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Inhibition of NAD(P)H:(Quinone-Acceptor) Oxidoreductase by Cibacron Blue and Related Anthraquinone Dyes: A Structure-Activity Study†

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Received July 23, 1991; Revised Manuscript Received October 14, 1991

ABSTRACT: Cibacron Blue, a widely used ligand for affinity chromatography, is a potent inhibitor of NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2) (quinone reductase). This property has been exploited to purify quinone reductase, to identify its nucleotide-binding site, and to obtain diffraction-grade crystals of this enzyme [Prochaska, H. J. (1988) *Arch. Biochem. Biophys. 267*, 529-538; Ysern, X., & Prochaska, H. J. (1989) *J. Biol. Chem. 264*, 7765-7767]. To define the structural region(s) of the dye responsible for its inhibitory potency, Cibacron Blue was synthesized and the dye, its synthetic intermediates, and some analogues of these intermediates were crystallized as novel trialkylamine or choline salts. These compounds were characterized by proton NMR and mass spectrometry, and their inhibitory potencies were measured. Only two of the four ring systems of the Cibacron Blue molecule are required for potent inhibition. Acid Blue 25 [1-amino-4-(phenylamino)anthraquinone-2-sulfonic acid] is an inhibitor (K_i = 22 nM) almost as potent as Cibacron Blue (K_i = 6.2 nM). However, removal of any of the three substituents on the anthraquinone ring of Acid Blue 25 markedly reduced inhibitory potency. These results are consistent with the proposal that Cibacron Blue is primarily a mimic for the ADP fragment of mono- and dinucleotides. The difference absorption spectrum of the Acid Blue 25-quinone reductase complex was very different from that of the complex with Cibacron Blue. In contrast to other compounds tested, Procion Blue M-3GS, the electrophilic dichlorotriazine precursor of Cibacron Blue, was an irreversible inhibitor of quinone reductase (K_D = 16 nM, k_3 = 0.03 min^{-1}), and the inactivation was blocked by Cibacron Blue, a monochlorotriazine.

Cibacron Blue is a potent inhibitor of many nucleotide-dependent dehydrogenases and kinases (Dean & Watson, 1979; Lowe & Pearson, 1984), including NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2). QR[1] is a widely distributed flavoprotein that catalyzes obligatory two-electron reductions of quinones and protects cells against the toxicities of quinones (Benson et al., 1980; Ernster et al., 1987; Prochaska & Talalay, 1991). The observation that Cibacron Blue is a high-affinity ligand for QR is of interest for several reasons: (a) pure QR can be isolated in a single step from crude liver cytosols by Cibacron Blue-agarose chromatography (Prochaska, 1988; Sharkis & Swenson, 1989); (b) Cibacron Blue binds to the nucleotide folds of many proteins, and this property was used to identify correctly the glycine-rich consensus amino acid sequence involved in nucleotide binding to QR (Prochaska, 1988; Liu et al., 1989); and (c) Cibacron Blue cocrystallized with QR to provide crystals suitable for high-

† These studies were supported by Grants from the National Cancer Institute, Department of Health and Human Services (PO1 CA 44530), and from the American Cancer Society (SIG-3 and PRD-30M). Tory Prestera is a Trainee of the NIH Medical Scientist Training Program (T32 GM 07309).
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[1] Abbreviations: QR, quinone reductase, NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2), also known as DT-diaphorase or menadione reductase; MTT, 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide; FAB, fast atom bombardment mass spectrometry; CI/EI, chemical ionization/electron ionization mass spectrometry; C.I., *Colour Index*; bromaminic acid, 1-amino-4-bromoanthraquinone-2-sulfonic acid; ASSO, 1-amino-4-[(4-amino-3-sulfophenyl)amino]-anthraquinone-2-sulfonic acid; Acid Blue 25, 1-amino-4-(phenylamino)anthraquinone-2-sulfonic acid; PIC, paired ion chromatography; TLC, thin layer chromatography.

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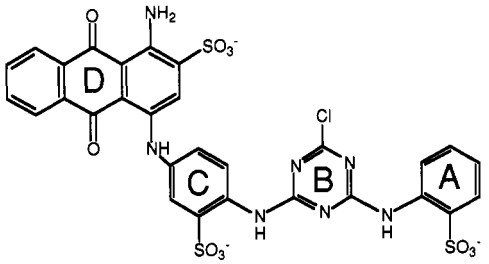
resolution X-ray diffraction (Ysern & Prochaska, 1989).

The name Cibacron Blue[2] designates a family of sulfonated, polycyclic, reactive dyes containing both the anthraquinone and the triazine ring systems. The structure of Cibacron Blue, with the four ring systems labeled A–D (Hanggi & Carr, 1985), is



The serendipitous discovery that the dye component of Blue Dextran [a high molecular weight polymer shown to contain Cibacron Blue by Böhme et al. (1972)] bound to pyruvate kinase during gel filtration (Haeckel et al., 1968) aroused interest in this dye. Subsequently, many enzymes were purified by Cibacron Blue affinity chromatography [see reviews by Dean and Watson (1979) and Lowe and Pearson (1984)].

The mechanism of binding of Cibacron Blue to various proteins is unclear. Böhme et al. (1972) suggested that rings C and D of the Cibacron Blue molecule bore similarities to ATP and demonstrated with models that the 1-amino group on ring D and the 3-sulfonate group on ring C could serve as conformational mimics for the amino group of the adenine ring and γ-phosphate group of ATP, respectively. Subsequently, Stellwagen (Thompson et al., 1975; Thompson & Stellwagen, 1976; Stellwagen, 1977) proposed that Cibacron Blue was a conformational analogue of the entire NAD structure and suggested that it might be a diagnostic probe for the "dinucleotide binding fold." However, difficulties have been encountered with these suggestions. Some proteins known to contain dinucleotide folds did not bind to Cibacron Blue affinity columns, whereas other proteins apparently lacking this structural feature did bind (Beissner et al., 1979). Furthermore, kinetic and affinity chromatography experiments (Böhme et al., 1972; Beissner & Rudolph, 1978a,b; Monaghan et al., 1982; Bornmann & Hess, 1977) with various NAD(H)- and ATP-dependent enzymes established that only the C and D ring portions of the Cibacron Blue molecule were required for inhibition and binding. Later, Stellwagen and colleagues (Liu et al., 1984) also concluded that the C and D rings were most important for binding, whereas the remainder of the molecule served as a "benign spacer" between the ligand and the chromatographic support. In apparent contrast, Burton et al. (1988b, 1990) showed that the terminal (A) ring of Cibacron Blue interacts specifically with horse liver alcohol dehydrogenase and that structural modifications of this ring could alter the affinity of the dye for the enzyme by a factor of almost 3000. In addition, the *meta*-sulfonated isomer of Cibacron Blue did not enter crystals of this enzyme, whereas a mixture of the *meta*- and *para*-sulfonated isomers did (Biellman et al., 1979). Nevertheless, X-ray crystallography indicated that the conformation of the C and D rings of the bound dye resembled that of the ADP portion of bound NADH but differed markedly in the remainder of the molecule. It is therefore unclear what portions of the Cibacron Blue molecule bind to various enzymes and whether the dye molecule and the dinucleotide binding folds are conformationally similar.

Despite these uncertainties, this dye is a very sensitive spectroscopic and kinetic probe for the binding sites of enzymes. Difference spectra obtained with Cibacron Blue distinguished between the taut, relaxed, oxidized, and dissociated forms of glutamine synthetase (Federici & Stadtman, 1985; Federici et al., 1985). Furthermore, since Cibacron Blue exhibited unique spectral changes in hydrophobic and hydrophilic environments (Subramanian, 1982), such difference spectra may be used to interpret the properties of protein binding sites.

The present studies were designed to elucidate the interaction of Cibacron Blue with QR, to determine which portions of the Cibacron Blue molecule are required for high-affinity binding to the enzyme, and to obtain insight into the environment in which the dye and its analogues are bound.

