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Induced Circular Dichroism in Enzyme–Dye Complexes: Lactic Dehydrogenase–Bromphenol Blue†

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ABSTRACT: Bromphenol blue (BPB) binds to bovine lactic dehydrogenase (LDH). We have characterized the interaction with bovine H_4 LDH by using difference spectroscopy, equilibrium dialysis, steady-state enzyme kinetics, and circular dichroism. Binding to the enzyme leads to a red shift and hyperchromism in the visible band of bromphenol blue. Equilibrium dialysis indicates that three types of sites exist on each subunit, with K_d values of 5.8×10^{-5} M, 3.4×10^{-4} M, and 1.3×10^{-3} M. Steady-state kinetic studies show that the dye is a competitive inhibitor with respect to both the coenzyme, reduced nicotinamide adenine dinucleotide (NADH), and the substrate, pyruvate. When ν, the number of dye molecules bound per tetramer, is less than ~0.75, the circular dichroism (CD) of the LDH–BPB complex exhibits a single positive band in the visible region, near 600 nm, and three bands of alternating sign in the 300–400-nm region. At higher ν, the visible band splits into a positive band at longer wavelengths and a weaker negative band on the short-wavelength side. Both lobes increase in amplitude as ν increases. Only small changes are observed in the near-ultraviolet bands. Bromphenol blue forms ternary complexes with LDH saturated with NADH or adenosine 5′-monophosphate (AMP), the CD spectra of which have a negative 600-nm band. Bromphenol blue also binds to LDH modified at the essential thiol by *p*-(hydroxymercuri)benzoate, and the CD spectrum of this complex has a positive band at 600 nm. The two tight binding sites for bromphenol blue are identified as the coenzyme-binding (C) site and the substrate-binding (S) site, in order of increasing K_d. Two possible interpretations are suggested for the splitting of the visible band and the ν-dependent CD amplitudes. Dye molecules bound to the coenzyme- and substrate-binding sites could account for the couplet, while ligand-induced conformational changes must be invoked to explain the variation in amplitude. Alternatively, exciton coupling between dyes bound to the same or different subunits could explain the long-wavelength CD behavior.

Although X-ray diffraction studies are yielding detailed descriptions of protein structure in the solid state, we must continue to improve techniques for structure determination in solution. By the very nature of the phenomenon, circular dichroism (CD)[1] is aptly suited for such structural investigations. We report here studies that were designed to broaden our knowledge of the CD of enzyme-bound chromophores.

One method of exploring the enzyme active site uses molecular probes that are bound (covalently or noncovalently) to the active site. By studying the properties of the enzyme-associated probe, we can determine various physical and chemical properties of the protein environment surrounding the probe. The CD of an enzyme-bound chromophore contains information about the conformation or configuration of the chromophore itself and the stereochemical properties of the binding site. Moreover, it can aid in the identification of the chromophore if that is in doubt.

All proteins have CD spectra in the ultraviolet region due to the transitions of the peptide backbone and side-chain residues. The CD bands due to the protein itself are called *intrinsic* Cotton effects. However, many proteins have CD bands at wavelengths that do not overlap with the intrinsic Cotton effects. These bands are due to enzyme-bound chromophores such as coenzymes, prosthetic groups, metal ions, substrates, inhibitors, etc. and are called *extrinsic* Cotton effects.

Extrinsic Cotton effects are due to the inherent dissymmetry of the enzyme-bound chromophore (an *inherent* effect) and/or to the interactions of the chromophore with the encompassing dissymmetric environment (*interactive* effects). The inherent effects are those which the free chromophore would exhibit if its conformation were identical with that of the enzyme-bound form. The interactive effects result from protein–ligand interactions or ligand–ligand interactions. The main problem in interpretation of the CD of enzyme-bound chromophores is distinguishing between the inherent and the interactive effects.

We have studied the CD of enzyme–dye complexes for several reasons. (1) Glazer (1970) has noted the propensity of dyelike molecules to bind to functional sites on proteins. (2) The dyes considered here have an extensively conjugated but twisted π-electron system which creates strong inherent effects. Thus, we may be able to assume that interactive effects are not dominant (perhaps even negligible), particularly for transitions at energies far from those of the protein transitions and when the number of dyes bound per protein is small. (3)

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[1] Abbreviations used: CD, circular dichroism; LDH, lactic dehydrogenase; BPB, bromphenol blue; PMB, *p*-(hydroxymercuri)benzoate; DEAE, diethylaminoethyl; NADH, reduced nicotinamide adenine dinucleotide; AMP, adenosine 5′-monophosphate.

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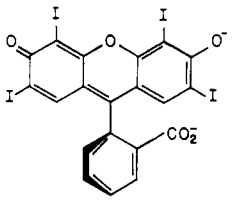
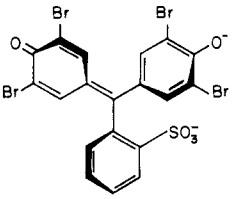
Chart I



Tetraiodofluorescein



Bromphenol Blue

π-Conjugated systems are ideal for performing semiempirical molecular orbital calculations to predict the sign and magnitude of CD bands as a function of the conformation of the dye. (4) Dye molecules usually have several transitions that lie at energies far from those of the protein. This multiplicity of dye transitions allows us to consider more than one electronic transition in an enzyme–dye complex and thus obtain more information. (5) Wassarman & Lentz (1971) studied the binding of tetraiodofluorescein to dogfish M_4 lactic dehydrogenase (LDH) and showed by X-ray diffraction that the dye was bound to the adenine portion of the coenzyme-binding site. We initially began our studies on bovine H_4 LDH complexes with tetraiodofluorescein but turned to bovine H_4 LDH–bromphenol blue complexes because bromphenol blue possesses a twisted chromophore whereas the chromophore in tetraiodofluorescein is planar (Chart I). However, the two dyes are very similar.

