Spectroscopic Studies of Cibacron Blue and Congo Red Bound to Dehydrogenases and Kinases. Evaluation of Dyes as Probes of the Dinucleotide Fold†

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ABSTRACT: Cibacron Blue and Congo Red have been shown to bind tightly to nucleotide-binding enzymes, and the former dye has proven useful in affinity chromatography of such enzymes. The conformation of such dyes when bound to enzymes is of interest because it has been suggested that their affinity for an enzyme results from an ability to mimic the conformation of the bound cofactor. The interaction of Cibacron Blue and Congo Red with several dehydrogenases (bovine lactic dehydrogenase, equine, and yeast alcohol dehydrogenase) and kinases (porcine adenylate kinase and yeast phosphoglycerate kinase) and aldolase has been studied by induced circular dichroism (CD), binding measurements, and competition with the appropriate coenzymes or substrates. To examine the sensitivity of the interaction to perturbations of the dye structure, we also studied Benzopurpurin 4B, a dimethyl analogue of Congo Red, and Cibacron Blue linked to dextran or Sepharose matrices. We have concluded that the dyes under consideration are not highly specific analogues of nucleotides or coenzymes. These dyes do not assume a single unique conformation on different enzymes possessing the dinucleotide fold. In most cases, however, the induced CD indicates that for closely related proteins or small variations in dye structure the conformation of the bound dye is quite similar. Despite the lack of a unique conformation for these dyes, our studies indicate that in addition to their usefulness in affinity chromatography, Cibacron Blue and Congo Red are valuable conformational probes for nucleotide-binding enzymes.

Aromatic dye molecules tend to bind preferentially to the active-site regions of globular proteins (Glazer, 1970). This may be the result of nonspecific interactions, such as a general hydrophobicity, or the result of a specific arrangement of charged groups, hydrophobic regions, and hydrogen-bond donors or acceptors. In either case, dyes bound at enzyme active sites have been used to obtain information about the enzyme in a variety of ways, including (1) deriving and interpreting X-ray diffraction structures (Wassarman & Lentz, 1971; Einarsson et al., 1974; Pai et al., 1977), (2) affinity chromatography (Böhme et al., 1972; Easterday & Easterday, 1974; Thompson et al., 1975; Baird et al., 1976; Stellwagen, 1977), (3) removal of coenzyme from the enzyme (Thompson et al., 1976), (4) as a direct spectroscopic probe (Perrin & Hart, 1970; Brand & Gohlke, 1972; Towell, 1977), and (5) through kinetic and binding studies (Krakow, 1965; Glazer, 1970; Jacobsberg et al., 1975; Thompson & Stellwagen, 1976; Bornmann & Hess, 1977; Kumar & Krakow, 1977).

The principal advantage of using dye molecules is that their visible absorption bands are well separated in energy from the region where most proteins absorb, and the perturbation of these bands on binding to the protein provides a convenient experimental monitor of the dye–protein interaction. Because of their similarity to the biological heterocyclic bases, dyes have been used as analogues to nucleotide mono-, di-, and triphosphates, NAD, acetyl-CoA, and folic acid (Thompson et al., 1975; Jacobsberg et al., 1975; Baird et al., 1976). Specificity has been demonstrated by selective competition with the natural coenzyme, activation analogous to cofactor activation, and by noting the similarity of binding to related enzymes (Stellwagen, 1977; Jacobsberg et al., 1975; Baird et al., 1976; Thompson et al., 1975; Thompson & Stellwagen, 1976). However, the questions of how closely the bound dye mimics bound coenzyme and how specific any particular dye molecule might be for binding to protein structural features have not been completely answered. To our knowledge the only studies of these questions are the kinetic studies of Wilson (1976), Bornmann & Hess (1977), and Beissner & Rudolph (1978) on dyes related to Cibacron Blue. These studies indicate that the dyes are not highly specific coenzyme analogues, since only a part of the Cibacron Blue structure is required to compete with the coenzyme, but they do not directly address the extent to which the dyes bound to different enzymes have similar conformations.

One objective of this study was to address these questions for two dyes, Cibacron Blue and Congo Red, bound to several related enzymes. Cibacron Blue has been suggested as a ligand specific for enzymes containing the dinucleotide fold because it can assume a conformation resembling that of bound nucleotide coenzymes, in which the terminal aromatic rings and the sulfonate groups mimic the bases and phosphates of the coenzymes, respectively (Thompson et al., 1975; Stellwagen, 1977; Stellwagen & Baker, 1976). We have recently suggested Congo Red, another aromatic sulfonated dye, as a coenzyme analogue (Edwards & Woody, 1977).

LADH[1] and LDH have the same topology in the six-stranded parallel β-sheet domain referred to as the dinucleotide fold, which forms part of the coenzyme binding site. NAD(H) has a similar extended conformation when bound to these two enzymes (Rao & Rossmann, 1973; Ohlsson et al., 1974; Rossmann et al., 1974, 1975; Buehner, 1975). The crystal structure of YADH has not yet been determined, but the strong sequence homology with LADH (Eklund et al., 1976) suggests that the NAD(H) binding site of YADH will closely resemble those of LADH and LDH. PGK also has a six-stranded parallel β sheet in the region of nucleotide binding

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[1] Abbreviations used: CD, circular dichroism; LADH, liver alcohol dehydrogenase; H_4-LDH, beef heart lactic dehydrogenase; M_4-LDH, beef muscle lactic dehydrogenase; PGK, phosphoglycerate kinase; YADH, yeast alcohol dehydrogenase.

