The interaction of yeast hexokinase with Procion Green H-4G

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1. A number of reactive triazine dyes specifically and irreversibly inactivate yeast hexokinase at pH 8.5 and 33°C. Under these conditions, the enzyme is readily inactivated by 100 μ M-Procion Green H-4G, Blue H-B, Turquoise H-7G and Turquoise H-A, is less readily inactivated by Procion Brown H-2G, Green HE-4BD, Red HE-3B and Yellow H-5G and is not inactivated at all by Procion Yellow H-A. 2. The inactivation of hexokinase by Procion Green H-4G is competitively inhibited by the adenine nucleotides ATP and ADP and the sugar substrates D-glucose, D-mannose and D-fructose but not by nonsubstrates such as D-arabinose and D-galactose. 3. Quantitatively inhibited hexokinase contains approx. 1 mol of dye per mol of monomer of mol.wt. 51000. The inhibition is irreversible and activity cannot be recovered on incubation with high concentrations (20 mM) of ATP or D-glucose. 4. Mg^{2+} protects the enzyme against inactivation by Procion Green H-4G but enhances the rate of inactivation by all the other Procion dyes tested. In the presence of 10 mM- Mg^{2+} the apparent dissociation constant between enzyme and dye is reduced from 199.0 μ M to 41.6 μ M. Binding of the dye to hexokinase is accompanied by characteristic spectral changes in the range 560–700 nm. 5. Mg^{2+} promotes binding of yeast hexokinase to agarose-immobilized Procion Green H-4G but not to the other dyes tested. Elution could be effected by omission of Mg^{2+} from the column irrigants or by inclusion of MgATP or D-glucose, but not by D-galactose. These effects can be exploited to purify hexokinase from crude yeast extracts. 6. The specific active-site-directed binding of triazine dyes to yeast hexokinase is interpreted in terms of the crystallographic structure of the hexokinase monomer.

It has been known for at least a decade that aromatic dye molecules tend to bind preferentially to the active-site regions of globular proteins (Glazer, 1970). Thus, not surprisingly, dyes have been used as direct spectroscopic probes of enzyme structure since their visible absorption bands are well removed in energy from the region where most proteins absorb, and the perturbation of these bands provides a facile monitor of the dye-protein interaction (Perrin & Hart, 1970; Brand & Gohlke, 1972; Edwards & Woody, 1979). Furthermore, the remarkable similarity in terms of overall shape, charge and aromaticity of many dyes to the naturally occurring biological heterocycles such as nucleoside phosphates, NAD^+ , coenzyme A and folic acid has prompted the application of immobilized dyes in the purification of their complementary enzymes by affinity chromatography (Baird

et al., 1976; Ashton & Polya, 1978; Beissner & Rudolph, 1978; Dean & Watson, 1979; Lowe *et al.*, 1980*a,b*). In particular, the anthraquinone dye Cibacron Blue F3G-A and the azo dye Procion Red HE-3B have proved particularly suitable for the purification of a number of blood proteins, kinases, dehydrogenases and glycolytic enzymes by affinity chromatography (Dean & Watson, 1979; Lowe, 1979; Lowe *et al.*, 1980*b*). Furthermore, these reactive triazine-based dyes have been unequivocally demonstrated to bind at the nucleotide-binding site of kinases and dehydrogenases (Biellmann *et al.*, 1979; Edwards & Woody, 1979) and therefore to act as specific affinity labels (Witt & Roskoski, 1980; Clonis & Lowe, 1980).

Yeast hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1.) has proven a particularly elusive protein to purify by classical affinity chromatography on immobilized nucleotides (Craven *et al.*, 1974; Harvey *et al.*, 1974), sugar

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analogues (Trayer *et al.*, 1978) or triazine dyes (Wilson, 1976; Land & Byfield, 1979). However, under appropriate adsorption conditions, the enzyme may be bound to immobilized Cibacron Blue F3G-A and subsequently eluted with a change in pH (Easterday & Easterday, 1974) or ATP concentration (Beissner & Rudolph, 1978). Nevertheless, yeast hexokinase appears to display a considerably weaker affinity for this dye than do other common NAD⁺- or NADP⁺-dependent dehydrogenases (Beissner & Rudolph, 1978). The present work was initiated in order to establish whether other immobilized triazine-based dyes would prove more effective than Cibacron Blue F3G-A for the purification of yeast hexokinase. This report suggests that one dye, Procion Green H-4G, displays an apparently unique interaction with this enzyme.