Materials and Methods

The chemicals used in this study and their respective sources are as follows: Bovine H_4 LDH (type III), NADH, adenosine 5′-monophosphoric acid sodium salt (type II), sodium pyruvate (type II), oxamic acid, and *p*-(hydroxymercuri)benzoate (PMB) obtained from Sigma Chemical Co.; the sodium salts of bromphenol blue and tetraiodofluorescein from Eastman Kodak Co.; L-cysteine hydrochloride hydrate from Nutritional Biochemicals Corp.

Lactate Dehydrogenase. Bovine heart LDH was further purified by elution from a DEAE-cellulose column according to Pesce et al. (1964). This step removed the two extraneous proteins observed by electrophoresis that were typically present in the Sigma LDH preparation. Normally this preparation was low in isozymes and H_3M was usually not present in significant quantities after elution from the DEAE-cellulose column. In those preparations where the H_3M isozyme concentration was abnormally high, we separated the isozymes on a DEAE-agarose column (Bio-Rad), again employing a NaCl gradient. The purified LDH was crystallized, stored, and assayed according to Pesce and co-workers. The specific activity was determined to be ~365 units/mg by using a spectrophotometric protein determination, $\epsilon_{280} = 2.1 \times 10^5$ M^{-1} cm^{-1} (Pesce et al., 1964). The enzyme was readied by extensive dialysis against 0.1 M potassium phosphate buffer, pH 7.0. The same buffer was used in all experiments, and the temperature was always 20 °C, except in the steady-state kinetic studies which were performed at 25 °C.

Bromphenol Blue. Several tests for bromphenol blue purity (Franglen & Martin, 1954; Wilson, 1951; Kosheleva, 1956) revealed no detectable impurities, and, indeed, Eastman bromphenol blue has been shown to be pure in previous studies (Wilson, 1951). Eastman claims 97% purity in their dye, assuming $\epsilon_{593} = 8.04 \times 10^4$ M^{-1} cm^{-1} when dried properly. When bromphenol blue was dried to a constant weight, our results were 3–5% below their reported extinction coefficient. Kragh-Hansen et al. (1974) used the value of 7.8×10^4 M^{-1} cm^{-1} in their studies of bromphenol blue interaction with human serum albumin. Because of our inability to achieve a higher value than 8.04×10^4, this extinction coefficient was used to determine the concentration of the dye in 0.1 M potassium phosphate, pH 7.0, at 25 °C. The absorption of bromphenol blue varies with temperature, and all spectrophotometric studies were performed with both sample and reference cells thermostated.

Difference Spectroscopy. Bromphenol blue exhibits a red shift and hyperchromicity upon binding to bovine H_4 LDH. Difference spectroscopy was used to study binding to H_4 LDH in the following manner. We assume equilibrium between an LDH subunit and bromphenol described by the dissociation constant $K_d = [E][D]/[E{\cdot}D]$ where [E] is the concentration of unliganded subunits, [D] is the concentration of free dye, and [E·D] is the concentration of liganded subunits. When D_0, the total dye concentration, and E_0, the total subunit concentration, are such that $D_0 \ll E_0$, the fraction of dye bound is given by

$$x \simeq 1/(1 + K_d/E_0) \quad (1)$$

The difference in absorbance by the sample and reference solutions during a titration is given by

$$\Delta A = (\epsilon_{ED} - \epsilon_D)D_0x = D_0x\Delta\epsilon \quad (2)$$

Therefore, the slope of a ΔA vs. D_0 plot will be $S = x\Delta\epsilon$. Substituting for x from eq 1

$$S = \Delta\epsilon/(1 + K_d/E_0) \text{ or } S = \Delta\epsilon - (S/E_0)K_d \quad (3)$$

The $\Delta\epsilon$ was determined from the intercept by plotting S vs. S/E_0 (Foster et al., 1953). Once $\Delta\epsilon$ had been determined, data obtained at higher bromphenol blue concentrations could be used, since it was no longer necessary that $D_0 \ll E_0$. From these data, the number of dye molecules bound per tetramer, ν, and the free dye concentrations were calculated, and a Scatchard plot was made. The difference spectroscopy titrations were performed on a Cary 14 or 17 spectrophotometer with thermostated cell holders at 20 °C. Dye additions were made by using Hamilton microsyringes with Chaney adaptors.

