Studies on the Subunit Molecular Weight of Beef Heart Lactate Dehydrogenase†

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ABSTRACT: A recent study on the molecular weight of beef heart lactate dehydrogenase in 6.2 M guanidine hydrochloride at pH 2 (Millar, D. B., Frattali, V., and Willick, G. W. (1969), *Biochemistry 8*, 2416) concluded that there were eight subunits of 18,000 molecular weight each, rather than the commonly accepted four polypeptide chains of 35,000. Their results conflict with previous subunit studies conducted under milder conditions of pH, and thus to resolve this disagreement we have established: (i) the protein exists as a random coil in 6 M guanidine hydrochloride at both near-neutral and low pH; (ii) the tetrameric structure for lactate dehydrogenase is correct, as shown by three independent methods, including sedimentation equilibrium at pH 2 in 6 M guanidine hydrochloride. Optical rotatory dispersion measurements were made on the protein in 6 M guanidine hydrochloride–0.1 M 2-mercaptoethanol at pH 6.4 and 1, exhibiting levorotation typical of denatured proteins. The experimentally observed rotations agreed with those calculated for a lactate dehydrogenase random coil at specific wavelengths, and the Moffitt–Yang parameter, b_0, was nearly zero, which is characteristic of fully denatured proteins. Molecular weights of lactate dehydrogenase under native conditions and in 6 M guanidine hydrochloride at pH 6 and 2 were determined by sedimentation equilibrium; constituent polypeptide chain length was estimated by sodium dodecyl sulfate acrylamide gel electrophoresis and guanidine–agarose chromatography. All methods gave nearly the same values for the subunit weight of 34,500 ± 1500, which is in agreement with a tetrameric structure for the native molecule with a weight of 136,700 ± 2100. The most likely source of low molecular weights previously found is an impurity in commercially obtained enzyme, which we removed through chromatography in 6 M guanidine hydrochloride.

The isozymes of lactate dehydrogenase have been thoroughly studied over the past years. Data including amino acid compositions, peptide maps, and sedimentation studies in denaturing solvents (Pesce *et al.*, 1967; Appella and Markert, 1961) from many laboratories indicate a native structure comprising four polypeptide chains, each of molecular weight 35,000; a high-resolution X-ray crystallographic analysis of the dogfish muscle isozyme performed by Rossmann and his collaborators (Adams *et al.*, 1970) shows it is a tetramer. In contrast to this evidence is the recent report that 18,000 molecular weight subunits are the basic constituents of beef heart lactate dehydrogenase; this result was obtained from sedimentation equilibrium experiments under conditions of 6.2 M Gdn·HCl,[1] 0.1 M H_3PO_4–KH_2PO_4, and 0.01 M dithiothreitol, at pH 2 (Millar *et al.*, 1969). It was further asserted that since studies in this solvent at neutral pH gave a monomer molecular weight of twice their extrapolated value at low pH, Gdn·HCl was an ineffective dissociating agent under normal conditions. Attracted by these findings, we have reinvestigated quaternary structure in this protein and the effectiveness of Gdn·HCl denaturing conditions in its determination, both at near-neutral and low pH. Our partial duplication of experiments by Millar *et al.* (1969), suggests some sources for their anomalous results, while our studies as a whole amply demonstrate that this enzyme is composed of 35,000 molecular weight subunits, which are fully denatured at pH 6 or 2 in 6 M Gdn·HCl.

Materials and Methods

Subunit studies were conducted on lactate dehydrogenase from beef heart (EC 1.1.1.27), purchased from Worthington Biochemical Corp. (code BHLDH, lot 9GB) as a 55% ammonium sulfate suspension; some of the native molecular weight data were collected on a special grade of the heart isozyme (code HLDH, lot 9GA) from the same source. Heico UltraPure Gdn·HCl was used after filtration of stock solutions through a fine fritted disk funnel; it was found to satisfy the criteria of purity established by Wong *et al.* (1971), without further purification, and therefore Gdn·HCl concentrations were accurately determined refractometrically on the basis of data from Kielley and Harrington (1960).

