Metal-ion-promoted binding of triazine dyes to proteins

The interaction of Cibacron Blue F3G-A with yeast hexokinase

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Bivalent metal ions, particularly Zn^{2+} and other members of the first-row transition series, promote irreversible inactivation of yeast hexokinase by Cibacron Blue F3G-A at a site competitive with both ATP and D-glucose. Difference spectroscopy indicates that the protein–dye dissociation constant is decreased from $250\mu\text{M}$ in the absence of metal ions to $<100\mu\text{M}$ in the presence of appropriate concentrations of metal ions, with specificity displayed in the sequence of $\text{Zn}^{2+} > \text{Cu}^{2+} > \text{Ni}^{2+} > \text{Mn}^{2+}$. Quantitative inactivation of yeast hexokinase leads to the incorporation of approx. 1 mol of Cibacron Blue F3G-A/mol of subunit of mol. wt. 51 000 in both the presence and the absence of metal ion. These results suggest the formation of a highly specific ternary complex involving enzyme, dye and metal ion at the active-site region of the enzyme, and correlate well with the known effects of metal ions in promoting the binding of hexokinase to immobilized Cibacron Blue F3G-A.

The emergence of Cibacron Blue F3G-A and other triazine dyes as multispecific ligands for affinity chromatography has led to new and improved techniques for the purification of proteins (Lowe, 1979a; Lowe *et al.*, 1981b). In particular, immobilized Cibacron Blue F3G-A has been shown to be an effective absorbent for the purification of nicotinamide nucleotide-dependent dehydrogenases, kinases, glycolytic enzymes, blood proteins and an increasing number of other proteins (Dean & Watson, 1979; Lowe *et al.*, 1981b). In addition, the high affinity and chemical reactivity of triazine dyes has made them particularly useful as direct spectroscopic probes of active-site structure (Brand & Gohlke, 1972; Edwards & Woody, 1979), as irreversible active-site-directed affinity labels (Clonis & Lowe, 1980; Witt & Roskoski, 1980; Clonis *et al.*, 1981) and as ligands in novel electrochemical sensors (Lowe, 1979b).

Until recently, however, the use of multivalent metal ions to enhance binding of proteins to immobilized triazine-dye affinity adsorbents had largely escaped attention. Some effect of Mg^{2+} on the binding of a number of synthetases (Moe & Piskiewicz, 1976; Mori & Cohen, 1978; Chen & Somberg, 1980), phosphodiesterases (Ashton &

Polya, 1978; Morrill & Thompson, 1979; Morrill *et al.*, 1979) and isocitrate dehydrogenase (Nagoaka *et al.*, 1977) had been noted, although more detailed studies with yeast hexokinase (Clonis *et al.*, 1981) emphasized the importance of these effects. A wider range of metal-ion-promoted effects involving the binding of a number of proteins to a range of immobilized triazine dyes has now been demonstrated in both conventional affinity chromatography (Hughes *et al.*, 1982) and in high-pressure liquid affinity chromatography (Small *et al.*, 1981). It has been found that metal ions of the first-row transition series, such as Zn^{2+} , Co^{2+} , Mn^{2+} and Cu^{2+} , and, to a lesser extent the group-IIa ions, Ca^{2+} and Mg^{2+} , promote the binding of proteins to immobilized dye adsorbents. The present study was undertaken to establish the nature of metal-ion-mediated interactions between triazine dyes and enzymes and demonstrates that enzyme–dye binding under these conditions originates through specific ternary-complex formation.

Experimental

Materials

Cibacron Blue F3G-A was obtained from Ciba-Geigy (U.K.) Yeast hexokinase [ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1; type C-300; 320

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units ($\mu\text{mol}/\text{min}/\text{mg}$) was from Sigma, and ATP (disodium salt) and phosphoenolpyruvate were from Boehringer. All other chemicals and reagents were supplied by BDH.

Enzyme assay

Yeast hexokinase activity was monitored by the method of Garland & Randle (1962) as described previously (Hughes *et al.*, 1982). One unit of enzyme activity is defined as that amount of enzyme which catalyses the conversion of 1 μmol of NADH/min at 37°C.

Difference spectroscopy

Cibacron Blue F3G-A was purified by preparative t.l.c. as described by Hughes *et al.* (1982). Yeast hexokinase (0.5 ml) was dialysed overnight against 200 vol. of 0.01 M-Tris/HCl buffer, pH 7.3, and diluted with the same buffer to give a final protein concentration of 1 mg/ml. Difference spectra were recorded on a Pye-Unicam SP8-100 double-beam spectrophotometer after the addition of purified Cibacron Blue F3G-A (1 mM stock solution in 0.01 M-Tris/HCl buffer, pH 7.3). Dye (1 mM stock) was added to the test cuvette containing protein (20 μM), giving final dye concentration of 0–100 μM , and measured against a reference cuvette containing an equal concentration of dye in buffer. Spectral titrations were repeated in the presence of different metal ions (0–5 mM). Dissociation constants (K_D) were calculated from double-reciprocal plots of ΔA_{680} versus dye concentration, by using weighted regression analysis (Hughes *et al.*, 1982). It was assumed that, under the conditions used for difference spectroscopy, little or no irreversible binding of the dye to the enzyme had occurred since no time-dependent absorbance changes were observed.

