Reassociation of Lactic Dehydrogenase from Pig Heart Studied by Cross-Linking with Glutaraldehyde

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Cross-linking with glutaraldehyde has been successfully applied in order to analyze the kinetics of reassociation of oligomeric enzymes (R. Hermann, R. Rudolph, and R. Jaenicke Nature **277**, 243−245 (1979)). In the present study the assembly of lactic dehydrogenase from pig heart is investigated using this approach.

In order to eliminate perturbations caused by excessive folding reactions, acid dissociation was performed in the presence of 0.8 M Na_2SO_4 at 0 °C. Under optimum conditions complete cross-linking of the tetrameric enzyme was achieved in less than 2 minutes.

Cross-linking during reconstitution proves the dimer to be the only intermediate of reassociation. The dimer → tetramer transition is found to be rate-limiting for both reassociation and reactivation, suggesting the tetramer to be the enzymatically active species.

The presence of monomers during reconstitution indicates that tetramer formation is preceded by a fast monomer-dimer equilibrium.

The kinetic model describing the experimental data

$$4\,M \overset{K}{\rightleftharpoons} 2\,D \overset{k}{\rightarrow} T$$
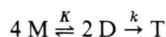
is characterized by an equilibrium constant $K = 3 \pm 1 \cdot 10^7$ liter $\cdot$ mol^{-1}, and a second-order rate constant $k = 1.4 \pm 0.2 \cdot 10^4$ liter $\cdot$ mol$^{-1} \cdot$ s^{-1}.

Introduction

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 and the aqueous environment [1]. *In vitro* reconstitution reflects the post-translational process insofar as the final product of reconstitution after intermediary dissociation is found to be indistinguishable from the initial native state [2].

Reconstitution of oligomeric enzymes implies association in addition to the previously mentioned folding process. The native quaternary structure requires both folding and association to be properly coordinated [3−5].

The investigation of association reactions with conventional methods is rendered difficult because of the potential complexity of the process of association, and perturbations caused by competing aggregation reactions [6]. Fast chemical cross-linking was therefore proposed as a tool to study association reactions of oligomeric proteins [7]. Applying this method we were able to show that the dimer → tetramer transition is rate-limiting for the reconstitution of lactic dehydrogenase from skeletal muscle; at the same time evidence was presented for a dissociation-association equilibrium between dimers and monomers [8]. Since reactivation was found to parallel tetramer formation, it was concluded that the dimeric intermediates are enzymatically inactive.

In the present study the cross-linking technique has been applied to investigate the reassociation of the heart muscle isoenzyme of lactic dehydrogenase. For this enzyme, reactivation after acid dissociation has been previously shown to be characterized by sigmoidal reactivation kinetics indicating a complex mechanism of reconstitution involving rate-determining consecutive folding and association steps [9]. Studies on various aspects of this reconstitution were unable to answer the question as to whether the rate-limiting association belongs to the formation of dimers, trimers, or tetramers [3, 4]. The present approach proves unequivocally that dimers are the only intermediates of reassociation. Their association to form the native tetramer represents the rate-

determining step for both reconstitution and reactivation. As in the case of the isoenzyme from skeletal muscle, tetramer formation is shown to be preceded by a dissociation-association equilibrium between dimers and monomers.

Materials and Methods

Substances. Lactic dehydrogenase from porcine heart muscle, lysozyme, and NADH were purchased from Boehringer, Mannheim, glutaraldehyde (purissimum, 25% (w/v), aqueous solution) from Fluka, Basel. Reagents for polyacrylamide gel-electrophoresis were obtained from Serva, Heidelberg; all other reagents (A-grade purity) from Merck, Darmstadt. Quartz bidistilled water was used throughout.

Standard buffer was 0.1 M sodium phosphate pH 7.6, containing 1 mM EDTA, saturated with N$_2$.

Stock solutions of the enzyme were prepared by dialysis at 4 °C in the presence of 1 mM dithioerythritol against standard buffer, or 0.1 M Na$_2$SO$_4$ plus 1 mM EDTA, pH 7.6.

