Inactivation of yeast hexokinase by Cibacron Blue 3G-A: spectral, kinetic and structural investigations

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Yeast hexokinase, a homodimer (100 kDa), is an important enzyme in the glycolytic pathway. Although Cibacron Blue 3G-A (Reactive Blue 2) has been previously shown to inactivate yeast hexokinase, no comprehensive study exists concerning the nature of interaction(s) between hexokinase and the blue dye. A comparison of the computer-generated three-dimensional (3D) representations showed considerable overlap of the purine ring of ATP, a nucleotide substrate of hexokinase, with the hydrophobic anthraquinone moiety of the blue dye. The visible spectrum of the blue dye showed a characteristic absorption band centred at 628 nm. The visible difference spectrum of increasing concentration of the dye and the same concentrations of the dye plus a fixed concentration of hexokinase exhibited a maximum, a minimum and an isobestic point at 683, 585, and 655 nm respectively. The visible difference spectrum of the blue dye and the dye in 50% ethylene glycol showed a maximum and a minimum at 660 and 570 nm respectively. The visible difference spectrum of the blue dye in the presence of the dye and hexokinase modified at the active site by pyridoxal phosphate, iodoacetamide and *o*-phthalaldehyde was devoid of bands characteristic of the hexokinase–blue dye complex. Size-exclusion-chromatographic studies in the absence or presence of guanidinium chloride showed that the enzyme inactivated by the blue dye was co-

eluted with the unmodified enzyme. The dialysis residue obtained after extensive dialysis of the gel-filtered complex, against a buffer of high ionic strength, showed an absorption maximum at 655 nm characteristic of the dye–enzyme complex. Inactivation data when analysed by ‘Kitz–Wilson’-type kinetics for an irreversible inhibitor, yielded values of 0.05 min^{−1} and 92 μM for maximum rate of inactivation (k_s) and dissociation constant (K_d) for the enzyme–dye complex respectively. Sugar and nucleotide substrates protected hexokinase against inactivation by the blue dye. About 2 mol of the blue dye bound per mol of hexokinase after complete inactivation. The inactivated enzyme could not be re-activated in the presence of 1 M NaCl. These results suggest that Cibacron Blue 3G-A inactivated hexokinase by an irreversible adduct formation at or near the active-site. Spectral and kinetic studies coupled with an analysis of the 3D representations of model compounds corresponding to the substructures of the blue dye suggest that 1-amino-4-(*N*-phenylamino)anthraquinone-2-sulphonic acid part of the blue dye may represent the minimum structure of Cibacron Blue 3G-A necessary to bind hexokinase. Hydrophobic interactions between the blue dye and the active site of hexokinase appear to play a major role during inactivation of the latter.

INTRODUCTION

Monochloro- and dichloro-triazine dyes have been used to purify a number of enzymes (Böhme et al., 1972; Travis and Pannell, 1973; Easterday and Easterday, 1974; Wilson, 1976; Dean and Watson, 1976; Stellwagen, 1977; Angal and Dean, 1978). Glazer (1970) suggested that aromatic-dye molecules tend to bind preferentially globular proteins. Rossmann et al. (1975) proposed that a ‘dinucleotide fold’, a supersecondary structure evolved and conserved in kinases and dehydrogenases, serves to bind nucleotide cofactors. Kinetic, spectroscopic and X-ray diffraction studies (Einarsson et al., 1974; Ashton and Poyle, 1978; Biellmann et al., 1979; Edwards and Woody, 1979) suggested that parts of triazine dyes mimic nucleotide cofactors of kinases and dehydrogenases. These dyes can therefore be used as immobilized pseudo-affinity ligands to purify these enzymes. Cibacron Blue 3G-A (Reactive Blue 2), a polynuclear monochlorotriazine dye (Figure 1) was shown to bind to a number of glycolytic enzymes that possess a ‘dinucleotide fold’ (Stellwagen et al., 1975; Thompson and Stellwagen, 1976; Stellwagen, 1977). Wilson (1976) and Beissner and Rudolph (1978) presented some data to show that Reactive Blue 2 was an inhibitor of the phosphotransferase activity of yeast hexokinase. Hexokinase was shown

not to bind to Reactive Blue 2 immobilized on dextran (agarose or Sepharose) matrix, and this was attributed to the absence of a ‘dinucleotide fold’ in this enzyme (Stellwagen et al., 1975). However, Easterday and Easterday (1974) purified yeast hexokinase by affinity chromatography on Sepharose 6B matrix covalently linked to Reactive Blue 2. Comprehensive reports describing either the spectral properties of hexokinase–Reactive Blue 2 complex or the nature of interactions between the blue dye and hexokinase are absent.

