Henkens, R. W., & Turner, S. R. (1973) *Biochemistry 12*, 1618.
Henkens, R. W., & Turner, S. R. (1979) *J. Biol. Chem. 254*, 8110–8112.
Ikai, A. (1971) Ph.D. Thesis, Duke University, Durham, NC.
Ikai, A., & Tanford, C. (1973) *J. Mol. Biol. 73*, 145.
Ikai, A., Fish, W. W., & Tanford, C. (1973) *J. Mol. Biol. 73*, 165.
Jullien, M., & Baldwin, R. L. (1981) *J. Mol. Biol. 145*, 265–280.
Kaminsky, L. S., Miller, V. J., & Davison, A. J. (1973) *Biochemistry 12*, 2215–2221.
Kawaguchi, H., & Noda, H. (1977) *J. Biochem. (Tokyo) 81*, 1307.
Knapp, J. A., & Pace, C. N. (1974) *Biochemistry 13*, 1289.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Lapanje, S. (1978) in *Physicochemical Aspects of Protein Denaturation*, Wiley, New York.
Levitt, M. (1981) *J. Mol. Biol. 145*, 251–263.
Margoliash, E., & Frohwirt, N. (1959) *Biochem. J. 71*, 570.
Mattoon, J. R., & Sherman, F. (1966) *J. Biol. Chem. 241*, 4330.
McLendon, G., and Smith, M. (1978) *J. Biol. Chem. 253*, 4004.
Montgomery, D. L., Leung, D. W., Smith, M., Shalit, P., Faye, G., & Hall, B. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 541.
Myer, Y. P. (1968) *Biochemistry 7*, 765–776.
Privalov, P. L., & Khechinashvili, N. N. (1974) *J. Mol. Biol. 86*, 665.
Ridge, J. A., Baldwin, R. L., & Labhardt, A. M. (1981) *Biochemistry 20*, 1622–1630.
Schejter, A., & George, P. (1964) *Biochemistry 3*, 1045.
Schellman, J. A. (1978) *Biopolymers 17*, 1305.
Sherman, F., & Stewart, J. W. (1978) in *Biochemistry and Genetics of Yeast* (Bacila, M., Horecker, B. L., & Stoppani, A. O. M., Eds.) p 273, Academic Press, New York.
Sherman, F., Stewart, J. W., Parker, J. H., Inhaber, E., Shipman, N. A., Putterman, G. J., Gardisky, R. L., & Margoliash, E. (1968) *J. Biol. Chem. 243*, 5446.
Sober, H. A. (1970) in *Handbook of Biochemistry* (Sober, H. A., Ed.) 2nd ed., p J278, Chemical Rubber Co., Cleveland, Ohio.
Stellwagen, E. (1968) *Biochemistry 7*, 2893–2898.
Tanford, C. (1962) *J. Am. Chem. Soc. 84*, 4240.
Tanford, C. (1969) *Adv. Protein Chem. 24*, 1.
Tsong, T. Y. (1974) *J. Biol. Chem. 249*, 1988–1990.
Tsong, T. Y. (1975) *Biochemistry 14*, 1542.
Tsong, T. Y. (1976) *Biochemistry 15*, 5467.

Reconstitution of Lactic Dehydrogenase from Pig Heart after Reversible High-Pressure Dissociation†

Klaus Müller, Hans-Dietrich Lüdemann, and Rainer Jaenicke*

ABSTRACT: Lactic dehydrogenase from pig heart was reversibly dissociated to monomers at high hydrostatic pressure. As shown by equilibrium measurements making use of the yield of reactivation and high-pressure fluorescence emission spectra, the dissociation/association can be described by a consecutive dissociation/unfolding mechanism according to $N \rightleftharpoons 4M' \rightleftharpoons 4M^*$ [Müller, K., Lüdemann, H.-D., & Jaenicke, R. (1981) *Biophys. Chem.* (in press)]. The extent and rate of dissociation depend on pressure as well as on the conditions of the solvent. Maximum yields of reconstitution are achieved under anaerobic conditions, after dissociation by 20-min incubation at 1200 bar in 0.2 M Tris-HCl, pH 7.6, in the presence of 1 mM ethylenediaminetetraacetic acid and 10 mM dithioerythritol. At concentrations $c \leq 25$ μg/mL (≤0.72 μM), reconstitution amounts to ~90%. The product of reconstitution is indistinguishable from the enzyme in its initial native state, as far as its physicochemical and enzymological properties are concerned. Based on the long-term stability of the enzyme under optimum reconstitution conditions, the kinetics of reactivation and renaturation after decompression were measured at 2 μg/mL $< c <$ 25 μg/mL. The sigmoidal kinetics can be quantitatively described by an irreversible sequential uni-bimolecular mechanism, assuming inactive subunits [Jaenicke, R. (1979) *FEBS-Symp. 52*, 182–198]; the respective first- and second-order rate constants under optimum conditions of reconstitution are $k_1 = (1.5 \pm 0.1) \times 10^{-3}$ s^{-1} and $k_2 = (3.5 \pm 0.2) \times 10^3$ M^{-1} s^{-1} (20 °C). As shown by independent evidence from cross-linking experiments, the rate-limiting association reaction is the dimerization of dimers generating the native tetramer. In accordance with the above-mentioned sequential dissociation/unfolding mechanism, the sigmoidicity of the kinetic traces is more pronounced after pressure deactivation under strongly denaturing conditions. Under these conditions, the yield and rate of reactivation are decreased.

