Conformational Drift of Dissociated Lactate Dehydrogenases

Lan King and Gregorio Weber*

Department of Biochemistry, School of Chemical Sciences, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

Received December 11, 1985; Revised Manuscript Received February 24, 1986

ABSTRACT: Bovine and porcine lactate dehydrogenases, in solutions of 0.1–10 μM at neutral pH, dissociate into monomers upon application of hydrostatic pressures of up to 2 kbar. The dissociation was determined from observations of the polarization of fluorescence under pressure in seeming equilibrium conditions and by occasional hybridization experiments of the H_4 and M_4 isozymes. Decompression is followed by the rapid association of the monomers into tetramers and by slow, and sometimes incomplete, return of the enzymic activity. The dissociation curves obtained on compression and decompression differ, indicating that association results in partial loss of subunit affinity. These phenomena are attributed to a slow conformational drift that follows the loss of contact of the monomers with each other and to an even slower reversal of the drift that takes place upon reassociation.

A number of oligomeric proteins, as well as the indefinite aggregates formed by actin, myosin, and tubulin, are dissociated by the application of pressure. [For review, see Heremans (1982) or Weber & Drickamer (1983).] Jaenicke and his co-workers (Schade et al., 1980; Mueller et al., 1981a,b) have carried out a detailed study of the effect of pressure upon the enzymic activity of lactate dehydrogenase (LDH). They reported that when enzyme solutions at micromolar concentrations were incubated at high hydrostatic pressures (<2 kbar), the enzyme activity, *measured after pressure release*, showed a monotonic decrease as the incubation pressure was raised. On the assumption that the enzyme activity recorded on decompression represented accurately the degree of dissociation of the system into monomers at the incubation pressure, Jaenicke et al. calculate standard volume changes upon dissociation of −500 mL/mol (M_4) and −360 mL/mol (H_4). The slow recovery of enzyme activity that they observed in the decompressed samples appeared in agreement with their assumption without, however, providing conclusive proof. We have carried out a series of observations on lactate dehydrogenase solutions subjected to hydrostatic pressures in the range of 1 bar to 2 kbar. We show below that when the protein dissociation under pressure and the enzyme activity after decompression are both measured, they reveal a more complex situation than that deduced from enzymic activity alone: Upon release of pressure, the monomers associate "immediately" into an aggregate with the volume of the original tetramer but with diminished catalytic activity. In time, if exposure to high pressure is not excessively long, full catalytic activity is regained by the reconstituted tetramer. Additionally, intermediate degrees of dissociation are reached at different pressures when this is raised to 2 kbar and then lowered toward atmospheric pressure. These phenomena of hysteresis and eventual recovery of the original properties clearly indicate the time-dependent nature of the dissociation and association reactions of the tetramer. The slow regain of catalytic activity after tetramerization shows that catalysis must depend upon conformational details that are independent, to a large extent, of those that ensure the reassociation of the isolated subunits.

Measurements of Dissociation under Apparent Equilibrium Conditions. To determine experimentally the standard free energy change of a chemical reaction, it becomes necessary to determine the proportions of reactants and products *under conditions of thermodynamic equilibrium*. Application of this concept in its most stringent form would render unfeasible the determination of the free energy change of any reactions of a protein in water solution, since all practical experience shows their limited stability. Fortunately, we can relax the necessary conditions of *indefinitely stable* thermodynamic equilibrium on noticing that the measured free energy changes cannot be appreciably different if products and reactants are "infinitely stable" or change into other forms very slowly in comparison with those changes that belong to the reaction under study. To detect these characteristic changes, some property of the system is followed in time after an initial alteration of its state variables: pressure, temperature, or chemical composition. As the system approaches a stable condition, the observed property changes over a period that we designate as a characteristic "stabilization time", t_s. The system may be considered stabilized when the measured property shows no change, within the limits of experimental error, for a further period of time which we designate as the "test time", t_t. Evidently the ratio t_t/t_s is a figure of merit of the stability of the system. The larger its value the more likely the system is to approach the ideal condition of thermodynamic equilibrium, though practical purpose limits this figure of merit to a small numerical value. We consider $t_t/t_s = 1$ as satisfactory, provided that the change in the observed property upon change in the state variable is at least a few times the experimental error in its determination. When these criteria are satisfied, we consider that *practical thermodynamic equilibrium* has been reached, in the sense that the derived thermodynamic parameters may be expected to approach those that would obtain under ideal conditions.

