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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
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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.

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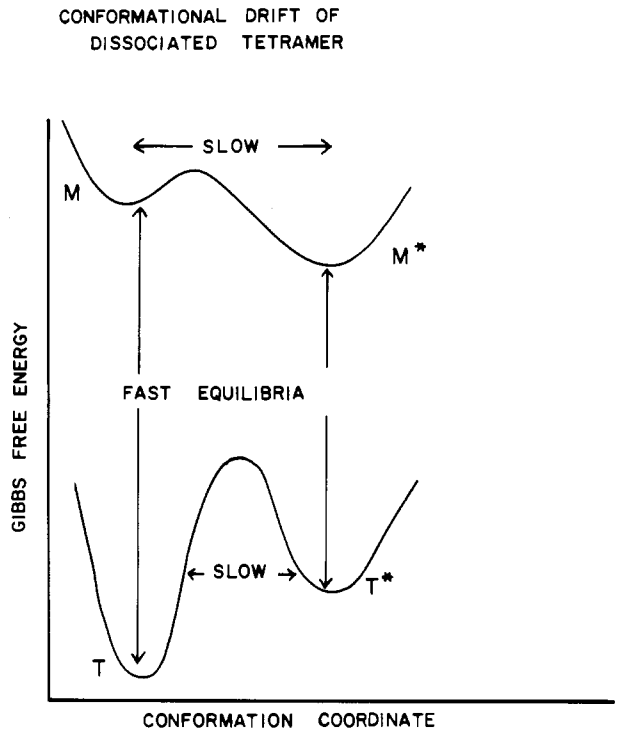

FIGURE 1: Free energy scheme of the conformational drift of lactate dehydrogenase. At low temperature dissociation is facilitated (Figure 2) and the conversion of drifted into native tetramer is hindered by the large energy barrier.

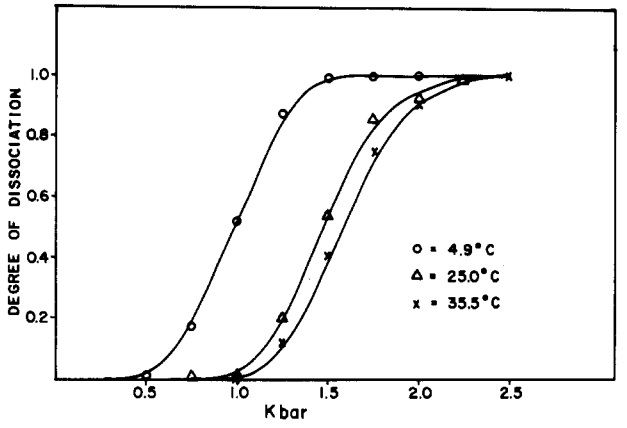

FIGURE 2: Plots of calculated degree of dissociation of porcine LDH from polarization of the intrinsic protein fluorescence. Protein concentration 1.1 μM, pH 7.6.

(*1*) *Cold Hybridization.* Table I shows the distribution of isozymes after equal amounts of H_4 and M_4 at micromolar concentrations were mixed and stored in the refrigerator (4.3 °C) for 5 and 12 days, respectively. No preferential formation of H_2M_2 was observed, indicating that the monomer was the main species involved in the hybridization equilibria. The control samples, kept for the same period at room temperature, showed no detectable hybridization.

(*2*) *Temperature Effect on Pressure Dissociation.* Figure 2 shows plots of the degree of dissociation vs. pressure for solutions of LDH. The results clearly indicate that dissociation is promoted by lowering the temperature. $p_{1/2}$ is shifted from 1470 to 970 bars as the temperature is lowered from 24 to 4.9 °C. Temperature effects become smaller above 25 °C. At 35.5 °C $p_{1/2}$ is 1590 bars. Above 36 °C $p_{1/2}$ does not appear to change. Investigation at higher temperatures is rendered difficult because the increased local rotations of the tryptophan residues makes the polarization measurements less reliable.