Equilibrium Dialysis. One-milliliter acrylic dialysis chambers and regenerated cellulose tubing were purchased from Arthur Thomas Company. The two halves of the dialysis chamber were separated by a membrane prepared according to Jacobsberg et al. (1975). An experiment was started by placing 1 mL of an LDH solution on one side and 1 mL of buffer on the other, injecting bromphenol blue into the buffer side, placing in a 20 °C Forma water bath and shaker, and gently agitating. Preliminary experiments with buffer solutions showed complete equilibration within 24 h. In the 24-h runs, the LDH in the control cell typically showed a 5–10% decrease in specific activity and a 2% increase in 280-nm absorbance. Equilibration times of 12, 24, and 36 h were used to demonstrate that equilibration was being achieved and that the loss of LDH activity was not significantly affecting the bromphenol blue binding. The binding data were analyzed by using the computer program ADAIR (Cornish-Bowden & Koshland, 1970) that was kindly given to us by Professor Daniel Koshland, University of California, Berkeley, CA.

Steady-State Enzyme Kinetics. The LDH inhibition studies were performed on a Cary 14 spectrophotometer with a 0.1-absorbance slide wire and thermostated cuvette holders at 25 °C. The reactions were initiated by mixing microliter amounts of an LDH solution, which was kept on ice, into the pyruvate–NADH solution with a plunger basket. The total volume of the reaction mixture was 2.2 mL in a 1-cm path length cuvette. The initial velocity was measured within the first 10% of the total ΔA by using a Hewlett-Packard desk top calculator

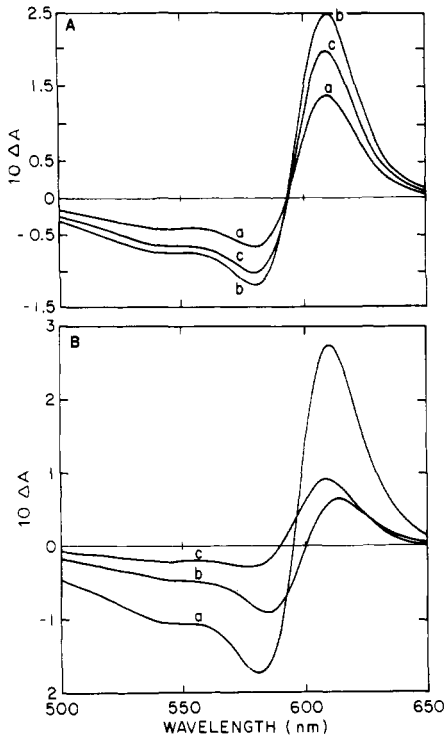


FIGURE 1: Difference spectra for binding of BPB to H_4 LDH. (A) Curve a, LDH + BPB, $\nu = 0.59$; curve b, LDH + BPB, $\nu = 1.25$; curve c, same as curve b after addition of NADH (99% saturation). All path lengths are 1 cm. (B) Curve a, LDH + BPB, $\nu = 2.9$ (l = 0.5 cm); curve b, LDH + BPB, $\nu = 4.8$ (l = 0.1 cm); curve c, same as curve b after addition of NADH (99% saturation). Initial enzyme concentration was 3.3×10^{-6} M H_4 LDH. Buffer was 0.1 M potassium phosphate, pH 7.0, at 20 °C.

and digitizer. The data were plotted on double-reciprocal plots (each point is the average of two consecutive runs). The K_I values were determined from the x intercept of a replot of slope vs. total dye concentration. (Whereever data are fitted to a linear equation in this paper, a nonweighted least-squares criterion has been used.)

Enzyme Modification. LDH was modified with PMB as described by DiSabato & Kaplan (1963). In preliminary experiments, we demonstrated an elimination of 94% of the LDH activity and a regain of 83% by adding 10 μL of a 1.7 M L-cysteine solution. The control solution in these experiments lost 10% activity after the addition of the same amount of cysteine solution.

CD Studies. The CD measurements were made on a JASCO ORD/UV 5 spectropolarimeter with a Sproul SS-20 CD modification or a JASCO J-40 C spectropolarimeter. Both instruments were calibrated with an aqueous solution of (+)-10-camphorsulfonic acid according to Cassim & Yang (1969). The molar ellipticity was calculated by using the formula (Moscowitz, 1960)

$$[\Theta] = 100\psi/(lm)$$

where ψ is the measured ellipticity in degrees, m is the molar concentration of bound dye, and l is the path length in centimeters.

Results

The difference spectrum of the H_4 LDH–bromphenol blue solution is shown in Figure 1. The S vs. S/E_0 plot yielded $\Delta\epsilon_{610} = 3.42 \times 10^4$ M^{-1} cm^{-1} which was used to calculate ν and the concentration of free dye for a Scatchard plot, which yielded the number of binding sites/tetramer, $n = 4.0$, and $K_d = 3.9 \times 10^{-5}$ M. When NADH is added to 99.9% saturation, assuming a $K_d = 4 \times 10^{-7}$ M for LDH–NADH complexes (Anderson & Weber, 1965), we observed a decrease in the intensity of the difference spectrum, but the isosbestic point remained at 593 nm (curve c, Figure 1A). However, subsequent addition of oxamate, a pyruvate analogue, to the LDH–BPB–NADH solution abolished the difference spectrum.