The heart isoenzyme of lactic dehydrogenase has been reversibly dissociated to inactive subunits under a variety of denaturing conditions (Teipel & Koshland, 1971; Levi & Kaplan, 1971; Chilson et al., 1966; Jaenicke, 1978). Special attention has been focused on the acid dissociation of the enzyme (Anderson & Weber, 1966; Levitzki, 1972; Jaenicke, 1974; Vallee & Williams, 1975; Rudolph et al., 1977a) since this method provides high yields of reconstitution.

In this study, the reconstitution of lactic dehydrogenase from pig heart after deactivation, denaturation, and dissociation at elevated hydrostatic pressure is reported. The influence of the conditions of high-pressure deactivation on the kinetics of reconstitution is studied with special emphasis on the com-

† From the Institut für Biophysik und Physikalische Biochemie, Universität Regensburg, D-8400 Regensburg, West Germany. *Received February 5, 1981.* This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

0006-2960/81/0420-5411$01.25/0

parison of the different modes of denaturation.

Materials and Methods

For *substances*, *stock solutions*, and *enzyme assays*, the respective experimental details were as described previously (Müller et al., 1981).

High-Pressure Dissociation. High-pressure experiments made use of the quench cell described by Schade et al. (1980b). Details regarding the time and pressure of incubation at 20 °C are given in the text. The solvents used were Tris-HCl (I = 0.16 M), Tris–H_2SO_4 (I = 0.12 M), and Tris–H_3PO_4 (I = 0.12 M), respectively, each containing 1 mM ethylenediaminetetraacetic acid (EDTA)[1] and 10 mM DTE. The pH was kept constant at 7.6. The enzyme concentration during high-pressure incubation was 0.65 ± 0.07 μM in all experiments; concentrations refer to the subunit molecular weight of 35 000.

Reconstitution. The kinetics of reactivation after pressure release were analyzed in a concentration range of 45 nM ≤ c ≤ 720 nM. Solutions were diluted immediately (~30 s) after decompression to adjust the respective final concentration. Aliquots of these dilutions were taken at defined time intervals and assayed for enzymatic activity; the first assay was started within 45 s after pressure release. Solutions of native enzyme diluted to the same final concentration were used as references to determine the yield of reactivation. Final values of reactivation were measured after a reactivation time of up to 150 h. These final values were used for the computer fit of the time course of reactivation, applying the irreversible uni-bimolecular kinetic model (Jaenicke, 1978, 1979; Jaenicke & Rudolph, 1980). Measurements of the recovery of native fluorescence intensity after high-pressure denaturation made use of the high-pressure fluorescence cell described elsewhere (Müller et al., 1981).

Results

Equilibrium Measurements. (A) High-Pressure Deactivation, Denaturation, and Dissociation. Measurements characterizing the behavior of lactic dehydrogenase from pig heart exposed to the action of high hydrostatic pressure were reported previously (Müller et al., 1981). As shown by hybridization with LDH-M_4 and subsequent gel electrophoresis, deactivation of LDH-H_4 is parallelled by monomerization. This is corroborated by high-pressure fluorescence measurements. The high-pressure equilibrium of active tetramers and inactive dissociated subunits is reached after long incubation (~2 h at 300 bar, ~10 min at 1000 bar). At p ≤ 1000 bar, dissociation and deactivation are found to be fully reversible. At pressures beyond 1000 bar, partial irreversibility of pressure denaturation occurs. The yield of reconstitution at p = 1 bar depends on the pressure and the time of deactivation.

(B) Yield of Reactivation. By the application of common approaches of denaturation/renaturation (e.g., guanidine-HCl or acid pH), the yield of reconstitution of a variety of enzymes has been shown to depend on enzyme concentration (Teipel & Koshland, 1971; Jaenicke, 1974; Jaenicke & Rudolph, 1977; Rudolph et al., 1977a, 1979). Figure 1 illustrates the behavior of LDH-H_4 in the case of the reconstitution after high-pressure incubation. Two features deserve consideration: (a) the yield of reconstitution depends on the conditions of deactivation;

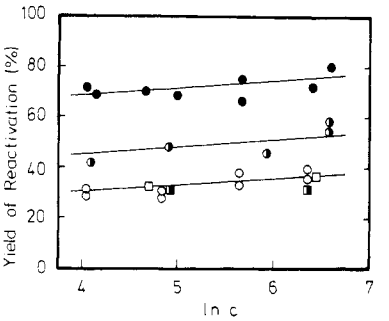

FIGURE 1: Yield of reactivation of lactic dehydrogenase from pig heart as a function of total enzyme concentration (in nanomolar). Deactivation was at 20 °C in Tris buffer, pH 7.6, plus 1 mM EDTA and 10 mM DTE at c_{LDH} = 23 ± 2 μg/mL (0.65 μM); reactivation was at 20 °C and 1 bar, with a reactivation time up to 150 h. Tris-HCl, I = 0.16 M, deactivation at (○) 2000 (20 min), (●) 1200 (20 min), and (◑) 1200 bar (15 ± 2 h); (◨) Tris–H_3PO_4, I = 0.12 M, deactivation at 2000 bar (20 min); (□) Tris–H_2SO_4, I = 0.12 M, deactivation at 2000 bar (20 min).