Experimental Procedures

Materials

NADH was from Pharmacia (Piscataway, NJ); 1-aminoanthraquinone and metanilic acid (3-aminobenzenesulfonic acid) were from Lancaster Synthesis (Windham, NH). Centricon microconcentrators were from Amicon (Danvers, MA). 1-Amino-4-bromoanthraquinone-2-sulfonic acid (bromaminic acid) was a gift of Peter Carr, Department of Chemistry, University of Minnesota, and was originally supplied by Pfaltz and Bauer (Waterbury, CT). 1-Aminoanthraquinone-2-carboxylic acid was a gift of the BASF Corp. (Parsippany, NJ). The Cibacron Blue sample labeled "Cibacron Blue 3G-A" used previously (Prochaska, 1988) and purified for inhibitor studies in this paper was the gift of Dr. H. P. Striebel, Ciba-Geigy (Basel, Switzerland). We refer to this sample, which had been stored at room temperature for several years, as "Ciba-Geigy Cibacron Blue" in this paper. Another sample of Cibacron Blue 3GA was obtained from Sigma (catalogue no. C9534). All other chemicals were obtained either from Sigma (St. Louis, MO), Aldrich (Milwaukee, WI), or J. T. Baker (Phillipsburg, NJ). Pure rat liver QR was prepared according to Prochaska (1988).

General Methods

Thin Layer Chromatography. Thin layer chromatography was performed on silica gel plates. Solvent systems used were either 4:3:2:1 (v/v) *tert*-butyl alcohol/methyl ethyl ketone/water/ammonium hydroxide (Federici & Stadtman, 1985) designated system 1, or 3:3:2:2 (v/v) 2-propanol/water/ethyl acetate/1-butanol, which is modified from Lowe and Pearson (1984) and is designated system 2.

Determination of Extinction Coefficients. All extinction coefficients were determined in water at concentrations below 20 μM to minimize deviations from Beer's law (Subramanian, 1982).

[2] The nomenclature for Cibacron Blue dyes is complex and confusing. Although the names Reactive Blue 2 and Basilen Blue E-3G, as well as Cibacron Blue with suffixes 3G, 3GA, 3G-A, F3G-A, and F-3GA have been used to describe these dyes, the relationship between name and structure is inconsistent with respect to the sulfonic acid isomerism at the A ring. For example, Sigma markets Cibacron Blue 3GA as the ring A ortho isomer, whereas the mixture of the ring A meta and para isomers is sold as Basilen Blue E-3G. However, as pointed out by Burton et al. (1988a), Cibacron Blue 3GA is shown in the *Colour Index* to be a mixture of the ring A meta and para isomers, whereas the ring A ortho isomer is termed Cibacron Blue F-3GA. We have demonstrated here that a sample labeled "Cibacron Blue 3G-A" obtained from Ciba-Geigy and used in our laboratory (Prochaska, 1988) is predominantly the ring A ortho isomer. Clearly, the *Colour Index* nomenclature is used incorrectly or inconsistently. Here, we shall refer to the ring A ortho isomer simply as Cibacron Blue. We recommend that future references to Cibacron Blue dyes be accompanied by explicit information on chemical structure and that the terminal ring isomerism be established by the methods described in this paper.

Paired Ion Chromatography. The purity of the recrystallized compounds was assessed by paired ion high-pressure liquid chromatography (PIC) (Hanggi & Carr, 1985) on an analytical μBondpack C_{18} reverse-phase column (250 × 4 mm) from Waters Associates (Milford, MA). The column was equilibrated with 50% solvent A (5 mM tetrabutylammonium chloride, 10 mM potassium phosphate buffer, pH 7.0) and 50% methanol, at a flow rate of 1.5 mL per min. Samples of dyes (1 mM) in 0.5 M tetrabutylammonium chloride were injected, and the concentration of methanol was increased to 100% during 20–30 min. The absorbance was monitored at 254 nm. Preparative PIC of synthetic Cibacron Blue was performed on a Partisil M9 ODS-2 reverse-phase column (1 × 50 cm) from Whatman (Hillsboro, OR).

Mass Spectrometry. Fast atom bombardment (FAB) mass spectrometry was performed by positive ion fast atom bombardment with a cesium iodide gun on a Kratos Concept 1H mass spectrometer (Manchester, England). All $(M + H)^+$ ion values reported in the following section are for the sulfonic acids rather than their salts. All samples gave $(M + H)^+$ ions and matched the expected molecular weights within 0.1%. Samples also gave $(M + H)^+$ ions plus or minus hydrogen radicals. This phenomenon has been observed with other aromatic dyes (Gale et al., 1986). Consequently, it was not possible to use the isotope abundances for identificational purposes. Chemical ionization/electron impact ionization (CI/EI) mass spectrometry of bromaminic acid (VII) and Acid Blue 25 (IX) was performed on a VG 70-S mass spectrometer (Manchester, England).

NMR Spectroscopy. Proton NMR spectra were obtained in either D_2O or methanol-d_4 at 300 MHz on a Bruker WM 300/WB NMR spectrometer or at 600 MHz on a Bruker AM600 NMR spectrometer.

Synthesis, Purification, and Crystallization of Cibacron Blue, Its Precursors and Their Analogues

The structures of compounds used in this study and methods for their crystallization are given in Table I. Synthetic procedures[3] and pertinent physical data for these compounds are summarized below:

1-Aminoanthraquinone-2-carboxylic acid (V) was recrystallized from nitrobenzene. λ_{max} = 497.5 nm, a_m = 6150 M^{-1} cm^{-1}; FAB *m/e* 267.9 $(M + H)^+$.

1-Aminoanthraquinone-2-sulfonic acid (VI) was synthesized by a modification of the procedure of Grossmann (1952), and the course of the reaction was monitored by analytical TLC in solvent system 1. For this synthesis, 1.15 g of 1-aminoanthraquinone, 600 μL of chlorosulfonic acid, and 700 μL of tripropylamine were stirred in dry 1,1,2,2-tetrachloroethane at 120 °C for 24 h, after which an additional 600 μL of chlorosulfonic acid were added and the reaction mixture was stirred at 120 °C for an another 24 h. Water (75 mL) and 1 g of sodium carbonate were added, and the mixture was then dried in a rotary evaporator. Water (150 mL) was added, the mixture was filtered, and the filtrate received 16 g of sodium chloride to salt out the sulfonated product. The crude product (38% yield) was recrystallized as the triethylammonium salt from hexane/methyl ethyl ketone (Mickan et al., 1969). Purity by PIC 99+%; λ_{max} = 473.5 nm, a_m = 6590 M^{-1} cm^{-1}; FAB *m/e* 304 $(M + H)^+$; ^1H NMR (300 MHz, D_2O) δ 7.83–7.79 (d, *J* = 8.0 Hz, 1 H), 7.75–7.69 (t, *J* = 7.65, 7.62 Hz, 2 H), 7.62–7.50 (m, 2 H), 7.13–7.10 (d, *J* = 7.8 Hz, 1 H).

1-Amino-4-bromoanthraquinone-2-sulfonic acid (bromaminic acid) (VII) was recrystallized as the triethylammonium salt from methanol or methyl ethyl ketone/chloroform (Mickan et al., 1969). Purity by PIC 99+%; λ_{max} = 485 nm, a_m = 6820 M^{-1} cm^{-1}; FAB *m/e* 382, 384 $(M + H)^+$, CI/EI exact *m/e* 381.9391 $(M + H)^+$ (calculated 381.9385); ^1H NMR (300 MHz, D_2O) δ 7.86 (s, 1 H), 7.62–7.43 (m, 4 H).