Earlier investigations in our laboratory showed that inactivation of yeast hexokinase by *o*-phthalaldehyde (OPH) (Puri and Roskoski, 1988a; Puri et al., 1988) and 2-aminothiophenol (Puri and Roskoski, 1988b) was mainly due to hydrophobic interactions between these ligands and the active site of the enzyme. More recently we demonstrated that interactions between Cibacron Brilliant Red 3B-A, a monochlorotriazine dye, and the hydrophobic environment of the active-site of hexokinase played an important role during the inactivation of the latter by the red dye (Puri and Roskoski, 1993). Superposition of computer-generated three-dimensional (3D) representations of ATP and Reactive Blue 2 (Figure 2) revealed that the latter might bind to the nucleotide-binding region of the enzyme. Therefore, kinetic, spectral and size-exclusion-chromatography studies were

Abbreviations used: PLP, pyridoxal phosphate; OPTH, *o*-phthalaldehyde; 3D, three-dimensional.

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Figure 1 Chemical structures and 3D representations of the ligands

Chemical structures and their 3D representations were made by CHEMDRAW and CHEM 3D programs (Cambridge Scientific Computing, Cambridge, MA, U.S.A.) respectively for the Macintosh SE computer. Standard bond lengths and bond angles stored in the computer programs were used in creating these illustrations. No modifications were made to minimize structural error (due to non-bonded interactions) in the 3D representations generated by using standard parameters of the computer program. The sulphonic acid residues in the representations are shown in their un-ionized form.

commenced to examine the nature of interaction(s) between Reactive Blue 2 and hexokinase. Model compounds corresponding to the substructures of the blue were examined by the computer-generated 3D representations and used in kinetic and spectral studies to learn about the minimum structural requirements for the blue dye to inactivate hexokinase.

MATERIALS AND METHODS

Cibacron Blue 3G-A (Reactive Blue 2 or Procion Blue H-B; Color Index 61211, Ashton and Poyla, 1978) and Nylomine Blue A-G (Acid Blue 25; Color Index G2055, Ashton and Poyla, 1978), yeast hexokinase, glucose-6-phosphate dehydrogenase, sugars, nucleotides and BSA were obtained from Sigma. Bromaminic acid was obtained from Pfaltz and Bauer. Throughout the present paper the dyes are identified by their commercial names shown in parentheses. Cibacron and Procion are trademarks of Ciba-Geigy and ICI respectively. Concentrations of Reactive Blue 2, Acid Blue 25 and bromaminic acid were computed from spectrophotometric measurements by using millimolar absorption coefficients of (ϵ_{mM}) 11.6 (622 nm), 13.0 (610 nm) and 6.57

(483 nm) respectively (Ashton and Poyla, 1978; Beissner and Rudolph, 1978). All other chemicals used were of analytical reagent grade or better.

Commercial samples of yeast hexokinase were analysed and assayed as described previously (Puri et al., 1988; Puri and Roskoski, 1988a). Values of 100 kDa and 50 kDa for the dimeric and monomeric hexokinase (Schultze and Colowick, 1969; Derechin et al., 1972) respectively were used in the calculations. Proteins were determined either by the method of Lowry et al. (1951), or by using a specific absorption of $0.947 \text{ cm}^2 \cdot \text{mg}^{-1}$ (Hoggett and Kellett, 1976). Spectra were recorded on a Gilford Model 2600 spectrophotometer by using matched cuvettes of 1 cm pathlength. Samples of Iodoacetamide-, pyridoxal phosphate (PLP)- and OPTH-modified hexokinase were prepared by the method of Puri et al. (1988).