(b) within the concentration range 2 μg/mL ≤ c ≤ 25 μg/mL, the yield of reactivation is only slightly decreased at low concentrations, proving intermediates of reconstitution to be effectively protected against irreversible modification (e.g., oxidation). On the other hand, the plateau value of the yield of reactivation at high concentrations indicates that the folding of the monomers does not significantly interfere with their assembly to form the native quaternary structure. In the case of irreversible modification and rate-determining isomerization reactions, one would expect a significant decrease of the reactivation yield at low and high enzyme concentrations [cf. Teipel & Koshland (1971), Jaenicke (1974), and Jaenicke & Rudolph (1977)]. The absence of an optimum profile under the given conditions is consistent with the results obtained in the case of acid deactivation of LDH-H_4 (Rudolph et al., 1977a) and high-pressure deactivation of LDH-M_4 (Schade et al., 1980a). Slight deviations of the given yields (Figure 1) from previous data (Müller et al., 1981) result from the repetitious handling of the enzyme solution during the investigation of the reconstitution kinetics.

(C) Characterization of the Reconstituted Enzyme. In order to interpret the reconstitution and its kinetics in terms of the elementary processes involved in the folding of oligomeric enzymes in vivo, the characterization of the products of denaturation and renaturation, and their comparison with the native state, is required. Provided that products of side reactions [i.e., high molecular weight aggregates; cf. Zettlmeissl et al. (1979)] are removed, previous analyses have shown that reconstituted enzymes are indistinguishable from the native protein in its initial state (Jaenicke et al., 1975; Jaenicke & Rudolph, 1977, 1980; Jaenicke, 1979).

The following procedure was applied to prepare pure reconstituted LDH-H_4 after deactivation at elevated pressure and reactivation at normal atmospheric pressure: samples of LDH-H_4 (25 μg/mL, i.e., 0.72 μM) in oxygen-free 0.2 M Tris-HCl buffer, pH 7.6, plus 1 mM EDTA and 10 mM DTE were pressurized for 20 min at 20 °C; after decompression to ambient pressure and reconstitution for 1 day at 20 °C, the samples were collected and concentrated to ~0.3 mg/mL by ultrafiltration (Amicon Diaflo with PM 10 filters). Removal of unspecific aggregates was achieved by Millipore filtration (0.45 μm) and subsequent centrifugation (48000g, 20 min).[2]

[1] Abbreviations used: DTE, 1,4-dithioerythritol; EDTA, ethylenediaminetetraacetic acid; k_i, rate constants; LDH, lactic dehydrogenase (EC 1.1.1.27) (H_4 and M_4 refer to isoenzymes from heart and skeletal muscle, respectively); p, hydrostatic pressure; Tris, tris(hydroxymethyl)aminomethane; N, native tetrameric state of LDH; M′ and M*, monomeric states of LDH.

[2] Occasionally, low molecular weight aggregates were observed; these cannot be separated by this technique unless an additional gel filtration step is applied [cf. Rudolph et al. (1977b)].

Table I: Characterization of Lactic Dehydrogenase from Pig Heart in Its Native and Renatured States[a]

enzyme state	sp act.[b]	s_{20}[c] (S)[c]	$\theta_\lambda \times 10^{-3}$[d] 207 nm	$\theta_\lambda \times 10^{-3}$[d] 222 nm	F_{rel}[e]	K_{av}^0[f]
native tetramer	330 ± 15	6.0 ± 0.1	10.6 ± 1.0	12.5 ± 1.0	100 ± 5	0.144 ± 0.007
renatured after pressure incubation at 2000 bar (20 min)	305 ± 10	6.0 ± 0.1	10.7 ± 1.0	12.8 ± 1.0	105 ± 5	0.144 ± 0.007
renatured after pressure incubation at 1200 bar (20 min)	310 ± 10	6.0 ± 0.1	11.1 ± 1.0	12.5 ± 1.0	97 ± 5	0.144 ± 0.007

[a] Tris-HCl buffer, pH 7.6, I = 0.16 M, plus 1 mM EDTA and 1–10 mM DTE. [b] Specific activity, IU/mg; concentration of the renatured enzyme determined according to Bradford (1976). [c] Sedimentation coefficient, s_{20} (S), from sedimentation velocity experiments (Beckman Instruments Model E); c_{LDH} = 0.3 mg/mL. [d] Ellipticity, θ, at 207 and 222 nm (deg·cm²·dmol⁻¹) (Roussel-Jouan Dichrographe II); c_{LDH} = 0.09–0.13 mg/mL. [e] Relative fluorescence emission at 345 nm (λ_{exc} = 285 nm) in arbitrary units (Hitachi Perkin-Elmer, MPF 44A); c_{LDH} = 25–45 μg/mL. Maximum excitation is found to be unchanged: 282 ± 2 nm. [f] $K_{av}^0 = V_p/(V_t - V_0)$ from gel filtration on Sephacryl S 200 column (1.6 × 86 cm); elution profiles analyzed by F_{rel}, determination of the void volume (V_0 = 62 mL) by use of dextran blue.