1,4-Diaminoanthraquinone-2-sulfonic acid (C.I. 62000) (VIII) was synthesized by refluxing 1.9 g of bromaminic acid, 1.25 g of sodium carbonate, 1.0 g of sodium sulfite, 20 mL of ammonium hydroxide, and 75 mg of cupric chloride in 63 mL of water at 100 °C for 1 h. The product was precipitated by addition of 20% (w/v) NaCl and recrystallized as the tripropylammonium salt from acetone/hexane. Purity by PIC 99+%: λ_{max} = 566 nm, a_m = 11300 M^{-1} cm^{-1}; FAB *m/e* 318.9 $(M + H)^+$; ^1H NMR (300 MHz, D_2O) δ 7.82–7.78 (m, 2 H), 7.60–7.57 (m, 3 H), 7.39 (s, 1 H).

1-Amino-4-(phenylamino)anthraquinone-2-sulfonic acid (Acid Blue 25, C.I. 62055) (IX) was synthesized by stirring 1 g of bromaminic acid, 0.6 g of sodium carbonate, 0.5 g of sodium sulfite, 7 mL of aniline, and 45 mg of cupric chloride in 30 mL of water under nitrogen for 48 h at room temperature. The product was treated with 300 mg of activated charcoal and purified by extraction of the triethylammonium salt into chloroform. The compound was recrystallized from either diethyl ether/methanol or acetone/hexane. Purity by PIC 99%; λ_{max} = 601 nm, a_m = 12400 M^{-1} cm^{-1}; FAB 395 $(M + H)^+$, CI/EI exact *m/e* 395.0710 $(M + H)^+$ calculated (395.0702); ^1H NMR (300 MHz, D_2O) δ 7.78 (s, 1 H), 7.60–7.51 (m, 2 H), 7.40–7.29 (m, 4 H), 7.18–7.12 (m, 1 H), 7.04–7.00 (d, *J* = 8.2 Hz, 1 H).

Much improved yields of IX were obtained by refluxing the reaction mixture for 1–2 h. Choline chloride was then added in excess, and the product was extracted into 1-butanol, leaving yellow impurities in the aqueous phase. The solvent was then removed and the product recrystallized from 0.5 M choline chloride in methanol.

1-Amino-4-[(3-carboxyphenyl)amino]anthraquinone-2-sulfonic acid (X) was synthesized by stirring 1.0 g of bromaminic acid, 0.6 g of sodium carbonate, 0.5 g of sodium sulfite, 1.0 g of 3-aminobenzoic acid, and 50 mg of cupric chloride in 30 mL of water under nitrogen for 16 h at room temperature. The product was treated with 300 mg of activated charcoal and salted out with 20% NaCl. The compound was recrystallized as the tripropylammonium salt from hexane/acetone by vapor diffusion in a hexane tank. Purity by PIC 99+%; λ_{max} = 597.5 nm, a_m = 14200 M^{-1} cm^{-1}; FAB *m/e* 438.9 $(M + H)^+$; ^1H NMR (300 MHz, D_2O) δ 7.72 (s, 1 H), 7.71–7.62 (m, 2 H), 7.59–7.55 (d, *J* = 7.4 Hz, 1 H), δ 7.51 (s, 1 H), 7.47–7.42 (m, 2 H), 7.39–7.33 (t, *J* = 8.1, 7.8 Hz, 1 H), 7.20–7.15 (d, *J* = 7.4 Hz, 1 H).

1-Amino-4-[(4-amino-3-sulfophenyl)amino]anthraquinone-2-sulfonic acid (ASSO) (XI) was synthesized by modifications of the method of Hanggi and Carr (1985). The reaction was carried out for 60 h rather than 8 h, and the charcoal treatment step was omitted. The tripropylammonium salt of ASSO was extracted into chloroform and recrystallized from acetone/hexane either at 4 °C overnight or by vapor diffusion in a hexane tank at 25 °C. Purity by PIC 98%; λ_{max} = 626.5 nm, a_m = 12900 M^{-1} cm^{-1}; FAB *m/e* 489.9 (M +

[3] The exceptional utility of alkylamines as counterions to purify and crystallize sulfonic acid dyes was only recognized after we had already synthesized several compounds. Since precipitation with NaCl did not accomplish significant purification of the dyes, and the precipitates are intractable, we recommend that the "salting-out" steps described in some of the preparations be replaced by extraction of alkylamine salts into organic solvents [cf., synthetic procedures for Acid Blue 25 (IX)].

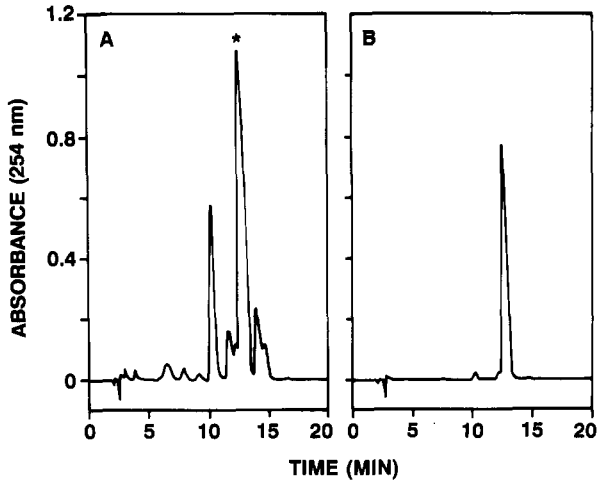


FIGURE 1: Paired ion chromatography (PIC) of the Ciba-Geigy sample of Cibacron Blue. Samples were dissolved in water, mixed with an equal volume of 1 M tetrabutylammonium chloride solution, and injected onto the reverse-phase column. A gradient was run from 50% 5 mM tetrabutylammonium chloride, 10 mM potassium phosphate, pH 7.0/50% methanol to 100% methanol over 30 min, at a rate of 1.5 mL/min. The gradient was stopped after 18 min in panel A and after 16 min in panel B, and the solvent was increased to 100% methanol. (A) PIC of 100 μg of crude Ciba-Geigy Cibacron Blue. The most abundant component, marked with an asterisk (*), was purified by TLC and recrystallized. (B) PIC of 38 μg of the recrystallized material.

$H)^+$; ^1H NMR (300 MHz, D_2O) δ 7.78–7.74 (m, 1 H), 7.72–7.68 (m, 1 H), 7.64 (s, 1 H), 7.53–7.44 (m, 3 H), 7.14–7.05 (br d, J = 7.2 Hz, 1 H), 6.97–6.87 (br d, J = 8.4 Hz, 1 H).

Procion Blue M-3GS (XII) was synthesized from ASSO (XI) and cyanuric chloride (Hanggi & Carr, 1985). The salted out product was recrystallized from 0.5 M choline chloride in methanol. Purity by PIC 90%; λ_{max} = 599 nm, a_m = 13 900 M^{-1} cm^{-1}; FAB m/e 637, 639 $(M + H)^+$; ^1H NMR (300 MHz, D_2O) δ 7.92 (s, 1 H), 7.80–7.66 (m, 3 H), 7.66–7.57 (m, 3 H), 7.19–7.08 (br d, J = 7.2 Hz, 1 H).

Cibacron Blue (XIII) was synthesized by adding a 4-fold excess of aniline-2-sulfonic acid, which had been neutralized with sodium carbonate, to a purified sample of XII. The solution was heated at 38 °C for 20 h. The pH was maintained between 7.5 and 5.0. The product was purified by PIC. Purity by PIC 95+%; ^1H NMR (300 MHz, CD_3OD) δ 8.57–8.38 (br, 1 H), 8.38–8.30 (m, 3 H), 8.25 (s, 1 H), 7.91–7.86 (d, J = 7.8 Hz, 1 H), 7.83–7.79 (t, J = 3.6, 3.5 Hz, 2 H), 7.77–7.73 (d, J = 2.5 Hz, 1 H), 7.68–7.55 (br, 1 H), 7.47–7.37 (br, 1 H), 7.21–7.13 (br t, J = 6.7 Hz, 1 H).