Experimental

Materials

Yeast hexokinase (type C-300, 310 units/mg), glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: NADP⁺ 1-oxidoreductase, EC 1.1.1.49; type VII; baker's yeast; 315 units/mg) and yeast enzyme concentrate (type II) were obtained from Sigma. The nucleotides ATP (disodium salt), ADP (disodium salt) and AMP (disodium salt) were from Boehringer. The sugars D-glucose, D-arabinose, D-galactose, D-mannose and D-fructose and all other reagents including urea (Aristar) were from BDH.

The triazine dyes were a generous and much appreciated gift from Dr. C. V. Stead, I.C.I. Organics Division, Blackley, Manchester, U.K. T.l.c. of dye samples on silica plates (DC Fertigfolien F1500 LS 254 Kieselgel; Schleicher & Schüll, Dassel, Germany) developed in butan-1-ol/acetic acid/water (4:1:5, by vol.; upper phase), butan-2-ol/propan-1-ol/ethyl acetate/water (4:3:2:1, by vol.) or butan-2-ol/propan-1-ol/ethyl acetate/water (2:4:1:3, by vol.) revealed the presence of minor contaminating species detectable under visible or u.v. light. Dyes purified by preparative t.l.c. behaved similarly to unpurified dyes and consequently all dyes were used without purification in these studies. Dyes are referred to in this paper by their commercial names (I.C.I.) and Colour Index (1971) constitution number where appropriate. Procion Blue H-B (I.C.I.) is chemically identical to Cibacron Blue F3G-A (Ciba-Geigy).

Enzyme assays

Yeast hexokinase activity was followed by the coupled reduction of NADP⁺ at 340 nm and 25°C with glucose 6-phosphate dehydrogenase. The reaction mixture contained the following in a total assay volume of 1 ml: Tris/HCl buffer, pH 7.5, 0.1 mmol; D-glucose, 5 μ mol; ATP, 3 μ mol; NADP⁺, 0.7 μ mol;

MgCl₂, 10 μ mol; yeast glucose 6-phosphate dehydrogenase, 3 units, and yeast hexokinase, 0–0.03 units. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 μ mol of NADPH per min at 25°C.

Enzyme inactivation studies

Yeast hexokinase was inactivated essentially according to Clonis & Lowe (1980). The incubation mixture contained in 1 ml total volume at 33°C: Tricine {*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine}/NaOH buffer pH 8.5, 100 μ mol; Procion dye, 100 nmol; hexokinase, 0.28 units. The rate of inactivation was followed by periodically removing samples (50 μ l) for assay of enzyme activity. In addition, the inactivation of yeast hexokinase by Procion dyes was performed in the presence of MgCl₂ (10, 20 or 50 μ mol), ATP (0.1–20 μ mol), ADP (10 or 20 μ mol), D-glucose (0.025–20 μ mol) and D-mannose, D-fructose, D-galactose or D-arabinose (20 μ mol).

Dye concentrations were determined spectrophotometrically at $\lambda_{\max}$ using the following molar absorption coefficient values determined in distilled water: Procion Yellow H-A (C.I. 13245), $\lambda_{\max}$ 385 nm, ϵ 8900 litre·mol⁻¹·cm⁻¹; Procion Yellow H-5G (C.I. 18972), $\lambda_{\max}$ 410 nm, ϵ 8000 litre·mol⁻¹·cm⁻¹; Procion Red HE-3B, $\lambda_{\max}$ 530 nm, ϵ 30 000 litre·mol⁻¹·cm⁻¹; Procion Blue H-B (C.I. 61211), $\lambda_{\max}$ 620 nm, ϵ 7700 litre·mol⁻¹·cm⁻¹; Procion Blue MX-R (C.I. 61205), $\lambda_{\max}$ 600 nm, ϵ 4100 litre·mol⁻¹·cm⁻¹; Procion Green H-4G, $\lambda_{\max}$ 675 nm, ϵ 57 400 litre·mol⁻¹·cm⁻¹; Procion Green HE-4BD, $\lambda_{\max}$ 630 nm, ϵ 20 800 litre·mol⁻¹·cm⁻¹; Procion Turquoise H-7G, $\lambda_{\max}$ 66 nm, ϵ 30 550 litre·mol⁻¹·cm⁻¹; Procion Turquoise H-A, $\lambda_{\max}$ 666 nm, ϵ 45 800 litre·mol⁻¹·cm⁻¹; Procion Brown H-2G, $\lambda_{\max}$ 410 nm, ϵ 10 000 litre·mol⁻¹·cm⁻¹.

