# Effect of Coenzyme on the Bis(8-anilino-1-naphthalenesulfonate)-Linked Association of Beef Muscle Lactic Dehydrogenase†

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ABSTRACT: Binding studies using (bis(8-anilino-1-naphthalenesulfonate) (bis(ANS)) previously showed that muscle type lactic dehydrogenase ($M_4$) from either beef or chicken undergoes a ligand-linked self-association while heart type lactic dehydrogenase ($H_4$) does not.

Sedimentation, fluorescence, and circular dichroism measurements have resolved the 10–12 primary bis(ANS) binding sites of beef $M_4$ into two functionally distinct classes. The binding of 4 equiv of AcPyADH reverses the self-association of beef $M_4$ with the concurrent release of 3–4 equiv of bis(ANS). Seven to eight bis(ANS) molecules remain bound at saturating coenzyme concentrations. The three to four displaceable bound dye molecules are characterized by a high fluorescence quantum yield and intense circular dichroism bands. The seven to eight residual bis(ANS) molecules have a low fluorescence yield and weak circular dichroism bands. The two kinds of binding sites have similar affinities for bis(ANS) in the absence of coenzyme.

That lactic dehydrogenase is a tetramer and that the five commonly occurring isozymes are the different combinations of two kinds of subunit, H ("heart" type) and M ("muscle" type), are well known (Appella and Markert, 1961; Cahn *et al.*, 1962; Markert, 1963; Pesce *et al.*, 1964). Tissue isozyme patterns show that "heart" type lactic dehydrogenase participates in aerobic metabolism while the "muscle" type functions principally in glycolysis. Differing sensitivities to substrate inhibition constitute the largest known difference between the catalytic properties of the two subunit types and are considered physiologically important (Kaplan and Cahn, 1962; Markert, 1968; Bishop *et al.*, 1972).

The stoichiometry of NADH binding to lactic dehydrogenase is consistent with the tetramer hypothesis. At pH 7.4, beef $H_4$ has four independent, equivalent sites while beef $M_4$ exhibits cooperative binding explained by molecular relaxation effects (Anderson and Weber, 1965). The affinities of beef $M_4$ and $H_4$ for the coenzyme are similar. The three hybrid lactic dehydrogenases have intermediate coenzyme binding properties.

Bis(8-anilino-1-naphthalenesulfonate) (bis(ANS))[1] is a dimer with the essential fluorescence characteristics of 8-anilino-1-naphthalenesulfonate (ANS) (Rosen and Weber, 1969). Unlike ANS, bis(ANS) adsorbs readily to lactic dehydrogenase in 0.1 M potassium phosphate, pH 7 (20°). Binding studies using bis(ANS) showed that $M_4$ from either beef or chicken undergoes a ligand-linked self-association while $H_4$ does not (Anderson, 1971). Apparently the tetramer is the associating unit. The addition of excess NADH reverses the association. This is the first example of reversible polymerization beyond the tetramer in lactic dehydrogenase and reveals one of the larger differences between the two parent enzymes.

Thus bis(ANS) binding is a useful addition to established lactic dehydrogenase isozyme techniques since it reflects properties not measured by the other methods. In addition, the self-association of $M_4$ could be physiologically significant if a metabolite or naturally occurring effector also stabilizes the polymers.

This report deals with the effect of coenzyme on the reversible association of beef $M_4$ and with the resolution of the 10–12 primary bis(ANS) binding sites into two functionally distinct classes. It contains fluorescence, circular dichroism, and sedimentation measurements on the binding of bis(ANS) to $M_4$ in the presence of reduced 3-acetylpyridine adenine dinucleotide (AcPyADH). The dissociation constant for the binding of AcPyADH to lactic dehydrogenase is 50 times smaller than the dissociation constant for NADH (Anderson, 1965, unpublished data). This strong binding is advantageous in stoichiometry determinations and in competition experiments.