Analysis and Purification of Cibacron Blue. On TLC, the Cibacron Blue sample obtained from Ciba-Geigy (see Materials) consisted of four major blue components, designated bands A–D in order of increasing R_f, as well as one red and one brown component (experiment not shown). Band D predominated and could be converted to band B by warming in water under acidic or basic conditions (i.e., band B was probably the monohydroxytriazine derivative of Cibacron Blue). When the crude dye was analyzed by PIC, four major and several minor peaks were resolved (Figure 1A). The most abundant component [asterisk (*), Figure 1A] comigrated with band D on TLC.

Band D (XIII) of the Cibacron Blue sample was isolated by preparative TLC in solvent system 2 (Lowe & Pearson, 1984) and recrystallized from 0.5 M choline chloride in methanol. Figure 1B shows the PIC of recrystallized Cibacron Blue. Pure band D had exactly the same retention time on PIC as both the Cibacron Blue that we synthesized and the major band of the commercial Sigma Cibacron Blue 3GA; mixtures of synthetic Cibacron Blue and purified band D eluted as one peak on PIC. Purity by PIC 95%; λ_{max} = 614 nm, a_m = 15 000 M^{-1} cm^{-1}; FAB m/e 773.9 $(M + H)^+$; ^1H NMR (600 MHz, D_2O) δ 8.05 (s, 1 H), 8.01–7.95 (t, J = 6.1, 6.9 Hz, 3 H), 7.89 (s, 1 H), 7.86–7.79 (d, J = 7.6 Hz, 2 H), 7.78–7.71 (m, 2 H), 7.52–7.47 (t, J = 6.4, 6.4), 7.29–7.24 (t, J = 7.5, 7.4, 1 H), 7.09–6.99 (br s, 1 H). ^1H NMR (300 MHz, CD_3OD) δ 8.47–8.40 (br, 1 H), 8.41–8.30 (m, 3 H), 8.18 (s, 1 H), 7.90–7.87 (d, J = 7.7 Hz, 1 H), 7.82–7.78 (t, J = 3.6, 4.3 Hz, 2 H), 7.78–7.74 (d, 2.5 Hz, 1 H), 7.65–7.51 (br, 1 H), 7.46–7.41 (br d, J = 8.6 Hz, 1 H), 7.23–7.17 (br t, J = 6.6, 8.6 Hz, 1 H). The 300-MHz proton NMR spectrum of Cibacron Blue in D_2O is shown in Figure 2.

For the isolation of band D by TLC, solvent system 2 was used because of indications that solvent system 1 could react with the dye. Difficulty was also encountered with the elution step. When the silica plates were eluted with water, large amounts of extraneous material (presumably binder) also dissolved in the water. The elution of contaminants from the TLC plates, rather than bleaching of the dye by silica, may in fact account for the decrease in the apparent a_m of these dyes observed by Burton et al. (1988a). Therefore, the pure dye was obtained by crystallization of the choline salt.

Crystallization of Dyes. The smaller dye sulfonates were recrystallized as the triethylammonium or tripropylammonium salts (Dusza et al., 1968; Mickan et al., 1969). The use of alkylamines as counterions for sulfonates also aided purification since these salts could be extracted from aqueous solution into organic solvents such as chloroform.

Cibacron Blue and Procion Blue M-3GS were crystallized as choline salts. Indeed, choline salts proved to be useful for the crystallization of a wide variety of dyes and dye precursors. In this procedure, the crude salt was dissolved in a minimum of boiling 0.5 M choline chloride in methanol, filtered, and allowed to cool to room temperature, 4 °C, and finally −20 °C.

Enzyme Assays

Determination of QR Concentration and Specific Activity. QR concentrations were determined by the method of Bradford (1976) as well as by measuring the absorbance of enzyme-bound FAD [a_m = 11 300 M^{-1} cm^{-1} at 450 nm; the subunit molecular weight of QR is 31 000 (Haniu et al., 1988)]. The two methods agree well (Prochaska, 1988). The specific activities of QR preparations were measured in 1.0-cm light path cuvettes by following the initial rate of oxidation of NADH at 340 nm in 3.0-mL assay systems containing 25 mM Tris-HCl (pH 7.4), 0.01% Tween 20, 0.067% bovine serum albumin, 200 μM NADH, and 50 μM menadione (added as a 7.5 mM solution in acetonitrile).

Determination of Inhibitory Constants (K_i) and Types of Inhibition in Microtiter Plates. To determine the type of inhibition and the K_i of each dye for QR, the rate of MTT reduction was followed for 2 min at 610 nm and at 25 °C in 96-well microtiter plates using a kinetic microtiter platereader (UV_{max}, Molecular Devices, Palo Alto, CA). Each well contained, in a final volume of 0.3 mL, 21 mM Tris-HCl (pH 7.4), 25 μM menadione, 0.25 mg/mL of MTT (a_m = 11 300 M^{-1} cm^{-1}), 0.0083% Tween 20, and concentrations of NADH and dye that were varied. The MTT assay for QR has been described (Prochaska & Santamaria, 1988).

Serial dilutions of the dye were first made across columns of the plate. Anthraquinone (I) and 1-aminoanthraquinone (II) were dissolved in dimethyl sulfoxide and diluted so that

the final concentration of dimethyl sulfoxide in the assay was less than 3% for I and 0.1% for II. Anthraquinone-2-carboxylic acid (IV) and 1-aminoanthraquinone-2-carboxylic acid (V) were dissolved in a slight molar excess of sodium hydroxide. All other dyes were diluted in distilled water. The final volume of the dye solution in each well was 50 μL, and the last two columns on the plate received 50 μL of dye-free diluent for determination of control activities. Then 50 μL of an NADH solution in 25 mM Tris-HCl (pH 7.4) and 0.01% Tween 20 were added to each well such that the final concentrations of NADH in the assay would be 300, 150, 90, and 60 μM, respectively. The reaction was initiated by the addition of 200 μL of a solution containing an appropriate quantity of QR (diluted in 30% glycerol), 37.5 μM menadione, 0.3 mg/mL MTT, 0.01% Tween 20, and 25 mM Tris-HCl (pH 7.4). No nonenzymatic MTT reduction was observed either in the absence or presence of the dyes. K_i values were determined by the method of Dixon (1953), and secondary transformations (Segal, 1975) were used to confirm competitive inhibition.

Concentration-Dependent Irreversible Inhibition of QR by Procion Blue M-3GS (XII) and Protection by Cibacron Blue (XIII). Serial dilutions of Procion Blue M-3GS (XII) in 30% glycerol, 17.5 mM Tris-HCl (pH 7.4), and 0.0075% Tween 20 were prepared in microtiter plates such that the final volume was 50 μL. QR was added in 100 μL of the above buffer to each well [3–10 milliunits (1.5–6.5 ng), in various experiments]. The plates were incubated at 38 °C, and at the appropriate time intervals 50 μL of a 2% bovine serum albumin solution (1 mg) was added to stop the reaction. The reduction of MTT at 610 nm was then measured for 2 min after addition of 100 μL of assay buffer containing 600 μM NADH, 75 μM menadione, 0.9 mg/mL of MTT, 25 mM Tris-HCl (pH 7.4), and 0.01% Tween 20 to each well. The k_3 and K_D values were determined according to Kitz and Wilson (1962).

In a separate experiment, QR (0.4 unit) in 30% glycerol, 17.5 mM Tris-HCl (pH 7.4), and 0.0075% Tween 20 was incubated at 25 °C with either no additions, 4 μM Cibacron Blue, 2.6 μM Procion Blue M-3GS (XII), or 4.0 μM Cibacron Blue and 2.6 μM Procion Blue M-3GS in a final volume of 150 μL. At appropriate times, 5-μL aliquots were removed and assayed in a 3.0-mL system containing 200 μM NADH, 25 μM menadione, 0.1% bovine serum albumin, 0.3 mg/mL MTT, 0.01% Tween 20, and 25 mM Tris-HCl (pH 7.4).