RESULTS

Visible difference spectra of Reactive Blue 2 in the presence of hexokinase and ethylene glycol

Owing to the absence of spectroscopic data on the nature of

Figure 2 Schematic illustration of the superposition of 3D representations of ATP and Reactive Blue 2

3D representations of ATP and Cibacron Blue 3G-A were created by using CHEMDRAW and CHEM 3D programs (Cambridge Scientific Computing) as described in the legend to Figure 1.

interactions between hexokinase and Reactive Blue 2, we chose to address this problem at the very outset of extended investigations. The visible spectrum of Reactive Blue 2 exhibited a broad absorption band centred at 628 nm (literature value 622 nm; Ashton and Poyla, 1978). Visible difference spectra of increasing concentration of the blue dye in the presence of the same concentrations of the dye plus a fixed concentration of hexokinase showed a maximum, a minimum and an isobestic point at 683, 585 and 655 nm respectively (Figure 3a). Visible difference spectrum of the dye and the dye in 50% ethylene glycol exhibited an absorption maximum and minimum at 660 and 570 nm, respectively (Figure 3b). These results suggest that Reactive Blue 2 forms a single complex with hexokinase and that this conjugation involves hydrophobic interactions between the ligand and enzyme.

Visible difference spectrum of Reactive Blue 2 in the presence of hexokinase modified at the active site by iodoacetamide, PLP or OPTH

The visible difference spectra of Reactive Blue 2 and the dye plus hexokinase modified at the active site by iodoacetamide, PLP

Figure 3 Visible difference spectrum of Reactive Blue 2 and Reactive Blue 2 plus hexokinase and ethylene glycol

(a) The sample cuvette contained hexokinase (20 μ M), 10 mM Tris/HCl, pH 7.5 and Reactive Blue 2 (60, 76 and 92 μ M) in the order of increasing absorbance. The reference cuvette contained Reactive Blue 2 (60, 76 and 92 μ M) in 10 mM Tris/HCl, pH 7.5. (b) The sample cuvette contained Reactive Blue 2 (53 μ M) and 10 mM Tris/HCl, pH 7.5, and the reference cuvette contained Reactive Blue 2 (53 μ M) plus 50% ethylene glycol and 10 mM Tris/HCl, pH 7.5. A baseline difference spectrum of the enzyme and the buffer were recorded from 500 to 800 nm to ensure that there was no absorption due to the enzyme protein in this region. The difference spectrum of Reactive Blue 2 and Reactive Blue 2 in the same buffer also showed no absorption in the 500–800 nm region. Spectra were recorded at 25 $^{\circ}$ C.

Figure 4 Size-exclusion chromatography of hexokinase modified by Reactive Blue 2

(a) Hexokinase (0.372 mg/150 μ l or 21.8 μ M) in 10 mM Tris/HCl, pH 7.5, was applied to a Sephadex G-25 column (0.45 cm $\times$ 18.5 cm) pre-equilibrated with the same buffer. The column was eluted by the same buffer at a rate of 3.5 min/fraction (1 ml). Fractions were assayed for phosphotransferase activity ($\circ$) and absorption at 280 nm ($\bullet$). (b) Hexokinase (21.8 μ M) was incubated with Reactive Blue 2 (150 μ M) at 25 $^{\circ}$ C until 90% of the phosphotransferase activity was lost. The incubation mixture was then applied to the column and the column eluted as described above. The fractions were monitored for phosphotransferase activity ($\circ$) and absorption at 280 ($\bullet$) and 680 ($\triangle$) nm.

and OPTH were devoid of any absorption bands in the region 500–800 nm which contains absorption bands characteristic of the dye–enzyme complex. The results show that the blue dye interacts with amino acid residues at or near the active site of hexokinase.