## Experimental Section

*Enzyme.* Beef muscle lactic dehydrogenase was prepared by the method of Pesce *et al.* (1964). Before the experiments, the enzyme was thoroughly dialyzed against 0.2 M potassium phosphate (pH 7) to give solutions containing 10–20 mg/ml of protein. Concentrations were determined using the published molar extinction coefficient (Pesce *et al.*, 1964).

*Bis(ANS).* The bis(ANS) was prepared by a modification of the procedure of Rosen and Weber (1969).

*AcPyADH.* AcPyADH was prepared by the procedure of Rafter and Colowick (1957) using horse liver alcohol dehydrogenase in place of yeast alcohol dehydrogenase.

*Fluorescence Measurements.* Fluorescence measurements were obtained with the Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer. The details of the measurements were described by Anderson (1971).

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[1] Abbreviations used are: AcPyADH, reduced 3-acetylpyridine adenine dinucleotide; ANS, 8-anilino-1-naphthalenesulfonate; bis(ANS), bis(8-anilino-1-naphthalenesulfonate); $\bar{n}$, moles of bis(ANS) bound per mole (140,000 g) of lactic dehydrogenase.

TABLE I: Sedimentation Velocity Studies on Beef $M_4$ in Solutions Containing Bis(ANS) and AcPyADH.[a]

| [Bis(ANS)] (M) | [AcPyADH] (M) | [AcPyADH]:[LDH] (moles:mole) | $s_{20,w}$ (S) Peak 1 | $s_{20,w}$ (S) Peak 2 |
|---|---|---|---|---|
| 0 | 0 | 0 | 6.91 | |
| $7.2 \times 10^{-4}$ | 0 | 0 | 19.50 | |
| $7.2 \times 10^{-4}$ | $1.2 \times 10^{-4}$ | 2 | 7.12 (44.4%)[b] | 13.26 (55.6%)[b] |
| $7.2 \times 10^{-4}$ | $2.4 \times 10^{-4}$ | 4 | 7.18 | |
| $7.2 \times 10^{-4}$ | $4.8 \times 10^{-4}$ | 8 | 7.17 | |

[a] Conditions: 8.4 mg/ml of beef $M_4$ in 0.1 M potassium phosphate, pH 7 (20°). Rotor speed: 52,000 rpm; schlieren optics. [b] Corrected for radial dilution.

TABLE II: Use of Scanner to Measure Displacement of Bound Bis(ANS) by AcPyADH.[a]

| [AcPyADH] (M) | [AcPyADH]:[LDH] (moles:mole) | $\bar{n}$[b] | $\Delta\bar{n}$ | $s_{20,w}$ (S) |
|---|---|---|---|---|
| 0 | 0 | 10.7 | | 17.12 |
| $1.4 \times 10^{-5}$ | 2 | 9.5 | 1.2 | |
| $3.5 \times 10^{-5}$ | 5 | 7.8 | 2.9 | |
| $7.2 \times 10^{-5}$ | 10 | 7.45 | 3.25 | 7.04 |

[a] Conditions: 1 mg/ml of beef $M_4$ and $10^{-4}$ M bis(ANS) in 0.1 M potassium phosphate, pH 7 (20°). Rotor speed: 48,000 rpm. [b] $\bar{n}$ = moles bis(ANS) bound per 140,000 g of $M_4$. Estimated error in $\bar{n} = \pm 0.4$.

*Circular Dichroism.* The circular dichroism spectra were recorded on the Jasco Model CD-SP circular dichroism recorder and spectrophotometer. The path length used was 1 cm. The direct contribution of the protein was eliminated by measurement of the difference between the enzyme–bis(ANS) mixture and the equivalent solution of enzyme alone. This correction is significant at wavelengths below 310 nm. Except when otherwise indicated, the values of $(\epsilon_l - \epsilon_r)$ were calculated using the total bis(ANS) concentrations.