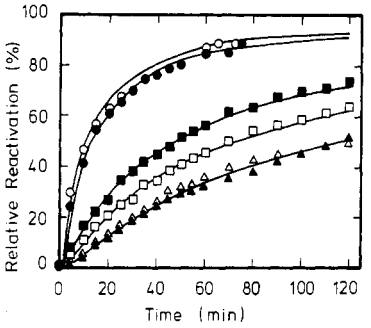

FIGURE 2: Kinetics of reactivation of LDH-H_4 after deactivation for 20 min at 1200 bar (residual activity ~10%). Deactivation and reactivation were at 20 °C in Tris-HCl buffer, pH 7.6, I = 0.16 M, plus 1 mM EDTA and 10 mM DTE. Reactivation was at the following enzyme concentrations (nanomolar): (○) 586, (●) 423, (■) 101, (□) 70, (△) 43, (▲) 41. Solid lines were calculated according to an irreversible uni-bimolecular mechanism with k_{uni} = 1.5 × 10^{-3} s^{-1} and k_{bi} = (3.5 ± 0.2) × 10^3 M^{-1} s^{-1}.

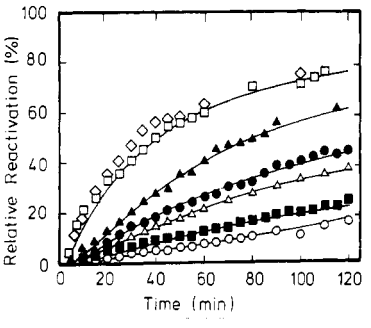

FIGURE 3: Kinetics of reactivation of LDH-H_4 after deactivation at 2000 bar for 20 min (closed symbols) or at 1200 bar for 15 ● 2 h (open symbols, residual activity ~5%). Tris-HCl buffer, pH 7.6, I = 0.16 M, plus 1 mM EDTA and 10 mM DTE; c_{LDH} (nanomolar) values were (◇, □) 340, (▲) 206, (●) 100, (△) 62, (■) 40, and (○) 25. Solid lines were calculated according to an irreversible uni-bimolecular mechanism with k_{uni} = (0.35 ± 0.05) × 10^{-3} s^{-1} and k_{bi} = (1.3 ± 0.1) × 10^3 M^{-1} s^{-1}.

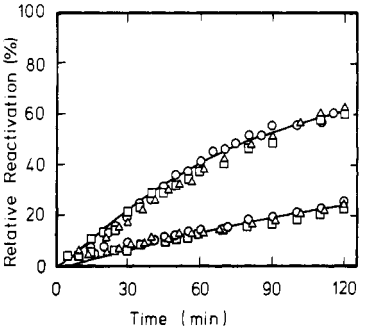

FIGURE 4: Kinetics of reactivation of LDH-H_4 after deactivation at 2000 bar for 20 min. Tris buffer, pH 7.6, plus 1 mM EDTA and 10 mM DTE. Concentration of the reactivating enzyme was 206 (upper line) and 40 nM (lower line). Buffers: Tris–H_3PO_4, I = 0.12 M (□); Tris–H_2SO_4, I = 0.12 M (△); Tris-HCl, I = 0.16 M (○). Solid lines were calculated as in Figure 3.

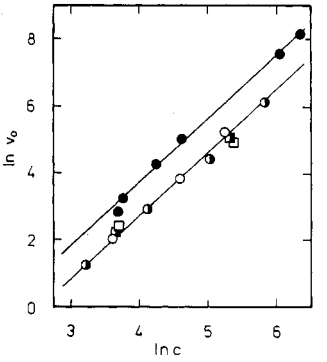

FIGURE 5: Determination of the reaction order for the reactivation of LDH-H_4 after pressure incubation. Enzyme concentration in nanomolar. Deactivation and reactivation as in Figures 2–4; symbols as in Figure 1. Full lines calculated for an irreversible uni-bimolecular mechanism with the rate constants summarized in Table II.

As indicated from the data summarized in Table I, the initial native state and the renatured state of LDH-H_4 after pressure denaturation and reconstitution are identical, regarding their physicochemical and enzymological properties.

Kinetic Measurements. (A) Kinetics of Reactivation. As mentioned previously, the yield of reactivation under optimum conditions of reconstitution depends on the pressure and on the incubation time (with respect to the pressure of deactivation). There is no significant effect of enzyme concentration on the yield of reactivation (cf. Figure 1), so that kinetic studies may be performed in the given concentration range by applying a constant value for the final yield of reconstitution. Figures 2–4 illustrate the kinetics of reactivation after pressure deactivation at p = 1200 and 2000 bar, respectively. In general, the time course of the reconstitution reaction depends on the enzyme concentration. A double-logarithmic plot of the initial velocity vs. enzyme concentration (Figure 5) gives a linear relationship with a slope n = 1.8 ± 0.1. At high concentrations, the apparent reaction order is reduced. Both this observation and the fact that the kinetics are characterized by sigmoidal profiles suggest the mechanism underlying the kinetics of reactivation to be complex.