Tetramer–Monomer Equilibria. The dissociation of a tetramer into smaller particles may be expected to occur in two stages: tetramer → dimer and dimer → monomer. If we call the degree of dissociation of the tetramer into dimers a ($1 > a > 0$), the degree of dissociation of the dimer into monomers b ($1 > b > 0$), and the total protein concentration, expressed as tetramer molarity, C, the equilibrium concentrations of tetramers, dimers, and monomers are respectively

$$[T] = (1 - a)C \qquad [D] = 2a(1 - b)C$$
$$[M] = 4abC \tag{1}$$

and the dissociation constants of tetramer and dimer, respectively, KT and KD, are

$$KT = 4a^2(1 - b)^2C/(1 - a)$$
$$KD = 8ab^2C/(1 - b)^2 \tag{2}$$

The character of the dissociation is determined by the relative

0006-2960/86/0425-3632$01.50/0

values of KT and KD. Equilibrium between tetramer and monomers is defined by the relation, from eq 1

$$K = [M]^4/[T] = 256a^4b^4C^3/(1 - a)$$
$$K = (KT)(KD)^2 \quad (3)$$

In a tetramer formed by identical subunits it seems reasonable to suppose that the affinities of the particles for each other are determined by the extent of the contacts; breaking down of the tetramer into dimers would then involve a change in free energy approximately twice that involved in the breaking of the dimer into monomers, and we accordingly expect $KD = KT^{1/2}$. In these circumstances if K is micromolar or less, the dissociation of the dimer will be virtually complete at all concentrations at which there is appreciable dissociation of the tetramer into dimers, and the dissociation will be described with reasonable approximation by eq 3 with $b = 1$. Because the dimensions of K are those of a concentration raised to the third power, it is convenient to replace it by the characteristic concentration $C_{1/2}$ at which $a = 0.5$. From the last equation we find $C_{1/2}^3 = K(0)/32$ giving (3) the new form:

$$C_{1/2}^3 = 8a^4C^3/(1 - a) \quad (4)$$

The dilution curves for the tetramer–monomer equilibria, in the absence of any conformational drift, have a characteristic span of only 1.59 log units in comparison with a span of 2.86 log units for the dimer–monomer case. As a result the experimental demonstration of the existence of a conformational drift by the decrease of the logarithmic span (Xu & Weber, 1982) is more difficult to achieve for tetramers than for dimers. Additionally (King & Weber, 1986) the value of $C_{1/2}$ for LDH is at least as small as 10^{-11} M which precludes an accurate determination by measurements of fluorescence polarization as a function of dilution.

Materials and Methods

Lactate dehydrogenase from bovine heart (H_4) was prepared by the procedure of Pesce et al. (1964). Lactate dehydrogenase from porcine heart (H_4) and the M_4 isozymes were a commercial preparation from Boehringer. Conjugation of the proteins with 2-(dimethylamino)naphthalene-5-sulfonyl chloride (2,5-DNS) was done by the method of Herron and Voss (1981). Enzyme activity was determined by following the rate of decrease in absorbance at 340 nm as NADH was oxidized by pyruvate. The degree of dissociation was determined from the values of the polarization of the intrinsic protein fluorescence or the polarization of the fluorescence of the dansyl fluorochrome. The method of measurement and the procedure of calculation of the degree of dissociation are given by Paladini and Weber (1981). It should be noted that in these calculations it is assumed that the original particles and the dissociated products are characterized by constant polarization values and that the computed intermediate degrees of dissociation may be seriously in error if this assumption does not apply. In a plot of degree of dissociation vs. pressure that includes $p_{1/2}$, the value of p at which $a = {}^1/_2$, an estimate of the standard volume change, dV^0, in the dissociation into monomers is obtained from the slope of the plot at $p_{1/2}$ by application of the equation

$$-d \ln (C_{1/2})/dp = (10/3)da/dp = dV^0/RT \quad (5)$$

or from the pressure span, $\langle dp \rangle$, as already discussed (Weber, 1986). For a tetramer–monomer equilibrium, at 25 °C

$$dV^0 = 272.34/\langle dp \rangle \quad (6)$$

if dV^0 is independent of pressure.

Table I: Distribution of Isozymes after Pressure Incubation of H_4 and M_4

(A) Equal Amounts at 0.9 kbar

isozyme	starting amount	distribution at incubation times (min) of 11	84	190
M_4	0.5	0.29	0.27	0.12
M_3H	0	0.15	0.12	0.17
M_2H_2	0	0.12	0.22	0.42
MH_3	0	0.16	0.23	0.15
H_4	0.5	0.29	0.17	0.14

(B) Unequal Amounts

isozyme	starting amount	distribution at incubation times (min) of 24 (0.9 kbar)	10 (2 kbar)
M_4	0.36	0.16	0
M_3H	0	0.13	0.15
M_2H_2	0	0.17	0.30
MH_3	0	0.22	0.40
H_4	0.64	0.32	0.15

Results

Conclusive proof of the validity of eq 4 for our case would demand the demonstration that at all degrees of dissociation only the tetramer and monomer are present and that under high pressures the dissociated species is indeed the monomer. The following observations are considered strong evidence for the exclusive tetramer–monomer equilibrium:

(1) The curves of fluorescence polarization vs. pressure do not show the existence of an intermediate step that could be identified as a region in which the dimer is the predominant species.