Difference spectral titrations

Difference spectra and difference spectral titrations were performed at 25°C in 10 mm lightpath cuvettes in a Pye– Unicam SP.1800 double-beam spectrophotometer. Protein solution (1 ml; 0.4 mg; 8 nmol of hexokinase previously dialysed overnight against 1 litre of 30 mM-Tris/HCl buffer, pH 7.5) and protein solvent (1 ml, 30 mM-Tris/HCl, pH 7.5) were placed in the sample and reference cuvettes respectively and the baseline difference spectrum was recorded. Identical volumes (5–20 μ l) of a concentrated solution of Procion Green H-4G (0.5 mM) were added to both cuvettes and the difference spectra were recorded after each addition. The spectral titration was repeated in the presence of 10 mM-MgCl₂ throughout. Furthermore, identical volumes (5 μ l) of a concentrated solution of MgCl₂

(100 mM) were added to a sample cuvette containing hexokinase (1 ml; 0.4 mg; 8 nmol) and Procion Green H-4G (7.5 μ M) and a reference cuvette containing only dye (7.5 nmol; 1 ml). Difference spectra were recorded after each addition and in all cases dissociation constants were calculated according to Thompson & Stellwagen (1976) and deduced from weighted linear regression analysis of double reciprocal plots using a Hewlett–Packard 9810A programmable calculator.

Stoichiometry of Procion Green H-4G binding to hexokinase

Yeast hexokinase (0.4 mg; 1 ml) in 0.1 M-Tricine/NaOH buffer, pH 8.5, was inactivated with 100 μ M-Procion Green H-4G for 30 min at 33°C as described above. The dye-inactivated enzyme (<0.5% activity remaining) was applied to a column (1.3 cm $\times$ 11.0 cm) of Sephadex G-25 (fine) equilibrated with distilled water, and fractions (1.5 ml) were collected at 20 ml/h. The void-volume peak containing Procion Green H-4G-modified inactive hexokinase was diluted 10-fold with 8 M-urea (Aristar) and the absorbance at 674 nm was determined spectrophotometrically in a Gilford system 2600 microprocessor-controlled UV-VIS spectrophotometer. The protein concentration was determined by the method of Lowry *et al.* (1951). A known concentration of Procion Green H-4G in distilled water was similarly diluted with 8 M-urea to determine λ_{max} . 674 nm and ϵ 51 400 litre $\cdot$ mol⁻¹cm⁻¹ under these conditions.

Dye immobilization

Triazine dyes were covalently attached to Sepharose 4B as follows (Lowe *et al.*, 1980a): to 5 g of exhaustively washed (2 litres of distilled water) Sepharose 4B was added 50 mg of dye in water (5 ml) followed by NaCl (1 ml; 22% w/v) to give a final concentration of 2% (w/v) NaCl. The gel slurry was slowly tumbled at ambient temperature (20–24°C) for 30 min prior to adding solid Na₂CO₃ to a final concentration of 1% (w/v). The gels were tumbled gently for 5 days at 20–24°C and then washed exhaustively with water until no dye was evident in the washings. The gels were stored moist at 0–4°C in sealed vials.

Immobilized dye concentrations were determined by acid hydrolysis of the gels (Beissner & Rudolph, 1978; Lowe *et al.*, 1980a). Moist gel (30 mg) was transferred to 5 M-HCl (0.6 ml), incubated at 37°C for 5 min and 2.5 M-sodium phosphate buffer, pH 7.5 (2.4 ml) was added. Dye concentrations were determined spectrophotometrically at λ_{max} , using the molar absorption coefficients quoted above.