Detailed analyses of the reconstitution properties of a number of dehydrogenases after deactivation in various de-

Table II: Rate Constants of Reactivation of LDH-H_4 according to an Irreversible Uni-bimolecular Kinetic Mechanism of Reconstitution

deactivation conditions	rate constants of reactivation k_{uni} ($\times 10^{-3}$ s^{-1})	k_{bi} ($\times 10^3$ M^{-1} s^{-1})
1200 bar (20 min)[a]	1.50 ± 0.10	3.5 ± 0.2
1200 bar (15 h)[a]	0.35 ± 0.05	1.3 ± 0.1
2000 bar (20 min)[a]	0.35 ± 0.05	1.3 ± 0.1
1 M glycine, pH 2.3 (5 min)[b]	1.45 ± 0.45	5 ± 1
6 M guanidine-HCl, pH 2.3 (5 min)[b]	1.45 ± 0.45	5 ± 1
6 M guanidine-HCl, pH 7.6 (5 min)[b]	1.45 ± 0.45	5 ± 1
6 M urea, pH 2.3 (5 min)[b]	1.45 ± 0.45	5 ± 1

[a] Tris buffer, pH 7.6, I = 0.16 M, plus 1 mM EDTA and 10 mM DTE, 20 °C; reactivation after pressure incubation induced by pressure release. [b] Deactivation in the presence of 1 mM EDTA and 0.1 mM DTE, 20 °C; reactivation by dilution in 0.2 M phosphate buffer, pH 7.6, plus 1 mM EDTA and 0.1–10 mM DTE [cf. Rudolph et al. (1977a)].

naturants were reported earlier from this laboratory (Jaenicke, 1979; Jaenicke & Rudolph, 1980). In accordance with a sequential folding and assembly model, the observed concentration-dependent reactivation profiles were fitted by an irreversible consecutive mechanism consisting of one unimolecular and one bimolecular rate-limiting step. In general, perfect fit of the kinetic data was achieved by assuming inactive reactants in the reassembly process.

As indicated by the agreement of the experimental data, and the respective calculated profiles given in Figures 2–4, the same kinetic mechanism is found to be sufficient to quantitatively describe the reconstitution of LDH-H_4 after high-pressure dissociation and deactivation. By correction of the reactivation data with respect to the residual activity of the enzyme (starting from the equilibrium of dissociation), the reactivation kinetics after 20 min of deactivation at p = 1200 bar (residual activity ~10%) turn out to be identical with the reactivation kinetics after acid dissociation at pH 2.3 (Rudolph et al., 1977a). If deactivation is performed at higher pressure (2000 bar), or during a longer time of incubation (15 h at 1200 bar), the kinetics of reconstitution are slower, and the lag phase in the beginning of the reactivation is more pronounced (Table II). Both findings are compatible with the effect of high pressure on the yield of reactivation (Müller et al., 1981) which suggests slow conformational changes within the dissociated monomers to occur after long incubation at $p \geq 1000$ bar. Apparently, this unfolding is also reflected by the decreased rate of reconstitution. Similar effects have been found for acid dissociation after long incubation at low pH (R. Hermann, G. Zettlmeissl, R. Rudolph, and R. Jaenicke, unpublished experiments).

As taken from Figure 4, exchange of the monovalent chloride ion against the bivalent phosphate or sulfate ion does not change the reactivation profile after dissociation at 2000 bar. This result is in agreement with the finding that in the case of LDH-H_4 no significant stabilization against pressure deactivation is induced by the above-mentioned bivalent buffer ions. Obviously, there is no preferential binding of HPO_4^{2-} or SO_4^{2-} to the enzyme species present at high-pressure equilibrium; on the other hand, specific ion binding to an intermediate of reconstitution cannot be involved in a rate-determining step on the pathway of reconstitution. The lack of ion specific effect is in contrast to the equilibrium properties of the isoenzyme from skeletal muscle (Schade et al., 1980b).

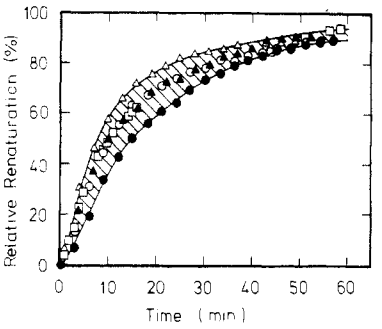

FIGURE 6: Kinetics of the recovery of native fluorescence (λ_{exc} = 285 nm, λ_{em} = 345 nm) of LDH-H_4 after pressure denaturation at c_{LDH} = 0.72 μM: (●) 700 bar (75 min); (○) 820 bar (40 min); (△) 900 bar (30 min); (□) 1000 bar (30 min); (▲) 1100 bar (20 min). The kinetic trace of renaturation after 20-min denaturation at 1200 bar coincides with (○). Renaturation at 1 bar was achieved under the same conditions as those given in Figure 2. Relative renaturation = $[I(t) - I(0)]/[I(N) - I(0)]$, with $I(N)$, $I(0)$, and $I(t)$ representing the fluorescence of the native tetramer, the renaturing enzyme at time t = 0, and the renaturing enzyme at time t, respectively [cf. Müller et al. (1981)]. The range of experimental error is given by the hatched area.