(2) The rotational relaxation times of DNS-protein conjugates, determined from Perrin plots of the polarization and lifetime of the fluorescence at 1 bar and at pressures of maximum dissociation, were 220 and 24 ns, respectively, for the porcine enzyme and 160 and 28 ns for the bovine enzyme. These times correspond respectively to compact tetramers and to monomers in which there is appreciable increase in the local motion of the fluorochrome.

(3) Evidence of dissociation into monomers which is equally strong, and independent of the spectroscopic observations, is given by the uniform appearance in time of the three mixed isozymes when the M_4 and H_4 isozymes are jointly subjected to pressure. Table IA shows the distribution of isozymes after equal amounts of H_4 and M_4 are mixed and subjected to a pressure of 0.9 kbar for various incubation times. If the dimer is the most important tetramer fragment, we should observe, at small degrees of dissociation, the predominance of H_2M_2 in comparison with MH_3 and M_3H, at the short incubation times (11 min). The result is the opposite, indicating that the monomer must be the predominant dissociated form even at low degrees of dissociation. Moreover, the H_2M_2 isozyme is shown to be the more stable form under pressure as its proportion at 84 and 190 min of incubation rises from 12%, at 11 min, to 22% and 42%, respectively. Table IB shows the distribution of isozymes after mixing different amounts of M_4 and H_4 isozymes. No evidence for the rapid appearance of H_2M_2 was found at either 0.9 or 2 kbar.

Time Course of the Dissociation of LDH. Figure 1 shows the changes with time of the polarization of the intrinsic fluorescence of bovine lactate dehydrogenase following a rapid rise in pressure from atmospheric to 2 kbar. From the original value of 0.124 the polarization of the fluorescence of the enzyme reaches slowly a stable value of 0.099 following a rapid

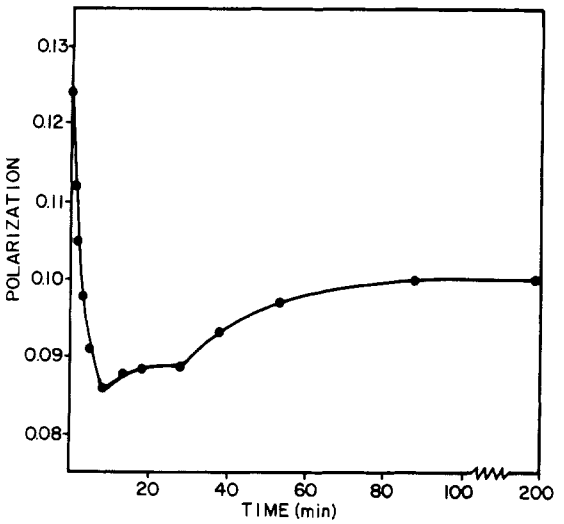

FIGURE 1: Time course of the polarization of the intrinsic fluorescence of bovine LDH-H_4 subjected to a pressure of 2 kbar. Enzyme concentration 3.4 μM. Buffer: 50 mM Tris-HCl, pH 7.6, plus 1 mM EDTA and 10 mM DTE, at 25 °C. Excitation at 280 nm through monochromator and Corning 7-54 filter. Emission filter WG-320.

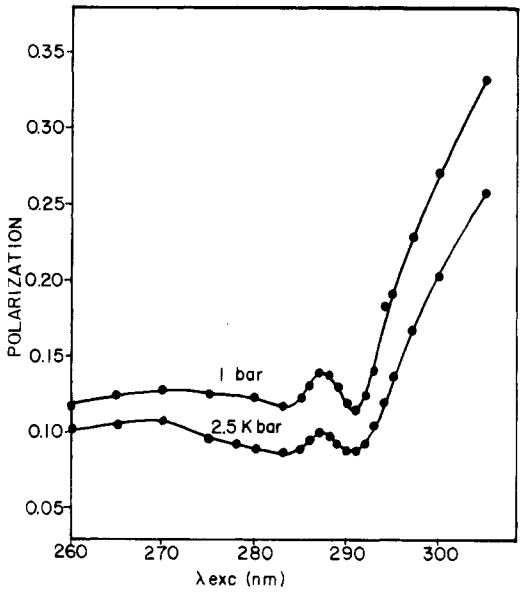

FIGURE 2: Excitation polarization spectrum of porcine LDH-H_4 at 1 bar and 2.5 kbar. Enzyme concentration 10 μM. Excitation bandwidth 1.67 nm. Other conditions as in Figure 1.