Chromatographic procedures

A sample containing yeast hexokinase (31 units; 0.1 mg) and bovine serum albumin (5.0 mg) in a total

volume of 0.5 ml was dialysed overnight at 0–4°C against 500 ml of 30 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-MgCl₂. A sample of the dialysed enzyme (100 μ l, 4.2 units, 13.5 μ g; 0.66 mg of bovine serum albumin) was applied to a column (4.5 mm $\times$ 32 mm) containing Sepharose 4B-bound triazine dye (0.5 g moist weight gel; 1.1 μ mol of Procion Yellow H-A/g, 2.7 μ mol of Procion Yellow H-5G/g, 1.0 μ mol of Procion Red HE-3B/g, 1.0 μ mol of Procion Blue H-B/g, 6.7 μ mol of Procion Blue MX-R/g, 2.5 μ mol of Procion Green HE-4BD/g or 2.6 μ mol of Procion Green H-4G/g) equilibrated with the same buffer at 23–24°C. Non-adsorbed protein was immediately washed off the column with buffer (10–12 ml) and elution was subsequently effected either by omitting the MgCl₂ from the irrigating buffer or with a pulse of ATP (10 or 20 mM, 5 ml total volume) or D-glucose (20 mM, 5 ml). Fractions (1.4 ml) were collected at a flow rate of 23 ml/h and assayed for enzyme activity. Eluant fractions were assayed for bovine serum albumin by absorbance at 280 nm and for hexokinase activity as described above.

Chromatography of crude yeast extract

A sample of yeast enzyme concentrate (50 mg; 1 ml of 30 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-MgCl₂) was dialysed overnight against 1 litre of 30 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-MgCl₂, at 0–4°C. A sample (400 μ l; 24.3 units of hexokinase, 10.5 mg of protein) was applied to a column (10 mm $\times$ 30 mm) containing Procion Green H-4G (2.5 g moist weight; 2.6 μ mol of Procion Green H-4G/g) equilibrated with 30 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-MgCl₂ at 24°C. Non-adsorbed protein was washed off with 12–13 ml of Tris/Mg²⁺ buffer and elution was effected by omission of MgCl₂ from the irrigating buffer and subsequently by a pulse (5 ml) of ATP (20 mM) in the Tris/Mg²⁺ buffer. Fractions (1.6 ml) were collected at a flow rate of 30 ml/h and assayed for protein (Lowry *et al.*, 1951) and hexokinase activity.

Results

Fig. 1 demonstrates that the inactivation of yeast hexokinase follows pseudo-first-order kinetics when the enzyme is incubated with a number of monochlorotriazinyl dyes of H- or HE- designation (Clonis & Lowe, 1980) at a concentration of 100 μ M in 0.1 M-Tricine/NaOH buffer, pH 8.5 at 33°C. In particular, hexokinase is rapidly inactivated by Procion Green H-4G, Blue H-B, Turquoise H-7G and Turquoise H-A, is less readily inactivated by Procion Brown H-2G, Green HE-4BD, Red HE-3B and Yellow H-5G and is not inactivated at all under these conditions by Procion Yellow H-A. A control sample of hexokinase, in the absence of triazine

Fig. 1. Time course for the inactivation of yeast hexokinase by several triazine dyes at pH 8.5 and 33°C. The rate of inactivation was followed by periodically removing samples (50 μ l) for assay of enzyme activity from the incubation mixture contained in 1 ml total volume at 33°C: Tricine/NaOH buffer, pH 8.5, 100 μ mol; yeast hexokinase, 0.28 units; triazine dye, 100 nmol. Procion Green H-4G (○); Blue H-B (●); Turquoise H-7G (△); Turquoise H-A (▲); Brown H-2G (□); Green HE-4BD (■); Red HE-3B (◐); Yellow H-5G (△); Yellow H-A (◑); no dye (◆).

dyes, displays no loss of activity over the same period. The reaction between an active-site directed reactive dye and an enzyme may be described mathematically by the equation:

$$\frac{1}{k_{\text{obs.}}} = \frac{1}{k_3} + \frac{K_D}{k_3} \frac{1}{[D]}$$

where $k_{\text{obs.}}$ is the observed rate of enzyme inactivation for a given concentration of dye, D , k_3 is the maximal rate of inactivation (min^{-1}) and K_D is the dissociation constant of the enzyme-dye complex (Clonis & Lowe, 1980; Witt & Roskoski, 1980). A plot of $1/k_{\text{obs.}}$ versus $1/[D]$ for the inactivation of hexokinase with several concentrations of Procion Green H-4G between 10 and 100 μM yields a straight line with a positive ordinate intercept, k_3 , of

Fig. 2. Effect of competing substrates on the time course for the inactivation of yeast hexokinase by Procion Green H-4G (100 μM) at pH 8.5 and 33°C. No addition (○); ADP, 20 mM (△); ATP, 20 mM (□); Mg^{2+} , 10 mM (●); control incubation minus dye (◆).