(*B*) *Kinetics of Renaturation.* The kinetics of renaturation were monitored by the increase of fluorescence intensity at λ_{em} = 345 nm (λ_{exc} = 285 nm) (Figure 6); the respective relaxations were determined at a constant enzyme concentration of c = 25 μg/mL (0.72 μM). Deactivation was achieved at 700 bar ≤ p ≤ 1200 bar; under these conditions, constant final values of fluorescence (corresponding to the equilibrium of denaturation) were obtained after 75 min at 700 bar and after 5 min at 1200 bar, respectively. The traces are identical within the ranges of experimental error; the yield of renaturation is 100% in both cases. The result is in agreement with the reaction scheme proposed previously to describe the pressure-induced dissociation (Müller et al., 1981). By comparison of the regain of the native fluorescence (Figure 6) with the corresponding kinetics of reactivation (Figure 2), both processes turn out to run strictly parallel. At higher enzyme concentrations, this has also been shown for the reconstitution after acid dissociation (Rudolph et al., 1977a).

Discussion

In a previous paper (Müller et al., 1981), pressure-induced structural changes at the level of the tertiary and quaternary structure of lactic dehydrogenase from pig heart were characterized. As shown by hybridization experiments involving the separate isoenzymes from heart and muscle, all five combinations, A_4, A_3B, A_2B_2, AB_3, and B_4, have been found to occur after pressure release and joint reconstitution.[3] The presence of the hybrids of the A_3B and AB_3 type, as well as the binomial distribution of all possible recombination products, proves that high-pressure dissociation yields the monomer as the predominant species. The binomial distribution clearly excludes a rapid equilibrium between monomers and dimers to be responsible for hybridization, since this reaction would lead to the preferred accumulation of the symmetrical A_2B_2 species which is not the case. Additional evidence supporting the view that high pressure induces monomerization comes from high-pressure fluorescence spectra: the fluorescence emission spectrum of the enzyme at high pressure is identical

[3] An earlier approach (Jaenicke, 1970) aiming at pressure-induced dissociation made use of joint pressure incubation of the two isoenzymes without reversible deactivation of the separate systems; this method does not allow unequivocal information regarding the state of association under pressure [cf. Jaenicke & Koberstein (1971) and Jaenicke et al. (1971)].

with the respective spectrum of homogeneous monomers produced by acid dissociation (pH ~2).

Cross-linking experiments with the enzyme from pig muscle (Schade et al., 1980a) also indicate dissociation to monomers, though the quantitative interpretation is difficult because of the rapid association to dimers occurring after reestablishing quasi-physiological conditions (Hermann et al., 1981). Evidence accumulated before proves that application of high pressure leads to dissociation of lactic dehydrogenase from pig heart to the monomer; a minor fraction of dimers as a by-product of dissociation may not be definitely excluded because of the limits of error of the applied population analyses (~5%). The effect of time and deactivation pressure on the yield of reactivation of lactic dehydrogenase from pig heart was found to obey an equilibrium scheme comprising dissociation and transconformation reactions:

$$N \rightleftharpoons 4M' \rightleftharpoons 4M^*$$

In this scheme, M′ represents the folded monomer produced at medium pressure ($p < 1$ kbar), while M* symbolizes the monomer generated at higher pressure ($p > 1$ kbar). Since the equilibrium $M' \rightleftharpoons M^*$ cannot be quantitatively determined by using the methods given, the calculation of the dissociation volume can only be achieved at pressures below 1 kbar, because under this condition a fully reversible equilibrium $N \rightleftharpoons 4M'$ is observed. In the case of lactic dehydrogenase from pig muscle (Schade et al., 1980a,b), the unfolding reaction $M' \rightarrow M^*$ is found to be negligible in the whole transition range of high-pressure deactivation ($p < 1.5$ kbar). In both isoenzymes, application of higher pressure shifts the previously given equilibria to the right-hand side, at the same time increasing the rate of dissociation.

In the present experiments, indirect evidence for the transconformation reaction, $M' \rightarrow M^*$, in the case of lactic dehydrogenase from pig heart at $p > 1$ kbar is gained from reconstitution experiments. There is an extended lag phase in the beginning of the reactivation reaction which reflects conformational changes after dissociation has taken place. As taken from the previous reactivation studies (Müller et al., 1981), the corresponding experimental conditions lead to the accumulation of M* in the above-mentioned scheme. Under the same conditions, a decrease of the rate of reconstitution is observed, in accordance with the hypothetical two-step mechanism of high-pressure denaturation. A similar interpretation of the kinetics of reconstitution after acid dissociation has been previously proposed by Vallee & Williams (1975).

The decreased rate of reactivation and the reduced reconstitution yield can be explained, if the reaction $M' \rightarrow M^*$ is considered to consist of two types of structural changes, i.e., partial exposure of hydrophobic interior parts of the structured monomers to the polar solvent and proline cis–trans isomerization or similar slow conformational changes. Proline cis–trans isomerization has been shown to be a rate-limiting step in certain protein-folding reactions (Brandts et al., 1975; Lin & Brandts, 1978; Baldwin & Creighton, 1980; Schmid & Baldwin, 1978; Schmid, 1980, 1981). It may also contribute to the rate-attenuating effects caused by long incubation at pressures beyond 1000 bar.