decrease down to 0.086. For porcine enzyme the polarizations oscillated between 0.087 and 0.092 and finally stabilized at 0.090. In this and other experiments described here the time course was followed to ensure a figure of merit of the stability, $t_s/t_t = 1$, in each case. We can then consider that the results reported in this study refer to states of "practical" thermodynamic equilibrium. Figure 1 shows that the approach to equilibrium is a complex process similar to that seen by Xu and Weber (1982, Figure 2) in enolase upon dilution at atmospheric pressure. The complexity of the equilibria involved in the dissociation is also shown by the drop, at the highest pressures, of the rotational relaxation time of the DNS conjugates below the value that corresponds to a compact monomer (see below). A reasonable interpretation of the time course displayed in Figure 1 is that dissociation itself takes place rapidly and that subsequent slow changes result from conformational adjustments of the separated particles.

Pressure Dependence of LDH Dissociation. The excitation–polarization spectra of the fluorescence of both porcine

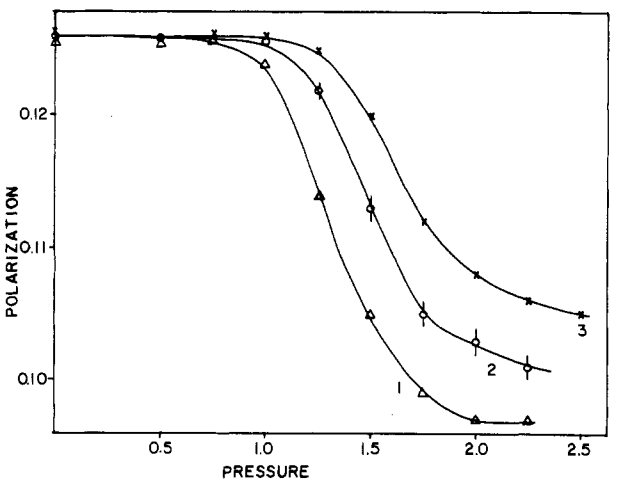

FIGURE 3: Plots of intrinsic fluorescence polarization of porcine LDH-H_4 vs. pressure at three concentrations: (1) 0.11, (2) 1.1, and (3) 11 μM. Other conditions as in Figure 1.

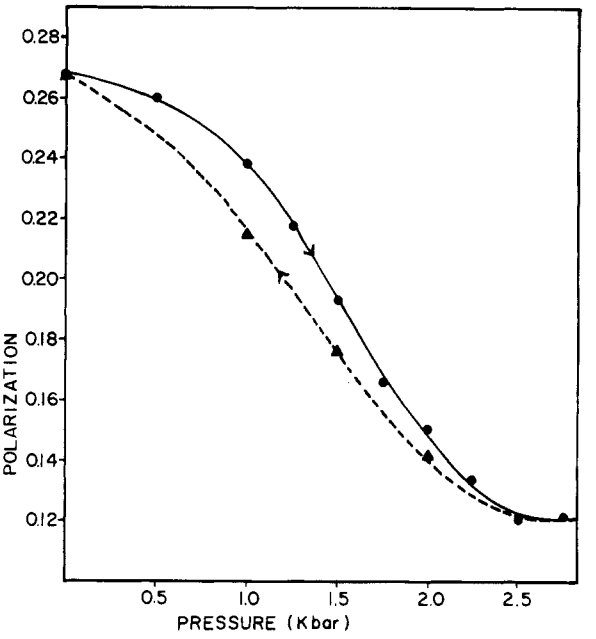

FIGURE 4: Plot of fluorescence polarization of 2,5-DNS conjugate of porcine LDH-H_4 containing 3.5 mol of label. Excitation at 410 nm. Enzyme concentration 2.8 μM, pH 7.6, 25 °C. The points on the dotted line correspond to observations on decompression.

and bovine LDH measured at 1 bar and 2.5 kbar were parallel over the range of excitation wavelengths of 260–305 nm, indicating no change in the relative contributions of the adjacent electronic transitions, L_a and L_b, in this pressure interval. This constancy of spectrum, shown in Figure 2, excludes the possibility that the observed polarization changes result from variation with pressure in the relative contributions of the overlapping electronic transitions rather than from changes in rotational rate of the fluorophore. Figures 3 and 4 illustrate the dependence of the degree of dissociation upon the pressure. Figure 3 shows the changes in intrinsic fluorescence polarization with pressure for solutions of porcine enzyme at three concentrations. The pressure span was similar for all of them, ~630 bars corresponding to $dV^0 = 430$ mL/mol (eq 6). Figure 4 shows the polarization changes induced by pressure upon a conjugate of porcine LDH with 3.5 residues of 2,5-DNS per mol. This conjugate showed $p_{1/2} = 1.45$ kbar and, from eq 5, $dV^0 = -170$ mL/mol. For bovine conjugates with $p_{1/2} = 2.33$–2.63 kbar, depending on concentration, $dV^0 = -220$ mL/mol. While the pressures of middissociation are very