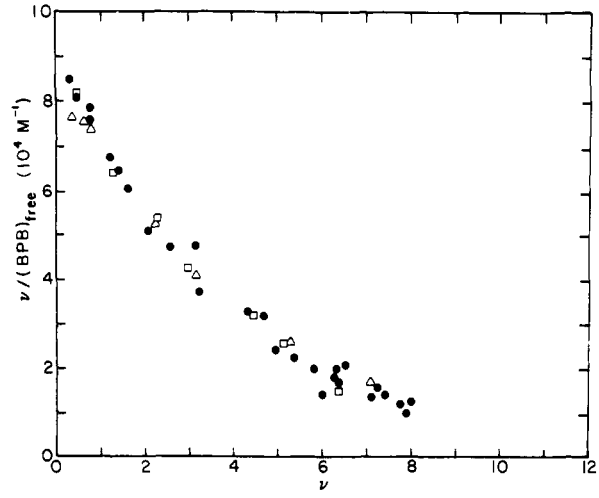


FIGURE 2: Scatchard plot of binding data from equilibrium dialysis data. The symbols represent different equilibration times: (△) 12; (●) 24; (□) 36 h. Buffer was 0.1 M potassium phosphate, pH 7.0, at 20 °C.

During a titration of LDH with bromphenol blue, the isosbestic point at 593 nm showed small red shifts with increasing ν, but it was not until ν values of 2 were exceeded that this red shift became readily apparent. When higher ν values were attained, we noted that the red shift was due to the negative peak growing faster than the positive peak, as shown in Figure 1B. However, when NADH was added to the LDH–dye solutions the isosbestic point shifted to shorter wavelengths (curve c, Figure 1B) and reestablished the type of difference spectrum observed at lower ν values, where the positive band has greater magnitude than the negative band. Due to the unstable isosbestic point, which either implies heterogeneous dye-binding sites or electronic interactions between bound dyes, a direct binding study using equilibrium dialysis was performed.

The results from the equilibrium dialysis studies are shown in the Scatchard plot in Figure 2. The titration curve (Klotz, 1974) in Figure 3 shows the computer fit to the binding data when three independent sites/subunit are assumed with K_d = 5.8×10^{-5} M, 3.4×10^{-4} M, and 1.3×10^{-3} M. Although the binding curve might also be interpretable in terms of a model with 12 sites/tetramer which exhibit negative cooperativity, such a model is difficult to reconcile with the tetrameric structure of lactic dehydrogenase. The dissociation constant derived from difference spectroscopy agrees reasonably well with the smallest dissociation constant from equilibrium dialysis. Using the two tightest binding sites from the equilibrium dialysis study (binding at the third site can be considered negligible when considering dye binding at ν values ≤ 2), we can show that as $\nu \rightarrow 0$, 15% of the total bromphenol blue bound is at the second tightest binding site. Therefore, our assumption in the difference spectroscopy experiments that all the dye was binding to homogeneous sites when $D_0 \ll E_0$ is a good approximation for $\nu < 2$.

Figures 4 and 5 show Lineweaver–Burk plots for bromphenol blue as an inhibitor of LDH. Although there is some scatter in the $1/v$ intercepts, the pattern of inhibition is best

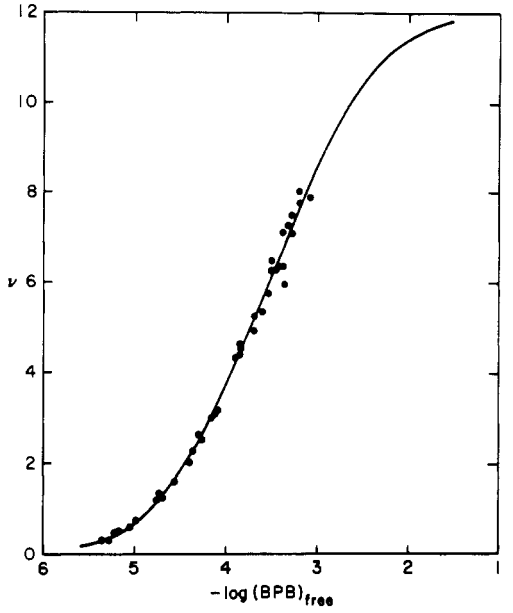


FIGURE 3: Equilibrium dialysis data. The curve was calculated by assuming three independent sites/subunit, with K_d values of 5.8×10^{-5} M, 3.4×10^{-4} M, and 1.3×10^{-3} M.

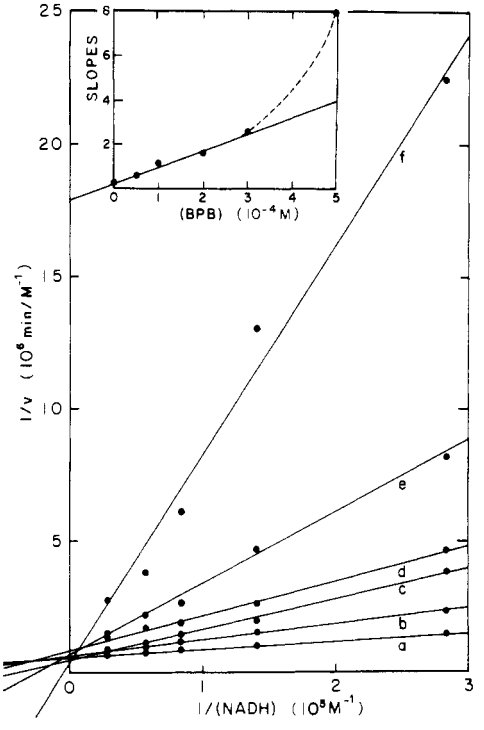


FIGURE 4: Steady-state inhibition of LDH with NADH as the variable substrate. Pyruvate concentration is 5.0×10^{-4} M. BPB concentrations are (a) 0, (b) 5, (c) 10, (d) 20, (e) 30, and (f) 50×10^{-5} M. The replot (insert) gives $K_I = 3.7 \times 10^{-5}$ M. Conditions are as in Figure 1, except $T = 25$ °C.

