Induced Circular Dichroism as a Probe of Cibacron Blue and Congo Red Bound to Dehydrogenases

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SUMMARY We have investigated the circular dichroism induced in Cibacron Blue and Congo Red upon binding to several dehydrogenases to probe the conformation of the bound dyes. The circular dichroism spectra of Congo Red are quite similar when the dye is bound to lactic dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and alcohol dehydrogenase but has bands of opposite sign when bound to cytoplasmic malic dehydrogenase. The circular dichroism spectra of Cibacron Blue bound to these same dehydrogenases are quite different from one another. Since circular dichroism is sensitive to the conformation of bound dye, these differences argue for at least local changes in dye conformation or environment when bound to different dehydrogenases. Congo Red appears to be less sensitive to these effects than Cibacron Blue.

INTRODUCTION Cibacron Blue (Fig. 1), the chromophore of Blue Dextran, has been suggested as a ligand specific for enzymes containing the dinucleotide fold and it can assume a conformation resembling that of the bound nucleotide coenzymes (1-3). The presence of the dinucleotide fold has been inferred in several enzymes of unknown structure which bind tightly to Cibacron Blue or Blue Dextran affinity columns, and which are eluted by low concentrations of coenzyme or substrate (3,4). However, Wilson has questioned the specificity of the dye-enzyme interaction and has shown that enzymes interact differently with Cibacron Blue and Blue Dextran (5).

Congo Red (Fig. 3) is another aromatic sulfonated dye which could mimic a bound nucleotide coenzyme. There is sufficient flexibility so that when bound to dehydrogenases the sulfonated naphthylamine rings could fit into the pockets which accomodate the adenine and nicotinamide rings of NAD. The polar and anionic sites on the dye do not correspond as closely to those on the coenzyme as those

Abbreviations H_4-LDH Beef Heart Lactic Dehydrogenase; M_4-LDH Beef Muscle Lactic Dehydrogenase; GAPDH Rabbit Muscle Glyceraldehyde-3-Phosphate Dehydrogenase; LADH Horse Liver Alcohol Dehydrogenase; cMDH Pig Heart Cytoplasmic Malic Dehydrogenase; CD Circular Dichroism; TLC Thin Layer Chromatography.

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on Cibacron Blue. However, the azo groups do have lone pair electrons which might hydrogen bond (cf. the ribofuranose oxygens of NAD) and the sulfonate group on one end of the dye might interact with the substrate site on dehydrogenases.

Absorption difference spectroscopy has been used to monitor Cibacron Blue interaction with enzymes (6-8). We have used another spectroscopic technique, circular dichroism, to study the conformation of Cibacron Blue and Congo Red when bound to several dehydrogenases which have the dinucleotide fold in the coenzyme binding region: LDH, LADH, GAPDH, and cMDH (9). Induced CD results from inherent dissymmetry (twisting) of the bound dye and from interactions of the dye with groups on the proteins (10-12).

MATERIALS AND METHODS Ammonium sulfate suspensions of lactic dehydrogenase (beef heart Type II and beef muscle Type X, E.C. No. 1.1.1.27), glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle, E.C. No. 1.2.1.12), and cytoplasmic malic dehydrogenase (pig heart, E.C. No. 1.1.1.37) were purchased from Sigma Chemical Co. Alcohol dehydrogenase (horse liver, E.C. No. 1.1.1.1) was obtained from Boehringer Mannheim. Both heart and muscle LDH were purified by chromatography on DEAE-cellulose (13) and shown to be pure H_4 and M_4 isozymes by gel electrophoresis. Both NADH (grade III disodium salt) and NAD (grade III) were purchased from Sigma Chemical Co.

Cibacron Blue F3GA was a generous gift of Dr. H. Bosshard of Ciba-Geigy, Basel, Switzerland and was chromatographed on a cellulose column using butanol:ethanol:water (2:1:1), which removes several impurities that can be identified by fluorescing or blue spots on paper chromatography and silica gel TLC, using the same solvent. Concentrations of this dye were measured by absorption at 610 nm using an extinction coefficient of 13.3 mM^{-1} cm^{-1} which was determined at 25°C in 10 mM Tris·HCl buffer, pH 7.5, at a concentration of less than 5 x 10^{-6} M. The dye had been dried for 2 weeks at room temperature over P_2O_5 in a vacuum.

