Biophysical Chemistry 11 (1980) 257–263

KINETICS OF RECONSTITUTION OF PORCINE MUSCLE LACTIC DEHYDROGENASE AFTER REVERSIBLE HIGH PRESSURE DISSOCIATION

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Received 20 September 1979

Porcine muscle lactic dehydrogenase can be reversibly dissociated into monomers at high hydrostatic pressure. The rate of dissociation depends on the conditions of the solvent (Schade et al., 1980, Biochemistry, in press). Maximum yields of reactivation are achieved after dissociation by 20 min incubation in 0.2 M Tris/HCl buffer or 0.2 M KCl at pH 7.6, in the presence of 10 mM dithioerythritol and 1 mM EDTA, provided that both dissociation and reassociation are performed under anaerobic conditions. At enzyme concentrations of the order of 1 μM reactivation amounts to $\geqslant$95%, the product of reactivation being indistinguishable from the enzyme in its initial native state. Based on the long-term stability of the enzyme under the optimum given conditions of reactivation, the kinetics of reconstitution after pressure release were investigated over a wide range of enzyme concentrations (1 nM $< c <$ 1 μM). The weakly sigmoidal kinetics may be described by an irreversible uni-bimolecular reaction scheme, corresponding to a sequential transconformation-association process. Assuming the protomers to be enzymatically inactive, the kinetic profiles may be fitted by one set of kinetic constants: $k_{uni} = 1.5 \times 10^{-2}$ s^{-1} and $k_{bi} = 7 \times 10^{3}$ s^{-1} M^{-1}, the association step belonging to either dimer or tetramer formation.

1. Introduction

Lactic dehydrogenase represents a stable tetrameric enzyme, the structure and enzymatic properties of which are characterized in great detail [1,2]. Therefore, the enzyme may be considered an appropriate model system for analyzing the *in vitro* association of oligomeric enzymes [3]. The specific assembly of subunits to form the native quaternary structure represents the final stage of self-organization in the process of translation, after folding and association of intrinsic globular domains within the nascent polypeptide chain. Reactivation and reassociation of porcine muscle lactic dehydrogenase after dissociation and deactivation at acid pH has been studied in great detail [4–6]. After formation of a dimeric intermediate, reactivation is parallelled by tetramer formation [6]. For acid denaturation, as for denaturation by urea or guanidine · HCl, refolding of the more or less randomized chains to a structured monomeric intermediate competes with the formation of inactive aggregates [7]. However, this side reaction can be more or less eliminated using specific dissociation-association conditions [8].

In a foregoing paper [9] it has been shown that the dissociation of porcine muscle lactic dehydrogenase under high hydrostatic pressure is completely reversible, pointing to a high degree of structure conservation within the pressure dissociated monomers. Comparing the reactivation of the product of pressure dissociation with the reactivation after dissociation by more randomizing denaturants may serve to evaluate the influence of residual structure of the isolated monomers on their reassembly. As becomes evident from the reactivation kinetics after pressure release, the reconstitution of procine muscle lactic dehydrogenase may be quantitatively described by sequential folding and association reactions involving inactive subunits. This is in agreement with previous findings with a variety of other oligomeric enzymes after acid dissociation and/or dissociation in 6 M guanidine · HCl. Contrary to these denaturants the dissociation and denaturation in the case of pressure induced deactivation is not affected by aggregation [7], in accordance with the above mentioned pressure dependent equilibrium of dissociation-association (cf. [9]).

2. Materials and methods

For *substances* and *enzyme assay* cf. [9].

2.1. Stability

Buffer solutions were thoroughly degassed and saturated with nitrogen. All operations were performed under anaerobic conditions; stock solutions were stored under nitrogen. To guarantee long term stability, especially in connection with reconstitution experiments at low concentrations, closed containers were used which were filled completely in order to minimize oxidation in the process of repeated sampling of probes.