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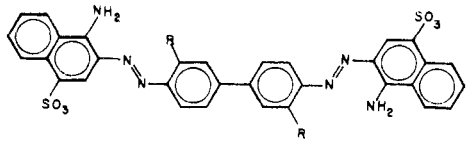
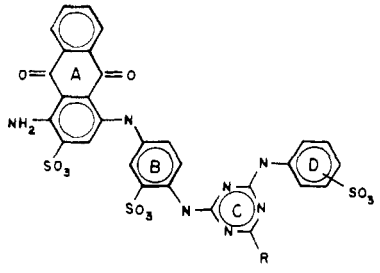
Chart I



R = -H CONGO RED

R = -CH_3 BENZOPURPURIN 4B



R = -Cl CIBACRON BLUE

R = -O-DEXTRAN BLUE DEXTRAN

R = -O-SEPHAROSE BLUE SEPHAROSE

which has a topology quite similar to that found in the dehydrogenases (Rossmann et al., 1974; Levitt & Chothia, 1976). A five-stranded parallel β-sheet domain topologically related to that found in the dehydrogenases is present in adenylate kinase (Schulz & Schirmer, 1974). The crystal structure of aldolase is not available, but from minimum base-change comparisons, secondary structure prediction, knowledge of particular residues, and binding to Blue Dextran columns, aldolase is predicted to contain a similar domain (Stellwagen, 1976).

Our primary method is direct spectroscopic investigation using induced CD in conjunction with binding and competition information. In addition to Cibacron Blue itself, Cibacron Blue covalently linked to dextran (Blue Dextran) or Sepharose (Blue Sepharose) was investigated, since these derivatives are important in affinity chromatography. A structural variant of Congo Red, Benzopurpurin 4B, was also studied (see Chart I). Molecular orbital calculations on the chromophores of Cibacron Blue and Congo Red (R. A. Edwards and R. W. Woody, unpublished experiments) have been helpful in our interpretation of the experimental spectra. The object of these theoretical calculations has been to determine whether twisting within the dye chromophore (inherent chirality) can account for the CD magnitudes observed and to assess the extent to which coupling of dye transitions to protein chromophores may contribute to the induced CD. Since these calculations will be described in detail elsewhere, we shall only refer to the relevant results in this paper.

We are able to conclude that there is not a single unique bound dye conformation for either dye which mimics the bound cofactor on different enzymes containing the dinucleotide fold. However, the induced CD does imply that in most cases, with closely related proteins or small variations in the dye chromophore, the bound dyes have quite similar conformations.

Materials and Methods

Chemicals. Congo Red and Cibacron Blue were purified and their concentrations determined as previously described (Edwards & Woody, 1977; Edwards, 1979). Benzopurpurin 4B was purified as described by Edwards (1979), and concentrations of this dye were determined by using the extinction coefficient used for Congo Red (5×10^4 M^{-1} cm^{-1} at 500 nm in 10 mM Tris-HCl, pH 7.5). Blue Dextran and Blue Sepharose CL-6B were purchased from Sigma Chemical Co., and concentrations of these dyes were determined by using the extinction coefficient of Cibacron Blue at the long-wavelength maximum, after correcting for light scattering [13.3×10^3 M^{-1} cm^{-1} at 610 nm in 10 mM Tris-HCl, pH 7.5 (Edwards, 1979)].

All enzymes and nucleotides used for competition or activity assays were from Sigma except hexokinase, which was obtained from Worthington Biochemical Corp. Substrates and buffer salts were of the best grade available from commercial sources, generally analytical grade.

Enzymes. All of the enzymes were purchased from commercial sources as ammonium sulfate suspensions except YADH, which was lyophilized. Purification (where necessary), removal of salt, and the determination of enzyme concentrations were carried out as described by Edwards (1979). Enzyme concentrations reported are subunit concentrations.

The buffer used for spectroscopic studies was 10 mM Tris-HCl, pH 7.5, for all of the enzymes except the kinases. For the kinases 50 mM potassium phosphate buffer, pH 7.5, was used because of occasional instability problems encountered with these enzymes in the low ionic strength Tris-HCl buffer.

All of the enzymes were shown to be active when dissolved in the buffer used for spectroscopy, with specific activities approaching those reported by the supplier. For all of the enzymes, except LDH, the enzyme–dye and enzyme–dye–cofactor solutions were assayed after the spectroscopic runs (which took several hours) with only small losses of activity.

Spectra. Absorption spectra were recorded on a Cary 118 spectrophotometer using path lengths from 0.1 mm to 5 cm, and CD spectra were run on a JASCO J-41C spectropolarimeter using a path length of 1 cm, except as noted below. Spectra were recorded at 25 ± 1 °C. Precautions were taken to minimize base line shifts, and the spectropolarimeter was calibrated with (+)-10-camphorsulfonic acid (Edwards, 1979). Theoretical spectra were plotted by assuming that the spectra are composed of Gaussian bands (Edwards, 1979).

No problems of photosensitivity were encountered with the Congo Red–enzyme CD spectra, indicating that there was no cis–trans isomerization of the azo bonds as a result of illumination.

The M_4-LDH plus Cibacron Blue solution clouded up while the CD spectrum was run. The spectrum for Benzopurpurin 4B bound to YADH increased over a period of hours, so it was recorded after equilibrium was established. On addition of NADH this spectrum also decreased over a period of ~1 h.

The CD spectra for enzyme bound to Blue Sepharose were run on the gel settled in a 0.5-mm cell. The settled gel does give a large amount of light scattering which may distort the amplitude and position of the CD bands, but we have minimized this problem by using a short path length (0.5 mm) which gives a maximum apparent absorbance (scattering plus true absorption) of less than 1.3 for the entire spectral region reported. The CD spectrum of the settled gel before enzyme addition was subtracted as base line. After adding enzyme, mixing, and allowing the gel to resettle, we measured the absorbance of the supernatant at 280 nm. From the free enzyme concentration in the supernatant and the total enzyme concentration, the bound enzyme concentration was approximated and used to calculate molar ellipticities.