Enzyme concentrations were determined spectrophotometrically using $A_{280 \, nm}^{0.1\%} = 1.40 \, cm^2 \cdot mg^{-1}$.

Lactic dehydrogenase activity was determined in standard buffer containing 0.74 mM pyruvate and 0.2 mM NADH. The oxidation of NADH was monitored at 366 nm using a recording Eppendorf spectrophotometer. The specific activity of the native enzyme at 25 °C was 360 ± 17 IU/mg.

Acid dissociation. Unless otherwise indicated, acid dissociation was performed by 1:10 dilution of the stock solution with 0.1 M sodium phosphate pH 2.3 containing 0.8 M Na$_2$SO$_4$. The enzyme was incubated under these conditions for 60 min at 0 °C. The dissociated monomers form loose aggregates at low pH in the presence of 0.8 M Na$_2$SO$_4$, as indicated by turbidity; upon transfer to pH 7.6 they are redissolved immediately. Hybridization with the isoenzyme from skeletal muscle proves that the enzyme is under the given conditions dissociated to the monomeric state [8].

Reconstitution of lactic dehydrogenase was initiated by 1:50 or 1:100 dilution with standard buffer at 20 °C. The kinetics of reactivation were analyzed by sampling aliquots at defined times. Reassociation was determined by cross-linking of aliquots of the reactivating enzyme with glutaraldehyde.

Cross-linking of native and reassociating lactic dehydrogenase was achieved by rapid mixing with glutaraldehyde. Unless otherwise indicated, the cross-linking reaction was quenched after 2 min by adding solid NaBH$_4$. This way the cross-linking products were stabilized by reduction of hydrolyzable imines to the corresponding amines. Excess NaBH$_4$ was destroyed by adding concentrated phosphoric acid. After addition of SDS, the samples were dialyzed against large volumes of 0.1 or 0.2% (w/v) SDS at 60 °C, in order to remove buffer salts and reactants.

To increase protein concentration, the samples were lyophilized and redissolved to a small volume, so that the protein concentration was 100–200 µg/ml. Water added to redissolve the protein contained ~ 50 µg/ml lysozyme as marker protein for the SDS polyacrylamide gel-electrophoresis.

SDS polyacrylamide gel-electrophoresis was performed as described previously [8] using proteins of known molecular weight for calibration. The following molecular weights were determined for the cross-linking products of porcine heart lactic dehydrogenase: tetramers 166 000, trimers 126 000, dimers 79 000, and monomers 33 000; range of error ± 15%.

Results and Discussion

Kinetics of reactivation

Reactivation of porcine heart lactic dehydrogenase after acid dissociation, is characterized by sigmoidal kinetic traces which have been previously described by a consecutive reaction sequence comprising first-order folding and second order association [9]. Sigmoidal reactivation after acid dissociation was confirmed in the present paper under slightly different experimental conditions (Fig. 1). Since the objective of the present study was to analyze association in the absence of additional folding reactions, a stabilizing salt was added during dissociation in order to prevent extensive unfolding of the monomers. Adding 0.8 M Na$_2$SO$_4$ during acid dissociation at 0 °C seems to preserve elements of native structure in the dissociated monomers, since it eliminates the sigmoidicity in the reactivation profiles (Fig. 1). Reactivation after dissociation under these conditions is determined by a second-order association reaction without perturbation by first-order folding, as shown by the concentration dependence of the rate (Fig. 2).