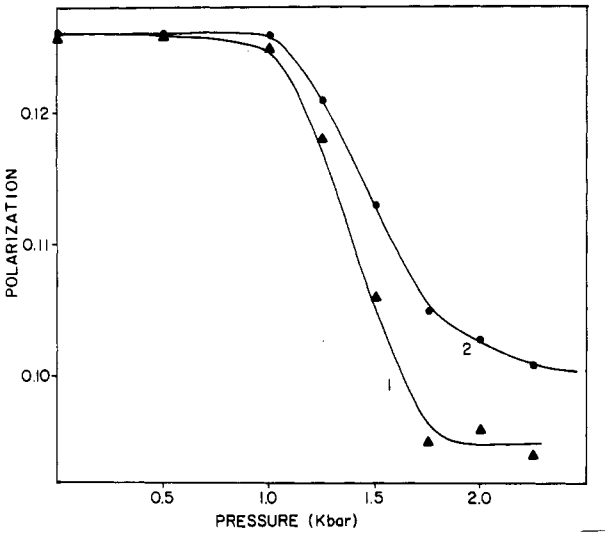

FIGURE 5: Plot of intrinsic fluorescence polarization of porcine LDH solution, 1.1 μM, excited at 280 nm and observed through two different cutoff filters: (1) Corning 0-52 and (2) Schott WG-320.

similar, the pressure spans, and the derived dV^0, of the plots in Figures 3 and 4 differ by a factor or 2 or more. The fluorescence polarization of the DNS conjugates is directly related to the overall rotation of the molecules, but the polarization of the intrinsic fluorescence is primarily determined by the local motion of the tryptophan residues, a difference that arises because the respective fluorescence lifetimes differ by an order of magnitude. The degree of dissociation calculated from the polarization of the fluorescence of conjugates can be expected to yield a valid figure of dV^0 but, as already discussed (Weber, 1985, Figure 6), this value may be in excess of the real standard change in volume upon dissociation, on account of the conformational drift. The reduced pressure span of the intrinsic fluorescence indicates that the main increase in the local motions of tryptophan occurs over a restricted range of degrees of dissociation. Thus, it gives proof of the change in structural properties of the particles, and therefore of their chemical potential, with the degree of dissociation. Further proof is given in Figure 5 which shows that the dissociation profile given by the polarization of the intrinsic fluorescence depends upon the spectral region observed: This can only arise if the multiple tryptophan residues in the protein change their rotational properties in different ways as dissociation proceeds. A remarkable feature of the curves in Figure 3 is the very small difference in middissociation pressures: a factor of 10 in the concentration would be expected (Weber, 1985, eq 13) to result in a difference of 400 bars for $dp_{1/2}$, but the observed value is barely one-third of this. We are unable at this stage to decide whether this deficit results from differences in compressibility of tetramer and monomers (Paladini & Weber, 1981) or from some less obvious cause.

Prompt Reassociation of Tetramers after Pressure Release. The polarization of the fluorescence of 2,5-DNS conjugates of both porcine and bovine enzyme recovered the atmospheric pressure values upon gentle release of the pressure, a procedure that took a few minutes. The average polarization of the tryptophan fluorescence was similarly recovered. The reassociation into tetramers soon after pressure release was confirmed by both gel filtration and gel electrophoresis. Additionally when H_4 and M_4 isozymes were hybridized under pressure, only the five isozyme bands were seen after pressure release.

Hysteresis Phenomena. The recovery of the original fluorescence polarization upon release of pressure took place