The CD titration was carried out by recording the whole visible CD spectrum after additions of Congo Red to H_4-LDH. The difference between the positive peak at 345 nm and the negative peak at 311 nm was determined as a function of dye

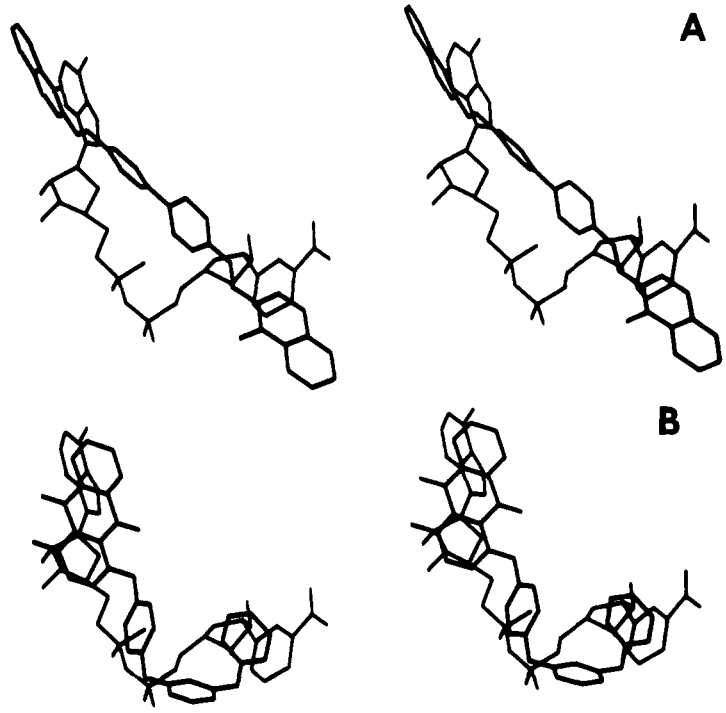


FIGURE 1: (A) Stereo drawing of Congo Red (in bold outline) superimposed on the conformation NAD assumes when bound to LDH in a ternary complex. (B) Stereo drawing of Cibacron Blue (in bold outline) in the conformation proposed by Thompson et al. (1975) superimposed on the conformation of NAD in the ternary complex.

concentration (Figure 3). These shorter wavelength peaks were used rather than the long-wavelength peak (see Figure 4) because by using the amplitude of a couplet the effect of any instrumental base line shifts on successive scans is decreased. The shorter wavelength bands also have less contribution from weak binding sites which may be occupied at the higher dye/enzyme ratios (Edwards & Woody, 1977).

The dissociation constant and number of binding sites were calculated by using an analytical parametric program which computed the intersection of direct linear plots (Eisenthal & Cornish-Bowden, 1974; Woosley & Muldoon, 1976). Only those experimental points for which the unbound dye and enzyme subunit concentrations exceeded 1 μM and were more than 5% of the total concentrations were selected for analysis of binding. Also, the free dye concentrations were required to be less than 5 μM to avoid aggregation.

Stereoscopic Drawings. Figure 1 was drawn on the microfilm plotter at the Colorado State University computer center by using the software package FORDYM developed by John H. Schock and routines we have written to give right- and left-eye views by translation of the object. The coordinates for NAD are from those of the ternary complex with LDH and pyruvate (Chandrasekhar et al., 1973). No hydrogen atoms are shown, and the sulfonates of the dye molecules are also omitted for simplicity.

Results

Dye Conformations. Congo Red and Cibacron Blue conformations are shown in Figure 1, which illustrates their similarity to the coenzyme NAD. The conformation shown for Cibacron Blue is that proposed by Thompson et al. (1975) to mimic NAD. [The stereo drawing for Cibacron Blue does not precisely correspond to the photograph of Thompson et al. (1975), differing mainly in the orientation of the phenyl ring D. This could be remedied by adjustment of the dihedral angles around the final nitrogen, which leads to a conformation which mimics NAD more closely.] π-Electron calculations have been performed (R. A. Edwards and R. W. Woody, unpublished results) by using this conformation, a second conformation suggested by the same group (Stellwagen, 1977), and other conformations which looked like potential NAD mimics. The conformation shown for Congo Red in Figure 1 is one of many used for calculations and is depicted because its calculated CD is nearly identical with the observed CD for this dye bound to H_4-LDH, which is the Congo Red–enzyme interaction we have studied most thoroughly.

Congo Red and Cibacron Blue Absorption. The absorption spectrum of Congo Red (Figure 2) has peaks at 500, 342, and 235 nm. At concentrations above 5 μM, a blue shift and hypochromism are observed, which implies self-aggregation (Edwards, 1979). The strong tendency of Congo Red to aggregate has been reported previously (Iyer & Singh, 1970; Yasunaga & Nishikawa, 1972). Benzopurpurin 4B has an absorption spectrum very similar to that of Congo Red (not shown) but does not show deviations from Beer's law up to 4×10^{-5} M.

Figure 2 also shows the calculated absorption spectrum of Congo Red. The energies of the calculated absorption bands agreed with the experimental energies very well, and the intensities agree to within a factor of 1.5. The long-wavelength band is actually composed of two transitions. The lowest energy transition is allowed, while the higher energy component is forbidden. Several transitions are involved in the band at 342 nm, and there is a multitude of higher energy transitions.

In the 420–480-nm range there are $n\pi^*$ transitions involving nonbonding orbitals composed of the σ lone pairs on the azo nitrogens. These $n\pi^*$ transitions are observed at $\sim$450 nm in several aromatic azo compounds, and their energy is not drastically influenced by substitution on the aromatic rings (Rau, 1973). However, hydrogen bonding between these orbitals and the amine hydrogens on the naphthyl rings may have a larger effect than is observed with most substituents. These $n\pi^*$ transitions will have small oscillator strengths but may have significant rotational strengths.