*Temperature Control.* The cuvet compartments were maintained at 20° by means of circulating water from a constant-temperature bath.

*Sedimentation Velocity.* Sedimentation experiments were conducted with the Spinco Model E ultracentrifuge using either the schlieren optical system or the scanner. The values of $s_{20,w}$ were calculated using the maximum ordinate positions of the gradient curves and the midpoints of the boundaries recorded by the scanner.

All experiments were conducted using 0.1 M potassium phosphate buffer (pH 7) prepared from glass-distilled water.

## Results

*Sedimentation Velocity Studies.* The following experiment established the minimum amount of AcPyADH needed to reverse the bis(ANS) induced association. A solution containing 8.4 mg/ml of beef $M_4$ and $7.2 \times 10^{-4}$ M bis(ANS) in 0.1 M phosphate (pH 7) was sedimented in a 12-mm double sector cell at a rotor speed of 52,000 rpm (20°). The schlieren pattern of this mixture contained a single peak with $s_{20,w}$ = 19.5 S. When 2 equiv of AcPyADH was added to the mixture, the schlieren pattern contained two peaks with sedimentation coefficients of 7.12 and 13.3 S. The addition of 4 or more equiv of AcPyADH caused the appearance of a single peak with $s_{20,w}$ = 7.17 S (Table I).

The scanner (Schachman, 1963) was then used to determine the concentration of bound dye in samples containing 1 mg/ml of enzyme, $10^{-4}$ M bis(ANS), and varying concentrations of AcPyADH. The monochromator was set to 420 nm. Comparison of the visible absorption spectra of AcPyADH (Pabst Circular OR-18, 1961) and bis(ANS) (Rosen and Weber, 1969) shows that the absorption of AcPyADH is almost negligible at this wavelength while the absorption of bis(ANS) is 40% of its maximum value. The corresponding molar extinction coefficients of AcPyADH and bis(ANS) are $0.5 \times 10^3$ and $6.6 \times 10^3$ $cm^{-1}$ $M^{-1}$, respectively. Each sample was sedimented at a rotor speed of 48,000 rpm until the boundary was well separated from the meniscus. The free dye concentration was calculated from the absorbance of the supernatant solution. The concentration of bound dye was then obtained by subtraction. The plateau region was not used in these calculations since binding causes an appreciable change in the absorbance of bis(ANS). The samples containing 5 and 10 equiv of AcPyADH were corrected for a small contribution (*ca.* 1 and 7%, respectively) of free coenzyme to the absorbance. Table II summarizes the values of $\bar{n}$, the average number of moles of bis(ANS) bound per 140,000 g of lactic dehydrogenase, and of $s_{20,w}$. These data show that the depolymerization obtained on the addition of AcPyADH is accompanied by the release of 3–4 equiv of bis(ANS). Seven to eight equivalents of dye remain bound.

The following fluorescence and circular dichroism measurements were designed to show whether the displaceable bis(ANS) molecules are spectroscopically distinct from the residual dye.

*Fluorescence.* A sample containing 1 mg/ml of beef $M_4$ and $10^{-4}$ M bis(ANS) was titrated with $10^{-3}$ M AcPyADH. To avoid excitation of AcPyADH, the exciting wavelength chosen was 430 nm. The fluorescence measured at 500 nm decreased linearly with a clear end point occurring on the addition of 4 equiv of AcPyADH (Figure 1). About 31% of the initial fluorescence remained at saturation. The shape and position of the emission spectrum, which has a corrected maximum between 510 and 520 nm, were invariant. Since the emission maximum of bound AcPyADH occurs at 450 nm, the constancy of the spectrum confirmed the absence of bound coenzyme fluorescence.