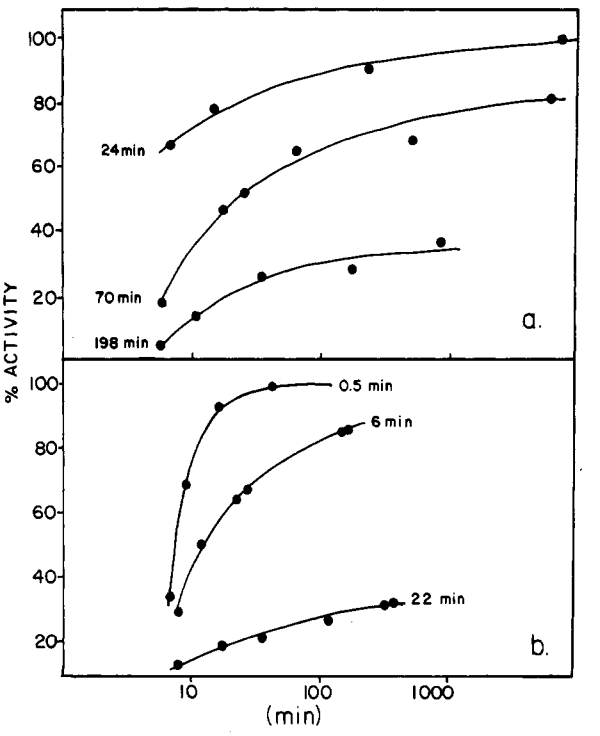

FIGURE 6: Enzyme activity following incubation for the times stated in the curves at pressures of 1.5 (a) and 2 kbar (b). The abscissa is the time after decompression in minutes.

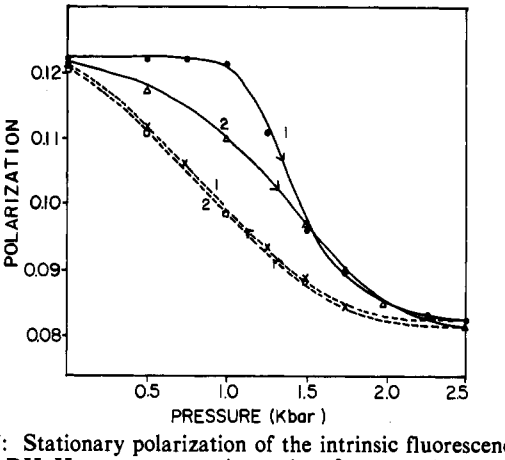

FIGURE 7: Stationary polarization of the intrinsic fluorescence of porcine LDH–H_4 on two successive cycles of pressure increase and release. The numbers indicate the order of performance of the cycles. The dotted lines correspond to decompression.

almost immediately, but the recovery of enzyme activity was in comparison extremely slow. Figure 6a shows that the time necessary for regain of activity of bovine LDH previously subjected to pressure of 1.5 kbar varied from 10 to more than 1000 min as the time of incubation at high pressure was raised from 3 to 70 min. For a longer time of incubation at high pressure, 198 min, enzyme activity was not fully recovered. Effects of the same nature were observed at the lower pressure of 1.0 kbar. At an incubation pressure of 2 kbar (Figure 6b) much activity was permanently lost after only 22 min of incubation. Figure 4 shows that the polarization values at equal pressures recorded on raising the pressure to 2 kbar and in lowering it again to atmospheric pressure differ significantly. The dissociation is systematically larger for the pressure decrease branch, indicating that some affinity has been lost during the high-pressure application. Figure 7 shows the result of an experiment in which the polarization of the fluorescence of porcine LDH was followed on two successive cycles of

pressure increase and release. Curve 2 corresponds to the cycle initiated after the pressure of 1 bar had been maintained for 1 h after completion of the first cycle. The forward branch of this cycle falls midway between the forward and reverse branches of the first cycle, indicating the incomplete recovery of the tetramers resulting from association after the first pressure cycle. The reverse branches of the first and second cycle coincided, and this reverse curve could be obtained in additional cycles, indicating that it corresponds to a state of final equilibrium between the completely drifted monomers and the corresponding associated tetramers. There are evident differences between the cycles of pressure application as observed through the DNS fluorescence (Figure 4) and the intrinsic protein fluorescence (Figure 7). With the 2,5-DNS conjugates the differences between forward and reverse branches are due directly to the loss of affinity of the monomers while similar differences in the polarization of the fluorescence of tryptophan arise mainly in the changes in local rotations of this residue in the original and reassociated tetramers.

Discussion

In distinction from the present case Xu and Weber did not observe any evident time-dependent effects in enolase: The conformational drift of the isolated monomers was inferred from the shortened span of concentrations over which the dissociation occurred. Pressure-dissociated enolase (Royer, unpublished observations) showed on reassociation at least 90% of the original enzyme activity. In lactate dehydrogenase we have a more complex situation: The conformational drift of the isolated monomers is evident from the low enzymic activity of the reassociated protein. A strong, though indirect, proof of the dependence of the chemical potential of the components upon the degree of dissociation is furnished by the character of the intrinsic fluorescence polarization shown in Figures 3 and 5.