characterized as competitive with respect to both NADH and pyruvate. The replots from these figures yielded K_I values of 3.7×10^{-5} M and 2.1×10^{-5} M, respectively. The nonlinearity of the inserted replot in Figure 4 and the slight noncompetitive effects in the double-reciprocal plots suggest multiple dye-binding sites.

The CD of the LDH–bromphenol blue complex at several ν values is shown in Figure 6. Five CD bands are observed above 300 nm: positive bands at 612 and 352 nm and negative bands at 582, 390, and 315 nm. The overlapping positive and negative CD bands in the 600-nm region apparently result

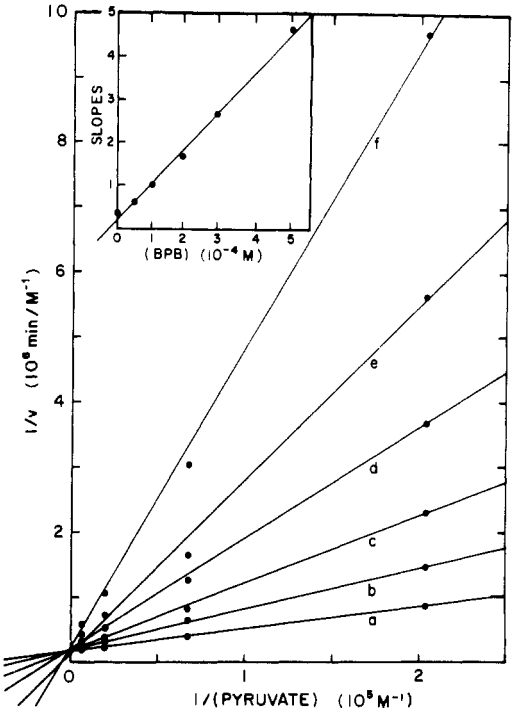


FIGURE 5: Steady-state inhibition of LDH with pyruvate as variable substrate. NADH concentration 3.2×10^{-5} M. BPB concentrations and conditions are as in Figure 5. The replot (insert) gives $K_I = 2.1 \times 10^{-5}$ M.

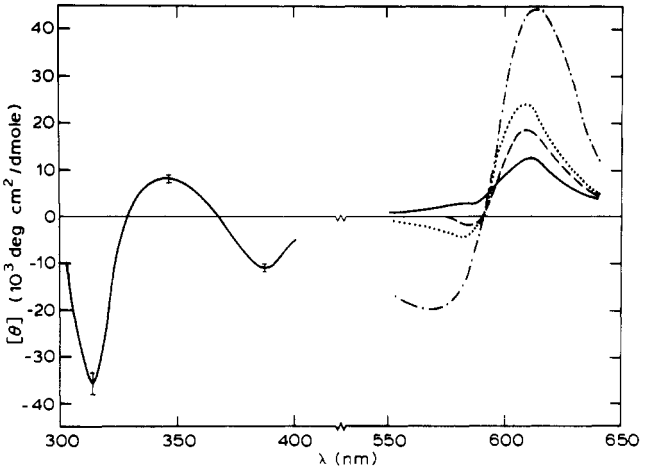


FIGURE 6: CD spectra of bovine H_4 LDH complexes with BPB at various ν values. For the long-wavelength band the values are as follows: $\nu = 0.39$ (—); $\nu = 0.83$ (- - -); $\nu = 1.24$ (···); $\nu = 4.70$ (-·-). The near-ultraviolet CD represents an average for five ν values ranging from 0.24 to 1.99. The error bars represent the standard deviations at the band maxima. The buffer was 0.1 M potassium phosphate, pH 7.0.

from a single electronic transition. The amplitude of the two long-wavelength peaks depends on the number of dye molecules bound. When $\nu \lesssim 0.75$, no negative band is observed near 580 nm. Instead, the long-wavelength band is positive throughout, although the shape of the CD band differs from that of the absorption band in such a way as to suggest the presence of a negative contribution on the short-wavelength side.