The relative quantum yields were calculated using this titration and the proportions of bound dye obtained from the sedimentation data. Let $q_E$ = relative quantum yield of displaceable dye (in arbitrary units) and $q_R$ = relative quantum yield of the residual bound dye. Sedimentation showed that 47% of the bis(ANS) was free after the addition of 10 equiv of AcPyADH. Since free bis(ANS) is virtually nonfluorescent (Rosen and Weber, 1969), $0.31 = 0.53q_R$. The fractional decrease in fluorescence corresponds to dissociation of 23.2% of the total dye. Thus $0.69 = 0.232q_E$. The ratio $q_E/q_R$ shows

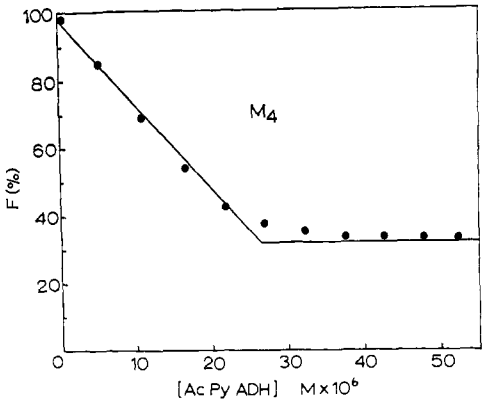


FIGURE 1: Fluorescence titration of beef $M_4$–bis(ANS) with AcPyADH. A mixture of 1 mg/ml of beef $M_4$ and $10^{-4}$ M bis(ANS) in a 1-cm cuvet was titrated with 10-$\mu$l increments of $10^{-3}$ M AcPyADH. After each addition of AcPyADH, the fluorescence intensity was recorded. Conditions: 20°; 0.1 M potassium phosphate (pH 7.0). Excitation at 430 nm. Emission at 500 nm. Bandwidth of excitation and emission was 5 nm.

that the quantum yield of the three to four displaceable bis(ANS) molecules is five times larger than the quantum yield of the bis(ANS) remaining bound at saturating AcPyADH concentrations.

*Circular Dichroism.* The free bis(ANS) molecule is symmetric and has no optically active absorption bands. Binding, however, induces asymmetry, and the adsorbate of beef $M_4$ with bis(ANS) has a distinctive circular dichroism spectrum with positive bands at 340 and 420 nm separated by a negative trough at 370 nm (Figure 2, curve 1). The circular dichroism spectrum of the complex of AcPyADH with either beef $M_4$ or $H_4$ has a strong positive band centered at 350 nm (Figure 3). The titration of a solution containing 1 mg/ml of enzyme and $8.6 \times 10^{-5}$ M bis(ANS) was followed at both 360 and 420 nm. The changes at 360 nm should largely reflect the binding of AcPyADH while the changes at 420 nm should reflect the dissociation of bis(ANS). Figure 2 illustrates the predicted linear changes in circular dichroism and end points occurring on the addition of 4 equiv of AcPyADH. Note the well-defined isodichroic points found at 350 and 380 nm.

The circular dichroism spectrum of the adsorbed dye present at saturating coenzyme concentration (Figure 4, lower curve) was obtained by subtracting the spectrum of a solution containing 1 mg/ml of enzyme and $3.85 \times 10^{-5}$ M AcPyADH from the spectrum of the solution containing 1 mg/ml of enzyme, $3.85 \times 10^{-5}$ M AcPyADH, and $8.6 \times 10^{-5}$ M bis(ANS). The values of $(\epsilon_l - \epsilon_r)$ were calculated using the concentration of residual dye measured in the sedimentation experiment.[2] The spectrum of the displaceable bis(ANS) (Figure 4, upper curve) was then obtained by subtracting the preceding difference spectrum from the spectrum of the initial solution, which contained 1 mg/ml of enzyme and $8.6 \times 10^{-5}$ M bis(ANS). The values of $(\epsilon_l - \epsilon_r)$ were calculated using the increase in free dye concentration produced by the addition of 5 equiv of AcPyADH.