The absorption spectrum of Cibacron Blue (Figure 2) shows a broad band with its maximum at 610–615 nm and another

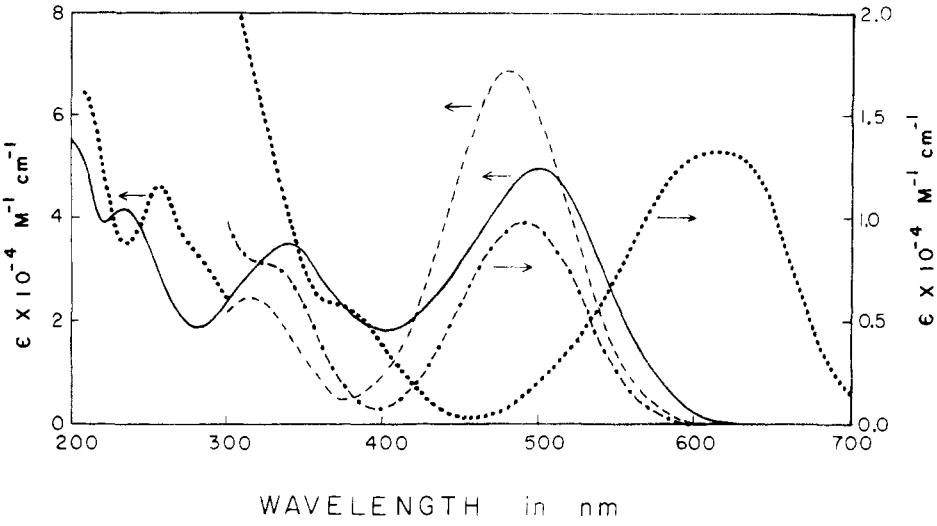


FIGURE 2: Absorption spectra of Congo Red and Cibacron Blue. Experimental spectrum of 1.3 μM Congo Red (—) and calculated spectrum for Congo Red (---). Experimental spectrum of 1.5 μM Cibacron Blue (···) and calculated spectrum for Cibacron Blue (-·-).

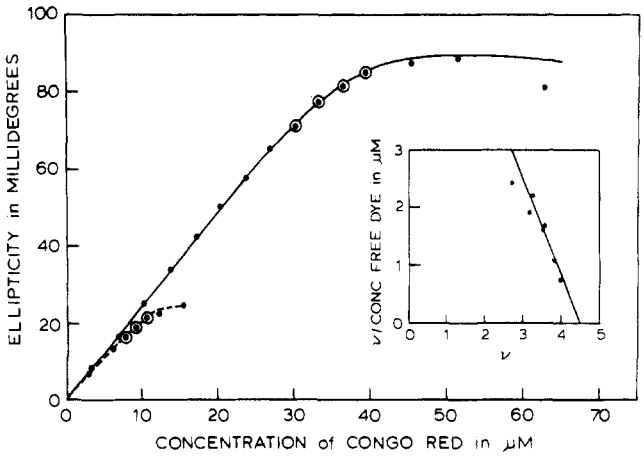


FIGURE 3: CD titration of H_4-LDH with Congo Red. The ellipticity is $\theta_{345} - \theta_{311}$. The upper curve is for 36 μM and the lower curve for 9.7 μM H_4-LDH before dilution. The insert is a Scatchard plot of the experimental points circled in the titration curves. The curves are drawn for the calculated K_D = 0.5 μM and n = 4.5.

band at 257 nm which has shoulders at approximately 280 and 375 nm. At concentrations above 5 μM, Cibacron Blue also shows a hypochromic deviation from Beer's law, which implies self-aggregation. Blue Dextran and Blue Sepharose give absorption spectra similar to that of the free dye, although the long-wavelength maxima (which are least affected by light scattering) are red-shifted by 5–10 nm.

The energies of the calculated absorption bands (R. A. Edwards and R. W. Woody, unpublished results) for Cibacron Blue (Figure 2) are larger than those observed experimentally, but the intensities agree well with experiment. [The parameters of Nishimoto & Forster (1966) which we have used in the molecular orbital calculations predict transition energies very well for most systems but systematically overestimate the energies in substituted anthraquinones. The error is accentuated by the long-wavelength character of the lowest transition.] Only one transition is involved in the long-wavelength band. There are several shorter wavelength transitions contributing to the 257-nm band, with one strong one calculated at 341 nm corresponding to the shoulder observed at 380 nm. All of the visible and near-UV transitions are localized on the anthraquinone and phenyl ring B.

Congo Red–Enzyme Interactions. We have investigated the binding of Congo Red to H_4-LDH by a CD titration (Figure 3). There are just over four dye molecules bound per tetrameric enzyme with a dissociation constant of 0.5 μM and no indication of cooperativity. Although the dissociation constant is slightly larger than that reported for NADH binding to beef H_4-LDH under different conditions [0.35 μM (Velick, 1958); 0.39 μM (Anderson & Weber, 1965)], competition as measured by induced CD indicates that under our conditions Congo Red binds to H_4-LDH in a simple competitive manner with a slightly larger affinity than NADH (Edwards & Woody, 1977).

As we have reported in a previous communication (Edwards & Woody, 1977), when Congo Red binds to LDH or ADH very similar CD spectra are induced which are negative at long wavelengths and show a positive–negative couplet between 300 and 400 nm (Figure 4). Rabbit muscle LDH induces a CD spectrum in Congo Red nearly identical with that induced by beef muscle LDH. Benzopurpurin when bound to H_4-LDH also gives this distinctive CD pattern which can be eliminated by NADH. There are differences in intensity (Table I) for the different enzymes, and between 400 and 450 nm no band is observed for H_4-LDH, whereas the M_4-LDH-, LADH-, and YADH-bound Congo Red spectra have either a positive