The dependence of the ellipticity per bound dye upon the number of dyes bound per tetramer is shown in Figure 7. The positive band near 610 nm shows a striking increase in amplitude for ν values up to ~ 2, beyond which it appears to level off. The negative 580-nm band does not appear until $\nu \gtrsim 0.75$ but increases in magnitude and appears to be continuing to

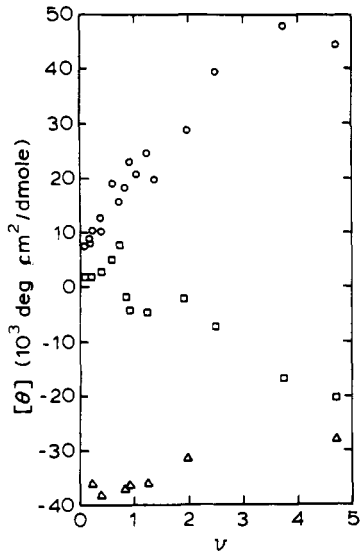


FIGURE 7: Dependence of molar ellipticity at three wavelengths on the number of dyes bound per tetramer. (○) Long-wavelength maximum near 610 nm; (□) 580 nm; (△) minimum near 315 nm.

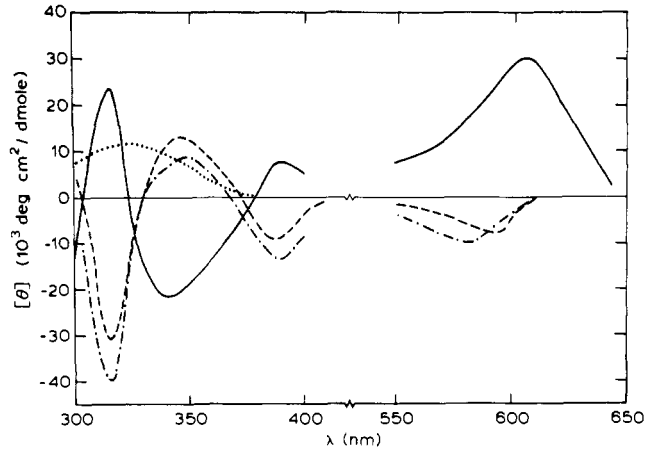


FIGURE 8: CD spectra of ternary complexes of BPB, LDH, and various substances. NADH (99% saturation with NADH, ν_{dye} = 1.23 assuming $K_d = 2.1 \times 10^{-5}$ M from enzyme kinetics) (- - -); NADH + oxamate (···); AMP (97% saturation with AMP, ν_{dye} = 1.45 with the above assumption) (– · –); PMB (94% inhibition, ν_{dye} = 3.46 assuming two sites/subunit with $K_d = 5.8 \times 10^{-5}$ M and 1.3×10^{-3} M) (—). Conditions are as in Figure 7.

increase at the highest ν values studied, though not very steeply. By contrast to the two long-wavelength bands, the ultraviolet CD bands show no perceptible dependence on the number of bound dye molecules over the range of 0–2 dyes bound, though a small decrease appears to set in at higher ν values.

When NADH was added to a LDH–bromphenol blue solution at a coenzyme concentration which would give 99.9% saturation in the absence of the dye, the CD spectrum (Figure 8) reflected ternary complex formation. The positive lobe in the long-wavelength region was eliminated, while the shorter wavelength pattern remained essentially unchanged, except for the increased CD at 340 nm which may be due to the bound NADH. NADH gives rise to a positive CD band at 345 nm in the binary LDH–NADH complex. When oxamate is added to the LDH–BPB–NADH mixture, the dye CD bands are lost and the positive 345-nm LDH–NADH CD band is intensified and blue shifted to ~322 nm. Such a blue shift and gain in intensity upon the addition of oxamate to the LDH–NADH complex was also observed in porcine M_4 LDH (Gurevich et al., 1972) and has been discussed by Towell (1977). In addition, the long-wavelength absorption maximum of the dye was blue shifted to 593 nm, the position of the free dye absorption, when oxamate was added.

Figure 8 also displays the CD spectrum of a LDH–bromphenol blue solution in which the LDH was 97% saturated with AMP, assuming $K_d = 5.5 \times 10^{-3}$ M (McPherson, 1970). Comparison of the CD spectra of the ternary complexes of LDH with bromphenol blue and either AMP or NADH shows that the adenylyl group of NADH is sufficient to evoke the observed effect.

The results of the experiment in which LDH was modified by reaction with PMB are also shown in Figure 8. This experiment shows that blocking the active-site essential thiol eliminates the negative CD band in the long-wavelength region.

Discussion

The purpose of the experiments described here is to characterize the LDH–bromphenol blue interaction, so that the sources of optical activity in the complex can be analyzed. We propose a simple model that is compatible with the data and will serve as a base from which more complex or alternative models can be formulated.

Our studies point to the presence of two relatively tight dye-binding sites/LDH subunit. This conclusion is supported by the binding studies, using difference spectroscopy and equilibrium dialysis. A third type of site is also observed in the equilibrium dialysis studies, but, because of the relatively high dissociation constant for this latter site (1.3×10^{-3} M), it will not be significantly populated under the conditions used in the optical experiments.

The two sites can be identified with the coenzyme-binding site and with the substrate-binding site on the basis of several lines of evidence. Bromphenol blue is essentially a competitive inhibitor with respect to the coenzyme. The inhibition constant 3.7×10^{-5} M agrees reasonably well with the dissociation constant for the tightest bromphenol blue binding site from equilibrium dialysis (5.8×10^{-5}), implying that this site coincides with the coenzyme-binding site. Bromphenol blue is also a competitive inhibitor with respect to the substrate, pyruvate. In this case, the inhibition constant is 2.1×10^{-5} M, much less than the equilibrium dissociation constant for the second class of dye-binding sites, 3.4×10^{-4} M. We propose that this difference arises as follows. In the steady-state kinetic experiment when bromphenol blue functions as an inhibitor vs. pyruvate, the dye binds to a subunit which has already bound NADH. In the case of equilibrium dialysis, the dye which binds to the substrate site is binding to a subunit to which zero or one dye molecule has bound at the coenzyme-binding site. The well-established conformational change triggered by NADH binding to LDH (Holbrook et al., 1975) presumably accounts for the substantially tighter binding of the dye in the presence of NADH.