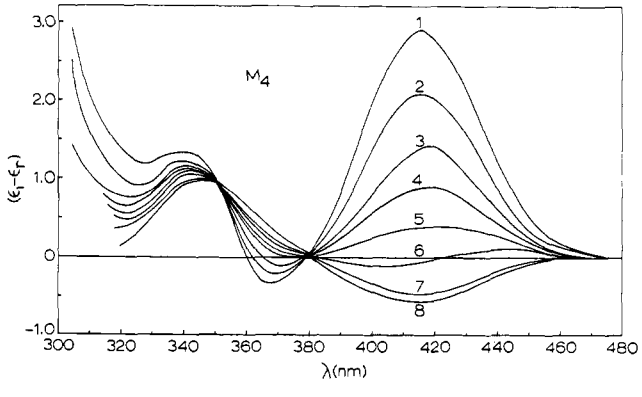


FIGURE 2: Effect of AcPyADH addition on the circular dichroism spectrum of the beef $M_4$–bis(ANS) adsorbate. Conditions: 1 mg/ml of beef $M_4$ and $10^{-4}$ M bis(ANS) in 0.1 M potassium phosphate, pH 7 (20°). The successive spectra were recorded at the following concentrations of AcPyADH: curve 1, 0; 2, $3.98 \times 10^{-6}$ M; 3, $7.93 \times 10^{-6}$ M; 4, $1.19 \times 10^{-5}$ M; 5, $1.57 \times 10^{-5}$ M. 6, $1.96 \times 10^{-5}$ M; 7, $2.34 \times 10^{-5}$ M; 8, $5.6 \times 10^{-5}$ M. The values of $(\epsilon_l - \epsilon_r)$ ($cm^{-1}$ $M^{-1}$) were calculated using the total bis(ANS) concentration.

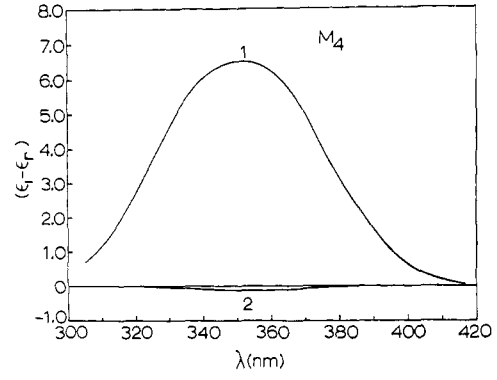


FIGURE 3: Circular dichroism spectra of AcPyADH. Curve 1: 1 mg/ml of beef $M_4$ + $1.5 \times 10^{-5}$ M AcPyADH. Curve 2: $10^{-4}$ M AcPyADH. The total AcPyADH concentration was used to calculate $(\epsilon_l - \epsilon_r)$ ($cm^{-1}$ $M^{-1}$). Conditions: 0.1 M potassium phosphate, pH 7 (20°).

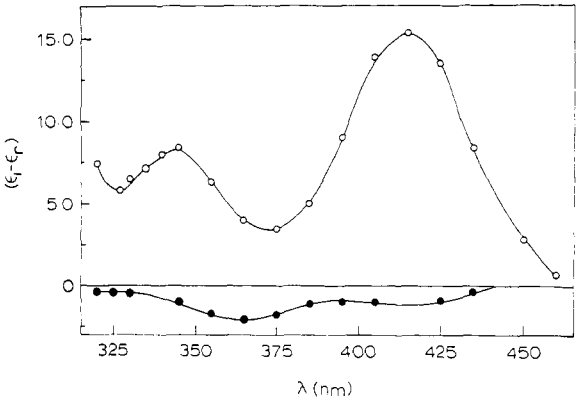


FIGURE 4: Circular dichroism difference spectra. The top curve (O) is the circular dichroism spectrum of the three to four displaceable bis(ANS) molecules. The lower curve (●) is the spectrum of the bound bis(ANS) present at saturating AcPyADH concentrations. The distribution of bound dye measured in the sedimentation experiment (Table II) was used in the calculation of $(\epsilon_l - \epsilon_r)$ ($cm^{-1}$ $M^{-1}$).