The observations of the polarization of both the intrinsic fluorescence and that of conjugates with 2,5-DNS demonstrate the overall reversibility of the dissociation of the protein by pressure. The dissociation characteristics deduced from these observations differ considerably from those suggested by Jaenicke and co-workers on the basis of enzymic activity alone. For example, at 0.7 μM concentration and a pressure of 0.5 kbar, porcine LDH shows no appreciable dissociation from polarization measurements. Mueller et al. (1981a) have shown that under these conditions the enzyme loses about half of its activity in 15 h, and we obtained a similar result. We believe that this inactivation is due to the microscopic behavior of the system under these conditions (King & Weber, 1986): At these inconspicuous degrees of dissociation the conformational drift must be quite limited owing to the short lifetime of the monomer. An incubation time of several hours, during which very many cycles of association and dissociation occur, is then necessary for the accumulation of an appreciable amount of inactive tetramer. As the pressure is increased and a larger fraction of the protein becomes dissociated, the lifetime of the monomers increases and a proportionally greater conformational drift takes place (Weber, 1986, Figure 5). This explains the observed correlation between pressure and time of incubation on one hand and loss of enzymic activity on the other. At protein concentrations of 1–10 μM and pressures of 2 kbar and higher the equilibrium is shifted almost completely to the side of the monomers, and a rapid conformational drift and inactivation follows. The loss in subunit affinity brought about by the monomer drift appears to reach a limit, in that succesive cycles of pressure application following periods of incubation at high pressure result in the same curve for the dependence of degree of dissociation upon pressure reversal. In contrast, the enzyme activity does not appear to reach a minimum value, and for the longer periods of incubation at high pressure part of the activity seems permanently lost. While the loss in conformation of the dissociated monomers can be readily inferred from the incomplete, time-dependent reversibility of the system, the polarization data, both of the intrinsic fluorescence and of DNS conjugates, give direct physical indication of the disorganization of the subunits upon dissociation: The rotational relaxation time of anhydrous spherical molecules of 140000 (tetramer) and 34000 (monomer) daltons are 130 and 33 ns, respectively. The values calculated from the spectral data for DNS conjugates at atmospheric pressure and high pressure are 220 and 24 ns, respectively. The excess from the unhydrated sphere represented by the atmospheric pressure value is due to hydration and hydrodynamic shape factors of the order observed for most globular proteins. The high-pressure value is, however, inferior to that corresponding to the anhydrous sphere and can only be explained by the existence of local fluorophore rotations independent from those of the particle as a whole. The loss of the polarization of the fluorescence of tryptophan provides an even more dramatic demonstration of the appearance of additional degrees of rotational freedom upon dissociation: For a fluorescence lifetime of 4 ns a change in rotational relaxation time from 130 to 33 ns should be followed by a change in fluorescence polarization of 7–8%, but the drop in fluorescence polarization upon increase of pressure is about 35%.

Our conclusion from these studies is that, upon dissociation, the lactate dehydrogenase monomers undergo a conformational drift with loss of both monomer affinity and the capacity to reconstitute an enzymically active tetramer. It is well-known that the free energy of subunit association can readily change in response to the binding of specific ligands, a phenomenon amply documented by the X-ray analysis of oxy- and deoxyhemoglobins. It is therefore not surprising that on separation the subunits should lose the conformation characteristic of the original aggregate. The unexpected result is the rapid reassociation and the final recovery of both subunit affinity and enzymic activity.

Apart from the interesting questions that regard the physical chemistry of these phenomena (Weber, 1986) there is the purely biological aspect: In what way does the limited stability of the dissociated species influence the formation, the function, and the replacement of oligomeric aggregates in the organism? The ability of high hydrostatic pressures to perturb reversibly the state of aggregation of oligomeric proteins makes it possible for the first time to address these questions both in vitro and in vivo and to expect rapid progress in the near future.

Registry No. Lactate dehydrogenase, 9001-60-9.

References

Heremans, K. (1982) *Annu. Rev. Biophys. Bioeng. 11*, 1–21.
Herron, J. N., & Voss, E. W. (1981) *J. Biochem. Biophys. Methods 5*, 1.
King, L., & Weber, G. (1986) *Biochemistry* (third paper of three in this issue).
Mueller, K. Leudemann, H.-D., & Jaenicke, R. (1981a) *Biophys. Chem. 14*, 101.
Mueller, K., Leudemann, H.-D., & Jaenicke, R. (1981b) *Biochemistry 20*, 5411.
Paladini, A. A., & Weber, G. (1981) *Biochemistry 20*, 2587.
Pesce, A., McKay, R. H., Stolzenbach, F., Cahn, R. D., & Kaplan, N. O. (1964) *J. Biol. Chem. 238*, 1753.