Results and Discussion

Synthesis, Purification, Crystallization, and Characterization of Cibacron Blue and Its Structural Analogues. Cibacron Blue and related polysulfonic acids are very water-soluble, and their isolation from aqueous reaction mixtures is usually performed by "salting out" procedures (Lowe & Pearson, 1984; Hanggi & Carr, 1985). We found this procedure to be unsatisfactory because the products were intractable, and the salting out procedures contributed very little to purification. We therefore prepared either the triethylammonium, tripropylammonium, or choline salts of the sulfonic acids. These salts can be extracted easily into organic solvents (e.g., chloroform) and then crystallized from such solvents. Table I lists 13 compounds (I–XIII) used in these studies and the salts and solvents involved in their crystallization. Compounds V–XIII were analyzed for purity by PIC and were all found to be greater than 95% pure except for Procion Blue M-3GS (XII), which was 90% pure. All compounds gave m/e values for the $(M + H)^+$ ions when subjected to FAB mass spectrometry and proton NMR spectra consistent with their structures. The use of ammonium salts as an aid to purification has made large quantities of refined and

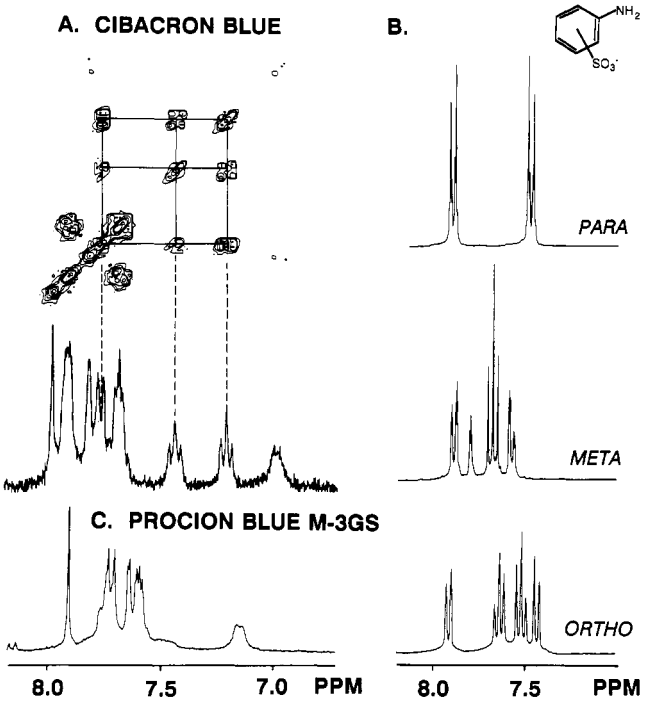


FIGURE 2: 300-MHz proton NMR spectra of Cibacron Blue and related compounds in D_2O. (A) 300-MHz COSY (homonuclear shift-correlated 2D NMR) spectrum of band D of Ciba-Geigy Cibacron Blue with the corresponding one-dimensional spectrum. The lines show interproton couplings. (B) Spectra of *para*-, *meta*-, and *ortho*-anilinesulfonic acids. (C) Spectrum of Procion Blue M-3GS (XII), the immediate precursor of Cibacron Blue (XIII).

well-characterized compounds available.

Determination of the Structure of Band D of Ciba-Geigy Cibacron Blue (XIII). Although many of the aromatic protons of Cibacron Blue are in similar chemical environments, proton NMR spectroscopy at 300 MHz provided unambiguous assignment of the controversial position[2] of the sulfonate group on ring A. The NMR spectra for Cibacron Blue (XIII), Procion Blue M-3GS (XII), and the three isomeric anilinesulfonic acids are shown in Figure 2. The obvious difference between the NMR spectra of Cibacron Blue (XIII; Figure 2A) and Procion Blue M-3GS (XII; Figure 2C) is the presence in the Cibacron Blue spectrum of two additional triplets at 7.4–7.5 and 7.1–7.3 ppm, with J values in the range of 6.3–7.5 Hz. Notably, only the spectrum of *ortho*-anilinesulfonic acid but not the other isomers shows (Figure 2B) two triplets with coupling constants in this range (J = 7.65–7.80 Hz) arising from the 4- and 5-protons on the anilinesulfonic acid ring. The remaining question was the location of the two doublets in the Cibacron Blue spectrum that originate from the 3- and 6-protons of ring A of anilinesulfonic acid. The [^{1}H]COSY spectrum of Cibacron Blue (Figure 2) shows that the two triplets were coupled to a doublet at 7.7–7.8 ppm, which integrated to two protons. The integration of this doublet was confirmed on a 600-MHz NMR instrument. These findings indicate that the two doublets arising from the 3- and 6-protons of the anilinesulfonic acid ring have the same chemical shift. We conclude that the major component (peak D) of the Ciba-Geigy Cibacron Blue sample was the ring A *ortho*-sulfonate isomer.

To confirm this conclusion, the *ortho*-sulfonate isomer of Cibacron Blue (XIII) was synthesized from Procion Blue M-3GS (XII) and 2-aminobenzenesulfonic acid. The synthetic Cibacron Blue sample was purified by preparative PIC and found to elute with purified band D of Ciba-Geigy Cibacron Blue on PIC. The 300-MHz proton NMR spectrum of the

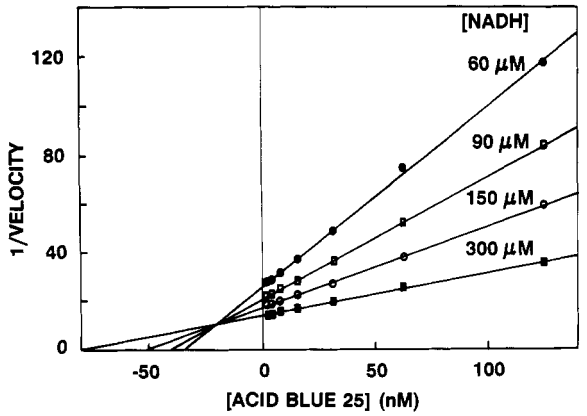


FIGURE 3: Inhibition of QR by Acid Blue 25 (IX) as a function of inhibitor and NADH concentrations. Initial rates of reduction of MTT (see Experimental Procedures) were measured over a range of inhibitor concentrations with 25 μM menadione in the presence of various concentrations of NADH (60–300 μM). The data are plotted by the method of Dixon (1953). The K_i value obtained in this set of assays is 28 nM (see Table I for means of several assays). The velocities are expressed as ΔA per min.

synthetic Cibacron Blue in CD_3OD was essentially identical to that obtained in CD_3OD for band D of the Ciba-Geigy sample (see Experimental Procedures). (The NMR spectra were obtained in CD_3OD because purification by PIC provided the tetrabutylammonium salt of the dye, which is not soluble in water.)

Inhibition of QR by Dyes and Dye Precursors. The microtiter plate assay provided rapid, precise, and highly reproducible results. The data from a typical experiment [with Acid Blue 25 (IX) as the inhibitor] shown in Figure 3 compare favorably with the results obtained from conventional cuvette assays [c.f., Figure 4 of Prochaska (1988)]. Furthermore, K_D values determined for selected compounds by other methods (discussed below) were identical (within experimental error) to the K_i values determined kinetically.

Cibacron Blue was synthesized in four steps from the anthraquinone ring with use of bromaminic acid (VII) as starting material (Hanggi & Carr, 1985). Intermediates and some analogues of intermediates were characterized. The inhibitory potencies of all compounds were measured with purified QR. Each of these compounds was also tested as a substrate of QR, but no NADH oxidation was observed. All the compounds were *linear* competitive inhibitors with respect to NADH when the menadione concentration was held constant. The inhibitory constants (K_i) are summarized in Table I.

The simplest compound tested was anthraquinone (I), which gave a K_i value of 50000 nM. Pure Cibacron Blue (XIII) gave a K_i value of 6.2 nM[4] and was therefore 8100 times more potent than anthraquinone.