Optical rotatory dispersion (ORD) measurements were made with a Cary Model 60 recording spectropolarimeter, using 1-cm quartz cells and solutions of about 1.5 mg of protein/ml. Stock solutions were prepared by dialyzing the $(NH_4)_2SO_4$ suspension of enzyme against 0.1 M KCl–0.001 M β-ME (pH 6.5); ORD samples were made by diluting portions of this into concentrated Gdn·HCl, which was then made 0.1 M in β-ME, reducing 6 hr at pH 8.6, and finally readjusting to pH 6.4 or 1. Concentrations of enzyme were obtained spectrophotometrically, using $E_{280}^{1\%} = 14.9$ (Pesce *et al.*, 1964). Data were analyzed in the manner described earlier (Tanford *et al.*, 1967), reported as Moffitt–Yang parameters, a_0 and b_0, and the reduced mean residue rotations

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[1] Abbreviations used are: Gdn·HCl, guanidine hydrochloride; β-ME, β-mercaptoethanol.

TABLE I: Optical Rotation of Lactate Dehydrogenase in 6.4 M Gdn·HCl.

Denaturing Conditions	$[m']_{589}$	$[m']_{400}$	$[m']_{300}$	Moffitt–Yang Parameters[a] a_0	b_0
Calculated from the amino acid composition[b] and intrinsic residue rotations[c]	−93	−243	−611		
6 M Gdn·HCl–0.1 M β-ME, pH 6.4	−86	−224	−570	−561	+3
6 M Gdn·HCl–0.1 M β-ME, pH 1	−87	−230	−581		

[a] λ_0 was arbitrarily set equal to 212 mμ. [b] Amino acid composition from Pesce *et al.* (1967). [c] Intrinsic residue rotations from Tanford (1968).

at selected wavelengths, $[m']_\lambda$. The $[m']_\lambda$'s expected from a lactate dehydrogenase random coil were calculated on the basis of intrinsic residue rotations (Tanford, 1968), applied to its established amino acid content (Pesce *et al.*, 1967).

Sodium dodecyl sulfate–acrylamide gel electrophoresis, according to Weber and Osborn (1969), and 6% agarose chromatography in 6 M Gdn·HCl (Fish *et al.*, 1969) were used as independent methods of determining subunit size. When sedimentation analysis of protein eluted from a Gdn·HCl column was planned, approximately 10 mg was initially applied, and the peak tube was diluted severalfold with solvent to yield about 0.2 mg of protein/ml, prior to dialysis.

A Spinco Model E analytical ultracentrifuge equipped with interference optics, having the temperature regulated at 25°, was used for sedimentation equilibrium studies. Protein solutions were prepared by dissolving about 6 μl of the ammonium sulfate suspension in 1 ml of solvent to give a 0.02% solution. For subunit studies, 6 M Gdn·HCl was the starting solvent, in which the enzyme was reduced 4 hr at pH 8.6, alkylated with iodoacetamide, and then adjusted to pH 6 or 2; the sample was then dialyzed against several changes of 6 M Gdn·HCl at the same pH with the final dialysis extending to four days. Studies of the native molecule were conducted in 0.1 M potassium phosphate buffer (pH 6.8) plus 0.1 M KCl after dialysis for 24 hr. The high-speed meniscus depletion method (Yphantis, 1964) was used in all experiments, and equilibrium was checked by comparing displacements of fringes recorded at least 2 hr apart; water blanks were run after all samples on the intact cell, but were usually found to yield negligible corrections. All calculations are based on the average of five fringes read from the photographic plate (Kodak, II-G Spectroscopic) with a Gaertner two-dimensional microcomparator.

The basic equation used in determining the apparent molecular weight, M_{app}, through sedimentation equilibrium is derived by Tanford (1961)

$$M_{app} = \frac{2RT}{\omega^2(1 - \bar{v}_2\rho)} \frac{\mathrm{d} \ln f}{\mathrm{d}r^2} \qquad (1)$$

where the partial specific volume, $\bar{v}_2$, is 0.74 cm³/g for lactate dehydrogenase (Millar, 1962), r is the distance fromt he rotor's center, ρ is the density of the solvent, ω is the speed in radians/sec, and f is the fringe displacement at a given radial position. Results obtained in 6 M Gdn·HCl at near-neutral pH must be corrected for preferential binding of Gdn·HCl to the protein (Hade and Tanford, 1967), which was accomplished by replacing $\bar{v}_2$ with the apparent partial specific volume, ϕ'_2, as discussed by Casassa and Eisenberg (1964); a ϕ'_2 equal to 0.73 cm³/g was used, which was indirectly determined by Castellino and Barker (1968) from membrane osmometry measurements.