2.2. Dissociation

High pressure experiments made use of the previously described quench cell [9] applying $p = 2$ kbar at 20°C for 20 min to provide complete dissociation. As solvents were applied: 0.2 M Tris/HCl pH 7.6 or 0.2 M KCl pH 7.6, both containing 10 mM dithioerythritol and 1 mM EDTA. During the high pressure dissociation step the enzyme concentration was 0.3 μM in all experiments.

2.3. Reactivation

Reactivation after pressure induced dissociation was investigated in a wide range of enzyme concentrations (1.5—250 nM $\hat{=}$ 0.05—9 μg/ml). To maintain a sufficiently high precision of the time and concentration dependent measurements, stock solutions (0.3 μM lactic dehydrogenase, 0.2 M Tris/HCl pH 7.6, 20°C) were incubated under standard conditions (20 min at 2 kbar); after pressure release the deactivated enzyme was removed from the autoclave, and diluted immediately with thermostated buffer to give the final concentration. From these dilutions aliquots were taken at defined time intervals, and assayed for enzymatic activity. Native enzyme solutions diluted to the same extent, and stored under identical conditions were used as references. The first assay was started within 40 seconds after pressure release.

The relaxations are corrected for the reactivation (~2%) occurring during the time between pressure release and enzyme dilution (<15 s). Computer analysis of the time and concentration dependent reactivation according to a uni-bimolecular reaction scheme [10,11] made use of final values determined after a reactivation time of up to 300 hours.

3. Results

3.1. Stability of the native enzyme

The relevance of reconstitution studies on monomeric or oligomeric enzymes depends in a critical way on their long-term stability. Therefore, the first objective of the present experiments must be the optimization of solvent conditions where denaturation does not interfere with reconstitution. It is well established for lactic dehydrogenases from various sources that decreasing enzyme concentration yields deactivation [12,13]. Previous experiments have shown that the mechanism underlying this deactivation must be complex. Only at $c < 1$ nM and after complete removal of coenzyme by charcoal treatment, irreversible dissociation can be detected. At higher concentrations (1 nM $< c < 1$ μM) chemical modification seems to be responsible for the observed deactivation [13]. The stability of the enzyme can be strongly enhanced by protection of sulfhydryl groups against oxidation upon addition of 10 mM dithioerythritol and 1 mM EDTA. A further increase in stability can be observed after degassing the buffer solutions and saturation with nitrogen; another important improvement is provided by preventing excessive air contact during the sampling of aliquots for the test assay. Using the previously mentioned precautions, the "concentration dependent deactivation" can be essentially eliminated; even at incubation periods up to 500 hours at enzyme concentrations as low as 1.4 nM, only ~25% deactivation can be observed.

3.2. High pressure deactivation and dissociation

The extent of reactivation of porcine muscle lactic dehydrogenase is determined by the parameters of the solution in the process of both deactivation and reactivation, hence the solvent conditions for pressure deactivation had to be optimized as well.

As previously shown [9], the rate and extent of high pressure dissociation of lactic dehydrogenase

strongly depends on the presence of coenzyme or specific ions. In 0.2 M Tris/HCl pH 7.6, complete deactivation of the apo-enzyme can be observed after 20 min incubation at 2 kbar and 20°C. This deactivation is parallelled by dissociation into monomers, as shown by crosslinking experiments and gel electrophoresis [9]. Long-term stability of these monomers at high pressure, as determined by the reversibility of deactivation, is enhanced by the addition of 10 mM dithioerythritol and 1 mM EDTA. Reactivation of porcine muscle lactic dehydrogenase after 20 min incubation at 2 kbar under these conditions exceeds 95%. Prolonged treatment at the given pressure (>1 h) leads to a decrease of the yield of reactivation, eventually caused by aggregation [14].