Addition to anthraquinone of a 1-amino group (II, K_i = 1500 nM) or a 2-sulfonic acid group (III, K_i = 3600 nM) increased the inhibitory potency 33- and 14-fold, respectively. Simultaneous presence of both 1-amino and 2-sulfonic acid groups on the anthraquinone ring (VI, K_i = 460 nM) further increased inhibitory potency. The combination of 1-amino and 2-sulfonic acid substituents is clearly of critical importance since the presence of both groups lowered the K_i value by a factor of more than 100 compared to the parent anthraquinone.

The further addition of a bromo substituent in the 4-position (VII, K_i = 120 nM) increased the affinity 4-fold as compared to VI. Substitution of an amino group in the 4-position to provide 1,4-diaminoanthraquinone-2-sulfonic acid (VIII, K_i = 460 nM) did not affect the inhibitory potency of the molecule for QR. However, the further addition of a phenyl ring to 1,4-diaminoanthraquinone-2-sulfonic acid (VIII) to give Acid Blue 25 (IX, K_i = 22 nM) markedly increased the inhibitory potency (IX is 21-fold more potent than VIII). When *meta*-sulfonic acid and *para*-amino groups were added to the aniline ring (ASSO, XI, K_i = 50 nM) inhibitory potency was decreased 2-fold compared to IX.

The further addition of a dichlorotriazine ring to XI to provide XII (K_i = 28 nM) increased the inhibitory potency only slightly (by a factor of 2). Attachment of the terminal *ortho*-anilinesulfonic acid to give Cibacron Blue then led to a further 4-fold increase in inhibitory potency (XIII, K_i = 6.2 nM). We also note that band B of crude Ciba-Geigy Cibacron Blue (i.e., the monohydroxytriazine congener of Cibacron Blue) was equipotent with Cibacron Blue in inhibiting QR, whereas the other components of the crude dye sample were far less potent (data not shown).

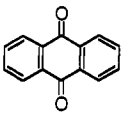
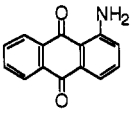
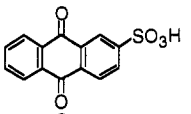
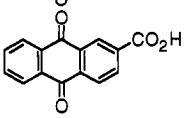
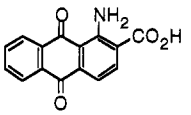
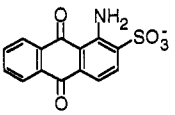
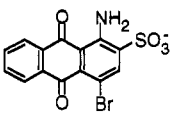
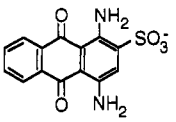
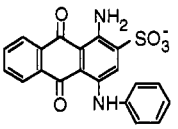
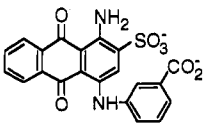
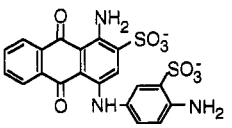
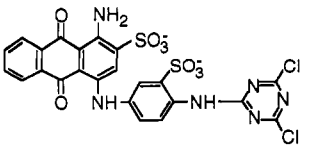
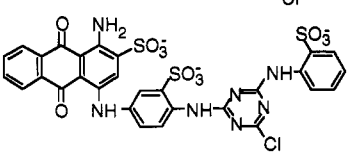
Since the synthetic plan adopted to synthesize Cibacron Blue was from ring D to ring A, the possibility remained that (a) the complete anthraquinone ring system was not required for potent inhibition and (b) that the A ring might itself inhibit the enzyme. Therefore, we examined the inhibition of QR by *ortho*-anilinesulfonic acid and 1,4-diaminobenzene-2-sulfonic acid. No inhibition of the enzymatic activity was observed with up to 50 μM final concentrations of either compound. These two compounds were tested both as mimics of the A ring of Cibacron Blue and as part of the aminosulfonic portion of the anthraquinone ring. The lack of any appreciable inhibition by either of these compounds indicates that (a) if the Cibacron Blue molecule had been built from the A to the D ring, simple potent inhibitors were unlikely to have been discovered, and (b) the anthraquinone moiety is essential for potent inhibition.

Finally, the question arose whether other anionic groups could substitute for the sulfonic acid. Substitution of the 2-sulfonate by a 2-carboxylate on the D ring increased the inhibitory potency 3–4-fold (compare IV with III; V with VI). In contrast, if a carboxylate group is added to the meta position of the C ring (X, K_i = 77 nM), there is a 3-fold decrease in inhibitor potency over Acid Blue 25 (IX), the unsubstituted C ring analogue. The presence of a sulfonic acid moiety on the C ring also decreased binding affinity relative to Acid Blue 25 (IX), but not as much as was observed with compound X. We conclude that substitution of a carboxylate for a sulfonic acid on the anthraquinone ring leads to an increase in inhibitory potency, whereas substitution of either a sulfonate or carboxylate group on the C ring leads to a decrease in inhibitory potency as compared to Acid Blue 25 (IX).

Irreversible Inhibition of QR by Procion Blue M-3GS (XII). Dichlorotriazine dyes are irreversible inhibitors of nucleotide-dependent enzymes (Clonis & Lowe, 1980) and have been used as affinity labels (Small et al., 1982). Monochlorotriazines are not irreversible inhibitors; rather, they can protect enzymes from reacting with dichlorotriazines (Clonis & Lowe,

[4] The K_i value for the inhibition of pure rat liver QR by Cibacron Blue was previously reported (Prochaska, 1988) to be 170 nM when measured in 3.0-mL assay systems in conventional cuvettes, whereas the value obtained in the present study in microtiter plates is consistently much lower (i.e., 6.2 nM). This discrepancy cannot be explained by the use of impure Cibacron Blue in earlier studies (as shown by subsequent experiments) but must arise from differences in the assay systems. The quinone reductase used by Prochaska (1988) was diluted with 0.1% bovine serum albumin before use, whereas in this study the enzyme was diluted with 30% glycerol. Since bovine serum albumin binds Cibacron Blue avidly, the presence of the albumin would be expected to lead to a higher apparent K_i value.

Table I: Structures, Crystallization Conditions, and Inhibition Constants (K_i) for Rat Liver Quinone Reductase, of Cibacron Blue, and Its Precursors and Their Analogues

compounds			crystalline salts		inhibition constants (K_i) (nM)[b]	relative potency of inhibition
no.	name	structure	cation[a]	solvent of crystallization		
I	anthraquinone		none	purchased sublimed	50000	1.0
II	1-aminoanthraquinone		none	1-butanol	1500	33
III	anthraquinone-2-sulfonic acid		none	nitrobenzene	3600	14
IV	anthraquinone–2–carboxylic acid		none	none	1300	35
V	1-aminoanthraquinone-2-carboxylic acid		none	nitrobenzene	110	460
VI	1-aminoanthraquinone-2-sulfonic acid		TEA	hexane/methyl ethyl ketone	460	110
VII	bromaminic acid		TEA	methanol	120	420
VIII	1,4-diaminoanthraquinone-2-sulfonic acid		TPA	acetone/hexane	460	110
IX	Acid Blue 25		TEA	acetone/hexane	22	2300
X	1-amino-4-[(3-carboxyphenyl)amino]-anthraquinone-2-sulfonic acid		TPA	acetone/hexane	77	650
XI	ASSO		TPA	acetone/hexane	50	1000
XII	Procion Blue M-3GS		choline	methanol	28	1800
XIII	Cibacron Blue		choline	methanol	6.2	8100

[a] TEA, triethylammonium; TPA, tripropylammonium. [b] Mean of at least three independent experiments.

1980). This difference can be attributed to the tremendous increase (about 1000-fold) in reactivity toward nucleophiles of dichlorotriazines compared with monochlorotriazine (Hildebrand, 1972). It was, therefore, of interest to determine

whether Procion Blue M-3GS (XII), the dichlorotriazine precursor of Cibacron Blue, would inhibit QR irreversibly. This analysis would also provide an independent measure of the binding affinity of this compound.