Inactivation studies

The inactivation of yeast hexokinase by Cibacron Blue F3G-A was based on the procedure of Cloniss & Lowe (1980). In a total incubation volume of 1 ml were: Tris/HCl buffer, pH 7.5, 10 μmol ; yeast hexokinase, 16.0 μg , 4.4 units; Cibacron Blue F3G-A, 100 nmol. The metal ions Co^{2+} , Ni^{2+} or Mn^{2+} (1 μmol), Cu^{2+} or Zn^{2+} (100 nmol), Mg^{2+} (100 μmol) and the substrates ATP (0–1 μmol) and D-glucose (0–5 μmol) were added where appropriate. The rate of inactivation was followed at 22°C by periodic removal of small samples (10 μl) for assay of enzyme activity.

The stoichiometry of protein–dye binding was determined by quantitative inactivation of the enzyme at 30°C as described above. In a reaction volume of 1 ml were: yeast hexokinase, 16.0 μg , 4.4 units; Cibacron Blue F3G-A, 2 μmol ; Zn^{2+} , 100 nmol. Quantitatively inactivated enzyme (Cloniss *et al.*, 1981) was precipitated from free dye by the

addition of 20 vol. of ice-cold ethanol (98%). The precipitate was removed by centrifugation for 10 min at 7000 rev./min in an MSE Minor S centrifuge, washed and re-centrifuged before reconstitution in 8 M-urea (1.0 ml). The A_{620} was measured versus an 8 M-urea blank and the concentration of bound dye determined by using a molar absorption coefficient for Cibacron Blue F3G-A in 8 M-urea of 8560 $\text{litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$. Protein concentration was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin (fraction V) as standard.

Results

Fig. 1 shows that the inactivation of yeast hexokinase with Cibacron Blue F3G-A at pH 7.3 and 22°C in the presence of a number of bivalent metal ions follows pseudo-first-order kinetics. Under these conditions, loss of activity with the dye alone in the absence of metal ions is relatively slow, whereas no activity is lost in control incubations minus the dye. The inclusion of metal ions in the incubation medium increases enzyme inactivation by Cibacron Blue F3G-A, with specificity displayed in the order $\text{Zn}^{2+} > \text{Cu}^{2+} > \text{Ni}^{2+} > \text{Co}^{2+} > \text{Mn}^{2+}$. The

Fig. 1. Time course for the inactivation of yeast hexokinase by Cibacron Blue F3G-A in the presence of several metal ions at pH 7.3 and 22°C

The total incubation volume of 1 ml contained Tris/HCl buffer, pH 7.3 (10 μmol), yeast hexokinase (16.0 μg , 4.4 units), Cibacron Blue F3G-A (100 nmol) and the metal ions Zn^{2+} (100 nmol, ○), Cu^{2+} (100 nmol, □), Ni^{2+} (1 μmol , ●), Co^{2+} (1 μmol , ■) or Mn^{2+} (1 μmol , △) where appropriate. Control incubations were performed without metal ions (▲) and without both dye and metal ion (○).

Fig. 2. Effect of competing substrates on the time course for the Zn^{2+} -promoted inactivation of yeast hexokinase by Cibacron Blue F3G-A at pH 7.3 and 22°C

The incubation medium of 1 ml total volume contained Tris/HCl buffer, pH 7.3 (10 μmol), yeast hexokinase (16.0 μg , 4.4 units), Cibacron Blue F3G-A (100 nmol) and Zn^{2+} (100 nmol) supplemented with 100 nmol ($\square$), 1 μmol ($\triangle$) or 5 μmol ($\bullet$) of D-glucose, or with 50 nmol ($\blacktriangle$) or 0.5 μmol ($\blacksquare$) of ATP. Control incubations were performed with no supplements ($\circ$) and in the presence of Zn^{2+} (100 nmol) but absence of dye ($\odot$).

natural ion, Mg^{2+} , required for enzyme activity, increased the rate of inactivation by Cibacron Blue F3G-A both in the absence of Zn^{2+} , as noted earlier (Clonis *et al.*, 1981), and in its presence. The metal-ion-mediated interaction of yeast hexokinase with Cibacron Blue F3G-A was progressively eliminated by the addition of increasing concentrations of the co-substrates, ATP and D-glucose. Fig. 2 demonstrates that the Zn^{2+} -mediated inactivation of yeast hexokinase is almost completely inhibited by 0.5 mM-ATP, with progressively smaller effects being observed at 0.05 mM-ATP respectively. This protective effect of ATP cannot be attributed to chelation of the metal ion by free ATP, since progressive protection by increasing concentrations of ATP is still observed even when the free metal ion concentration is maintained constant. Similarly, significant protection from inactivation by Cibacron Blue F3G-A is observed in the presence of 5 mM-D-glucose, with progressively less protection afforded in the presence of 1.0 mM- and 0.1 mM-D-glucose respectively. These results are comparable with those obtained for the inactivation of yeast hexokinase with the copper phthalocyanine dye, Procion Green H-4G (Clonis *et al.*, 1981).