Congo Red was purchased from Eastman Kodak and purified by salting it out of aqueous solution 5 times with sodium acetate, precipitating 20 times with dilute HCl, and recrystallizing once from 50% ethanol. This procedure removes several fluorescent impurities identified by the paper chromatographic technique of Bedaux et al. (14). Concentrations of Congo Red were determined from the following molar extinction coefficients calculated from data of Bedaux et al. (14): 5.00 x 10^4 M^{-1} cm^{-1} at 500 nm for low ionic strength or 4.15 x 10^4 M^{-1} cm^{-1} at 488 nm for 1/15 N phosphate buffer.

Absorption measurements were run on a Cary 17 or 118 spectrophotometer in 10 mM Tris·HCl or 1/15 N phosphate buffers. CD spectra were run on a Jasco J-41C spectropolarimeter calibrated with an aqueous solution of (+)-10-camphorsulfonic acid (15). All the CD spectra were run using 10 mM Tris·HCl pH 7.5 buffer at 25°C. The M_4 LDH plus Cibacron Blue solution clouded up while the CD spectrum was run. The CD spectrum of cMDH plus Congo Red increased over a period of minutes so the spectrum reported was recorded after equilibrium was established. Molar ellipticities per bound dye were calculated by assuming that all the dye is bound to the various enzymes under the conditions used.

RESULTS Marked deviation from Beer's law is observed for both Cibacron Blue and Congo Red at concentrations greater than 5 x 10^{-6} molar in 10 mM Tris buffer,

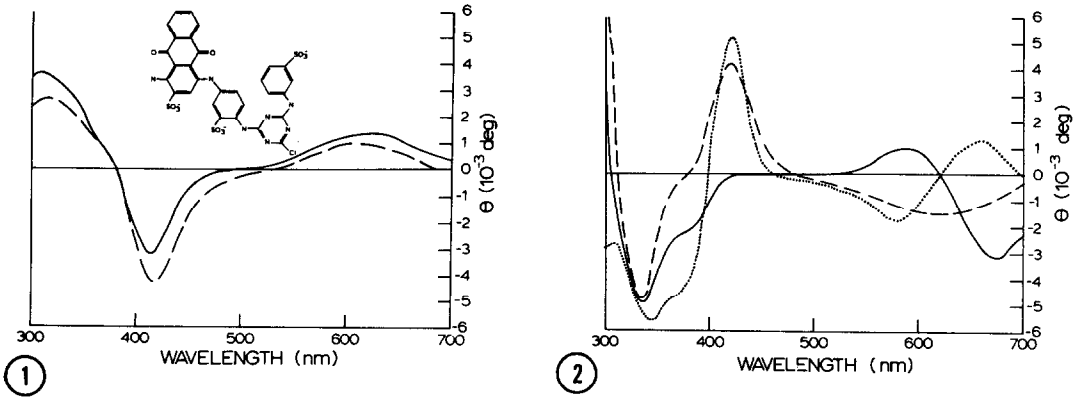


FIGURE 1. CD Spectra of Beef Heart or Beef Muscle Lactic Dehydrogenase plus Cibacron Blue, including the structure of Cibacron Blue
——— 12.6 μM H_4-LDH, 23 μM Cibacron Blue; $[\theta]_{630}=6\times10^3$ deg cm^2/dmole
— — 4.6 μM M_4-LDH, 11 μM Cibacron Blue; $[\theta]_{600}=10\times10^3$ deg cm^2/dmole

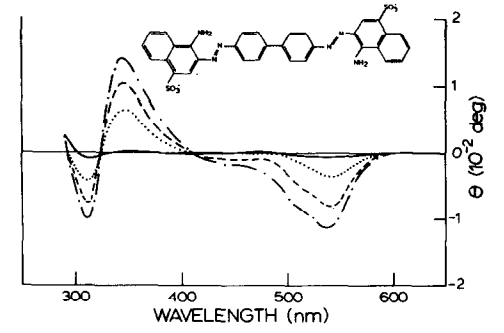
FIGURE 2. CD Spectra of Rabbit Muscle Glyceraldehyde-3-Phosphate Dehydrogenase, Horse Liver Alcohol Dehydrogenase, or Pig Heart Cytoplasmic Malic Dehydrogenase plus Cibacron Blue
——— 30 μM GAPDH, 120 μM Cibacron Blue; $[\theta]_{675}=-3\times10^3$ deg cm^2/dmole
— — 24.8 μM LADH, 10 μM Cibacron Blue; $[\theta]_{620}=-15\times10^3$ deg cm^2/dmole
····· 28 μM cMDH, 27 μM Cibacron Blue; $[\theta]_{660}=5.2\times10^3$ deg cm^2/dmole

a result of dye aggregation. The extinction coefficient determined for purified Cibacron Blue in dilute solution is 13.3 mM^{-1} cm^{-1}, falling between two previously determined values of 13.6 (7) and 12.3 (8).