Size-exclusion chromatography of hexokinase modified by Reactive Blue 2

Hexokinase lost 95% of its phosphotransferase activity when incubated with Reactive Blue 2 (160 μ M) in 1.5 h at 25 $^{\circ}$ C and at pH 7.5. Size-exclusion chromatography of an incubation mixture containing unmodified hexokinase on a Sephadex G-25 column showed the presence of a single peak of protein coincident with the peak of enzyme activity (Figure 4a). The elution profile of hexokinase modified by Reactive Blue 2 showed the presence of

Figure 5 Attempted re-activation of hexokinase inactivated by Reactive Blue 2

Hexokinase (20 μM) was incubated with Reactive Blue 2 (150 μM) in 100 mM Tris/HCl, pH 7.5, at 25 $^{\circ}\text{C}$. The total volume of the incubation mixture was 1 ml. Aliquots (25 μl) were withdrawn into an assay mixture (1 ml) and the enzyme activity remaining ($\circ$) at various times was determined. After 90 min, the ionic strength of the incubation mixture was increased by adding NaCl solution (1 M final concn.). Aliquots of the incubation mixture were withdrawn into the assay mixture and enzyme activity before ($\circ$) and after ($\bullet$) addition of NaCl solution was determined. A control incubation mixture without Reactive Blue 2 ($\blacksquare$) was identically treated.

Figure 6 Time course of inactivation of hexokinase by Reactive Blue 2

Hexokinase (40 μM), Reactive Blue 2 and 100 mM Tris/HCl, pH 7.5, were incubated in a total volume of 1 ml at 25 $^{\circ}\text{C}$. Aliquots (25 μl) of the incubation mixture were withdrawn into an assay mixture (1 ml) and residual enzyme activity was determined. The concentrations of the dye were as follows: $\square$, 0 μM ; $\blacksquare$, 38 μM ; $\circ$, 77 μM ; $\bullet$, 154 μM ; and $\triangle$, 192 μM . The primary plot (a) was constructed by plotting the logarithm of the percentage of enzyme activity remaining versus time. A double-reciprocal plot of apparent first-order rates versus concentration of Reactive Blue 2 is shown in (b). Control experiments showed that the concentrations of the dye in the assay mixture, following dilution of the inactivation mixture, had minimal effect on glucose-6-dehydrogenase, the coupling enzyme also present in the assay mixture. The lines shown in plots (a) and (b) were obtained by exponential and linear regression (Cricket graph V1.3; Macintosh computer) of the data respectively.

a protein peak (absorption at 280 nm) coincident with another peak corresponding to the modified hexokinase (absorption peak at 680 nm) (Figure 4b). The peaks corresponding to absorptions at 280 and 680 nm were coincident with the peak of enzymic activity (< 10% control; Figure 4b) remaining after chemical

modification by Reactive Blue 2. Another peak in the elution profile (Figure 4b) corresponds to the free dye. When a similar incubation mixture containing hexokinase modified by the blue dye was chromatographed over the same Sephadex G-25 column, equilibrated and eluted with a buffer containing 6 M guanidinium chloride, the fraction showing peaks of absorption at 280, 680 and 655 nm corresponded to the same fraction that contained unmodified dissociated enzyme (results not shown). When the fractions corresponding to the modified hexokinase were pooled and dialysed extensively against 100 mM Tris/HCl, pH 7.5, containing 1 M NaCl, the dialysis residue still showed absorption at 655 nm characteristic of the dye-enzyme complex. These results suggest that inactivation of hexokinase by Reactive Blue 2 is a consequence of the formation of either an irreversible complex or a covalent adduct.

Attempted re-activation of hexokinase inactivated by Reactive Blue 2 in buffers of high ionic strength

Addition of NaCl (1 M final concn.) to an incubation mixture containing hexokinase (< 10% enzyme activity compared with a control) modified by Reactive Blue 2 did not result in the restoration of the enzyme activity over a period of 1 h (Figure 5). These results corroborate the notion that hexokinase, during inactivation by Reactive Blue 2, undergoes irreversible modification.

Time course of inactivation of hexokinase by Reactive Blue 2

Inactivation of hexokinase by the blue dye (38–192 μM) was investigated at 25 $^{\circ}\text{C}$ and pH 7.5. Hexokinase lost about 90% of its activity in 45 min when incubated with 192 μM Reactive Blue 2. A plot of the logarithm of the percentage of enzyme activity remaining versus time showed linear relationships (Figure 6a). Apparent first-order rate constants were calculated from these plots as described previously (Puri and Roskoski, 1988b). The kinetics of inactivation of hexokinase by the blue dye were analysed by the procedure developed by Kitz and Wilson (1962) (eqns. 1 and 2 below) for an irreversible inhibition and described by the authors and other investigators previously (Witt and Roskoski, 1980; Puri and Roskoski, 1988a, 1988b):

