Fluorescence Polarization Studies of Conjugates of Beef Heart Lactic Dehydrogenase with 1-Dimethylaminonaphthalene-5-sulfonyl Chloride*

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ABSTRACT: Fluorescence polarization measurements of beef heart lactic dehydrogenase conjugated with 1-dimethylaminonaphthalene-5-sulfonyl chloride demonstrate that the enzyme preserves its size and shape throughout the protein concentration range of 3 μg/ml to 1 mg/ml in solutions containing 0.10 M potassium phosphate (pH 6 or 7) and in 0.10 M potassium phosphate plus 2 M NaCl (pH 6). The rotational relaxation time of lactic dehydrogenase obtained from independent measurements of polarization and lifetime is approximately 220 nsec at 25°. This value is independent of the degree of labeling in the range of 1.1–3.6 moles of 1-dimethylaminonaphthalene-5-sulfonate/mole of lactic dehydrogenase. There are no thermally activated ligand rotations in the conjugates of lactic dehydrogenase with 1-dimethylaminonaphthalene-5-sulfonate.

There is conflicting evidence regarding dissociation of beef heart lactic dehydrogenase in dilute neutral solutions of moderate ionic strength. Millar (1962) originally reported that beef heart lactic dehydrogenase which has a molecular weight near 140,000, dissociates into units of mol wt 72,000 at protein concentrations less than 1 mg/ml. The observations of Bernfeld *et al.* (1965) and Hathaway and Criddle (1966) appeared to support this conclusion whereas Appella (1964) and Pesce *et al.* (1964) were unable to detect dissociation.

One argument against dissociation is based on failure to obtain hybridization in dilute neutral solutions of lactic dehydrogenase (Kaplan, 1964; Massaro and Markert, 1968). However, two limitations of hybridization experiments must be kept in mind. (1) Subunits available from each of two distinct types of lactic dehydrogenase, which may differ in their tendencies to dissociate, are required. (2) Hybridization may reflect subunit exchange arising from a degree of dissociation which is not detectable in most molecular weight determinations.

Additional information on dissociation can be obtained from fluorescence polarization measurements of conjugates of lactic dehydrogenase with 1-dimethylaminonaphthalene-5-sulfonyl chloride. Dissociation of the labeled enzyme into monomers or dimers should result in a decrease in fluorescence polarization. The concentration range involved (<1 mg/ml) is suitable for polarization measurements, which can be conducted at protein concentrations 100 times more dilute than those examined by Millar.

I shall present the results of fluorescence polarization studies of beef heart lactic dehydrogenase conjugates varying amounts of 1-dimethylaminonaphthalene-5-sulfonate The rotational relaxation time, ρ, of the enzyme is calculated from independent measurements of polarization and lifetime, τ, under conditions where the molecular weight has been precisely determined. The measurements are then extended to protein concentrations as low as 3 μg/ml in order to reveal dissociation under the conditions examined by Millar.

Experimental Procedures

Electrophoretically pure beef H_4 was isolated chromatographically (Pesce *et al.*, 1964) from a preparation of beef heart lactic dehydrogenase obtained from Worthington Biochemicals.

The conjugates of lactic dehydrogenase with 1-dimethylaminonaphthalene-5-sulfonyl-chloride were prepared by the procedure of Anderson and Weber (1966) with two minor modifications: 10^{-3} M EDTA was included in the reaction mixture and the final concentration of acetone was reduced to 1.3%. Variation in the incubation period of the enzyme with 1-dimethylaminonaphthalene-5-sulfonyl chloride produced different degrees of labeling. Incubation for 1 hr and 20 min resulted in an average degree of labeling of 0.3 mole of 1-dimethylaminonaphthalene-5-sulfonate/mole of lactic dehydrogenase; 4 hr and 20 min, 1.1 moles of 1-dimethylaminonaphthalene-5-sulfonate/mole of lactic dehydrogenase; and 10 hr and 20 min, 3.6 moles of 1-dimethylaminonaphthalene-5-sulfonate/mole of lactic dehydrogenase. The preparation containing 2.5 moles of 1-dimethylaminonaphthalene-5-sulfonyl/mole of lactic dehydrogenase was obtained by the earlier procedure. These conjugates have similar fluorescence spectra with corrected emission maxima at 520 nm. Labeling caused no loss in catalytic activity.