Table I: Molar Ellipticities of Congo Red–Enzyme Complexes and Competition with Cofactors

enzyme	dye	wavelength (nm)	$[\theta] \times 10^{-4}$ (deg cm²/dmol)	competition[a]	
	calcd	490	−10.2		
H_4-LDH	Congo Red	525	−12.0	NADH	simple
H_4-LDH	Benzopurpurin	540	−7.0	NADH	simple
M_4-LDH	Congo Red	525	−7.3	NADH	simple
M_4-LDH[b]	Congo Red	520	−6.0	NADH	simple
LADH	Congo Red	490	−3.6	NADH	complex
YADH	Congo Red	510	−2.2	NADH	simple[c]
YADH	Benzopurpurin	530	−4.9	NADH	simple
adenylate kinase	Congo Red	530	+3.9	ADP or ATP	complex
PGK	Congo Red	510	+1.3	ATP or ATP-Mg^{2+}	simple
aldolase	Congo Red	540	+7.6	F-1,6-diP_i[d]	complex
aldolase	Benzopurpurin	550	+1.2	F-1,6-diP_i	complex

[a] Simple implies complete elimination of CD in a competitive manner. Complex implies that a ternary complex CD is observed. [b] Rabbit muscle LDH. [c] Not complete elimination of CD, but still appears to be competitive. [d] Fructose 1,6-diphosphate.

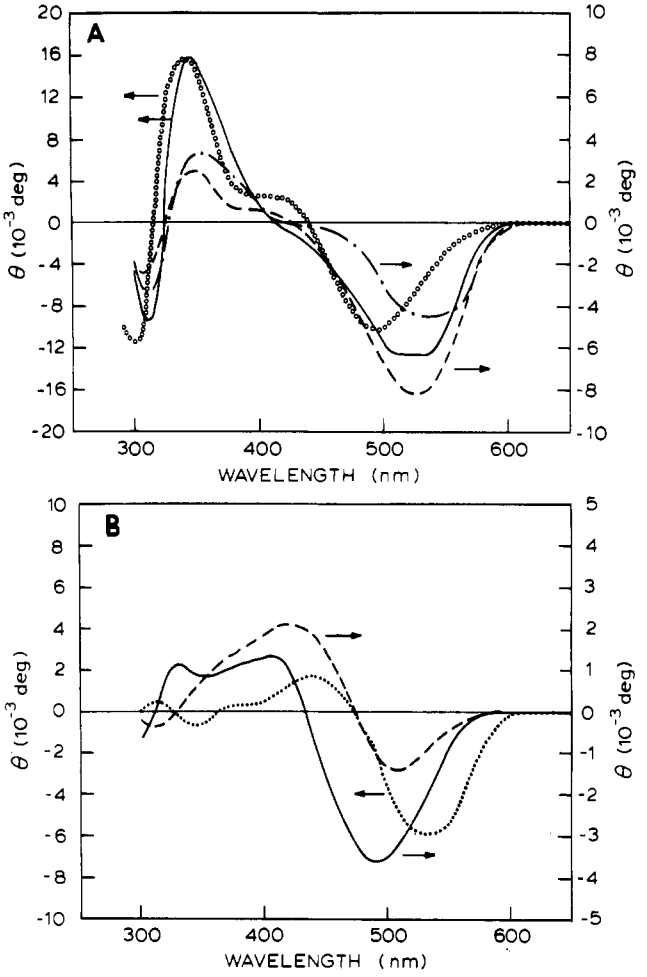


FIGURE 4: (A) CD spectra of Congo Red bound to LDH. 36 μM H_4-LDH, 10.4 μM Congo Red (—); 20 μM H_4-LDH, 6.3 μM Benzopurpurin 4B (-·-); 37 μM M_4-LDH, 11.5 μM Congo Red (---); calculated spectrum for Congo Red conformation of Figure 1 for 10 μM concentration (O). (B) CD spectra of Congo Red bound to ADHs. 44 μM LADH, 10 μM Congo Red (—); 43 μM YADH, 6.3 μM Congo Red (---); 45 μM YADH, 11.9 μM Benzopurpurin 4B (···).

shoulder or a band. Benzopurpurin bound to YADH gives a CD spectrum similar to that for Congo Red bound to YADH in the long-wavelength region but has a couplet of opposite sign between 300 and 400 nm.

Although Congo Red competes with NADH for binding to H_4-LDH, M_4-LDH, and YADH, its binding to LADH is somewhat more complex. Adding saturating levels of NADH changes, but does not eliminate, the induced CD of Congo Red bound to LADH (spectra not shown). This indicates that a ternary complex is formed between Congo Red, NADH, and LADH. The long-wavelength CD spectrum of the ternary complex is similar to that which is observed when the Congo Red/LADH active-site ratio exceeds unity. Under these conditions secondary sites would become occupied.

The CD spectra of Congo Red bound to adenylate kinase, PGK, and aldolase are quite different from those observed with the dehydrogenases (Figure 5), even though these enzymes have a nucleotide-binding domain similar to the dinucleotide fold. The long-wavelength band is positive for Congo Red bound to all of these enzymes, and a negative band is observed between 300 and 350 nm. However, differences between these spectra are evident in the $n\pi^*$ and 350–400-nm regions. Only in the case of ATP (and ATP-Mg^{2+}) with phosphoglycerate kinase do saturating levels of the cofactors (both ADP and ATP with and without Mg^{2+} were used with the kinases)

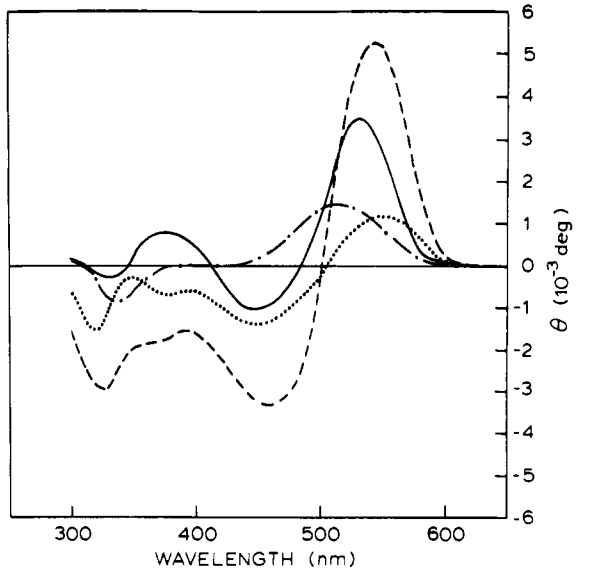


FIGURE 5: CD spectra of Congo Red bound to adenylate kinase, PGK, or aldolase. 30 μM adenylate kinase, 8.9 μM Congo Red (—); 60 μM PGK, 11.3 μM Congo Red (-·-); 25 μM aldolase, 7.0 μM Congo Red (---); 14 μM aldolase, 10 μM Benzopurpurin 4B (···).