On the other hand, slowing down of the reactivation kinetics may reduce the yield of reconstitution by favoring unspecific aggregation. In this connection, the previously mentioned exposure of interior hydrophobic residues or domains of the protein to the polar solvent may be important. Since hydrophobic interactions are weakened by high pressure (Kauzmann, 1959), aggregation during pressure incubation is inhibited (Jaenicke, 1971). After decompression, "irreversible aggregation" as a side reaction competing with reconstitution is enhanced, thus causing a decreased yield of reactivation. With the application of elevated hydrostatic pressure to oligomeric enzymes, remarkable differences in comparison with other methods of denaturation are observed: no "hysteresis" of the deactivation/reactivation transition is detectable, and (up to a certain limiting pressure) deactivation is found to be completely reversible, even after incubation over a long period of time. Beyond the limiting pressure, irreversible deactivation (Joly, 1965) becomes important. The given characteristics raise the question of whether there are significant differences regarding the reconstitution mechanism after high-pressure dissociation, compared to the reconstitution after dissociation and deactivation with other denaturants. LDH-H_4 has been the subject of detailed reconstitution studies after deactivation at low pH and/or in 6 M guanidine-HCl or urea (Jaenicke, 1974; Rudolph et al., 1977a). Under all conditions, sigmoidal reactivation kinetics were observed after reestablishing native conditions. The quantitative description of the kinetic traces is provided by the previously mentioned irreversible consecutive uni-bimolecular mechanism with one single set of kinetic constants, assuming catalytically inactive subunits (Jaenicke, 1978). As shown by the excellent fit of the kinetic traces in Figures 2–4 and by the kinetic data summarized in Table II, reactivation after high-pressure deactivation is characterized by the same kinetic pattern; the rate constants are of the same order of magnitude. This leads to the conclusion that the acquisition of the three-dimensional structure of LDH-H_4 does not depend on the pathway of denaturation/renaturation; obviously, there exists a common intermediate on this pathway, the folding and association of which is rate determining in the uni-bimolecular overall process of reconstitution.

The question of whether the association step belongs to the formation of the dimer or tetramer may be answered by cross-linking experiments (Hermann et al., 1979). For LDH-M_4, the kinetics of reactivation after acid dissociation were found to parallel tetramer formation (Hermann et al., 1979, 1981); the same holds for LDH-H_4 (Bernhardt, 1980).

These results, together with the observed uni-bimolecular mechanism of reconstitution, prove that the acquisition of the native tetrameric structure is necessary to generate enzymatic activity. In general, reconstitution of oligomeric enzymes in solution is characterized by a close correlation of folding and association; in all cases investigated so far, full catalytic function requires the assembly of the structured subunits to form the native quaternary structure.

Acknowledgments

The excellent cooperation of the mechanical workshop, especially G. Wiesener, M. Löffler, and R. Knott, is gratefully acknowledged. We thank Drs. R. Rudolph and F. X. Schmid for fruitful discussions and Dr. P. Bartholmes for help in performing gel chromatographic analyses.

References

Anderson, S., & Weber, G. (1966) *Arch. Biochem. Biophys. 116*, 207–223.

Baldwin, R. L., & Creighton, T. E. (1980) in *Protein Folding* (Jaenicke, R., Ed.) pp 217–260, Elsevier/North-Holland, Amsterdam.

Bernhardt, G. (1980) Thesis, Regensburg University.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Brandts, J. F., Halvorson, H. R., & Brennan, M. (1975) *Biochemistry 14*, 4953–4963.

Chilson, O. P., Kitto, G. B., Pudles, J., & Kaplan, N. O. (1966) *J. Biol. Chem. 241*, 2431–2445.