Polarization measurements were made on the apparatus described by Weber (1956). Excitation was

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with the 366-nm line isolated from a mercury arc by Corning glass filters CS7-60 and CS4-71. The emitted light passed through liquid filters of 2 M $NaNO_2$ and Corning glass CS3-72. The fluorescence lifetimes were determined by the phase-shift method using the cross-correlation phase fluorometer designed by Spencer and Weber (1968).

All other procedures were described by Anderson and Weber (1966).

Results

Rotational Relaxation Time. The rotational relaxation time at a specified temperature and viscosity is calculated from a plot of $1/P$ (reciprocal of polarization) against T/η (the ratio of the absolute temperature to the viscosity of the solvent) using the relationship

$$\rho = 3\tau \frac{\left(\frac{1}{P_0} - \frac{1}{3}\right)}{\left(\frac{1}{P} - \frac{1}{P_0}\right)}$$

where P_0 is the limiting polarization (Weber and Young, 1964).

The Perrin plot for beef heart lactic dehydrogenase containing 2.5 moles of 1-dimethylaminonaphthalene-5-sulfonate/mole of protein is given in Figure 1. The values of T/η were adjusted by both isothermal additions of sucrose and variation in temperature (Wahl and Weber, 1967). Since the directly measured lifetime of this conjugate is 17.2 ± 0.1 nsec, the relaxation time in water at 25° calculated from Figure 1 is 208 nsec. The conditions of this experiment coincide with those used in low-speed sedimentation equilibrium measurements which demonstrated that the enzyme is a single species of mol wt 138,000 (see Anderson and Weber, 1966). The relaxation time of an anhydrous sphere of this molecular weight, ρ_0, is 114 nsec (Anderson and Weber, 1966). Factors contributing to the relatively large value of ρ may include hydration, asymmetry, and preferential orientation of the emission oscillators with respect to the hydrodynamic ellipsoid (Weber and Anderson, 1968).

The excellent agreement between the results obtained isothermally and by variation in temperature answers two questions. The dissociation reported by Millar (1962) was strongly temperature dependent, the weight-average molecular weight changed from *ca.* 140,000 at 5° to *ca.* 95,000 at 20° when the protein concentration was 1 mg/ml. Such a temperature-dependent equilibrium would cause a discrepancy in the results obtained by the two methods of varying T/η. Clearly there is no evidence for a temperature-dependent dissociation. The second conclusion is that there are no thermally activated ligand rotations such as those found in conjugates of γ-globulin with 1-dimethylaminonaphthalene-5-sulfonate (Wahl and Weber, 1967).

Similar values of ρ were found for the other preparations. The conjugate containing 1.1 moles of 1-dimethylaminonaphthalene-5-sulfonate/mole of protein gave τ = 16.5 nsec and ρ = 228 nsec while the conjugate with 3.6 moles of 1-dimethylaminonaphthalene-5-sulfonate/mole of protein gave τ = 16.6 nsec and ρ = 220 nsec. Previous measurements had provided an estimate of ρ of 188 nsec (Anderson and Weber, 1966). However, direct measurements of τ were not then available.