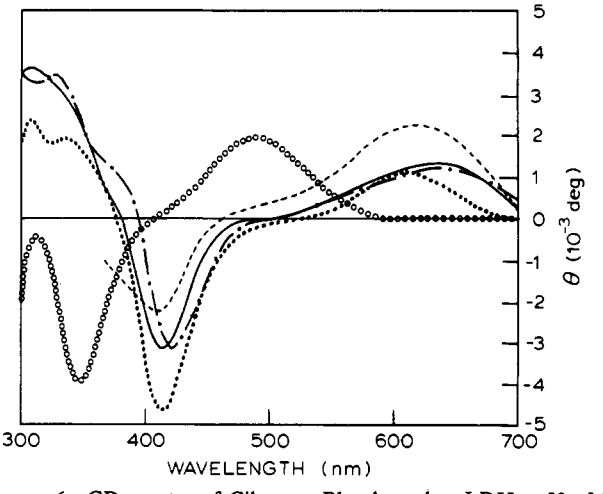


FIGURE 6: CD spectra of Cibacron Blue bound to LDHs. 50 μM H_4-LDH, 23 μM Cibacron Blue (—); 39 μM H_4-LDH, 21 μM Blue Dextran (-·-); 18 μM M_4-LDH, 11 μM Cibacron Blue (···); 850 μM M_4-LDH bound to Blue Sepharose (---); calculated spectrum for Cibacron Blue conformation of Stellwagen (1977) for 10 μM concentration (O).

completely eliminate the induced CD in the visible region, implying that in the other cases dye–enzyme–cofactor ternary complexes are formed. Throughout the visible region, Benzopurpurin bound to aldolase gives an induced CD of the same sign as Congo Red bound to the same enzyme.

Cibacron Blue–Enzyme Interactions. Figures 6–8 give the induced CD which results from binding of the Cibacron Blue chromophore to enzymes containing a dinucleotide fold. The Cibacron Blue chromophore has been shown to compete with the appropriate cofactor, for all of the enzymes used (Thompson et al., 1975; Thompson & Stellwagen, 1976; Wilson, 1976; Stellwagen, 1977). Saturating concentrations of the cofactor also eliminate the induced CD spectra for all of the Cibacron Blue–enzyme complexes, except that with aldolase (see Table II).

When the chromophore is bound to LDH (Figure 6), positive CD is induced at the longest wavelengths, negative CD near 420 nm, and positive CD again at shorter wavelengths. Although there are small differences in the intensities of the

Table II: Molar Ellipticities of Cibacron Blue-Enzyme Complexes and Competition with Cofactors

enzyme	dye	wavelength (nm)	$[\theta] \times 10^{-4}$ (deg cm^2/dmol)	competition[a]	
	calcd[b]	480	−0.6		
	calcd[c]	490	+1.9		
H_4-LDH	Cibacron Blue	630	+0.6	NADH	simple
H_4-LDH	Blue Dextran	640	+0.6	NADH	simple
M_4-LDH	Cibacron Blue	600	+1.0	NADH	simple
M_4-LDH[d]	Cibacron Blue	600	+1.3	NADH	simple
M_4-LDH	Blue Sepharose	620	+0.5 (2.0)[e]	NADH	simple
LADH	Cibacron Blue	620	−1.5	NADH	simple
LADH	Blue Dextran	600	−1.5	NADH	simple
LADH	Blue Sepharose	600	−1.5 (3.0)[e]	NADH	simple
YADH	Cibacron Blue	600	−0.4	NADH	simple
adenylate kinase	Cibacron Blue	630	+0.3	ADP or ADP-Mg^{2+}	simple
PGK	Cibacron Blue	680	+0.1	ATP or ATP-Mg^{2+}	simple[f]
aldolase	Cibacron Blue	610	−0.5	F-1, 6-diP_i[g]	complex
aldolase	Blue Dextran	660	+0.3	F-1, 6-diP_i	simple

[a] Simple implies complete elimination of CD in a competitive manner. Complex implies that a ternary complex CD is observed. [b] Conformation of Thompson et al. (1975), shown in Figure 1. [c] Conformation of Stellwagen (1977). [d] Rabbit muscle LDH. [e] Value in parentheses assumes that only one active site is occupied per enzyme molecule. [f] Not complete elimination of CD, but still appears to be competitive. [g] Fructose 1,6-diphosphate.

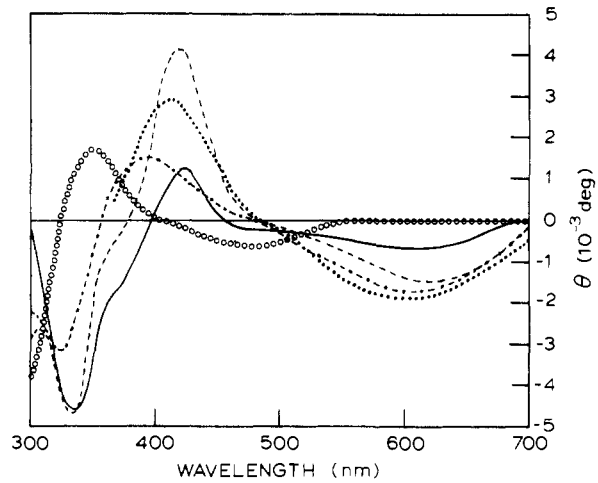


FIGURE 7: CD spectra of Cibacron Blue bound to ADHs. 50 μM LADH, 10 μM Cibacron Blue (- -); 18 μM LADH, 11.5 μM Blue Dextran (-·-); 200 μM LADH bound to Blue Sepharose (···); 43 μM YADH, 19.8 μM Cibacron Blue (—); calculated spectrum for Cibacron Blue assuming the conformation of Thompson et al. (1975) (shown in Figure 1) for 10 μM concentration (O).