The yield of reactivation of lactic dehydrogenase is decreased by extensive unfolding, since under this
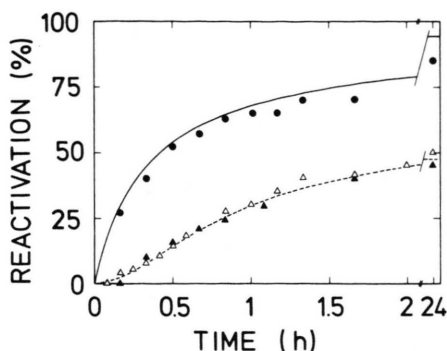
Fig. 1. Dependence of the kinetics of reactivation of por-cine heart lactic dehydrogenase (20 °C, enzyme concentra-tion 6.8 µg/ml) on the conditions of acid dissociation. Closed symbols: Reactivation by 1:50 dilution in standard buffer after 60 min of acid dissociation in 0.1 M sodium phosphate pH 2.2 plus 0.8 M Na$_2$SO$_4$ at 0 °C (●), and 37 °C (▲) respectively. Open symbols: Reactivation by 1:50 dilution in 0.2 M sodium phosphate buffer pH 7.6, after 5 min of acid dissociation in 0.2 M sodium phosphate pH 2.3 at 20 °C (△). All buffers contained 1 mM EDTA. The full line was calculated for tetramer formation accord-ing to Eqn. (2), with the rate constant and equilibrium constant determined in Fig. 5.
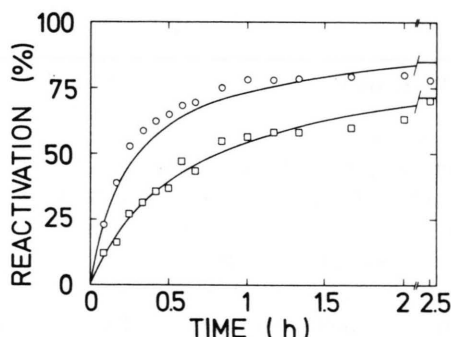

Fig. 2. Concentration dependence of the kinetics of reacti-vation of porcine heart lactic dehydrogenase after acid dissociation by 60 min incubation at pH 2.3, 0 °C, in 0.1 M sodium phosphate plus 0.8 M Na$_2$SO$_4$ plus 1 mM EDTA. Reactivation at 20 °C by dilution in standard buffer at 4.4 µg/ml (□), and 8.7 µg/ml (○) enzyme concentration. Residual Na$_2$SO$_4$ concentration was 0.04 M. Full lines were calculated for tetramer formation according to Eqn. (2) with the rate constant and equilibrium constant deter-mined in Fig. 5.

condition formation of noncovalent "wrong aggre-gates" competes with refolding [6]. On the other hand, the yield of reactivation is found to be increased if unfolding of the acid dissociated mono-mers is reduced, *e. g.* in the presence of Na$_2$SO$_4$ (Table I). The stabilizing effect of Na$_2$SO$_4$ vanishes if dissociation is performed at elevated temperature:

Table I. Yield of reactivation of porcine heart lactic de-hydrogenase after acid dissociation in the presence of varying amounts of Na$_2$SO$_4$. Reactivation (24 h, 20 °C, 6.8 µg/ml enzyme concentration) by dilution with standard buffer pH 7.6, after 60 min incubation in 0.1 M phosphate buffer pH 2.3 (0 °C) in the presence of given amounts of Na$_2$SO$_4$.

[Na$_2$SO$_4$] [mol/l]	Reactivation yield [%]
0	50
0.2	57
0.4	77
0.6	93
0.8	97
1.0	100

acid incubation at 37 °C in the presence of Na$_2$SO$_4$ results in the same sigmoidal kinetics with low yield as were previously observed after acid dissociation at low temperature, in the absence of the stabilizing salt (*cf.* Fig. 1).*

In the following cross-linking study, experimental conditions were chosen such that major unfolding of the acid dissociated monomers was prevented: 60 min incubation at pH 2.3, 0 °C, in the presence of 0.8 M Na$_2$SO$_4$. Thus a clear-cut correlation of reactivation and reassociation is rendered possible, without significant perturbations due to refolding.

Cross-linking of native lactic dehydrogenase

Chemical cross-linking with bifunctional reagents as a tool in order to study protein association has to fulfil three requirements: (i) intramolecular (inter-subunit) cross-linking of associated particles must be quantitative; (ii) intermolecular cross-linking of particles which are not in direct contact in a given quaternary structure must be negligible; (iii) intra-molecular (intersubunit) cross-linking must be fast compared to the association reaction under con-sideration.