0.14 min^{-1} and a slope corresponding to a dissociation constant (K_D) of 199.0 μM . To determine the stoichiometry of chromophore binding, hexokinase was completely inactivated by Procion Green H-4G and the dye-enzyme complex was resolved from free dye by gel filtration on Sephadex G-25, since the free dye binds tenaciously to the gel. The molar ratio of dye to enzyme subunit was determined by measuring the dye spectrophotometrically in 7.3 M-urea and the protein by the procedure of Lowry *et al.* (1951). Under these conditions, the molar ratio of Procion Green H-4G to subunit of mol.wt. 51000 was 1.1. The activity of the stoichiometrically inhibited enzyme could not be recovered by subsequent incubation of the dye-enzyme conjugate with ATP (20 mM) or D-glucose (20 mM).

For substantiation of the contention that Procion Green H-4G is an active-site-directed reagent, hexokinase was incubated with the green dye in the presence of substrates. Thus, as Fig. 2 demonstrates, the rate of dye inactivation is significantly retarded in the presence of 20 mM-ADP and almost

completely extinguished by 20 mM-ATP. Interestingly, the presence of 10 mM-Mg²⁺ in the incubation medium reduces the rate of inactivation of yeast hexokinase by 100 μ M-Procion Green H-4G to approx. 20% of the rate in the absence of Mg²⁺. Of the dyes tested, this protective effect of Mg²⁺ appears to be unique to the inactivation by Procion Green H-4G. Indeed, the presence of 10 mM-Mg²⁺ in the incubation medium enhances the rate of inactivation of hexokinase by 100 μ M-Procion Yellow H-5G, Yellow H-A, Green HE-4BD, Turquoise H-7G, Blue MX-R, Turquoise H-A, Red HE-3B and Blue H-B by 3.7-, 3.0-, 2.0-, 1.8-, 1.5-, 1.2- and 1.2-fold respectively.

The effect of Mg²⁺ is to increase the affinity of Procion Green H-4G for yeast hexokinase. Thus, determination of k_{obs} at a number of dye concentrations in the presence of 10 mM-Mg²⁺ allows the apparent dissociation constant, K_D , to be determined from the slope of the double reciprocal plot. In the presence of 10 mM-Mg²⁺, K_D is reduced from 199.0 μ M to 41.6 μ M and k_3 , the maximal rate of inactivation at saturating dye concentration, from 0.14 min⁻¹ to 0.04 min⁻¹. Difference spectral titrations of yeast hexokinase (8 μ M) with Procion Green H-4G in the presence of 10 mM-Mg²⁺ indicate a dissociation constant for the green dye of 42.5 μ M under these conditions (Thompson & Stellwagen, 1976). Fig. 3 illustrates the effect of increasing Mg²⁺ concentration on the difference spectra of Procion Green H-4G (7.5 μ M) and hexokinase (8.0 μ M) in the region 560–720 nm. The increase in absorbance at 700 nm is consistent with a dissociation constant of 2.4 mM for Mg²⁺.

The protective effect of adenine nucleotides and Mg²⁺ on the inactivation of hexokinase by Procion Green H-4G is also paralleled by a similar effect with some sugars. Thus, Fig. 4 demonstrates that both 20 mM-D-glucose and D-mannose completely protect yeast hexokinase against inactivation by Procion Green H-4G. Some protection is afforded by 20 mM-D-fructose, whilst 20 mM-D-arabinose and D-galactose do not significantly alter the rate of inactivation. D-Glucose, D-mannose and D-fructose are catalytically active substrates for yeast hexokinase with Michaelis constants (K_m) of 1×10^{-4} M, 5×10^{-5} M and 7×10^{-4} M respectively (Colowick, 1973). On the other hand, D-galactose ($K_m > 5 \times 10^{-2}$ M) and D-arabinose display minimal catalytic activity (Colowick, 1973). Since competitive inhibitors characteristically reduce the rate of enzyme inactivation by active-site-directed agents according to the equation (Clonis & Lowe, 1980):

$$\frac{1}{k_{\text{obs}}} = \frac{1}{k_3} + \frac{K_D}{k_3} \cdot \frac{1}{[D]} \left(1 + \frac{[I]}{K_i} \right)$$

where [I] is the concentration of competitive inhibitor and K_i its dissociation constant, pro-