The spectral titration of yeast hexokinase by purified Cibacron Blue F3G-A produces a red shift

Fig. 3. Effect of metal ions on the dissociation constant of Cibacron Blue F3G-A for yeast hexokinase

Spectral titrations were performed in 0.01 M-Tris/HCl buffer, pH 7.3, at various concentrations of Zn^{2+} ($\circ$), Cu^{2+} ($\bullet$), Ni^{2+} ($\triangle$), Co^{2+} ($\blacktriangle$) and Mn^{2+} ($\square$).

in the region of the maximum absorption wavelength of the dye from 620 to 680 nm, owing to the formation of a protein-dye complex. Protein-dye dissociation constants (K_D) were calculated by weighted regression analysis of double-reciprocal plots of the change in absorbance (ΔA_{680}) versus dye concentration. In the absence of metal ions the affinity of Cibacron Blue F3G-A for yeast hexokinase was low, with a measured K_D of 250 μM . The presence of different metal ions at various concentrations increased the affinity of the dye for the enzyme by 2–3-fold, giving K_D values below 100 μM at appropriate concentrations (Fig. 3). Specificity was also demonstrated with regard to the metal ion (Zn^{2+} , Cu^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+}), whereas Mg^{2+} and Ca^{2+} had no apparent effect.

The stoichiometry of Cibacron Blue F3G-A/hexokinase binding was determined by quantitative irreversible inactivation of the enzyme by Cibacron Blue F3G-A followed by ethanol precipitation of the protein-dye conjugate. The molar ratio of dye bound to protein was determined spectrophotometrically in 8 M-urea and found to be 0.99 mol of dye/mol of subunit of mol. wt. 51000. When the inactivation was permitted to proceed in the presence of Zn^{2+} (0.1 mM), the molar ratio remained essentially unchanged at 1.1 mol of dye/mol of subunit of mol. wt. 51000.

Discussion

The inclusion of bivalent metal ions, particularly those of the first-row transition series, increases the affinity of yeast hexokinase for Cibacron Blue F3G-A. Difference spectroscopy indicates that the protein-dye association constant is decreased by metal ions in the sequence Zn^{2+} , Cu^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+} . This order of metal ion

specificity closely parallels the order observed during conventional affinity chromatography involving the metal ion-promoted binding of the enzyme to immobilized Cibacron Blue F3G-A, $\text{Zn}^{2+} > \text{Cu}^{2+} > \text{Ni}^{2+} > \text{Co}^{2+} > \text{Mn}^{2+}$ (Hughes *et al.*, 1982). However, the relatively low affinity of metal ions for Cibacron Blue F3G-A alone, with specificity $\text{Ni}^{2+} > \text{Co}^{2+} > \text{Mg}^{2+} > \text{Ca}^{2+} > \text{Zn}^{2+} > \text{Cu}^{2+}$ (Hughes *et al.*, 1982), suggests that the specificity observed in the present work must originate from the presence of all three components in a highly specific ternary complex involving enzyme, dye and metal ion.

The results indicate that the ternary complex is formed at the active site of the enzyme. Thus, bivalent metal ions, particularly Zn^{2+} and other members of the first-row transition series, promote irreversible inactivation of hexokinase by Cibacron Blue F3G-A at a site competitive with both ATP and D-glucose. Increasing concentrations of these co-substrates afford progressive protection from inactivation, with the inclusion of 1 mM-ATP almost fully protecting the enzyme from covalent modification by the dye. Furthermore, quantitative inactivation of hexokinase leads to the incorporation of 1 mol of Cibacron Blue F3G-A/mol of subunit of mol. wt. 51000. These observations closely parallel the inactivation of yeast hexokinase by Procion Green H-4G (Clonis *et al.*, 1981), except that the relatively small size of Cibacron Blue F3G-A makes it more likely that this dye binds predominantly at the nucleotide-binding site rather than traversing both nucleotide and sugar sites, as was suggested in the former case. This proposal is supported by the fact that 1 mM-D-glucose afforded almost quantitative protection against inactivation of hexokinase by Procion Green H-4G, but only partial protection against inactivation by Cibacron Blue F3G-A. Further differences in the behaviour of these two triazine dyes were apparent in the metal ion requirement. The inactivation of hexokinase by Procion Green H-4G displayed marked specificity for protection by Mg^{2+} , Sr^{2+} and Ca^{2+} , with little or no effect observed with metal ions of the first-row transition series. In contrast, the inactivation of yeast hexokinase with Cibacron Blue F3G-A, and apparently some other triazine dyes (Clonis *et al.*, 1981), is promoted by metal ions, particularly those of the transition series, with little or no effect observed with group-IIa metal ions. These observations support the view that the metal ion-promoted adsorption of proteins to triazine-dye affinity adsorbents is due to highly specific ternary-complex

formation. These conclusions may be exploited to manipulate the behaviour of proteins in conventional affinity chromatography (Hughes *et al.*, 1982) and high-pressure liquid affinity chromatography (Lowe *et al.*, 1981a; Small *et al.*, 1981).

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