$$1/K_{\text{app.}} = (K_d/k_3)(1/[\text{dye}]) + (1/k_3) \quad (2)$$

E corresponds to the free enzyme, whereas E $\cdots$ dye and E dye correspond to Michaelis–Menten-type complex and irreversible adduct respectively. A double-reciprocal plot (Figure 6b) of the apparent first-order rate constants versus concentration of the dye yielded a straight line with a positive intercept. These results show that inactivation of hexokinase by the blue dye followed saturation kinetics involving information of a Michaelis-type complex before formation of an irreversible complex (Witt and Roskoski, 1980). The maximum rate of an irreversible complex-formation, k_3 , and the dissociation constant for the product, K_d , were 0.05 min^{-1} and 92 μM respectively.

Effect of sugar and nucleotide substrates on the inactivation of hexokinase by Reactive Blue 2

Hexokinase was completely protected by 10 mM glucose from inactivation against Reactive Blue 2 (Figure 7a). Glucose (1 mM) was also very effective and far more potent than fructose (1 mM) in affording protection to hexokinase against inactivation by Reactive Blue 2. The product of the hexokinase reaction, glucose

Figure 7 Effect of sugars and nucleotides on the inactivation of hexokinase by Reactive Blue 2

(a) Hexokinase (40 μ M), Reactive Blue 2 (154 μ M) and 100 mM Tris/HCl, pH 7.5, were incubated at 25 $^{\circ}$ C in the presence of the following: $\square$, buffer; $\blacksquare$, 10 mM glucose; $\circ$, 1 mM glucose; $\triangle$, 1 mM fructose; $\blacktriangle$, 1 mM glucose 6-phosphate. A control experiment ($\square$) in the absence of any ligand was carried out over the same time period under identical conditions. Aliquots were withdrawn into an assay mixture as described above and residual enzyme activity was determined. (b) Hexokinase (40 μ M), Reactive Blue 2 (154 μ M) and 100 mM Tris/HCl, pH 7.5, were incubated at 25 $^{\circ}$ C in the presence of the following: $\triangle$, buffer; $\blacksquare$, 10 mM ATP; $\circ$, 10 mM ADP; and $\bullet$, 10 mM AMP. A control experiment ($\square$) in the absence of any ligand was carried out over the same time period under identical conditions. Aliquots were withdrawn into an assay mixture as described above and the residual enzyme activity was determined. The data were plotted as the logarithm of the percentage of maximum activity remaining of a control taken as 100%. The lines shown in the Figure were obtained by regression of the data (see the legend to Figure 6).

6-phosphate, provided little protection to hexokinase against inactivation.

The nucleotide substrate, ATP (10 mM), provided more protection than AMP (10 mM), ADP (10 mM), and ITP (10 mM) to hexokinase against inactivation (Figure 7b). MgATP (10 mM) provided nearly the same protection as ATP (10 mM), suggesting that MgATP and free ATP bound to the enzyme at the same site. The results show that sugars were superior to nucleotides in providing protection to hexokinase against inactivation by Reactive Blue 2.

Inactivation of yeast hexokinase by Acid Blue 25

Acid Blue 25 [1-amino-4-(*N*-phenylamino)anthraquinone-2-sulphonic acid] (Figure 1) constitutes an important hydrophobic segment of the chemical structure of Reactive Blue 2. It was found to be an inhibitor of the phosphotransferase activity of hexokinase. Gel-filtration studies of the incubation mixture containing hexokinase inactivated by Acid Blue 25 in the absence and presence of 6 M guanidinium hydrochloride showed that this dye bound reversibly to the enzyme (result not shown). The visible spectrum of Acid Blue 25 exhibited a maximum at 612 nm (literature value 610 nm; Ashton and Poyla, 1978) characteristic of the dye chromophore. The visible difference spectra of an increasing concentration of the dye in the presence of the same

concentrations of the dye and a fixed amount of hexokinase showed a maximum, a minimum and an isobestic point at 650, 582 and 605 nm respectively. The visible difference spectrum of Acid Blue 25 and the dye in 50% ethylene glycol exhibited a maximum and a minimum at 660 and 570 nm respectively.