To test whether the cross-linking capacity of glutaraldehyde satisfied these requirements, the fixation of porcine heart lactic dehydrogenase in its native tetrameric state was studied. Quantitative cross-linking of the native enzyme is achieved by

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* A similar change from hyperbolic to sigmoidal reac-tivation kinetics is observed for porcine skeletal muscle lactic dehydrogenase under slightly different conditions: hyperbolic reactivation relaxations occur after short incu-bation at acidic pH (even without Na$_2$SO$_3$ stabilization) [10]; after more drastic unfolding, *e. g.* in 6 M guani-dine · HCl, sigmoidicity is observed (G. Zettlmeißl *et al.*, in preparation).
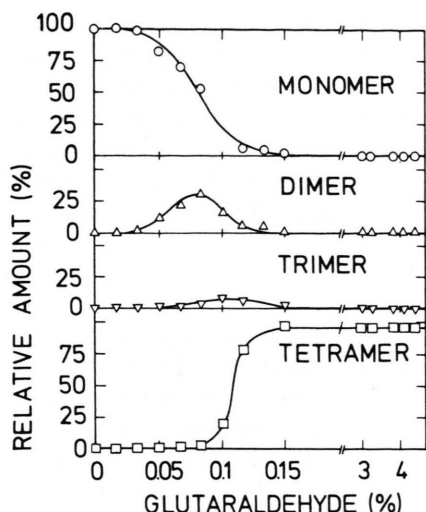
Fig. 3. Influence of glutaraldehyde concentration on the cross-linking of native porcine heart lactic dehydrogenase (enzyme concentration 4.7 μg/ml). Cross-linking at 20 °C by 2 min incubation in standard buffer, in the presence of varying amounts of glutaraldehyde. The relative amounts of monomers, dimers, trimers, and tetramers were determined by densitometry after SDS polyacrylamide gel-electrophoresis. At glutaraldehyde concentrations > 0.5% (w/v) about 4% octamers could be detected.

2 min incubation in the presence of ≧ 0.15% (w/v) glutaraldehyde (Fig. 3). Under these conditions, only minute amounts of octamers are formed as artifacts by intermolecular cross-linking. The rate of the cross-linking reaction critically depends on the glutaraldehyde concentration (Fig. 4). In the presence of 0.17% (w/v) glutaraldehyde the reaction is complete after ~ 2 min. In the presence of even higher glutaraldehyde concentrations used in the kinetic studies (see below), cross-linking should be several orders of magnitude faster than reassociation.

As illustrated in Fig. 3 and 4, glutaraldehyde is well suited for providing quantitative and unperturbed cross-linking of porcine heart lactic dehydrogenase after exceedingly short incubation. The formation of tetramers during reassociation can therefore by analyzed kinetically by trapping the enzyme in its actual quaternary structure during reconstitution. In this context one has to consider that the quantitative cross-linking of native tetramers does not a priori guarantee the unperturbed fixation of intermediates of reconstitution. As shown for the isoenzyme from porcine skeletal muscle [11], these intermediates (which supposedly consist of loose complexes) may be dissociated rather than tied

together, due to changes in the polarity of the solvent, induced by the addition of the bifunctional reagent. For this reason, each oligomeric system has to be specifically optimized regarding the respective cross-linking conditions. Quantitative glutaraldehyde fixation depends on the number and the reactivity of lysine side chains and their topography on the protein surface. Systematic studies with different enzymes have shown that general rules cannot be given. For example, porcine skeletal muscle lactic dehydrogenase requires three times as much glutaraldehyde for complete cross-linking within 2 min [8], compared to the isoenzyme from pig heart, although the latter shows a lower lysine content [12]. The closely related mitochondrial malic dehydrogenase is found to be inaccessible to complete cross-linking, while the cytoplasmatic enzyme reacts completely under conditions similar to those applied in the present experiments [13].