Moderate to strong CD is induced in the visible region upon binding either Cibacron Blue or Congo Red to dehydrogenases. The molar ellipticities for the long-wavelength band of Cibacron Blue range from 3 x 10^3 to -15 x 10^3 deg cm^2/dmole (Fig. 1,2). Those for Congo Red are about an order of magnitude larger, ranging from -2.2 x 10^4 to -12 x 10^4 deg cm^2/dmole (Fig. 3,5).

The CD spectra which result when Cibacron Blue is bound to the five dehydrogenases show no systematic pattern. Beef M_4-LDH and H_4-LDH induce almost identical CD spectra (Fig. 1) but LADH, GAPDH, and cMDH induce very different spectra (Fig. 2).

When the coenzyme NADH (NAD in the case of GAPDH) was added to any of the enzyme-dye complexes, the induced CD spectra were decreased as the coenzyme displaced the dye from the enzyme. Figure 3 illustrates this competition of NADH and Congo Red for binding to H_4-LDH. The NADH concentration exceeded the Congo



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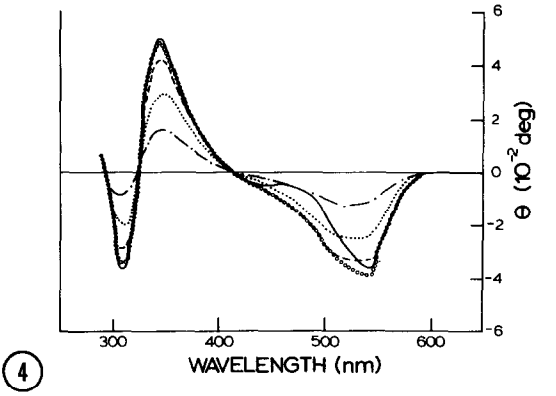

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FIGURE 3. CD Spectra of Beef Heart Lactic Dehydrogenase with Congo Red plus NADH, including the structure of Congo Red
—·— 2.2 μM H_4-LDH, 14.4 μM Congo Red
– – – 2.2 μM H_4-LDH, 14.4 μM Congo Red, 6.2 μM NADH
····· 2.2 μM H_4-LDH, 14.2 μM Congo Red, 30.5 μM NADH
——— 2.1 μM H_4-LDH, 13.7 μM Congo Red, 118 μM NADH

FIGURE 4. CD Spectra of Beef Heart Lactic Dehydrogenase plus Congo Red
—·— 9.0 μM H_4-LDH, 10.4 μM Congo Red, $[\theta]_{525}$=-12.0x10^4 deg cm^2/dmole
····· 8.9 μM H_4-LDH, 20.4 μM Congo Red; – – – 8.7 μM H_4-LDH, 30.0 μM Congo Red
ooooooo 8.6 μM H_4-LDH, 39.4 μM Congo Red; ——— 8.4 μM H_4-LDH, 51.3 μM Congo Red

Red concentration before the induced CD spectrum had been decreased to approximately half its original value, indicating that Congo Red has a slightly larger affinity for LDH than does NADH.

As Congo Red is added to H_4-LDH the induced CD spectrum increases until the dye concentration is more than four times the enzyme concentration (Fig. 4). The stoichiometry indicated is one dye molecule per subunit. When the dye concentration exceeds the enzyme subunit concentration, there are changes in the CD, particularly a decrease in amplitude around 475 nm. This change must reflect a second site on the enzyme to which dye binds more weakly and which gives a different induced CD spectrum.

The CD spectra induced in Congo Red by LDH, LADH, and GAPDH (Fig. 4,5) are quite similar to one another. On the other hand, when Congo Red is bound to cMDH the CD bands are similar in position but opposite in sign (Fig. 5).