Fig. 3. Difference spectral titration of yeast hexokinase with Procion Green H-4G as a function of Mg²⁺ concentration

Identical volumes (5 μ l) of a concentrated solution of MgCl₂ (100 mM) were added to a sample cuvette containing yeast hexokinase (1 ml; 0.4 mg; 8 nmol) and Procion Green H-4G (7.5 nmol) and a reference cuvette containing only dye (7.5 nmol; 1 ml) in 30 mM-Tris/HCl buffer, pH 7.5. (a) Difference spectra were recorded after each addition; (b) the difference absorbance at 700 nm as a function of the total Mg²⁺ concentration.

gressive protection by increasing concentrations of ATP and D-glucose may be used to estimate values of K_D and K_i . Progressive protection of enzyme inactivation by Procion Green H-4G was observed with D-glucose concentrations increasing from 25 μ M to 10 mM and by ATP concentrations increasing

Fig. 4. Effect of competing sugars on the time course for the inactivation of yeast hexokinase by Procion Green H-4G (100 μ M) at pH 8.5 and 33°C

No addition (O); D-galactose (▲); D-arabinose (□); D-fructose (●); D-mannose (△); D-glucose (■); control incubation minus dye (◆). The concentration of each sugar was 20 mM.

from 100 μ M to 20 mM. The dissociation constants for the dye (K_D) and the competitive substrates ATP and D-glucose (K_I) were calculated to be 196–202 μ M, 10.3 mM and 65 μ M respectively. The latter values are in good agreement with dissociation constants for these substrates determined by gel filtration (Noat *et al.*, 1969).

In view of the unique Mg^{2+} -potentiated interaction between Procion Green H-4G and yeast hexokinase, attempts were made to exploit these phenomena in affinity chromatography. In the first instance, columns of Procion Blue H-B, Yellow H-5G, Red HE-3B and Green H-4G (Fig. 5a) equilibrated with 30 mM-Tris/HCl, pH 7.5 at 23–24°C, failed to adsorb yeast hexokinase. However, in the presence of 10 mM- $MgCl_2$ in the irrigating buffers, quantitative binding of yeast hexokinase to immobilized Procion Green H-4G was achieved (Fig. 5b). The enzyme passed unretarded through all the other immobilized dye adsorbents and appeared quantitatively in the void volume along with bovine

Fig. 5. Affinity chromatography of yeast hexokinase on agarose-immobilized Procion Green H-4G

A sample of dialysed yeast hexokinase (100 μ l; 4.2 units; 13.5 μ g) and bovine serum albumin (0.66 mg) was applied to a column (4.5 mm $\times$ 32 mm) of Sepharose 4B-bound Procion Green H-4G (0.5 g moist weight gel; 2.6 μ mol of Procion Green H-4G/g of gel) equilibrated with (a) 30 mM-Tris/HCl buffer, pH 7.5, and (b,c,d) 30 mM-Tris/HCl buffer, pH 7.5, containing 10 mM- $MgCl_2$ at 24°C. Non-adsorbed protein was washed off the column with buffer (10–12 ml) and elution effected as indicated above. Hexokinase activity (●).

serum albumin. Hexokinase quantitatively adsorbed to immobilized Procion Green H-4G in the presence of 10 mM- $MgCl_2$ could be eluted with 10 mM-ATP (80% recovery), 20 mM-ATP (100% recovery) (Fig. 5c) or 20 mM-D-glucose (55% recovery) (Fig. 5d). Adsorbed hexokinase could not be released with

Fig. 6. *Chromatography of a crude yeast extract on immobilized Procion Green H-4G*

A dialysed sample of yeast enzyme concentrate (400 μ l; 24.3 units of hexokinase; 10.5 mg protein) was applied to a column (10 mm $\times$ 30 mm) of immobilized Procion Green H-4G (2.5 g moist weight gel, 2.6 μ mol of Procion Green H-4G/g) equilibrated with 30 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-MgCl₂. Non-adsorbed protein was washed off with 12–13 ml of buffer and elution was effected by omission of MgCl₂ from the irrigating buffer and subsequently by a pulse (5 ml) of ATP (20 mM) in the Tris buffer. Fractions (1.6 ml) were collected at 30 ml/h and assayed for protein (○) and hexokinase activity (●).