Table II: Dependence of Cold Inactivation on Time of Storage and Protein Concentration

concn (μM)	days of storage at 4 °C	activity (%)
0.36	1	67
0.36	7	36
0.28	10	12
0.28	20	7
11	10	70
2.2	10	34
0.28	10	12
0.07	10	7

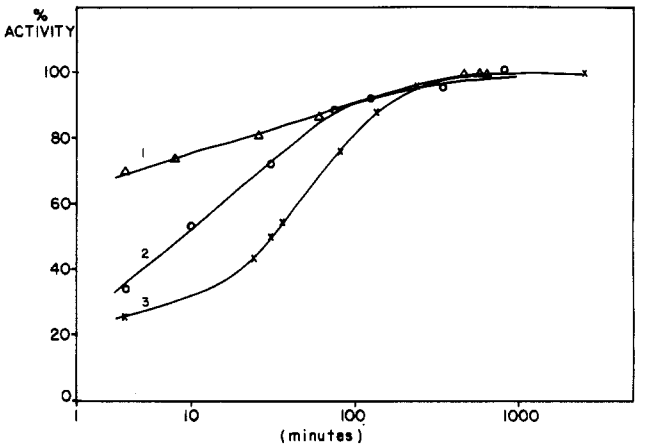

FIGURE 3: Time dependence (logarithmic scale, minutes) of the room temperature reactivation of the enzyme activity of solutions of porcine LDH in Tris buffer, pH 7.6, after incubation for 7 days in the cold room. Protein concentrations: (1) 11, (2) 2.2 and (3) 0.11 μM.

The pressure dissociation curves at the different temperatures are nearly parallel, indicating similar pressure spans and therefore similar volume changes upon dissociation at the three temperatures. The difference in $p_{1/2}$ at temperatures of 4.9 and 25 °C, $dp_{1/2}$, is 0.5 kbar so that taking $dV° = -200$ mL mol^{-1} (King & Weber, 1986) $dp_{1/2}dV°$, the difference in free energy of tetramer association at the two temperatures, equals 2.4 kcal/mol. Thus, $C_{1/2}$ increases by a factor of about 60 at the lower temeperature. This large change is responsible for the almost qualitative differences observed in the stability of the enzyme activity at cold room and room temperatures.

(*3*) *Cold Inactivation.* Table II shows the inactivation of LDH after various periods of storage in the cold room. The enzyme lost 33% of its original activity during the first day of storage in the cold room. A further 31% of activity was lost in the following 7 days. After 20 days the enzyme had lost most of the original activity. Table II, which refers to incubation in the cold room for 10 days, shows that cold inactivation is concentration dependent. It is noticeable that the activity remained high (70%) for the sample with the highest concentration, 11 μm. Both time dependence and concentration dependence of the cold inactivation provided independent evidence of the existence of a microscopic association–dissociation cycle that results in a modified form of the protein.

(*4*) *Reactivation of LDH after Cryoinactivation.* The plots of Figures 3 and 4 show that several hours to days are necessary for the return of full activity after cold-incubated samples (0.11–11 μm) are stored at room temperature. Figure 4 shows the activity recovery of samples of very low concentration (4.4–280 nM) that had been previously stored in the cold room for 10 days. The activity of the more diluted samples was not completely recovered even after 15 days at room temperature. The dilution curve of the 280 nM sample was determined by measuring the degree of polarization of

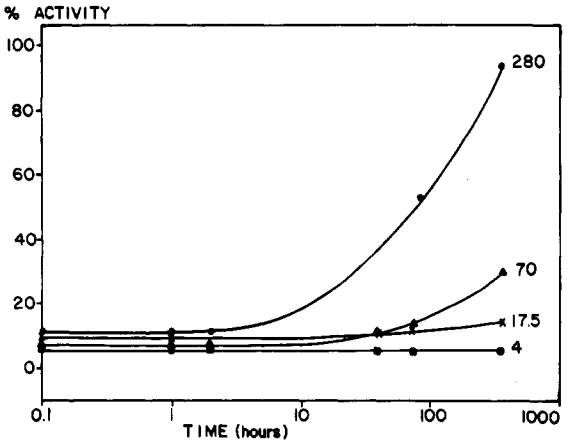

FIGURE 4: Time dependence (logarithmic scale, hours) of the room temperature reactivation of the enzymic activity of solutions of porcine LDH in Tris buffer. Samples were kept in the cold room for 10 days previous to the experiments, at the nanomolar concentrations shown in the figure.

