mL (Bartholmes et al., 1973), the cross-linking procedure was tested

by using native LDH-$M_4$. This is expected to yield *one* band after $NaDodSO_4$–polyacrylamide gel electrophoresis, showing the migration characteristics of a protein of $M_r \sim 140\,000$. In fact, this is found to be true after 2-min incubation in the presence of ~1% (w/v) glutaraldehyde. When the time required for complete cross-linking is compared with the rate of reconstitution in the given range of enzyme concentrations, this "fixation time" turns out to be negligible. Thus, glutaraldehyde may be assumed to be suitable to study protein association.[3]

Cross-linking of reconstituting LDH-$M_4$ by glutaraldehyde yields monomers, dimers, and tetramers. Above a critical concentration, the relative proportions of the three species were only insignificantly affected by the concentration of the bifunctional reagent. Therefore, the observed amount of dimers may be taken to represent the actual dimer concentration during reconstitution. No trimeric intermediates were observed, in accordance with previous hybridization experiments (Levitzki, 1972; Tenenbaum-Bayer & Levitzki, 1976). The instability of trimeric intermediates can be visualized on the basis of the "dimer of dimers" structure of the enzyme deduced from the X-ray structure (Holbrook et al., 1975). In the trimer, two subunits are expected to be joined by relatively strong interactions, while the third subunit which is only loosely attached would be prone to dissociation.

The reactivation rate after acid dissociation in the presence of $Na_2SO_4$ shows the same dependence on enzyme concentration as observed after acid dissociation in the presence of glycine. This has been previously described by a simple bimolecular reaction (Rudolph & Jaenicke, 1976; Jaenicke & Rudolph, 1980). Analysis by chemical cross-linking reveals a more complex kinetic pattern, with dimers and monomers present during the whole reconstitution process. Both monomers and dimers disappear upon tetramer formation, which in turn coincides with reactivation.

The most plausible kinetic model to fit the observed association pattern consists of a fast dissociation–association equilibrium comprising the monomer and the dimer, followed by the rate-limiting association of the dimer to the tetramer. Relaxation curves for the changes in monomer, dimer, and tetramer concentration, computed for an equilibrium constant $K = 3 \times 10^8$ L mol$^{-1}$ and a rate constant $k = 3.15 \times 10^4$ L mol$^{-1}$ s$^{-1}$, show a striking agreement with the experimental data. Slight deviations of the initial monomer and dimer concentrations from the theoretical curves (cf. Figure 6) may be caused by additional folding reactions of the monomers influencing the initial dimer formation. On the other hand, the association of the monomers cannot be as fast as assumed in the present model which implies instantaneous equilibration although the monomer to dimer association of LDH turns out to be much faster than the subsequent dimerization of the dimers. Its rate constant can be guessed from the equilibrium constant $K$. When 100–1 s is assumed as a reasonable range for the half-time of the dimer dissociation, $(2 \times 10^6)$–$(2 \times 10^8)$ L mol$^{-1}$ s$^{-1}$ is an estimate for the rate constant of the monomer association reaction. Obviously, this figure is close to the value calculated for diffusion-controlled association. For comparable molecules, association rates ranging from $10^5$ to $10^9$ L mol$^{-1}$ s$^{-1}$ have been calculated, depending on the respective steric requirements (Gutfreund, 1972; Osborn & Hollaway, 1974; Bothwell & Schachman, 1974). The high rate of the monomer association may be determined by relatively unspecific geometrical restrictions of this process. This conclusion is supported by the observed dissociation–association equilibrium, which suggests that the dimers only form a loose complex.

The dimeric intermediate must be enzymatically inactive since reactivation occurs during the final association of dimers to tetramers. Tetramer formation seems to require a higher geometrical specificity for productive dimer collisions. This process is characterized by a rather high energy of activation of 242 kJ mol$^{-1}$ (Rudolph et al., 1977a).

Rate constants for association reactions may cover a wide range, as demonstrated by the fast monomer to dimer and the slow dimer to tetramer association of LDH. The rates cannot be correlated with the size of the associating particles since the *monomer* association of procine muscle mitochondrial malic dehydrogenase has a reaction velocity similar to *dimer* association of lactic dehydrogenase (Jaenicke et al., 1979). *Monomer* association of triosephosphate isomerase, on the other hand, was found to be very fast, with a rate constant close to the value expected for a diffusion-controlled reaction (Zabori et al., 1980). In all three cases, the subunit molecular weights are of the same order of magnitude.

As demonstrated by the present study, the analysis of association processes by chemical cross-linking may be successfully applied to the elucidation of the complete association mechanism of a tetrameric enzyme like lactice dehydrogenase. The method seems highly promising in the analysis of the association characteristics of multicomponent systems of higher complexity.

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[3] Analogous experiments using dimethylsuberimidate instead of glutaraldehyde were unsuccessful (Vogel, 1980); similarly, studies on the subunit association of creatine kinase gave only poor yields, even after long reaction times (Bickerstaff et al., 1980).

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# Purification and Characterization of *Escherichia coli* Formamidopyrimidine-DNA Glycosylase That Excises Damaged 7-Methylguanine from Deoxyribonucleic Acid†

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ABSTRACT: A DNA glycosylase that excises 7-methylguanines with alkali-opened imidazole rings (formamidopyrimidines) from DNA has been purified more than 8000-fold from *Escherichia coli* cell extracts. The enzyme does not cleave 3-methyladenine, uracil, and intact 7-methylguanine from DNA. In assays containing pyrimidine analogues like oxauracil, 2,4,6-triaminopyrimidine, 2,5,6-triamino-2-hydroxypyrimidine sulfate, formamidopyrimidine, and 5-nitroso-2,4,6-triaminopyrimidine, only the last two compounds showed end product inhibition of the enzyme. The enzyme has been named formamidopyrimidine-DNA glycosylase. It has a molecular weight of 30000 and a Stokes radius of 26.4 Å. The enzyme prefers double-stranded to single-stranded DNA and is stimulated by the presence of 0.1 M KCl in the reaction mixture.

The formation of 7-methylguanine (7-meGua)[1] adducts is the predominant reaction observed when DNA is treated with $Me_2SO_4$ or MMS (Lawley & Shah, 1972b). The presence of these adducts does not interfere with DNA replication (Prakash & Strauss, 1970). There is also no evidence of miscoding when the copolymers uridylic acid and 7-methylguanylic acid are transcribed in vitro by *Escherichia coli* RNA polymerase (Ludlum, 1970). Although the slow rate of release of 7-MeGua ($t_{1/2} \simeq 150$ h) at 37 °C and pH 7.0 (Brooks & Lawley, 1963; Lawley & Orr, 1970; Singer, 1979) had earlier been taken to indicate that enzymatic mechanisms for 7-MeGua removal are not essential (Lawley & Warren, 1976), the discovery of bacterial and animal DNA glycosylases that excise these adducts from DNA has now been reported (Laval et al., 1981; Singer & Brent, 1981; Margison & Pegg, 1981% Cathcart & Goldthwait, 1981).

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[1] Abbreviations used: MMS, methyl methanesulfonate; $Me_2SO_4$, dimethyl sulfate; AP, apurinic–apyrimidinic; FAPY, formamidopyrimidine, shortened form of 2,6-diamino-4-hydroxy-5-(*N*-methylformamido)pyrimidine; EDTA, ethylenediaminetetraacetic acid; 1 × SSC, 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); 7-MeGua, 7-methylguanine; 7-MeGuo, 7-methylguanosine; 3-MeAde, 3-methyladenine; DNA, deoxyribonucleic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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