Hermann, R., Rudolph, R., & Jaenicke, R. (1979) *Nature (London) 277*, 243-245.
Hermann, R., Jaenicke, R., & Rudolph, R. (1981) *Biochemistry* (in press).
Jaenicke, R. (1970) in *Pyridine Nucleotide Dependent Dehydrogenases* (Sund, H., Ed.) pp 71-90, Springer-Verlag, Berlin, Heidelberg, and New York.
Jaenicke, R. (1971) *Eur. J. Biochem. 21*, 110-115.
Jaenicke, R. (1974) *Eur. J. Biochem. 46*, 149-155.
Jaenicke, R. (1978) *Naturwissenschaften 65*, 569-577.
Jaenicke, R. (1979) *FEBS-Symp. 52*, 182-198.
Jaenicke, R., & Koberstein, R. (1971) *FEBS Lett. 17*, 351-354.
Jaenicke, R., & Rudolph, R. (1977) *FEBS-Symp. 49*, 351-367.
Jaenicke, R., & Rudolph, R. (1980) in *Protein Folding* (Jaenicke, R., Ed.) pp 525-548, Elsevier/North-Holland, Amsterdam.
Jaenicke, R., Koberstein, R., & Teuscher, B. (1971) *Eur. J. Biochem. 23*, 150-159.
Jaenicke, R., Engelhard, M., Kraus, E., & Rudolph, R. (1975) *Biochem. Soc. Trans. 3*, 1051-1054.
Joly, M. (1965) *A Physico-chemical Approach to Protein Denaturation*, Academic Press, New York.
Kauzmann, W. (1959) *Adv. Protein Chem. 14*, 1-63.
Levi, A. S., & Kaplan, N. O. (1971) *J. Biol. Chem. 246*, 6409-6417.
Levitzki, A. (1972) *FEBS Lett. 24*, 301-304.
Lin, L.-N., & Brandts, J. F. (1978) *Biochemistry 17*, 4102-4110.
Müller, K., Lüdemann, H.-D., & Jaenicke, R. (1981) *Biophys. Chem.* (in press).
Rudolph, R., Heider, I., Westhof, E., & Jaenicke, R. (1977a) *Biochemistry 16*, 3384-3390.
Rudolph, R., Heider, I., & Jaenicke, R. (1977b) *Biochemistry 16*, 5527-5531.
Rudolph, R., Zettlmeissl, G., & Jaenicke, R. (1979) *Biochemistry 18*, 5572-5575.
Schade, B. C., Lüdemann, H.-D., Rudolph, R., & Jaenicke, R. (1980a) *Biophys. Chem. 11*, 257-263.
Schade, B. C., Rudolph, R., Lüdemann, H.-D., & Jaenicke, R. (1980b) *Biochemistry 19*, 1121-1126.
Schmid, F. X. (1980) in *Protein Folding* (Jaenicke, R., Ed.) pp 387-400, Elsevier/North-Holland, Amsterdam.
Schmid, F. X. (1981) *Eur. J. Biochem.* (in press).
Schmid, F. X., & Baldwin, R. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4764-4768.
Teipel, J. W., & Koshland, D. E., Jr. (1971) *Biochemistry 10*, 792-805.
Vallee, R. B., & Williams, R. C. (1975) *Biochemistry 14*, 2574-2580.
Zettlmeissl, G., Rudolph, R., & Jaenicke, R. (1979) *Biochemistry 18*, 5567-5571.

Sulfhydryl and Histidinyl Residues in the Flavoenzyme Alcohol Oxidase from *Candida boidinii*†

Thomas H. Cromartie

ABSTRACT: The flavoenzyme alcohol oxidase from *Candida boidinii* has about eight sulfhydryl residues per 75 000 molecular weight subunit. The enzyme is inactivated by treatment with *p*-(chloromercuri)benzoate at pH 7.5, but full enzymatic activity can be restored by treatment with excess β-mercaptoacetic acid. Complete inactivation of the enzyme requires reaction of all eight sulfhydryl residues per subunit. For any particular subunit, however, inactivation is an all-or-nothing event; i.e., slow reaction of a first sulfhydryl residue with *p*-(chloromercuri)benzoate is immediately followed by reaction of all remaining sulfhydryl residues of that subunit. The rate of inactivation shows saturation kinetics with respect to *p*-(chloromercuri)benzoate concentration, and substrates and products slow the rate of inactivation. The enzyme is also inhibited by treatment with Hg^{2+}, Cu^{2+}, and Ag^{+}, but no inactivation was found with *N*-ethylmaleimide, acrylonitrile, 5,5′-dithiobis(2-nitrobenzoate), or methyl methanesulfonate. Apparent time-dependent inactivation of alcohol oxidase with iodoacetate was found to be reversible on removal of excess inhibitor. Proposals for the function of sulfhydryl groups in alcohol oxidase are discussed. Alcohol oxidase is also rapidly inactivated by diethyl pyrocarbonate. The pH dependence of inactivation and absorbance changes on modification suggest that histidine residues are modified. Total inactivation of the enzyme occurs on reaction of four histidine residues per FAD coenzyme, but only one of these residues appears to be catalytically essential. [1-^{14}C]Diethyl pyrocarbonate was used to demonstrate that only histidine residues were modified. In the presence of either of the competitive inhibitors acetaldehyde or acetate, diethyl pyrocarbonate reacts with only 3 equiv of histidine in a fashion which suggests that the catalytically essential histidine is at the active site of the enzyme. Modification of alcohol oxidase with diethyl pyrocarbonate slightly alters the visible absorbance spectrum of the flavin coenzyme and prevents reduction by substrate methanol, consistent with a role for histidine at an early stage in catalysis.

There has been increasing interest in the use of microorganisms to produce protein for supplementing animal feed because the microorganisms can be rapidly produced by aerobic fermentation from raw materials which cannot be used directly by mammals (Hamer & Hamden, 1979). Methanol now appears to be promising as a growth substrate, and several bacteria and yeasts which can grow on methanol as sole carbon and energy source have been isolated (Sahm, 1977; Cardemil, 1978). Under such growth conditions, several yeasts of the *Candida* species have been found to produce a soluble alcohol

† From the Department of Chemistry, University of Virginia, Charlottesville, Virginia 22901. *Received February 3, 1981.* This work was supported in part by institutional grants from the University of Virginia partially funded by a Biomedical Sciences Support Grant from the National Institutes of Health and in part by a grant from Research Corporation.

0006-2960/81/0420-5416$01.25/0