Although the dissociation equilibrium at 1 kbar is shifted almost completely to the inactive monomer [9], the rate of dissociation of the apoenzyme is very slow at this pressure ($\tau_{1/2}$ = 1.4 h). Long-term incubation of up to 120 h does not cause any appreciable irreversible deactivation under this condition, and at least 95% reactivation can be obtained applying an enzyme concentration of 0.3 μM.

To ensure complete dissociation at a reasonably fast rate with minimum perturbation by irreversible loss of activity, dissociation was achieved by 20 min incubation at 2 kbar in the subsequent reactivation studies.

3.3. Yield of reactivation

Applying the precautions given in the foregoing paragraphs, sufficiently high reactivation yields could be obtained in the concentration range under consideration (1 nM < c < 1 μM). The concentration dependence of the yield of reactivation at the given concentrations is illustrated in fig. 1. Under the given conditions of optimum stability, and at concentrations as low as 1.4 nM, the yield of reactivation after pressure deactivation could be raised to ~65% (relative to the not inactivated native enzyme under identical conditions). In contrast to the concentration dependence of the reactivation after acid denaturation [4,5] the yield increases continuously over the concentration range without showing an optimum profile. Most probably the side-reaction due to oxidation becomes insignificant at high concentrations of the enzyme, were yields close to 100% can be observed. These high yields are to be expected, since the pressure induced

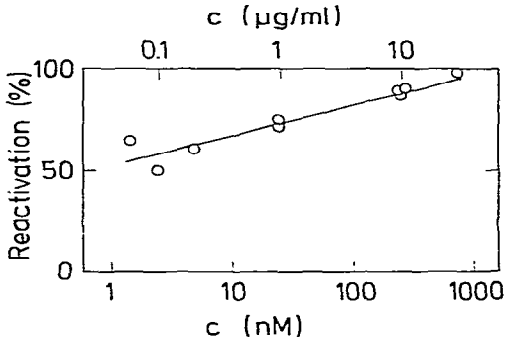

Fig. 1. Yield of reactivation of porcine muscle lactic dehydrogenase as a function of enzyme concentration. Reactivation in 0.2 M Tris/HCl buffer pH 7.6, 10 mM dithioerythritol, 1 mM EDTA at 20°C after deactivation by 20 min incubation at 2 kbar.

dissociation of lactic dehydrogenase is a true equilibrium transition [9], not superimposed by preferential aggregation, as observed for the dissociation of this enzyme by such denaturants as hydrogen ions, guanidine · HCl, or urea.

3.4. Characterization of reconstituted enzyme

A necessary requirement for kinetic studies connected with the correlation of folding and association of oligomeric enzymes after dissociation and deactivation in a given denaturant is the reconstitution of the unperturbed *native* quaternary structure. As shown in table 1 the final product of renaturation and the enzyme in its initial native state may be compared directly using available physico-chemical and enzymological criteria. No significant differences can be detected; the reactivated enzyme is homogeneous re-

Table 1
Comparison of native porcine muscle lactic dehydrogenase with the reconstituted enzyme after deactivation by 20 min incubation at 2 kbar, c = 0.87 μM. Reactivation at 20°C in 0.2 M Tris/HCl buffer pH 7.6, 10 mM dithioerythritol, 1 mM EDTA. Reactivation yield: 80%.

State of the enzyme	$s_{20,w}$ (S)	$M_{s,D}$	$K_{M,pyruvate}$ (μM)
native	7.49 ± 0.06	140 000 ± 4000	250 ± 40
reconstituted	7.54 ± 0.06	142 000 ± 4000	245 ± 40

garding its tetrameric state.

3.5. Kinetics of reactivation

The optimization of the reconstitution conditions yields a reactivation of >65% even at extreme dilution; this permits kinetic studies in a wide range of enzyme concentrations. Figs. 2 and 3 illustrate the respective reactivation kinetics in 0.2 M Tris/HCl pH 7.6 and 0.2 M KCl, respectively. The patterns in both solvents turn out to be identical.