Concentration-dependence of inactivation of hexokinase by Acid Blue 25 showed saturation kinetics, suggesting that the inactivation process involved Michaelis-Menten kinetics. Pseudo-first-order rates of inactivation were obtained from the primary plot of the logarithm of the enzyme activity remaining versus period of incubation of the enzyme with the dye. The double-reciprocal plot of the apparent first-order reaction rates of inactivation of hexokinase (ordinate) and concentration of Acid Blue 25 (abscissa) yielded values of 0.175 min^{-1} and 0.35 mM for k_3 and K_d respectively. Glucose (10 mM), ATP (10 mM) and MgATP (10 mM) afforded 100, 37 and 40% protection to hexokinase against overall inactivation by Acid Blue 25 respectively.

The results show that Acid Blue 25 also formed a unique complex with hexokinase. The dye interacted with the hydrophobic environment of the active site of the hexokinase during its inactivation.

Inactivation of hexokinase by bromaminic acid

Bromaminic acid (1-amino-4-bromoanthraquinone-2-sulphonic acid) (Figure 1), a commercially available dye, closely resembles the anthraquinone core of Reactive Blue 2 and Acid Blue 25. Bromine in bromaminic acid, unlike chlorine in the 6-chloro-2,4-diamino-1,3,5-triazine moiety present in Reactive Blue 2, is unreactive. Thus bromaminic acid is devoid of elements of chemical reactivity necessary for covalent-bond formation. Bromaminic acid was found to be a poor inhibitor of hexokinase. Concentration-dependence of inactivation of hexokinase showed that it lost about 84% of the phosphotransferase activity when incubated with 14.4 mM bromaminic acid for 20 min at 25 $^{\circ}$ C, pH 7.5. The kinetics of inactivation were too slow to be followed meaningfully.

Visible spectrum of bromaminic acid showed the presence of an absorption band at 485 nm. Visible difference spectrum of increasing concentration of bromaminic acid and the same concentrations of the dye plus a fixed concentration of hexokinase showed the absence of an isobestic point. Gel filtration of an incubation mixture containing hexokinase inactivated by bromaminic acid over a Sephadex G-25 column confirmed that there was no complex formed between this dye and hexokinase during inactivation.

These results show that inactivation of hexokinase by bromaminic acid was due to non-specific interactions between the dye and enzyme.

DISCUSSION

Absorptions in the visible region of the spectra of Cibacron dyes are well separated in energy from those bands corresponding to protein absorption. Binding of proteins to these dyes causes perturbations in their chromophore, leading to a bathochromic shift (red shift) of the maximum corresponding to their chromophoric absorption. The visible difference spectrum of a free dye and the dye bound to an enzyme has been previously used to investigate formation of a dye-enzyme complex (Thompson and Stellwagen, 1976; Johnson et al., 1980; Federici et al., 1985; Puri and Roskoski, 1993) and conformational changes resulting from dye-protein interactions (Edwards and Woody, 1979). Comparison of the visible difference spectrum of the dye in aqueous

buffers and of the dye in aqueous organic solvents (50 % ethylene glycol or 50 % dioxan) with the visible difference spectrum of the dye and dye bound to an enzyme has been shown to provide important information concerning the nature of polarity of the environment of dye-enzyme complex-formation (Thompson and Stellwagen, 1978; Subramanian and Kaufman, 1980; Puri and Roskoski, 1993). Such comparisons have also been used by the authors to probe the polarity of sites occupied in enzymes by ligands other than Cibacron dyes (Puri et al., 1985a, 1985b).