20 mM-D-galactose (Fig. 5d) although omission of MgCl₂ from the irrigating buffers effected a prompt release of the enzyme with 60–70% recovery of enzyme activity (Fig. 5b).

The Mg²⁺-dependent adsorption of hexokinase to immobilized Procion Green H-4G may also be exploited for the purification of the enzyme from crude yeast extracts. Fig. 6 illustrates the chromatography of a crude yeast extract (24.3 units of hexokinase; 10.5 mg of protein) on a Procion Green H-4G column (2.5 g moist weight gel) equilibrated with 30 mM-Tris/HCl, pH 7.5, containing 10 mM-MgCl₂ at 24°C. Under these conditions, however, only 7.5% of the hexokinase activity was eluted on removal of the Mg²⁺ from the irrigating buffer, although a further 87.2% of the activity was released by a pulse of 10 mM-MgCl₂/20 mM-ATP. The hexokinase eluted with the ATP pulse had a specific activity of 15.9 units/mg, an increase of nearly 7-fold over that in the crude yeast extract.

Discussion

Several nucleotide-dependent enzymes have now been shown to be susceptible to chemical modification by Cibacron Blue F3G-A (Apps & Glead,

1976; Witt & Roskoski, 1980) and other reactive triazine dyes (Clonis & Lowe, 1980). Thus, whilst lactate dehydrogenase is significantly inactivated only by the more reactive dichlorotriazinyl dyes (Clonis & Lowe, 1980), this report demonstrates that yeast hexokinase can be inactivated by both monochlorotriazinyl (Procion H- and HE- range) and dichlorotriazinyl (Procion MX) dyes (Clonis & Lowe, 1980). Yeast hexokinase appears to be especially readily inactivated by three copper phthalocyanine-containing monochlorotriazinyl dyes, Procion Green H-4G, Turquoise H-7G and Turquoise H-A.

Procion Green H-4G inhibits yeast hexokinase in a time-dependent fashion. The hyperbolic dependence of inhibition on dye concentration, the protection against inhibition afforded by specific adenine nucleotides and sugar substrates, the lack of reactivation after inhibition and the approximately equimolar ratio of bound dye to enzyme subunit in the quantitatively inhibited enzyme are characteristic of active-site-directed irreversible inhibitors (Kitz & Wilson, 1962; Clonis & Lowe, 1980; Witt & Roskoski, 1980). The rate of inactivation of yeast hexokinase by Procion Green H-4G is decreased in the presence of adenine nucleotides (ATP and ADP)

and catalytically active sugars (D-glucose, D-mannose and D-fructose) but is unaffected by low-affinity catalytically inactive sugars such as D-galactose and D-arabinose. These observations are entirely consistent with the known kinetic constants for these substrates (McDonald, 1955; Colowick, 1973). It seems likely that dye binding may traverse both the nucleotide- and sugar-binding domains on the enzyme. X-ray crystallographic studies on yeast hexokinase (Steitz *et al.*, 1973) have shown that the enzyme subunit folds into two rather distinct lobes rather like that observed for 3-phosphoglycerate kinase (Blake & Evans, 1974). The lobes carry the binding sites for MgATP and D-glucose with the terminal γ -phosphate moiety of ATP, which is transferred to the sugar during the catalytic reaction, being located in the space between the two closely juxtaposed binding sites. The proximity of the MgATP- and glucose-binding sites is also supported by the observation that the transfer of the terminal phosphate from ATP to D-glucose proceeds by an in-line transfer without formation of a phosphoryl-enzyme intermediate (Blättler & Knowles, 1979). It is not unreasonable, therefore, to suppose that Procion Green H-4G, which is over twice the size of ATP, may bind across both nucleotide- and sugar-binding sites. This proposal would account for the protection from inactivation by ATP and D-glucose and for the ability of both substrates to elute the enzyme from an immobilized Procion Green H-4G adsorbent. Dye occupation of coenzyme and substrate binding sites has also been suggested with the interaction between immobilized Cibacron Blue F3G-A and lactate dehydrogenase (Stellwagen, 1977), 3-phosphoglycerate kinase (Stellwagen, 1977) and adenylosuccinate synthetase (Clonis & Lowe, 1981). Alternatively, crystallographic studies on yeast hexokinase have demonstrated unequivocally that glucose binding induces a conformational change comprising a large relative motion of the two lobes of the enzyme with a consequent closure of the interlobar cleft (Anderson *et al.*, 1979). It is not inconceivable, therefore, that the protective effect of D-glucose and analogous sugars is due to the conformational change and consequent alteration in Procion Green H-4G binding to the nucleotide-binding lobe.