Ternary complexes of bromphenol blue, LDH, and NADH can be formed, as shown by difference spectroscopy and CD. The difference absorption and CD spectra of the dye–LDH complex are significantly altered upon the addition of NADH but not eliminated (Figures 1 and 8). By contrast, the addition of oxamate, a pyruvate analogue, to the ternary complex eliminates both the difference absorption spectrum and the CD spectrum due to the dye. This experiment strongly supports the identification of the second dye site as overlapping the substrate-binding site. However, we cannot conclusively rule out the alternative interpretation that the formation of the strong LDH–NADH–oxamate ternary complex causes a conformational change, which eliminates dye binding at a locus removed from the active site.

An interesting study that parallels the work reported here was published by Wassarman & Lentz (1971), who studied the interaction of dogfish M_4 LDH with tetraiodofluorescein. Tetraiodofluorescein and bromphenol blue are structurally similar (see Scheme I), and the results of difference spectroscopy, binding studies, and steady-state inhibition studies for the bovine H_4 LDH–bromphenol blue and dogfish M_4 LDH–tetraiodofluorescein systems are very similar. Wassarman and Lentz reported that saturation of the dogfish muscle LDH–tetraiodofluorescein solution with NADH did not eliminate the difference spectrum, which is what we observe in the bovine H_4 LDH–bromphenol blue complex. We added tetraiodofluorescein to bovine H_4 LDH, and the resulting difference spectrum was basically the same as that observed by Wassarman and Lentz. Saturation with NADH also failed to eliminate the difference spectrum, but subsequent addition of oxamate did abolish it. However, using X-ray diffraction, Wassarman and Lentz observed that tetraiodofluorescein bound to dogfish M_4 LDH only at the adenine portion of the coenzyme-binding site. A subsidiary dye-binding site of low occupancy may have been below the level of detection. It is also possible, however, that intermolecular contacts interfere with dye binding at the substrate site or that there are subtle differences in conformation between the solid state and solution which decrease the affinity of the substrate site for tetraiodofluorescein. The existence of a second dye-binding site at the substrate site is consistent with the X-ray evidence for an anion-binding site at this location (Adams et al., 1973).

The CD behavior of the bromphenol blue–LDH complexes also fits a model in which the dye binds at the sites which normally bind the coenzyme and the substrate. We identify the strongest binding site with the coenzyme site, based on the inhibition constant for bromphenol blue with respect to NADH and upon the analogy with tetraiodofluorescein binding (Wassarman & Lentz, 1971). Thus, the simple positive CD band seen in LDH–bromphenol blue complexes at low ν values indicates that binding of the dye to the coenzyme-binding site gives rise to a positive CD band at long wavelengths. This assignment is also consistent with the CD of bromphenol blue bound to PMB-modified enzyme, where the substrate-binding site is blocked.

The sign of the induced CD for bromphenol blue bound to the substrate-binding site is less certain. If we attribute the splitting of the visible CD band solely to the heterogeneity of binding sites, it follows that dye bound to the substrate site has a negative CD band near 600 nm, which is also consistent with our observations on the ternary complexes with NADH and AMP.

There are two difficulties with this simple model, however. One is the increase in the amplitude of the long-wavelength band with increasing ν values (Figure 7). The second is the very small change in the molar ellipticity of the near-ultraviolet bands with ν values of <2. The increase in amplitude in the 600-nm region could be due to a conformational change in the LDH triggered by binding one or two dyes/tetramer. There is no evidence for cooperativity in dye binding from the equilibrium dialysis studies or from steady-state kinetics. Although the interpretation of the binding data in terms of three independent sites/subunit is probably not unique, it is consistent with the tetrameric nature of LDH. However, small intersubunit interactions are not excluded by the binding data. The most serious defect with this explanation, however, is that the postulated conformational change has no significant effect on the amplitude of the three near-ultraviolet transitions.

A possible explanation for the strong ν dependence of the 600-nm band and the absence of corresponding changes in the 300–400-nm band is exciton interaction between bound dyes, either on the same subunit or on different subunits. In this model, the difference in behavior between the visible band and the near-ultraviolet bands results from the large difference in oscillator strengths. The intense visible band, with an oscillator strength near unity, permits coupling among dyes bound at different sites, while the weakly allowed near-ultraviolet bands would not be subject to such interactions.

The results of exciton calculations (R. W. Woody, unpublished results) indicate that this mechanism is potentially capable of operating in the BPB–LDH system, even among dyes bound on different subunits, and could account for the apparent splitting of the long-wavelength CD band in the binary dye–enzyme complexes. The absence of splitting in the long-wavelength band of dye bound in ternary complexes or to the PMB-modified enzyme argues that the exciton splitting may involve dyes bound to different sites on the same subunit. Further experimental work with these systems as well as additional studies of the binary enzyme–dye complexes will be necessary to distinguish among the possible interpretations of the complex behavior in this system.