Kinetics of reassociation

The combined use of fast cross-linking with glutaraldehyde and subsequent SDS polyacrylamide gel-electrophoresis allows the quantitative analysis of the reassociation of oligomeric enzymes [7]. Reconstitution of porcine skeletal muscle lactic dehydrogenase which was previously investigated by a
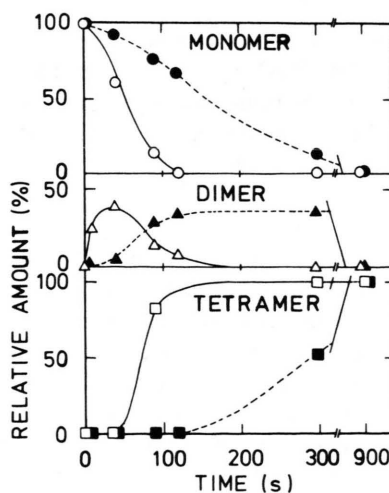

Fig. 4. Kinetics of the cross-linking of native porcine heart lactic dehydrogenase by glutaraldehyde. Cross-linking at 20 °C, 10.8 μg/ml enzyme concentration, in standard buffer; incubation during the given times in the presence of 0.08% (w/v) (closed symbols), or 0.17% (w/v) (open symbols) of glutaraldehyde, respectively.
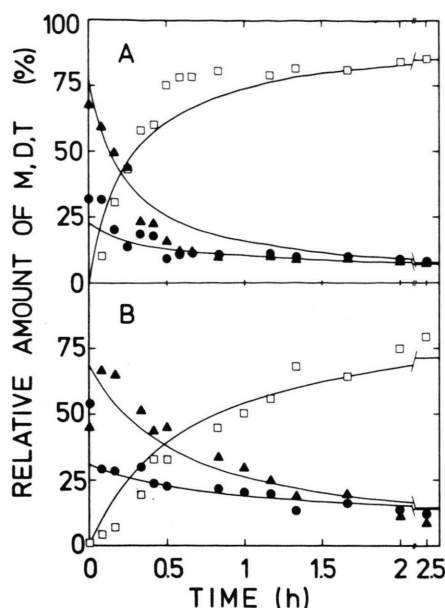
Fig. 5. Kinetics of reassociation of porcine heart lactic dehydrogenase, determined by cross-linking with glutaraldehyde during reconstitution. Reassociation at 8.7 μg/ml (A) and 4.2 μg/ml (B) enzyme concentration, by dilution in standard buffer (20 °C), after acid dissociation as described in Fig. 2. Cross-linking was performed at 20 °C by 2 min incubation in the presence of 0.67% (w/v) glutaraldehyde. Full lines are calculated for the disappearance of monomers (●) and dimers (▲), and formation of tetramers (□), according to Eqn. (2) with $K = 3 \cdot 10^7 \, \text{l} \cdot \text{mol}^{-1}$ and $k = 1.4 \times 10^4 \, \text{l} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$.

Alternative mechanisms based on incomplete dissociation of dimers, or on a heterogeneous population of monomers with respect to the reassociation characteristics, might account for the presence of dimers and monomers during reconstitution as well.

The most simple kinetic mechanism consistent with the observed reassociation pattern is as follows

$$4 \, \text{M} \overset{K}{\rightleftharpoons} 2 \, \text{D} \overset{k}{\to} \text{T}. \tag{1}$$
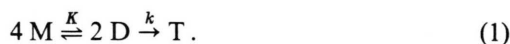
In this scheme, M, D, and T represent monomers, dimers, and tetramers, respectively; K stands for the equilibrium constant of the dimer-monomer equilibrium, and k for the rate-limiting association of dimers to tetramers. For the given kinetic model the following integrated form of the rate equation has been determined [8]:

$$\frac{3\sqrt{\dfrac{1}{2}[M_0] + \dfrac{1}{16\,K} - 2\,[T]} - \dfrac{1}{4\sqrt{K}}}{3\left(\sqrt{\dfrac{1}{2}[M_0] + \dfrac{1}{16\,K} - 2\,[T]} - \dfrac{1}{4\sqrt{K}}\right)^3} \tag{2}$$

$$= k \cdot t + \frac{3\sqrt{\dfrac{1}{2}[M_0] + \dfrac{1}{16\,K}} - \dfrac{1}{4\sqrt{K}}}{3\left(\sqrt{\dfrac{1}{2}[M_0] + \dfrac{1}{16\,K}} - \dfrac{1}{4\sqrt{K}}\right)^3}.$$

The equilibrium constant and the rate constant fitting the present data (*cf.* Fig. 5) were determined as described earlier [8]. Using $K = 3 \times 10^7$ ($\text{l} \cdot \text{mol}^{-1}$) and $k = 1.4 \times 10^4$ ($\text{l} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$) the distribution of monomers, dimers, and tetramers during reconstitution was calculated according to Eqn. (2). Comparison of the calculated relaxations with the experimental data shows satisfactory agreement thus corroborating the proposed kinetic model.

Tetramer formation runs parallel to reactivation indicating that for porcine heart lactic dehydrogenase neither the monomer nor the dimeric intermediates of reassociation show enzymatic activity.

As reported recently, cross-linking experiments with the isoenzyme from porcine skeletal muscle closely resemble the present reconstitution pattern [8]. For this isoenzyme the occurrence of inactive dimers as intermediates of reconstitution has been confirmed independently by hybridization experiments [8], as well as limited proteolysis during reassociation [14]. Similarly, the reconstitution from

variety of techniques (including cross-linking) has been quantitatively described by a kinetic model comprising a fast dissociation-association equilibrium of inactive dimeric intermediates, followed by their rate-limiting association to the active tetramer [8].

Reconstitution of porcine heart lactic dehydrogenase after acid dissociation in the presence of 0.8 M Na$_2$SO$_4$ shows a strikingly similar reassociation pattern (Fig. 5). As in the case of the isoenzyme from skeletal muscle, only dimers are found as intermediates of reconstitution; these must be formed in a rapid reaction during the transfer to reconstitution conditions. The association of the intermediary dimers into tetramers is found to be the rate-limiting step in the overall process of reconstitution. Since a certain amount of monomers is present throughout the whole reconstitution reaction, a dissociation-association equilibrium between dimers and monomers is suggested.

dimeric intermediates in the dissociation of lactic dehydrogenase has been shown to be governed by the same rate constants reported for the overall reconstitution, starting from denatured monomers, again suggesting the rate-determining step in the reassociation reaction to be the dimerization of inactive dimers [15].

Conclusions

Fast cross-linking of reconstituting oligomeric proteins by glutaraldehyde, and subsequent analysis by SDS ployacrylamide gel-electrophoresis has been shown to be an excellent method for studying the kinetics of protein self-assembly.

Reassociation of porcine heart lactic dehydrogenase which was analyzed by this method in the present study exhibits a striking similarity to the previously determined reconstitution behaviour of the isoenzyme from porcine skeletal muscle.

The dimer → tetramer association is found to be the rate-limiting step in the reconstitution of both isoenzymes; evidence from the anomalous persistence of dimers and monomers during the whole process of reconstitution suggests a dissociation-association equilibrium of the dimeric intermediates and monomers to participate in the overall reaction. The rate constant for the dimer → tetramer association determined for lactic dehydrogenase from pig heart ($k = 1.4 \times 10^4 \, 1 \cdot mol^{-1} \cdot s^{-1}$) is found to be about half the respective rate constant for the isoenzyme from skeletal muscle. The monomer → dimer association must be very fast for both isoenzymes; the respective rate constants seem to be close to the value expected for diffusion controlled reactions (*cf.* [8]). A similar fast association process was described earlier for the assembly of triosephosphate isomerase [16].

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