The presence of a single isobestic point (cf. Thompson and Stellwagen, 1976; Johnson et al., 1980; Boyd et al., 1983; Federici et al., 1985; Puri and Roskoski, 1993) in the visible difference spectrum of the increasing concentration of Reactive Blue 2 and the dye bound to hexokinase suggested the formation of a unique dye-enzyme complex. This was further confirmed by spectral titration of hexokinase with Reactive Blue 2, which yielded a value of 2.93 ± 0.47 ($n = 3$) mol of the blue dye bound/mol of the enzyme. A favourable comparison between the visible difference spectrum of Reactive Blue 2 and that of the Reactive Blue 2 in 50 % ethylene glycol with the visible difference spectrum of the dye and dye bound to hexokinase suggested that the dye-binding region of the enzyme is located in a hydrophobic environment. Essential residues of hexokinase, e.g. thiol and ϵ -amino functions of cysteine and lysine residues respectively, were shown to be located in the hydrophobic cleft of the active site of hexokinase (Puri et al., 1988; Puri and Roskoski, 1988b). Chemical modification of hexokinase by iodoacetamide, PLP and OPTH under selective and controlled conditions renders the enzyme inactive, with concomitant covalent modification of the active-site residues (Puri et al., 1988; Puri and Roskoski, 1988b). The visible difference spectrum of Reactive Blue 2 and the dye in the presence of hexokinase modified at the active site by iodoacetamide, PLP and OPTH did not exhibit an isobestic point. These results are in agreement with the conclusion that Reactive Blue 2 forms a complex with hexokinase by interaction with residues located in the hydrophobic environment of the active site. Spectroscopic data presented here constitute the first ever definitive report of the formation of a complex between hexokinase and Cibacron Blue 3G-A.

When the hexokinase-Reactive Blue 2 complex, obtained by size-exclusion chromatography, was extensively dialysed against buffers of high ionic strength, the dialysis residue still showed absorption characteristic of the blue-dye-hexokinase complex. Attempts to re-activate hexokinase inactivated by Reactive Blue 2 by incubating the inactivation mixture with 1 M NaCl were unsuccessful. These results suggest formation of an irreversible complex or a covalent adduct between Reactive Blue 2 and hexokinase. The monochlorodiaminotriazine moiety in Reactive Blue 2 contains a far more nucleophilic chlorine than bromine in bromaminic acid (Figure 1). Although the monochlorodiaminotriazine moiety and anthraquinone ring systems present in Reactive Blue 2 and bromaminic acid respectively are both aromatic in character, the carbon-halogen bond in the former is far more reactive than the one present in bromaminic acid. This is due to the large negative inductive effect of the three nitrogen atoms present in the triazine ring system that renders the carbon-chlorine bond weak in Reactive Blue 2 compared with the carbon-bromine bond in the anthraquinone ring system in bromaminic acid. It is thus conceivable that the essential amino acid residues, containing a nucleophilic functional group in their side chain in hexokinase form covalent bond(s) with the monochlorodiaminotriazine moiety of Reactive Blue 2. This is reasonable in the light of the fact that Reactive Blue 2 immobilized on a dextran (agarose or Sepharose) matrix by covalent-bond formation between the chlorine of the monochlorodiamino-

triazine moiety of Reactive Blue 2 and hydroxy functions of a dextran (Ryan and Vestling, 1974) serves as pseudo-affinity ligand for the purification of a number of enzymes, including a variety of kinases (Easterday and Easterday, 1974).

Inactivation of hexokinase by Reactive Blue 2 followed 'Kitz-Wilson' (1962)-type kinetics characteristic of an irreversible-complex or covalent-adduct formation (Borchardt et al., 1978; Witt and Roskoski, 1980; Puri and Roskoski, 1988b, 1993). Non-linear dependence of rates of inactivation of hexokinase on the blue-dye concentration (primary plot) and presence of a positive intercept and a straight line in the secondary plot (Figure 6b) are characteristic of the formation of a Michaelis type of dye-enzyme intermediate before formation of the final adduct. The rate of irreversible adduct formation, k_3 , and dissociation constant, K_3 , for hexokinase-Reactive Blue 2 adduct were 0.05 min^{-1} and $92 \mu\text{M}$ respectively. These values compare favourably with the values 0.1 min^{-1} and $120 \mu\text{M}$, and 0.13 min^{-1} and $100 \mu\text{M}$, for the inactivation of yeast hexokinase by Cibacron Brilliant Red 3B-A (Puri and Roskoski, 1993) and the catalytic subunit of cyclic AMP-dependent protein kinase by Cibacron Blue 3G-A (Witt and Roskoski, 1980) respectively. These inactivation reactions have been previously shown to form an irreversible complex between hexokinase and the inactivating ligands. Sugars afforded better protection than nucleotides against inactivation of hexokinase by Reactive Blue 2. Similar results have been obtained by us (Puri et al., 1988; Puri and Roskoski, 1988a, 1988b, 1993) and other workers (Jones et al., 1975; Otieno et al., 1975) during investigations of inactivation of hexokinase by ligands that form irreversible complexes. One explanation might be that, during the natural phosphorylation reaction catalysed by hexokinase, conformational changes induced by glucose juxtapose MgATP and glucose to allow phospho transfer (Bennett and Steitz, 1978; Steitz et al., 1981). In the absence of glucose, ATP or MgATP probably binds less than optimally to the active site of the enzyme, thus providing only partial protection against inactivation by Reactive Blue 2.