A double logarithmic plot of the initial velocities versus concentrations shows at lower concentrations the linear dependence with a slope of 2, characteristic for a bimolecular rate-limiting process (fig. 4). At higher concentrations deviations from the linear behaviour are observed. The decrease of the slope (i.e. of the reaction order) indicates complex kinetics with additional rate determining first order processes. As a result the reactivation relaxations are found to be slightly sigmoidal (figs. 2 and 3). Assuming an irreversible sequential uni – bimolecular reaction mechanism implying inactive subunits [3], one single set of rate constants k_{uni} and k_{bi} is found to be sufficient to quantitatively fit the observed time course in the whole range of enzyme concentrations [‡]:

$$k_{uni} = 1.5 \times 10^{-2}\,s^{-1}, \qquad k_{bi} = 7 \times 10^{3}\,s^{-1}\,M^{-1}.$$

The calculated full lines in figs. 2, 3, and 4 illustrate the quality of the fit. The value for k_{bi} is in fair agreement with the result of earlier reconstitution experiments after acid dissociation and deactivation [5]. However, the experimental conditions in the former set of data did not allow to detect the weak sigmoidicity of the kinetic profiles which is obvious in the present high pressure study: therefore, in previous work a simple bimolecular mechanism was considered sufficient to describe the results.

A similar description of the observed reactivation kinetics may be accomplished by a mechanism comprising a bimolecular association reaction followed by a unimolecular step. A distinction between these two alternatives should be possible based on the analysis of association intermediates by chemical crosslinking and subsequent gel-electrophoresis [6].

Since the experimental setup does not allow the determination of fast reactions, measurements cannot

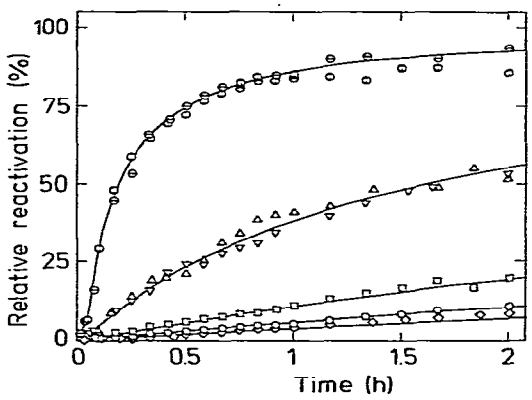

Fig. 2. Kinetics of reactivation of porcine muscle lactic dehydrogenase after deactivation by 20 min incubation at 2 kbar. Reactivation at 20°C in 0.2 M Tris/HCl buffer pH 7.6, 10 mM dithioerythritol, 1 mM EDTA at varying enzyme concentrations (nM): (⊖, ⊙) 290; (△, ▽) 29; (□) 6; (○) 3; (◇) 1.5. Solid lines are calculated according to an irreversible uni-bimolecular mechanism with $k_{uni} = 1.5 \times 10^{-2}\,s^{-1}$ and $k_{bi} = 7 \times 10^{3}\,s^{-1}M^{-1}$.

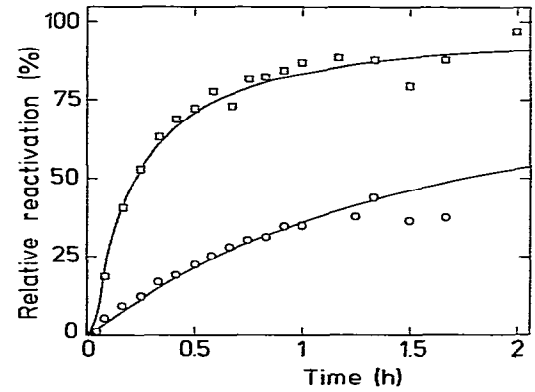

Fig. 3. Kinetics of reactivation of porcine muscle lactic dehydrogenase after deactivation by 20 min incubation at 2 kbar. Reactivation at 20°C in 0.2 M KCl pH 7.6, 10 mM dithioerythritol, 1 mM EDTA; enzyme concentrations were 290 nM (□) and 29 nM (○). Solid lines are calculated according to an irreversible uni-bimolecular mechanism with the rate constants derived from the reactivation kinetics in 0.2 M Tris/HCl buffer (cf. fig. 2).