Inherent chirality in bromphenol blue is due to rotameric states, and the conformation of the dye can be pictured as either a right- or left-handed propeller-like molecule. When free in solution, bromphenol blue is racemic and optically inactive unless a chiral solvent or solute is present to perturb the distribution of enantiomers. This effect is called the Pfeiffer effect (Pfeiffer & Quehl, 1931), and we propose that LDH perturbs the racemic BPB mixture by preferential binding of the rotational enantiomers. The loss in optical activity when the dye is displaced from the enzyme is due to rapid interconversion of the rotational enantiomers. Geiger & Wagnière (1973) have observed in chiral chromophores of C_2 symmetry a regularity that correlates the chirality with the sign of the lowest-energy CD band. In a right-handed chromophore with a long-wavelength transition of symmetry B (polarized perpendicular to the C_2 axis), the sign of the CD band is positive. Molecular orbital calculations on the chromophoric system of BPB (J. F. Towell and R. W. Woody, unpublished experiments) indicate that the long-wavelength transition of bromphenol blue is of symmetry B. If we assume that the twisted conjugated system of the dye will dominate the longest-wavelength CD band and thus neglect the effects of the sulfonated phenyl ring, then the chromophore of bromphenol blue has C_2 symmetry. Therefore, the sign of the long-wavelength band in bromphenol blue should reveal the handedness of the chromophore.

If we further assume that the inherent chirality of the bromphenol blue chromophore dominates the sign of the long-wavelength CD band, then it is possible to infer the chirality of the LDH-bound dye. In the complex, the data suggest that the LDH coenzyme-binding site has a higher affinity for right-handed bromphenol blue molecules. The chiral preference of the substrate site cannot be determined until the questions raised above are resolved.

It is not clear at present what factors determine the signs and magnitudes of the CD bands observed in bromphenol blue–enzyme complexes between 300 and 400 nm. In general, three or four bands of alternating sign are observed in this wavelength range. The only exception is in the case of the bovine M_4 LDH–bromphenol blue complex (Towell, 1977), where only two rather broad bands are observed in the near-ultraviolet. The near-ultraviolet CD bands are of com-

parable strength to the visible band(s), even though the oscillator strengths of the former are much smaller.

One feature of the CD spectrum of the ternary bromphenol blue–NADH–H_4 LDH complex (Figure 8) which is puzzling is that the contribution of the NADH 340-nm band appears to be very small. The binary LDH–NADH complex has a positive CD band centered at about 340 nm for bovine H_4 (J. F. Towell, unpublished experiments) and porcine M_4 (Gurevich et al., 1972), which has ~70% of the amplitude of the ternary LDH–NADH–oxamate complex. There is no indication of a positive band of this intensity in the ternary complex with bromphenol blue. It may be that the NADH binds in a different way in the ternary complex with bromphenol blue than it does in the ternary complex with oxamate or in the binary complex. It is possible that the dye bound at the substrate-binding site partly overlaps the nicotinamide binding site and that NADH is bound primarily through the AMP moiety. Such a binding mode, with the nicotinamide ring relatively free, would be expected to lead to negligible CD in the 340-nm reduced nicotinamide band.

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Mechanism of the Melibiose Porter in Membrane Vesicles of *Escherichia coli*†

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ABSTRACT: The melibiose transport system of *Escherichia coli* catalyzes sodium–methyl 1-thio-β-D-galactopyranoside (TMG) symport, and the cation is required not only for respiration-driven active transport but also for binding of substrate to the carrier in the absence of energy and for carrier-mediated TMG efflux. As opposed to the proton–β-galactoside symport system [Kaczorowski, G. J., & Kaback, H. R. (1979) *Biochemistry 18*, 3691], efflux and exchange of TMG occur at the same rate, implying that the rates of the two processes are limited by a common step, most likely the translocation of substrate across the membrane. Furthermore, the rate of exchange, as well as efflux, is influenced by imposition of a membrane potential ($\Delta\Psi$; interior negative), suggesting that the ternary complex between sodium, TMG, and the porter may bear a net positive charge. Consistently, energization of the vesicles leads to a large increase in the V_{max} for TMG influx, with little or no change in the apparent K_m of the process. It is proposed that the sodium gradient ($Na^+_{out} > Na^+_{in}$) and the $\Delta\Psi$ (interior negative) may affect different steps in the overall mechanism of active TMG accumulation in the following manner: the sodium gradient causes an increased affinity for TMG on the outer surface of the membrane relative to the inside and the $\Delta\Psi$ facilitates a reaction involved with the translocation of the positively charged ternary complex to the inner surface of the membrane.

A large body of evidence has accumulated indicating that chemiosmotic phenomena, as postulated by Mitchell (1961, 1966, 1968, 1973, 1977), are responsible for respiration-dependent active transport in membrane vesicles isolated from *Escherichia coli* (Kaback, 1974, 1976; Harold, 1976; Konings & Boonstra, 1977). According to this hypothesis, oxidation of electron donors that drive substrate accumulation leads to the development of a transmembrane electrochemical gradient

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