The apparently essential thiol of cysteine-243 is the most likely candidate for alkylation by the reactive triazine dye. It is known that of the four cysteine residues per monomer, alkylation of this single thiol causes complete inactivation of the enzyme with protection being afforded by substrates of both nucleotide and hexose classes (Otieno *et al.*, 1977).

The binding of some proteins to immobilized dye ligands is known to be dependent on the presence of certain cations, notably Na^+ or K^+ (Messenger &

Zalkin, 1979), Mg^{2+} (Moe & Piszkiwicz, 1976, 1979; Mori & Cohen, 1978), Ca^{2+} (Morrill & Thompson, 1979; Morrill *et al.*, 1979) and even Mn^{2+} (Fulton, 1980). Thus for example, the binding of isoleucyl- and glycyl-tRNA synthetases to immobilized Cibacron Blue F3G-A is enhanced in the presence of Mg^{2+} (Moe & Piszkiwicz, 1976, 1979). Similarly, yeast hexokinase binding to immobilized Procion Green H-4G is potentiated in the presence of Mg^{2+} , apparently by lowering the dissociation constant for the dye-enzyme interaction. Inclusion of 10 mM MgCl_2 in column irrigants promotes enzyme binding to immobilized Procion Green H-4G but not to adsorbents comprising immobilized Procion Blue H-B, Yellow H-5G and Red HE-3B. Unfortunately, lack of information on the structure of Procion Green H-4G precludes a detailed discussion on its mode of interaction with, and inactivation of, yeast hexokinase. Suffice it to say that, of the dyes tested, the Mg^{2+} -dependent interaction between yeast hexokinase and Procion Green H-4G is apparently unique. Indeed, of a number of divalent metal ions tested, only Mg^{2+} significantly protected yeast hexokinase against inactivation by Procion Green H-4G; markedly less protection was observed with Ca^{2+} and Sr^{2+} and no significant protection against inactivation was observed with the transition metal ions Mn^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} or Zn^{2+} (M. J. Goldfinch & C. R. Lowe, unpublished work). The fact that Mg^{2+} encourages the dye to bind more tightly to the enzyme, yet reduces the rate of irreversible inactivation, may be justified on the basis that the Mg^{2+} -dye complex may have a slightly different orientation on the enzyme than the dye alone. In this context, it is known that different divalent metal ions can alter the stereospecificity for the diastereomers of adenosine 5'-O-(2-thiotriphosphate) ($\text{ATP}\beta\text{S}$) by co-ordinating to either the O or S on the β -phosphate (Jaffe & Cohn, 1979). Presumably the binding of Mg^{2+} , and to a lesser extent Ca^{2+} and Sr^{2+} , to Procion Green H-4G induces a conformation which favours binding of the dye to the enzyme whilst at the same time moving the triazine ring away from the reactive nucleophile. However, it is not clear from the present results whether the metal ion acts as a bridge between the dye and enzyme or binds exclusively to the dye, and consequently the dissociation constant of 2.4 mM for Mg^{2+} deduced from the spectral titration of enzyme-dye complex with Mg^{2+} must be interpreted with caution. Nevertheless, Procion Green H-4G is a particularly suitable active site-directed affinity label by virtue of its unusually high molar absorption coefficient. Indeed, in general terms, the reactivity of the chlorotriazine group with nucleophiles, the high molar absorption coefficient of the chromophores, and their high stability and solubility, make reactive triazine dyes ideal 'ready-

made' affinity labels for general use with nucleotide-dependent enzymes (Clonis & Lowe, 1980).

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