[‡] Due to ambiguities in the determination of the final values of reactivation at low enzyme concentrations, systematic deviations from the given rate constants cannot be excluded. They may very well reduce the deviations from the simple second order mechanism [5].

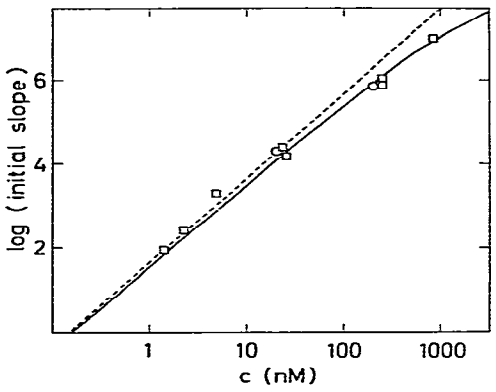

Fig. 4. Reaction order of the reactivation of porcine muscle lactic dehydrogenase calculated from the regain of activity after 5 min. Deactivation and reactivation were as described in fig. 2 (□) or fig. 3 (○). Dotted line: Theoretical line for a simple bimolecular reaction; full line: Calculated for an irreversible uni-bimolecular mechanism with the rate constants given in fig. 2.

be extended to higher concentrations where the deviations from second order are expected to become important. In the accessible concentration range the deviations from second order are just outside the range of experimental error.

Upon incubation at 1 kbar a dissociation-association

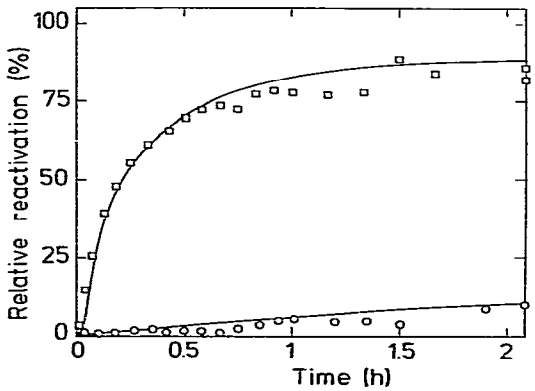

Fig. 5. Kinetics of reactivation of porcine muscle lactic dehydrogenase after partial deactivation (10% residual activity) by 24 h equilibration at 1 kbar. Enzyme concentrations were 260 nM (□) and 2.6 nM (○); relaxations are corrected for the residual activity at zero time. Solid lines are calculated for an irreversible uni-bimolecular mechanism with the rate constants given in fig. 2.

equilibrium is reached which is characterized by a residual activity of 10%, corresponding to the same amount of undissociated tetramers (cf. fig. 3 in ref. [9]). Correcting for this residual activity, the reactivation relaxations after dissociation at 1 kbar for 24 hours show the same kinetic pattern as observed after complete dissociation at 2 kbar (fig. 5). Since reactivation after dissociation at 1 or 2 kbar shows the same kinetics, additional unfolding (which might occur in the given pressure range) does not affect the reconstitution behaviour of the enzyme.