In order to learn about relative contributions of the substructures of the parent blue dye towards inactivation of hexokinase, we made use of the commercially available model compounds that most closely resemble these structures. A comparison of the computer-generated 3D representations of Reactive Blue 2 and Acid Blue 25 (Figure 1) show considerable similarity between the two and suggest that the latter might also act as an inhibitor of hexokinase. The visible difference spectrum of Acid Blue 25 and the dye bound to hexokinase showed the presence of an isobestic point characteristic of a single dye-enzyme complex-formation. A comparison of this spectrum with that of the dye and the dye in 50 % ethylene glycol suggested that complex-formation between Acid Blue 25 and hexokinase occurred in the hydrophobic environment of the enzyme. When rates of inactivation of hexokinase by Acid Blue 25 were analysed by Michaelis-Menton kinetics, values of 0.176 min^{-1} and $350 \mu\text{M}$ for k_3 and K_3 respectively were obtained. The results show that Acid Blue 25 binds less tightly to hexokinase than the parent blue dye. But, once bound, it inactivated hexokinase at a higher rate compared with that of the parent dye. When computer-generated 3D representations of Acid Blue 25 and 2,4-diamino-1,3,5-triazine are juxtapositioned, the combination mimics Reactive Blue 2 more than Acid Blue 25 alone (Figure 1). The rates of inactivation of hexokinase by equimolar mixtures of Acid Blue 25 and 2,4-diamino-1,3,5-triazine were slightly greater than those by Acid Blue 25 alone (results not shown). These results suggest that the 6-chloro-2,4-diamino-1,3,5-triazine moiety actually present in Reactive Blue 2 may serve two functions. First, by being present as a side chain in the substituted aminoanthraquinone

moiety (cf. Acid Blue 25), the chlorodiaminotriazine moiety may add to the overall hydrophobic character of Reactive Blue 2, thus contributing to its more efficient binding to the enzyme. Secondly, it provides a seat of chemical reactivity required for covalent-adduct formation with hexokinase. Although there is considerable resemblance between the computer-generated 3D representations of bromaminic acid (Figure 1) and Acid Blue 25 (Figure 1), the experimental evidence presented above suggested that bromaminic acid inactivated hexokinase by non-specific interactions. Thus it is reasonable to propose that a structure like Acid Blue 25, and not bromaminic acid, represents the minimum structure of Reactive Blue 2 necessary to bind hexokinase during its inactivation. This structural moiety present in Reactive Blue 2 may be involved in positioning the dye along the active site of hexokinase by hydrophobic interactions. Once the orientation of the dye with respect to the active site of hexokinase is stabilized, a covalent-bond formation may take place between the chlorodiaminotriazine moiety of the dye and essential residue(s) at or near the active site of the enzyme. Experimental evidence presented suggest, but do not prove, that formation of a covalent adduct between Reactive Blue 2 and hexokinase during its inactivation represents only one of the possibilities. The computer-generated 3D representations shown in Figure 1 merely represent some of the several possible conformations of the ligands that may exist in solution.

In conclusion, we have demonstrated that visible difference spectral, size-exclusion-chromatography and kinetic data, taken together, support the formation of a unique irreversible complex between Cibacron Blue 3G-A and the active site of hexokinase. Analysis of the structure and inactivation relationship suggest that 1-amino-4-(*N*-phenyl)aminoanthraquinone-2-sulphonic acid may represent the minimum structure of Cibacron Blue 3G-A necessary to bind hexokinase. Hydrophobic associations between the nucleotide-mimetic ligands and the nucleotide-binding region of hexokinase appear to play a major role during the inactivation of this enzyme.

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