Equation 1 does not adequately describe the equilibrium sedimentation pattern of a highly charged protein in a three-component system. This obtains because the protein and salt gradients become coupled through the Donnan effect, which leads to a shallower protein gradient than eq 1 predicts and therefore to a low molecular weight. The rigorous thermodynamic description of this situation was first derived by Scatchard and coworkers (Johnson *et al.*, 1954); treatment of the problem is also given by Tanford (1961), and recently it has been extended to the analysis of self-associating systems of high charge (Roark and Yphantis, 1971). In calculations of M_{app} for lactate dehydrogenase in 6 M Gdn·HCl at pH 2, the equation of Scatchard applies

$$M_{app} = \frac{2RT}{\omega^2(1 - \bar{v}_2\rho)} \frac{\mathrm{d} \ln f}{\mathrm{d}r^2} + \frac{1}{2} Z M_3 \left(\frac{1 - \bar{v}_3\rho}{1 - \bar{v}_2\rho} \right) \qquad (2)$$

where M_3 is the molecular weight of Gdn·HCl, $\bar{v}_3$ is its partial specific volume, equal to 0.76 cm³/g, and Z is the protein charge which has a maximum value of +40 under these conditions. Our use of eq 2 gives the maximal correction to M_{app}, since Z could be less than +40 due to counterion binding; use of $\bar{v}_2$ instead of ϕ'_2 in the first term on the right-hand side of eq 2 also maximizes M_{app}, but this is justifiable because repulsion from the highly charged protein should suppress preferential binding of guanidinium ions.

Results

The ORD spectrum of reduced lactate dehydrogenase in 6 M Gdn·HCl exhibits a smooth increase of levorotation from 600 to 250 nm and is devoid of any fine structure in the aromatic region, which is typical of ORD curves for randomly coiled polypeptides (Tanford *et al.*, 1967). As shown in Table I, the calculated and observed $[m']_\lambda$'s agree well, considering the sensitivity of this method. The slight increase of observed levorotation upon lowering the pH from 6.4 to 1 obeys the Kauzmann–Eyring rule (Kauzmann and Eyring, 1941) which is discussed by Schellman (1958), and states that influences restricting orientation about bonds will tend to increase the magnitude of optical activity; the increase in positive charge on the protein at pH 2 will cause a stiffening of the polypeptide chain which should produce greater levorotation, as is observed. It is noteworthy that the Moffitt–

TABLE II: Molecular Weight Data.

Figure[b]	Condn of Expt	Mol Wt: Using Eq 1[a]	Mol Wt: Using Eq 2[a]	Rpm (Sed Equil)
	Sedimentation equilibrium			
1	0.1 M KCl–0.1 M K_2PO_4, pH 6.8	139,850[c]		15,218
		139,000		16,202
		131,270		14,290
2a	6 M Gdn·HCl–0.1 M β-ME, pH 6 (reduction at 8.6)	34,460		43,981
2b	6 M Gdn·HCl, pH 2; peak tube off Gdn·HCl column	30,200	34,000	44,769
		31,300	35,200	52,645
	Gdn·HCl column chromatography on 6% agarose (reduced at pH 8.6 and alkylated with iodoacetic acid)	34,500		
	Dodecyl sulfate acrylamide gel electrophoresis	35,000		

[a] For sedimentation equilibrium data; all ln C *vs.* r^2 plots were linear over the full length of the solution column. [b] These figures were submitted with the manuscript and examined by reviewers; they are recorded on the microfilm version of this paper (see footnote 2). [c] The pure heart isozyme of lactate dehydrogenase was used for this experiment; all other data presented refer to enzyme (Worthington, grade BHLDH) which contained some of the muscle isozyme.

Yang parameter, b_0, is close to zero, which is generally considered diagnostic for random coils (Urnes and Doty, 1961).