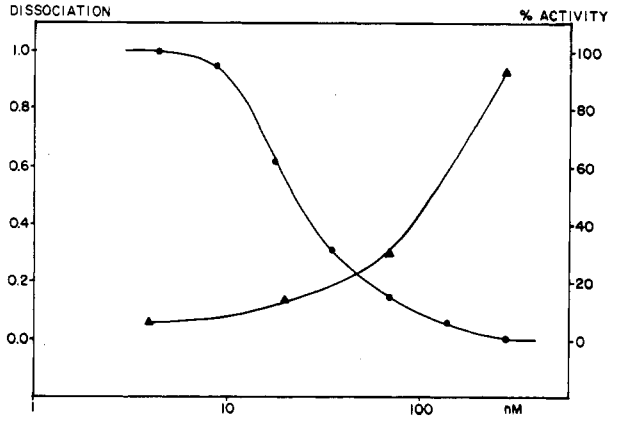

FIGURE 5: (●) Degree of dissociation vs. concentration of a sample of porcine LDH stored in the cold room for 10 days previous to the experiment at 280 nM concentration, as determined by measurements of polarization of the intrinsic fluorescence. (▲) The final activity recovery after 15 days at room temperature, of protein solutions stored at different concentrations.

the tryptophan fluorescence as done by Xu and Weber (1981) with enolase, and the results are shown in Figure 5. Because the sample had lost 90% of the original enzyme activity, we conclude that this dilution curve must closely represent the equilibrium between the drifted tetramer and drifted monomers. The logarithmic span of the dilution curve (Weber, 1986) is definitely smaller than 1.5 units, the normal value for a tetramer–monomer equilibrium. From the plot we determine $C^*_{1/2}$ = 23 nM. Samples below this concentration would not be expected to recover their enzymic activity even after prolonged incubation at room temperature, and this conclusion is substantiated by a plot, shown in the same figure, of the activity recovered by samples of protein concentration in the range of the dilution curve after 15 days of incubation at room temperature. Control samples of LDH that had not been stored in the cold could be diluted below the nanomolar range without appreciable decrease in fluorescence polarization, but the polarization of still lower concentrations could not be reliably measured. It is then apparent that $C_{1/2}$ is at least 2 orders of magnitude smaller than $C^*_{1/2}$.

Detection of the Microscopic Cycle at Atmospheric Pressure, Room Temperature, and Neutral pH. When gel filtration of a freshly prepared solution of porcine LDH at a concentration of 1.2 μM is carried out at room temperature, the elution profile shows a small amount of monomer, and a

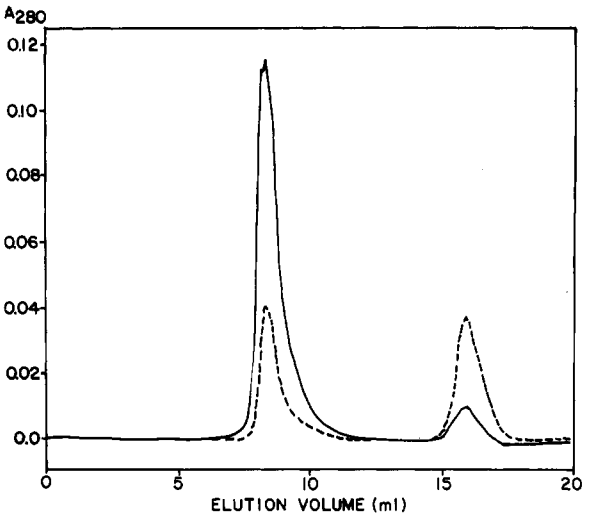

FIGURE 6: Gel filtration patterns of porcine LDH on a TSK-3000 column. (—) Sample diluted to 1.2 μM from stock solution (36 μM) 12 h before the run. (---) Sample diluted to 0.9 μM from stock solution 18 days before the run. Both samples were kept at room temperature.

larger amount is seen if the sample is previously kept at room temperature for a long time (Figure 6). If the tetramer isolated by this means is left for sometime at room temperature, a further small amount of monomer is seen to appear. These experiments indicate that under these conditions an equilibrium is established between tetramer and monomers and that the equilibrium between the forms *that are then present* is slow enough to permit column separation. When samples of LDH at concentrations of 7.2 and 0.9 μM were kept for 18 days at room temperature, a distinctly larger accumulation of monomer was seen in the sample at the lower concentration. Measurements of enzymic activity showed no appreciable deterioration of the samples of LDH over these times, indicating that the tetramers present were mostly of the native kind. On the other hand, if $C_{1/2}$, the concentration for half-dissociation of the native species, is on the order of 10^{-11} or smaller, a solution of micromolar concentration should show a degree of dissociation a given by the approximation

$$a = (C_{1/2}/2C)^{3/4} = 3 \times 10^{-3} \quad (1)$$

which would remain undetected in gel filtration. Therefore by eq 1 the monomer separated must be almost solely M*.