4. Discussion

In a previous investigation the pressure dependent dissociation transition of procine muscle lactic dehydrogenase has been analyzed in detail [9]. As a result, dissociation to monomers at high hydrostatic pressure could be unequivocally proven, using SDS polyacrylamide electrophoresis of the enzyme cross-linked before and after pressure application. This finding confirmed earlier indirect evidence from hybridization [15,16] and deactivation studies [14,15] which pointed to dissociation and deactivation of the enzyme under high hydrostatic pressure. The pressure induced dissociation is fully reversible over the whole dissociation range, indicating a true dissociation/association equilibrium, unperturbed by side reactions such as aggregation. Upon dissociation and denaturation of the enzyme by hydrogen ions, on the other hand, pronounced aggregation was observed in the transition range. At higher enzyme concentrations this aggregation may compete significantly with the refolding reaction leading to a decrease in the yield of reactivation [7]. The reason for this "irreversible deactivation" at high enzyme concentrations is the fact that the higher order of the aggregation reaction causes this side reaction to outrun the correct folding and association of the enzyme under consideration. In contrast to this observation, pressure dissociation can be reversed to more than 95% after pressure release, provided that the enzyme is protected against oxidation both under dissociating and reassociating conditions.

The complete absence of aggregation in the pressure-induced dissociation/association transition of procine muscle lactic dehydrogenase, and the resulting high yields of reactivation, may be tentatively explained by

a higher extent of structural order in the pressure dissociated monomers compared to the dissociation products obtained by applying stronger denaturants, e.g. pH 2 or 6 M guanidine · HCl. Exposure of hydrophobic groups upon more extensive randomization of the monomers is expected to promote the unspecific aggregation observed in the range of the dissociation transition or during reassociation. The absence of cis/trans isomerization of proline residues, claimed to be rate limiting in certain protein folding reactions [17], might be an alternative explanation for the complete reversibility of the pressure induced dissociation of porcine muscle lactic dehydrogenase.

Previous investigations have shown that the reactivation rate for several oligomeric enzymes is independent of the mode of denaturation; obviously the different extent of residual structure within the dissociated monomers does not affect the folding and association reactions which are rate limiting for the formation of the fully reconstituted oligomer from the randomized chains fluctuating around some preferential structures in the different denaturants [11,18,19]. This finding necessitates the same rate limiting steps on the pathway of folding and reassociation after dissociation in various denaturants.

Since pressure denaturation differs in a characteristic way from the conventional denaturation procedures regarding complete reversibility over the whole transition range, it would be of interest to determine whether the hypothesis of identical reaction mechanisms holds for pressure denaturation too. The observed correspondence of the reactivation rates after high pressure deactivation to the respective values for the reactivation after acid or guanidine denaturation is striking in view of the previously mentioned difference in the dissociation-association characteristics.

Based on the unperturbed reconstitution, a more stringent analysis of the kinetic data is rendered possible. As a result a kinetic model of higher complexity is suggested, including first-order processes in addition to the simple association scheme applied previously [5]. The fact that the mechanism underlying the reactivation of porcine muscle lactic dehydrogenase is of higher complexity than a simple second-order association reaction was suggested earlier, based on a population analysis of all species formed during reconstitution of the enzyme after acid dissociation [6]. The determination of three species, namely monomers, dimers as intermediates, and tetramers as the final state, necessitates a consecutive reaction mechanism.

There is good evidence from the kinetic experiments and their fit with the uni-bimolecular mechanism, that enzymatic activity of lactic dehydrogenase after high pressure deactivation is not generated unless both the transconformation and association processes have taken place. From this we may conclude that (as in the case of strong denaturants, like guanidine · HCl and/or acid) the formation of the correct backbone structure of the enzyme necessarily requires the acquisition of the native quaternary structure.

While the reconstitution process may be quantitatively described by the uni-bimolecular reaction scheme with the enzyme concentration as the only parameter, dissociation is determined solely by pressure. The rate and extent of deactivation increases with increasing pressure; above a certain limiting value irreversible denaturation becomes important [20]. This may be interpreted in general terms by the exposure of interior hydrophobic residues or domains of the enzyme molecule into the polar aqueous solvent leading to stable conformations different from the native state.

Acknowledgements

This investigation was supported by Grants of the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. Excellent cooperation of the mechanical workshop, especially Mr. G. Wiesener, Mr. R. Knott, and Mr. K. Zweckerl is gratefully acknowledged.

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