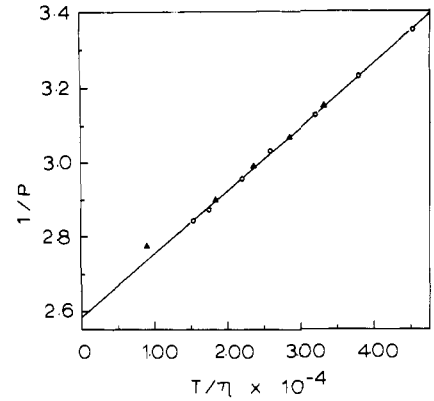

FIGURE 1: Plot of $1/P$ against T/η (°K/centipoise) for beef heart lactic dehydrogenase (H_4) conjugate containing 2.5 moles of 1-dimethylaminonaphthalene-5-sulfonate/mole of lactic dehydrogenase. The values of T/η were adjusted by addition of known amounts of sucrose to the solution at 25° (▲) or by variation of temperature in the range 0.2–38° (○). Conditions: 0.10 M potassium phosphate (pH 7.0) and 1 mg/ml of lactic dehydrogenase.

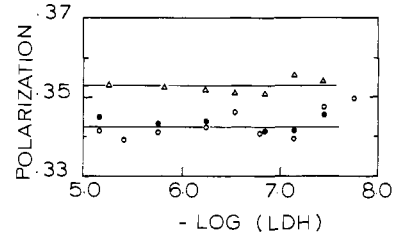

FIGURE 2: Demonstration of the constancy of the fluorescence polarization of beef heart lactic dehydrogenase conjugates in the protein concentration range 2×10^{-8}–7×10^{-6} M. Conditions: 0.10 M potassium phosphate (pH 7.0) (○), 0.10 M potassium phosphate (pH 6.0) (●), and 0.10 M potassium phosphate + 2 M NaCl (pH 6.0) (△) at 9°. (This conjugate contained 1.1 moles of 1-dimethylaminonaphthalene-5-sulfonate/mole of lactic dehydrogenase. The over-all increase in P observed in 2 M NaCl reflects principally the change in viscosity.)

Dilution Experiments. The fluorescence polarization of 1-dimethylaminonaphthalene-5-sulfonate-labeled beef heart lactic dehydrogenase is independent of protein concentration throughout the range 1 mg/ml to 3 μg/ml in solutions containing 0.10 M potassium phosphate (pH 6 or 7) and in 0.10 M potassium phosphate plus 2 M NaCl (pH 6) (Figure 2). Therefore I conclude that the enzyme does not dissociate appreciably under these conditions. Failure to detect dissociation in polarization measurements is possible only if the effects resulting from changes in symmetry exactly compensate those caused by the decrease in molecular weight. Previous studies of dissociation of lactic dehydrogenase at pH 2.5 showed that a 2.4-fold decrease in ρ occurred (Anderson and Weber, 1966).

Discussion

The fluorescence polarization data presented in this paper augment previous measurements of sedimentation (D. N. Raval and H. K. Schachman, 1965;[1] Pesce *et al.*, 1964) and light scattering (Appella, 1964) which indicated that beef heart lactic dehydrogenase does not dissociate appreciably in dilute neutral solutions. Thus three sets of independent measurements, involving different physical techniques, fail to substantiate Millar's (1962) observations. The fluorescence polarization measurements have two distinct advantages over the other methods used to detect dissociation. First, the sensitivity of fluorescence measurements permits examination of lactic dehydrogenase concentrations 100 times more dilute than those accessible to either the light-scattering or conventional sedimentation measurements. The second is that measurements in concentrated salt solutions are not directly affected by preferential interactions of the macromolecule with solvent.

The decrease in specific activity accompanying dilution of rabbit muscle lactic dehydrogenase (Bernfeld *et al.*, 1965) should be interpreted cautiously since the enzyme in dilute solutions is sensitive to surface denaturation. In the course of routine assays, using the procedures of Pesce *et al.* (1964) and Anderson and Weber (1966), I have found that the specific activity of either beef heart or beef muscle lactic dehydrogenase is independent of concentration in the approximate range of 2×10^{-10}–10^{-8} M.

The substrate-induced changes described by Hathaway and Criddle (1966) were not investigated since the fluorescence of NADH and absorption by high concentrations of pyruvate would interfere with polarization measurements.

[1] Personal communication to Anderson and Weber (1965).

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