Schade, B. C., Leudemann, H.-D., Rudolph, R., & Jaenicke, R. (1980) *Biophys. Chem. 11*, 257.
Weber, G. (1986) *Biochemistry* (first paper of three in this issue).
Weber, G., & Drickamer, H. G. (1983) *Q. Rev. Biophys. 16*, 89–112.
Xu, G.-J., & Weber, G. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5268–5271.

Conformational Drift and Cryoinactivation of Lactate Dehydrogenase

Lan King and Gregorio Weber*

Department of Biochemistry, School of Chemical Sciences, University of Illinois at Urbana–Champaign, Urbana, Illinois 61801
Received December 11, 1985; Revised Manuscript Received February 24, 1986

ABSTRACT: Solutions of porcine lactate dehydrogenase of micromolar concentration kept at 4 °C for several days lose the greater part of their enzymic activity but recover it when returned to room temperature. The rate of spoiling decreases and the rate of recovery increases with the concentration of the solutions. The decrease in tetramer stability in the cold is shown by experiments of pressure dissociation at various temperatures and confirmed because isozyme hybridizaton occurs in parallel with the inactivation at low temperature but is absent at room temperature. Cold-inactivated solutions contain tetramers that dissociate much more readily than those of the fully active solutions. It is postulated that cryoinactivation, like pressure inactivation, takes place through a cycle of dissociation, conformational drift [King, L., & Weber, G. (1986) *Biochemistry* (second paper of three in this issue)] and reassociation into inactive tetramers.

Jaenicke and his co-workers (Mueller et al., 1981) and ourselves (King & Weber, 1986) have observed the slow inactivation of micromolar solutions of lactate dehydrogenases subjected to relatively small hydrostatic pressures. Additionally we have shown in the previous paper (King & Weber, 1986) that at these pressures dissociation is, and remains, very small or undetectable by spectroscopic methods. The progressive inactivation over a long time is attributed to a microscopic cycle of dissociation into monomers, slow conversion of the free monomers into a conformationally different species, and reassociation of these into tetramers characterized by decreased subunit affinity and altered enzyme activity (drifted tetramers). The rate of accumulation of drifted monomers is proportional to the degree of dissociation. Therfore, it proceeds very slowly at low pressure and in a much shorter time at a pressure sufficiently high to achieve virtually complete dissociation. The inactive tetramer obtained in either case should be the same species, provided that the pressure itself is not an additional cause of drift. The drifted tetramer is a metastable species, but the first-order equilibrium between native and drifted tetramers involves an energy of activation sufficiently large to prevent rapid reconversion of the drifted into the native tetramer. The free energy relations between the various species are shown in Figure 1. The ordinate is the Gibbs free energy, and the abscissa is a conformational coordinate dependent upon structural differences. The scheme places the free energy of the monomers well above those of the tetramers and corresponds therefore to an equilibrium involving small proportions of the monomers with respect to the corresponding tetramers. This condition presupposes a total protein concentration conspicuously larger than the characteristic concentrations for half-dissociation of the native tetramer, $C_{1/2}$ and of the drifted tetramer, $C^*_{1/2}$. The horizontal first-order conversions, M ↔ M* and T ↔ T*, involving changes in conformation, are very much slower than the vertical changes, which correspond to the higher order processes T ↔ 4M* of association and dissociation of the tetramers. If all equilibria were equally fast, the stable forms would be T and M*, but the large energy barrier prevents the ready reconversion of T* into T and thus leads to accumulation of the drifted tetramer.

Table I: Distribution of Isozymes on Storage at Room Temperature and in the Cold

		4.3 °C for	
isozyme	room temp	5 days	12 days
M_4	0.36	0.30	0.19
M_3H	0	0.02	0.09
M_2H_2	0	0.05	0.07
MH_3	0	0.11	0.28
H_4	0.64	0.53	0.37
	room temp for 6 days		4.3 °C for 6 days
M_4	0.50		0.36
M_3H	0		0.10
M_2H_2	0		0.07
MH_3	0		0.12
H_4	0.50		0.35

Cryoinactivation through Microscopic Conformational Drift. We first realized the influence of the temperature upon the conformational drift through the observation that a sample that had been stored for several weeks in the cold did not show hysteresis in the fluorescence polarization experiments, thus behaving in a way similar to the sample that was incubated at medium pressure over a much shorter time. Lowering the temperature was found to shift the pressure dissociation curve to a lower pressure range. Later, hybridization of H_4 and M_4 isozymes was detected in samples of micromolar concentration stored in the cold room at 4 °C for several weeks. These long incubations in the cold resulted in enzyme inactivation, but this could be completely reversed by warming the samples to room temperature for a period that depended both on the concentration and on the duration of cold inactivation. All these experiments will be described in more detail.

0006-2960/86/0425-3637$01.50/0