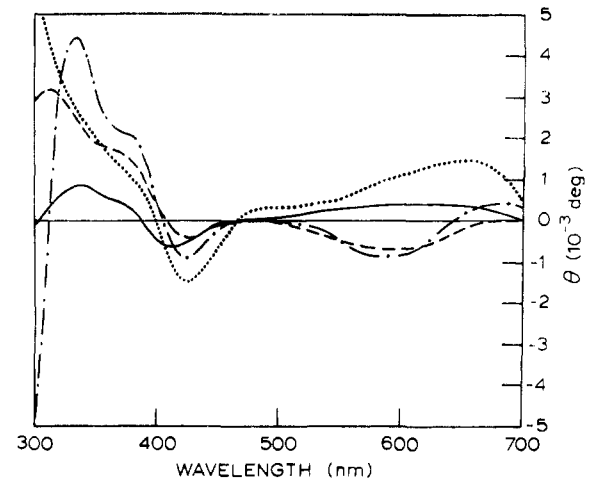


FIGURE 8: CD spectra of Cibacron Blue bound to adenylate kinase, PGK, or aldolase. 30 μM adenylate kinase, 11.5 μM Cibacron Blue (—); 58 μM PGK, 57 μM Cibacron Blue (-·-); 54 μM aldolase, 12.2 μM Cibacron Blue (- - -); 190 μM aldolase, 49 μM Blue Dextran (···).

various bands (see Table II), essentially the same CD spectra result from Cibacron Blue, Blue Dextran, or Blue Sepharose bound to heart or muscle LDH isozymes. This implies that the conformation of the bound chromophore is not changed to any large extent by the matrices or by the differences between heart and muscle LDH. Rabbit muscle LDH and beef muscle LDH induce nearly identical CD in Cibacron Blue (see Table II).

When the chromophore of Cibacron Blue is bound to ADH, the induced CD is negative at long wavelengths, positive in the 400–450-nm region, and negative again for the shorter wavelengths (Figure 7). The sign of each of these bands is opposite from that induced by LDH. The spectra for the modified chromophores with ADH from two species are quite similar.

Adenylate kinase, phosphoglycerate kinase, and aldolase induce weaker optical activity in Cibacron Blue (Figure 8) than the dehydrogenases induce. Cibacron Blue and Blue Dextran bound to adenylate kinase have the same CD spectrum (Blue Dextran spectrum not shown). However, for phosphoglycerate kinase and aldolase the spectra are different for the various chromophores, as we illustrate in the figure in the case of aldolase. This indicates some differences in the conformation of the bound Cibacron Blue resulting from conjugation to dextran. The spectrum of Blue Sepharose bound to aldolase is not shown because it is very weak, but it shows a positive CD near 420 nm and a negative CD in the 350–400-nm region, indicating a conformation different from either bound Cibacron Blue or Blue Dextran. Cibacron Blue also binds to bovine serum albumin, giving a moderately intense CD signal.

Generally, the modification of the chromophore of Cibacron Blue does not alter the CD induced by a particular enzyme, indicating little change in the bound conformation. However, there are exceptions, as illustrated by aldolase. In agreement with Wilson's (1976) finding that yeast hexokinase is inhibited by Cibacron Blue but not by Blue Dextran, we find that a solution of Blue Dextran and hexokinase (designated PII or B form) shows no CD other than that of free Blue Dextran whereas hexokinase does induce CD in Cibacron Blue (spectra not shown). This is consistent with differences in the conformational binding constraints.

Discussion

The CD spectrum of a bound chromophore contains information describing its dissymmetric conformation, but this is

complicated by interactions with dipolar or chromophoric groups in its environment (Blauer, 1974; Towell, 1977). If one or the other of these mechanisms is the dominant source of the induced CD in the bound dye chromophores, which are optically inactive in solution, it becomes much easier to extract conformational information.

Several converging lines of evidence indicate that the CD of Congo Red bound to dehydrogenases is caused primarily by the twisted conformation of the bound chromophore rather than by interaction with the protein. Figure 4A shows the CD calculated (R. A. Edwards and R. W. Woody, unpublished results) for Congo Red in the conformation presented in Figure 1. The good agreement with experiment should not be taken as evidence that the conformation shown in Figure 1 is the correct one for the LDH-bound dye. Other possible conformations could give rise to equally good agreement. The result does indicate, however, that a twisted Congo Red molecule can account for CD of the observed magnitude. Furthermore, model calculations of coupling with a tryptophan side chain (R. A. Edwards and R. W. Woody, unpublished results) indicate that such interactions are unlikely to be dominant in this system.

Similarities in the CD spectra observed for Congo Red bound to the various dehydrogenases are most easily explained as the result of similar dye conformations resulting from the dye mimicking the coenzyme NAD or some portion of it. NADH is bound to these dehydrogenases in a particular conformation (Buehner, 1975). The dye does not necessarily interact with the enzyme at all of the base, ribofuranose, and phosphate subsites, since binding at a limited number of these subsites could provide the required similarity in dye conformation. The adenine subsite binds organic anions quite readily, as discussed below. In contrast, there is no evidence that the nicotinamide subsite binds organic ions, and it may not interact with the dye either. The coenzyme fragments AMP, ADP, and ADP–ribose compete with Congo Red bound to H_4-LDH, indicating interactions at the adenine subsite.