Conclusions. The set of observations reported in this and the previous paper (King & Weber, 1986) together with the free energy diagram of Figure 1 permits us to form a clear picture of the cold inactivation and the thermal reactivation of LDH. The proposed sequence of events is in agreement with observations on the reactivation of oligomeric proteins carried out over many years. In oligomeric proteins both the formation of inactive aggregates after reversal of urea denaturation by dilution (Blond & Goldberg, 1985) and their temperature activation (Irias al., 1968) have been described. Dissociation has been long suspected as the cause of the cryoinactivation (Bock & Frieden, 1978), but the relation between the two phenomena has remained obscure because inactivation takes place in many instances at concentrations at which dissociation is inconspicuous. The demonstration that a microscopic cycle of dissociations and reassociations leads to the formation of metastable, enzymically inactive agggregates that are difficult to distinguish from the original ones by their physical properties can provide a satisfactory explanation for these cases. The increase in the rate of formation of the inactive aggregates by the application of relatively small pressures opens up the

possibility of testing the general validity of this explanation.

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

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Rat Growth Hormone Gene Expression Is Correlated with an Unmethylated CGCG Sequence near the Transcription Initiation Site†

Jeannine S. Strobl*,‡

Department of Pharmacology and Toxicology, West Virginia University Medical Center, Morgantown, West Virginia 26506

Priscilla S. Dannies

Department of Pharmacology, Yale University, New Haven, Connecticut 06520

E. Brad Thompson‡

Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77550

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ABSTRACT: The methylation status of the rat growth hormone (GH) gene was compared in DNA obtained from GH-producing and GH-nonproducing sources by digestion with three methylation-sensitive restriction enzymes. GH gene expression was correlated with an unmethylated *Tha*I site (CGCG) 144-bp upstream of the GH RNA transcription initiation site. This *Tha*I site was unmethylated in nine GH-producing subclones of the rat pituitary tissue culture cell line GH_3 and in greater than 50% of the DNA isolated from rat anterior pituitary, a gland containing GH-producing somatotroph cells as well as GH-nonproducing cells. In DNA prepared from GH-nonproducing tissues, e.g., rat spleen, kidney, liver, and brain, this *Tha*I site was entirely methylated. Furthermore, this site was entirely methylated in hybrid cells formed by the fusion of GH_3 cells with mouse fibroblasts in which GH production has been extinguished. DNA methylation at 10 other restriction sites located throughout the rat GH gene region failed to correlate with GH expression in GH-producing subclones of GH_3 cells as well as in GH-nonproducing $GH_3 \times LB82$ hybrid cells. We suggest that the conserved absence of methylation 144 bp 5′ of the RNA transcription initiation site of transcribed GH genes identifies a potential GH gene control region.

In eukaryotic DNA approximately 3-5% of the cytosines have undergone modification to 5-methylcytosine (Razin & Riggs, 1980). Although the function of DNA methylation in eukaryotes is not clear, the evidence is strong that specific cytosines are methylated in the 5′ flanking DNA of inactive genes. In many genes examined in eukaryotic tissues or cultured cells, DNA methylation occurs in a tissue-specific pattern. Methylcytosine is present 5′ of the nonexpressed genes while there is an unmodified cytosine in the expressed genes [for a review, see Yisraeli & Szyf (1984)]. Increases in gene transcriptional activity during development have also been correlated with decreases in gene methylation (Andrews et al., 1982; Bird et al., 1981; Colgan et al., 1982; Weintraub et al., 1981). More recently, DNA methylation changes 5′ of the albumin gene have been shown to accompany reversible gene activation and deactivation (extinction) in somatic cell hybrids (Ott et al., 1982, 1984).

In certain instances, experimentally induced alterations in gene methylation patterns have resulted in predictable changes in gene transcription. DNA methyltransferase activity is inhibited by 5-azacytidine (Taylor & Jones, 1982). Treatments with 5-azacytidine have resulted in DNA demethylation and activation of a number of genes including the rat GH[1] gene in GH_3 cells (Cherington & Tashjian, 1983; Lan, 1984). The chief limitation of this type of experiment is the inability to control the sites of demethylation. The influence of 5-methylcytosine within specific genes has been tested by in vitro methods to introduce 5-methylcytosine into cloned genes and gene transfer to study the effects of methylation on gene activity (Wigler et al., 1981; Stein et al., 1982; Vardimon et al., 1982). Methylation clearly depresses the activity of the globin

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* Author to whom correspondence should be addressed.

‡ Formerly of the Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, MD 20205.

[1] Abbreviations: GH, growth hormone; kbp, kilobase pairs; DMEM, Dulbecco's minimum essential medium; SSC, standard saline citrate, 0.14 M sodium chloride-0.015 M sodium citrate, pH 7.2; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; FCS, fetal calf serum; HS, horse serum; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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