Incubation of QR with Procion Blue M-3GS resulted in time- and concentration-dependent loss of enzymatic activity (not shown). Assuming a stoichiometry of binding of unity, the values of k_3 and K_D were calculated (Kitz & Wilson, 1962; Clonis & Lowe, 1980). The rate of covalent binding of the ligand to QR, k_3, was 0.03 ± 0.01 min^{-1}. This value is comparable to the rates of reaction of related dichlorotriazine dyes with other nucleotide-dependent enzymes (Clonis & Lowe, 1980). The value for K_D (16 ± 7.1 nM) is in reasonable agreement with the K_i value (28 nM) obtained by inhibitory kinetics.

In a separate experiment (not shown), incubation of QR with 2.6 μM Procion Blue M-3GS resulted in pseudo-first-order 85% loss of activity over 210-min period. However, complete protection was observed when 4.0 μM Cibacron Blue was also present. This suggests that Procion Blue M-3GS and Cibacron Blue bind to a common site, and thus the former might be useful as an affinity label for the nucleotide-binding site of QR.

Difference Spectra of QR and Acid Blue 25 (IX). Binding of Cibacron Blue to a number of enzymes leads to large changes in the absorption spectrum of the dye (Appukuttan & Bachhawat, 1979; Bull et al., 1981; Pompon et al., 1980; Barden et al., 1980; Subramanian & Kaufman, 1980). Model studies designed to relate these difference spectra to the environment of the dye have also been reported (Subramanian, 1982). The difference spectrum of Cibacron Blue upon binding to QR has been described (Prochaska, 1988). If partial structures of Cibacron Blue such as Acid Blue 25 (IX; C and D rings only) produced difference spectra similar to those of Cibacron Blue (XIII) when bound to QR, we could determine the stoichiometry of binding and the dissociation constant for this compound and also compare the difference spectra obtained with the two dyes.

Spectral titrations of QR with Acid Blue 25 and with Cibacron Blue (Figure 4) show that the difference spectra obtained with the two ligands are strikingly dissimilar. The spectrum of enzyme-bound Cibacron Blue exhibits two maxima at 580 and 626 nm, whereas that for Acid Blue 25 shows only one maximum, at 686 nm. The Cibacron Blue and Acid Blue 25 difference spectra displayed minima at 680 and 588 nm, respectively. While the difference spectrum of Cibacron Blue showed two isosbestic points, at 485 and 651 nm, the difference spectrum of Acid Blue 25 had only a single isosbestic point at 609 nm. Titration of QR with Cibacron Blue led to a decrease and 4-nm red shift of the FAD peak, whereas titration with Acid Blue 25 resulted in an *increase* and 3-nm *blue* shift. Another important distinction is that the magnitudes of the changes observed in the Acid Blue 25 titration were more than double those observed with Cibacron Blue.

One simple interpretation of the difference spectrum of the complex between Acid Blue 25 and QR suggests the participation of electrostatic interactions (Subramanian, 1982). In those studies, the interaction of Cibacron Blue with poly(L-lysine) showed a maximum at about 690 nm and a minimum at about 590 nm, which is similar to the observations with Acid Blue 25. In the model studies, however, the magnitude of the negative peak was approximately equivalent to the magnitude of the positive peak, whereas for the Acid Blue 25 titration of QR, the magnitude of the maximum peak was 5-fold greater than that of the minimum. It is questionable whether these

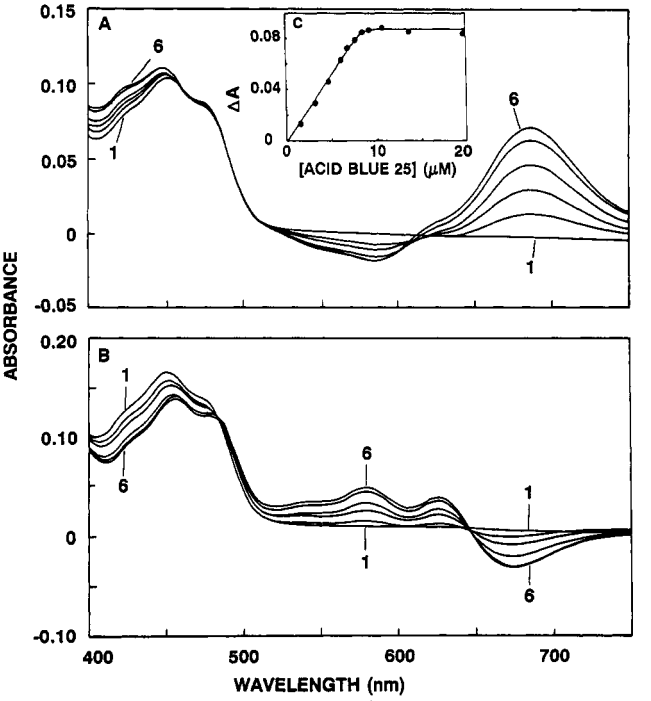


FIGURE 4: Spectroscopic titration of binding to QR of (A) Acid Blue 25 (IX) and (B) pure Cibacron Blue (XIII). Freshly purified QR [specific activity = 2300 μmol/(min·mg)] was washed extensively on a Centricon-30 microconcentrator with a solution containing 150 mM NaCl and 25 mM Tris-HCl (pH 7.4). Then 700 μL of a solution of enzyme containing 8.45 μM enzyme in the experiment shown in panel A and 14.6 μM enzyme in the experiment shown in panel B in this buffer was added to a cuvette. Another cuvette was prepared containing buffer only. After the visible spectrum of QR was recorded, sequential aliquots (0.5–2 μL) of Acid Blue 25 (A) or Cibacron Blue (B) were added to the blank and enzyme-containing cuvettes, and the difference spectra were recorded in a Perkin-Elmer Lambda 9 dual-beam spectrophotometer. Panel A shows the difference spectra with 0 (spectrum number 1), 1.53, 3.06, 4.59, 6.11, and 6.88 μM (spectrum number 6) Acid Blue 25. Panel B shows the difference spectra with 0 (spectrum number 1), 2.89, 5.77, 8.56, 11.5, 13.0 μM (spectrum number 6) Cibacron Blue. In panel C (inset) the changes in absorbance (ΔA) at 686 nm were plotted as a function of Acid Blue 25 concentration and computer-fitted to a curve describing the equilibrium binding equation of one dye-binding site per enzyme subunit and a K_D of 22 nM.

results can be generalized to Acid Blue 25 since the difference spectrum obtained for Cibacron Blue is strikingly different from that obtained for Acid Blue 25 under similar conditions. Nevertheless, it is clear that the spectral properties of anthraquinone dyes are exquisitely sensitive to environment.

When the changes in absorbance at 686 and 700 nm were plotted as a function of the concentration of Acid Blue 25, the data were consistent (as determined by a curve-fitting program) with binding of one molecule of dye to each subunit of QR with $K_D = K_i = 22$ nM.[5]

Conclusions

These studies were undertaken to identify the structural features of Cibacron Blue that are important for its powerful inhibition of QR. We synthesized Cibacron Blue from its component ring systems, purified and crystallized the dyes as salts with organic cations, and characterized these compounds by mass spectrometry and proton NMR. Two-dimensional proton NMR resolved the ambiguity of the position of the

[5] The accuracy of the K_D value determined by this method is limited because the enzyme and the dye concentrations used were in the micromolar range while the K_D value determined is in the nanomolar range.

sulfonate group on the terminal (A) ring of Cibacron Blue. Only two (D and C) of the four rings of Cibacron Blue are essential for high-affinity binding to QR. The introduction of 1-amino, 2-sulfonate (or carboxylate), and 4-anilino groups on the anthraquinone (D ring) lowers the K_i value for QR by a factor of more than 2000, whereas the remainder of the Cibacron Blue molecule exerts only minor effects on inhibition.