## Discussion

Beef muscle lactic dehydrogenase has at least two kinds of binding sites with similar affinities for bis(ANS). Three to four of the bound bis(ANS) molecules are essential for the self-association of beef $M_4$ and exhibit a high fluorescence quantum

[2] The amount of dye bound at $8.6 \times 10^{-5}$ M does not differ appreciably from the amount bound at $10^{-4}$ M.

yield and intense circular dichroism bands. The other seven to eight bound bis(ANS) molecules are apparently not essential for association and have a low fluorescence yield and weak circular dichroism bands. The two kinds of binding sites must have overlapping affinities for bis(ANS) in the absence of coenzyme since the circular dichroism spectrum of the adsorbate is nearly independent of the degree of saturation through $\bar{n}$ equals 12. The fluorescence time decay of these adsorbates previously demonstrated the existence of multiple components (Anderson, 1971).

The circular dichroism spectra of the adsorbates of bis(ANS) with the hybrid lactic dehydrogenases were resolved into the component spectra characteristic of the parent enzymes (Anderson, 1971). The diminished ability of the hybrids to undergo self-association was correlated with reduced binding of bis(ANS) to the M subunits. Since the intense circular dichroism bands of the essential dye molecules account largely for the characteristic shape of the spectrum of the beef $M_4$ adsorbate, the correlation is correct and applies specifically to the bound dye molecules which participate in association.

Since bis(ANS) is a dimer, it may possibly form a direct cross-link or bridge between lactic dehydrogenase molecules. On the other hand, it may have an indirect function such as stabilization of a conformation of the enzyme which undergoes self-association. The formation of a single intermolecular cross-link between M subunits requires 0.5 bis(ANS) molecule per subunit. Our stoichiometry, *ca.* one bis(ANS) per subunit, suggests that the dye has an indirect role.

The binding of 4 equiv of AcPyADH reverses the self-association of $M_4$ with the concurrent release of 3–4 equiv of bis(ANS). There are no spectroscopically distinguishable intermediates in the dissociation since clear isodichroic points were found in the titration of the adsorbate with AcPyADH. The binding of AcPyADH and the binding of the essential bis(ANS) molecules are apparently mutually exclusive or competitive processes. Possibly the binding sites for the two ligands overlap. Wassarman and Lentz (1971) found that 2,4,5,7-tetraiodofluorescein can occupy the NADH site of dogfish $M_4$. Competition may also occur when two ligands stabilize opposing conformations of a protein.

The preceding paragraph suggests that bis(ANS) is a competitive inhibitor with respect to the coenzyme. Activity measurements at the high protein concentrations used are not possible because of the high turnover number of lactic dehydrogenase. Full activity is immediately obtained on the $10^4$–$10^5$-fold dilution of the mixtures into the assay medium (Anderson, 1971). We conducted a few inhibition experiments at the assay concentration, 0.01 $\mu$g of enzyme/ml. We used a $10^{-4}$ M pyruvate solution in 0.1 M potassium phosphate, pH 7. No inhibition occurred when the concentrations of bis(ANS) and NADH were $10^{-6}$ and $10^{-4}$ M, respectively. A highly time dependent inhibition of activity resulted when the NADH concentration was reduced to $10^{-6}$ M, with the other concentrations held constant.

The reported experiments on beef $M_4$ and unpublished studies on dogfish (*Squalus acanthias*) $M_4$ demonstrate the same opposing effects of bis(ANS) and AcPyADH. Apparently the bis(ANS)-linked self-association reflects some essential characteristic of $M_4$ preserved throughout the vertebrate evolutionary line. We believe that it is important to determine whether the self-association is simply an isolated phenomenon obtained under artificial conditions or whether it may occur *in vivo* with some regulatory function.

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