The subunit molecular weights obtained by dodecyl sulfate–acrylamide gel electrophoresis and gel chromatography on 6% agarose in 6 M Gdn·HCl are in good agreement, giving values of 35,000 ± 2000 and 34,600 ± 2000, respectively. This is significant since they depend on different manifestations of polypeptide chain length, in that dodecyl sulfate normalizes proteins to a similar rodlike structure (Reynolds and Tanford, 1970) whereas concentrated Gdn·HCl causes formation of random coils. In each case an unknown polypeptide must have a conformation like the standards to give the correct molecular weight, and agreement between results from these distinct approaches and sedimentation equilibrium (which gives the molecular weight based on thermodynamic grounds rather than hydrodynamic properties) in 6 M Gdn·HCl at near-neutral pH (Table II) guarantees that the constituent polypeptide chains of lactate dehydrogenase are randomly coiled and hence nonaggregating, without resorting to extreme pH. The data of Fish *et al.* (1970) support these data further, as they used gel chromatography in dodecyl sulfate to assess molecular size and obtained a Stokes radius consistent with the same mass that we find. Likewise Castellino and Barker (1968) found this protein in 6 M Gdn·HCl–0.5 M β-ME has an intrinsic viscosity of 32.4 cm^3/g, which corresponds to a randomly coiled polypeptide chain of about 35,000, and observed a 40-fold increase in the second virial coefficient on going from a native to denatured protein. When the Worthington enzyme (code BHLDH) was chromatographed on 6% agarose in 6 M Gdn·HCl, whose pH was about 6, a minor component eluted with a molecular weight of 20,500 ± 1500, which amounted to 5–10% of the main peak. This was doubtless a contaminant and was the source of initial difficulties in sedimentation equilibrium studies at low pH.

Typical sedimentation equilibrium data are displayed in the figures, which are recorded on the microfilm copy of this manuscript;[2] both native (Figure 1) and denatured enzyme (Figure 2) are seen to give linear ln f *vs.* r^2 plots from 100 μ displacement to the base of the solution column, indicating homogeneity and the absence of any protein association or dissociation. The average weight for the native protein was found to be 136,700 ± 2100, from equilibrium sedimentation in 0.1 M phosphate buffer (pH 6.8) plus 0.1 M KCl; this agrees very well with values of 136,290 ± 1400 and 141,440 obtained by Castellino and Barker (1968) through membrane osmometry and sedimentation equilibrium, respectively. In order to obtain linear data at pH 2 in 6 M Gdn·HCl, it was necessary to use material which had first been fractionated by Gdn·HCl–agarose chromatography to remove a lower molecular weight impurity; when this precaution was not taken, a curved ln f *vs.* r^2 plot was found, with weight-average molecular weights considerably lower than for purified material.

In 6 M Gdn·HCl we found a molecular weight of 34,460 ± 1500 for the enzyme at pH 6, using commercial protein directly, but an average value of 30,700 ± 1500 for purified material at pH 2, both calculated with eq 1; the weight at pH 6 corresponds within experimental error to 36,180 ± 800 and 37,850–35,750 as found by Castellino and Barker (1968) with osmometry and equilibrium sedimentation in 6 M Gdn·HCl–0.5 M β-ME at pH 6. For the low pH data, adding a correction based on the second right-hand term of eq 2 to the value from eq 1 raises the weight by 1630 to 32,330 ± 1500. However by using eq 2 for the whole calculation, which takes into account suppression of preferential guanidinium binding by the highly charged protein in addi-

[2] Supplementary material describing sedimentation equilibrium ln f *vs.* r^2 plots (Figures 1 and 2a,b) will appear following these pages in the microfilm edition of this volume of the journal. Single copies may be obtained from the Business Operations Department, Books and Journals Division, American Chemical Society, 1155 Sixteenth St., N. W., Washington, D. C. 20036, by referring to code number BIO-72-1609. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche.

tion to the Donnan effect term, the value becomes 34,600 ± 1500, which is in total agreement with the weight at pH 6.

Discussion

The foregoing experiments present consistent evidence from several approaches for a native lactate dehydrogenase structure comprising tetramers of 35,000 molecular weight. ORD studies demonstrate the protein in 6 M Gdn·HCl–0.1 M β-ME satisfies the optical rotation criteria for a random coil at pH 6.4 and 1; thus there is no spectral evidence of residual structure which would mediate association between subunits at neutral pH. The agreement between values obtained from sedimentation equilibrium in 6 M Gdn·HCl, dodecyl sulfate gels, and Gdn·HCl–agarose column chromatography shows the polypeptide chain length of the subunits is that expected from their thermodynamically based molecular weight, and thus also supports the random coil nature of the subunits at low and near neutral pH. The sedimentation equilibrium values for runs at pH 6 and 2 are similarly in agreement, provided that for runs at low pH the sample was first purified on the Gdn·HCl column. Thus it is clear that 6 M Gdn·HCl denatures lactate dehydrogenase to a randomly coiled state and there are no grounds for suspecting any associations between subunits at near-neutral pH. In conjunction with a native molecular weight of about 139,000, our subunit weights prove the existence of a tetrameric structure for native protein beyond any doubt. The anomalous results of Millar *et al.* (1969), very likely stem from the impurity which we found in enzyme preparations that were the same commercial grade as they used.

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