Similar findings have been reported for other enzymes. Beissner and Rudolph (1978a) showed that ASSO (XI) was about as potent an inhibitor as Cibacron Blue (XIII) of rabbit muscle lactate dehydrogenase, pig heart malate dehydrogenase, yeast glucose-6-phosphate dehydrogenase, and yeast hexokinase. Bornmann and Hess (1977) found that, for a variety of kinases and dehydrogenases, only the C and D ring systems of Cibacron Blue are required for potent inhibition. Indeed, they showed that compounds containing only the C and D ring systems were the best and most consistent inhibitors for the enzymes tested. Furthermore, Monaghan and colleagues (1982) showed that Acid Blue 25 is as potent an inhibitor as Cibacron Blue for β-lactamase, an enzyme that is not nucleotide dependent.

Taken together, our work and that of others supports the notion of Böhme et al. (1972) that rings C and D of Cibacron Blue are ATP analogues and not the initial proposal by Stellwagen (1977) that Cibacron Blue in its entirety is a mimic for NAD. Interestingly, Wierenga et al. (1985) compared the crystal structures of seven dinucleotide folds and found that only the ADP-binding sites were significantly conserved. Indeed, these workers concluded that the "dinucleotide binding fold" was a misnomer and suggested that it be termed the "ADP-binding $\beta\alpha\beta$ fold." Perhaps by analogy, Cibacron Blue should be considered not as an "NAD" mimic but rather as an "ATP" mimic, a conclusion that must await confirmation from the crystal structure of the QR–Cibacron Blue complex.

ACKNOWLEDGMENTS

We are grateful to Lou Sing Kan and Albert S. Mildvan for advice on obtaining NMR spectra and their interpretation, to Joseph L. Kachinski, Jr., for the chemical and electron impact ionization mass spectra, and to Robert J. Cotter and Kim Monti for fast atom bombardment mass spectra, carried out at the Mid Atlantic Mass Spectrometry Laboratory, an NSF shared instrumentation facility. The NMR Facility of Johns Hopkins University School of Hygiene was established by an NIH Grant (GM 27512). We thank Gale Doremus for preparing the manuscript and the illustrations.

Registry No. I, 84-65-1; II, 82-45-1; III, 84-48-0; IV, 117-78-2; V, 82-24-6; VI, 138285-85-5; VII, 138285-86-6; VIII, 138285-87-7; IX, 138285-88-8; X, 138285-89-9; XI, 138285-90-2; XII, 138285-92-4; XIII, 138285-94-6; EC 1.6.99.2, 9032-20-6.

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Cation-Dependent Transition between the Quadruplex and Watson-Crick Hairpin Forms of d($CGCG_3GCG$)†

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Received June 4, 1991; Revised Manuscript Received October 10, 1991

ABSTRACT: The DNA oligonucleotide d($CGCG_3GCG$) can form either a Watson-Crick (WC) hairpin or a parallel-stranded quadruplex structure containing six G-quartet base pair assemblies. The exchange between these forms and single strands can be monitored using circular dichroism (CD). NMR results verified the assignment of specific CD bands to quadruplex and hairpin species, respectively. Cations stabilize the quadruplex in the order $K^+ > Ca^{2+} > Na^+ > Mg^{2+} > Li^+$ and $K^+ > Rb^+ > Cs^+$, indicating that K^+ has an optimum ionic radius for complex formation and that ionic charge affects the extent of ion-induced stabilization. The quadruplex is stable in the presence of 40 mM K^+ at micromolar DNA concentration and can be kinetically trapped as a metastable form when prepared at millimolar DNA concentration and then diluted into buffer containing 40 mM Na^+. The concentration of K^+ required to reverse the equilibrium from the hairpin to the quadruplex decreases sharply with increased DNA concentration. The quadruplex has an unusual pK_a of ca. 6.8, indicating that $C{\cdot}C^+$ base pairs are probably forming. This system provides insights into some of the detailed structural characteristics of a ["G4-DNA"·ion] complex and an experimental model for the recently proposed "sodium-potassium conformational switch" [Sen, D., & Gilbert, W. (1988) *Nature 334*, 364-366; Sen, D., & Gilbert, W. (1990) *Nature 344*, 410-414]. These results may help to explain the lack of cytidine residues in G-rich telomeric DNAs and suggest that methylation of GC-rich duplex DNAs in "GpC islands" may induce quadruplex formation within heterochromatin domains, resulting in reversible chromosomal condensation.

Telomeres occur at the ends of linear eukaryotic chromosomes and are necessary for faithful genomic heritability (Blackburn, 1986, 1991). Telomeric DNAs consist of repetitive sequences that are unique for a given organism. A G-rich 3′-terminal strand extends an estimated 12-16 nucleotides past the end of the complementary C-rich strand in the telomeric DNAs of organisms that have been analyzed (Blackburn, 1986; Blackburn & Szostak, 1984; Henderson & Blackburn, 1989). Repetitive G-rich telomeric sequences that are composed of G's and T's, G's and A's, or all three nucleotides are known to exist. Specific examples include T_2G_4 (*Tetrahymena*), T_4G_4 (*Oxytricha*), $TGTGTG_3$ (*Saccharomyces*), $AG_7AGAG_6AG_6$ (*Dictyostelium*), T_2AG_3 (*Homo sapiens*), and T_3AG_3 (*Arabidopsis*) (Forney et al., 1987; Richards & Ausubel, 1988; Roberts, 1988). Curiously, C residues occur very infrequently in the G-rich strands of telomeric DNAs (Forney et al., 1987; Richards & Ausubel, 1988; Roberts, 1988).

Electrophoretic chemical-probing experiments and spectroscopic studies have shown that telomeric DNAs can form a number of unusual structures that apparently contain G·G base pairs (Sundquist & Klug, 1989; Oka & Thomas, 1987; Williamson et al., 1989; Sen & Gilbert, 1990; Raghuraman & Cech, 1990; Hardin et al., 1991). The term "G-DNA" was coined for this class of structures (Cech, 1988). G-DNA structures can be classified into two general categories (Sen & Gilbert, 1990; Hardin et al., 1991), duplexes ("G2-DNA") and quadruplexes ("G4-DNA"). Duplex forms include both intramolecular hairpins and bimolecular helices, while quadruplexes can conceivably be constructed from 1, 2, 3, or 4 strands (Oka & Thomas, 1987; Sundquist & Klug, 1989; Sen & Gilbert, 1990; Hardin et al., 1991). Nondenaturing electrophoresis, NMR, and CD[1] results showed that d$(T_2G_4)_4$ can form different equilibrium mixtures of apparent 1-, 2-, or 4-stranded quadruplex species, depending upon the solution conditions (Hardin et al., 1991). Three-stranded G-DNA complexes could conceptually exist as an intramolecular triplex annealed (via G·G base pairing on both "sides") to a second strand. Strand polarities can be classified as parallel or antiparallel. Hairpin-containing complexes are constrained to having antiparallel strands. Strand polarity and nucleoside glycosidic torsion angles appear to be correlated in G-DNA quadruplexes. Syn and anti glycosidic torsion angles have been observed by NMR in the hairpin complex formed by d$(T_2G_4)_4$

† These studies were supported by grants from the North Carolina Research and Development Fund (No. 9223) and the National Institutes of Health (BRSG RR9223). Partial funding was also provided by the North Carolina Agricultural Research Service.
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[1] Abbreviations: CD, circular dichroism; DMS, dimethyl sulfate; KP, 20 mM potassium phosphate (pH 7) containing 0.1 mM EDTA; NaP, 20 mM sodium phosphate (pH 7) containing 0.1 mM EDTA; TSP, sodium 3-(trimethylsilyl)-1-propanesulfonate; Tris, tris(hydroxymethyl)aminomethane; WC, Watson-Crick.

0006-2960/92/0431-833$03.00/0