DISCUSSION Induced CD is a convenient method for showing competition between

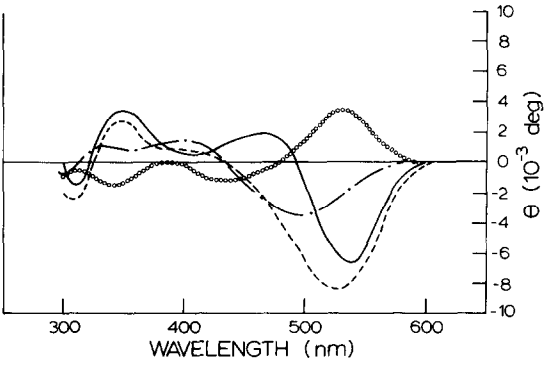

FIGURE 5. CD Spectra of Rabbit Muscle Glyceraldehyde-3-Phosphate Dehydrogenase, Horse Liver Alcohol Dehydrogenase, Beef Muscle Lactic Dehydrogenase, or Pig Heart Cytoplasmic Malic Dehydrogenase plus Congo Red
——— 30 μM GAPDH, 30 μM Congo Red, $[\theta]_{540}$=-2.2x10^4 deg cm^2/dmole
—·— 22 μM LADH, 10 μM Congo Red, $[\theta]_{490}$=-3.6x10^4 deg cm^2/dmole
– – – – 9.2 μM M_4-LDH, 11.5 μM Congo Red, $[\theta]_{525}$=-7.3x10^4 deg cm^2/dmole
oooooo 9 μM cMDH, 4.9 μM Congo Red, $[\theta]_{540}$=7.1x10^4 deg cm^2/dmole

the dyes and the coenzyme and for determining the stoichiometry of dye binding. Adding coenzyme to an enzyme-dye complex will, in the case of competitive binding, eliminate the induced CD since the free dye is optically inactive. The relative affinities of dye and coenzyme for the enzyme site can also be determined. Since nonspecific dye binding sites usually induce a distinct and often weak CD, it is possible to determine the stoichiometry of the tight binding site.

Induced CD is an excellent empirical indicator of similarity. The isoenzymes of lactic dehydrogenase induce nearly identical CD spectra in either dye used in this study, indicating a very similar conformation and environment of the bound dyes. This can also be applied to variations in the dye chromophore such as a comparison of Cibacron Blue and Blue Dextran in order to investigate the differential biospecificity of these ligands (5).

Although the marked differences in the induced CD spectra found when Cibacron Blue is bound to dehydrogenases do argue for differences in bound dye conformation, they do not necessarily rule out the possibility that the dye has a similar overall conformation mimicking the similar bound coenzyme conformations on these enzymes. The differences in induced CD may be the result of either dif-

ferences in local conformation or different interactions of the dye chromophore with groups on the proteins (10-12). The visible transitions of Cibacron Blue are localized on the anthraquinone and to a smaller extent the attached phenyl ring (Fig. 1), which implies that the local twist about the nitrogen connecting these groups is the determining factor for inherent CD.

The similar induced CD spectra of Congo Red bound to M_4-LDH, H_4-LDH, GAPDH, and LADH suggests that Congo Red has a very similar conformation on these enzymes and that interactive effects are not complicating the observed spectra. The strength of these CD bands also argues for an inherent source. However, Congo Red bound to cMDH, which also has the dinucleotide fold, gives a CD with bands of opposite sign. The visible transitions of Congo Red are not localized in any part of the chromophore but extend over the whole molecule, which implies that Congo Red bound to cMDH has an opposite sense of overall twist. There is sufficient flexibility in the dye chromophore to allow various conformations in the poorly matched central region on various enzymes even though the terminal rings have similar orientations. In contrast to all of the other dehydrogenases, when Congo Red was mixed with cMDH, the CD signal increased over a period of about 1 hr which may indicate a particularly poor accommodation of dye on the enzyme and the necessity of a conformational change.

Induced CD contains a wealth of information about the conformation and surrounding environment of an enzyme bound chromophore but the interpretation is still poorly understood (10,11). In this report we have begun the process of extracting this information for Congo Red and Cibacron Blue by noting empirical features of the spectra in light of what we know about the electronic transitions in these dyes. For bound Congo Red the large CD bands, similar CD patterns for four of the five enzymes known to have similar coenzyme binding regions, and the delocalization of the long wavelength electronic transitions indicate that Congo Red is a better CD probe than Cibacron Blue, which shows very different CD spectra when bound to similar dehydrogenases, generally weaker CD, and has localized long wavelength transitions.

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