The differences among these spectra in the 400–450-nm region are probably the result of $n\pi^*$ transitions. The CD of these localized transitions would be particularly sensitive to local differences in electronic structure and thus give different CD spectra for chromophores with quite similar overall conformation. The couplet observed between 300 and 400 nm is composed of several partially canceling bands. All of the conformations studied in our π-electron calculations (R. A. Edwards and R. W. Woody, unpublished results) gave this couplet, but the magnitude would be strongly dependent on the amount of cancellation, which is a sensitive function of the positions of the bands as determined by the chromophore conformation and the polarizability of its environment.

In the case of Cibacron Blue bound to any of the enzymes studied, the induced CD probably has significant contributions both from inherent chirality of the dye and from coupling to protein transitions. The magnitude of the induced CD in these cases is much smaller than that for the Congo Red complexes.

Figure 6 shows the CD calculated (R. A. Edwards and R. W. Woody, unpublished results) for the conformation of Cibacron Blue proposed by Stellwagen (1977). A conformation differing only in the dihedral angles between the anthraquinone ring and phenyl ring B with the linking nitrogen was illustrated by Thompson et al. (1975) and is shown in Figure 1. The CD calculated for this conformation is given in Figure 7. As in the case of Congo Red (see above), the good agreement of CD signs and amplitudes[2] with those for Cibacron Blue bound to various enzymes should not be taken to indicate that the assumed conformations correctly describe the bound dye. What these results do demonstrate is that because of the localized nature of the low-energy transitions in Cibacron Blue, a relatively small change in conformation can drastically alter the induced CD. Furthermore, calculations show that the effect of coupling could be comparable to the inherent chirality contributions in the case of Cibacron Blue.

Muscle LDHs from different species induce CD spectra in both dyes which are more nearly identical than do the heart and muscle isozymes from the same species. These results are consistent with the known similarities of LDH isozymes and demonstrate the sensitivity of induced CD as a phenomenological indicator of similarity. Although there are some differences between the CD induced by LADH and YADH, the similarities argue for marked homology in their binding sites. The crystal structure of YADH has not yet been solved, but our results imply that it has a binding site for coenzyme which binds the nucleotide in a conformation similar to that observed for other dehydrogenases. This prediction is consistent with that made from comparing primary structures (Eklund et al., 1976), which implies that the nucleotide-binding domain present in other dehydrogenases is also present in YADH.

We conclude from these considerations that there is not a unique conformation of either of these dyes when bound to enzymes which have a dinucleotide fold. For more closely related enzymes, a similar bound dye conformation is indicated. Congo Red assumes a similar conformation on more distantly related dehydrogenases than Cibacron Blue does, probably because Congo Red has fewer degrees of conformational flexibility and thus cannot adjust as well to the small differences in binding sites. When small changes are made in the dye chromophores, such as methylation or attachment to polysaccharide matrices, the conformation of bound dye is usually not drastically altered. This implies that the binding interactions are not seriously altered for at least the spectroscopically crucial parts of the dye molecules and thus lends validity to comparison of studies of the free chromophores and matrix-bound chromophores. Because there are exceptions to this generalization, induced CD can be used as a monitor of individual cases.

This study supports the intuitively justifiable idea that dye molecules are not highly specific analogues of coenzymes. Instead their several functional groups and conformational freedom allow them to insert aromatic rings into hydrophobic pockets while the other portions of the molecule and the protein assume conformations which maximize favorable and minimize unfavorable interactions. The binding of dyes by albumin to what are probably nonspecific organic anion-binding sites illustrates this point. On the other hand, since both enzyme active sites and dye molecules have limited conformational freedom, some specificity is achieved.

In a recent report, Beissner & Rudolph (1978) have advanced similar ideas from their observation that dehydrogenases and kinases are inhibited to comparable extents by Cibacron Blue and 1-amino-4-[(4′-aminophenyl)amino]-anthraquinone-2,3′-disulfonic acid (which contains the anthraquinone and phenyl ring B of Cibacron Blue). Beissner

[2] The marked blue shift of the theoretical curves in Figures 6 and 7 with respect to the experimental curves parallels the discrepancies in transition energies discussed in connection with the absorption spectrum of Cibacron Blue (see Results). The discrepancy is further enhanced in the CD spectra because the experimental CD spectra reflect the red shift which occurs upon binding to an enzyme, for which no correction has been made.

& Rudolph argue that the sulfonated anthraquinone and one phenyl ring are sufficient to mimic a nucleotide and that the other rings bind nonspecifically if the active site is large enough to accommodate them. From kinetic studies, which included both kinases and dehydrogenases and used a series of dyes analogous to portions of Cibacron Blue, Bornmann & Hess (1977) suggested that the adenosine moiety of ADP, ATP, and NAD, and not the dinucleotide as a whole, is mimicked by these dyes.

A wide range of organic molecules can compete with the coenzyme for binding to dehydrogenases. The X-ray crystallographic studies of Wassarman & Lentz (1971) and Einarsson et al. (1974) show that rather large dye molecules, such as tetraiodofluorescein, 5-iodosalicylic acid, and 1-anilino-8-naphthalenesulfonate, are accommodated by the adenine subsite on LDH and ADH. This structural information implies that the anthraquinone ring of Cibacron Blue or the naphthylamine ring of Congo Red could also be accommodated at the adenine subsite.

In summary, we have used induced CD to evaluate the hypothesis that specific dyes are accurate mimics of coenzyme conformation. We conclude that this is not likely to be the case, although the dyes studied (Cibacron Blue and Congo Red) do bind to closely related enzymes in a similar manner. Likewise, small perturbations on the dye structure do not usually alter the mode of binding to a given enzyme. Induced CD has proven to be a valuable probe of dye conformation at enzyme active sites, as well as a useful method for determining binding constants and stoichiometry. It can be anticipated that these dyes will be generally useful as spectroscopic probes in the study of nucleotide-binding systems.

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