The inhibition of glucokinase and glycerokinase from *Bacillus stearothermophilus* by the triazine dye Procion Blue MX-3G

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Glucokinase from *Bacillus stearothermophilus* was irreversibly inactivated by the reactive dichlorotriazinyl dye Procion Blue MX-3G at pH 8.0. The enzyme was protected from inactivation by the substrate MgATP. Kinetic data implied that the dye occupied the MgATP-binding site. The apparent K_m values for MgATP and D-glucose were found to be 70 μM and 210 μM respectively, and the K_d of the pure reactive dye was 16 μM ; 1 mol of the pure reactive dye bound to 1 mol of glucokinase subunit. The dye was shown to have potential as an affinity probe for glucokinase. Glycerokinase from the same bacterium was inactivated by Procion Blue MX-3G at high concentrations (5 mM), but only after a period of increased enzyme activity. Kinetic data indicated that the dye preferentially attacked the glycerol-binding site. The apparent K_m values for MgATP and glycerol were found to be 38 μM and 13 μM respectively, and 4 mol of reactive dye could be bound to 1 mol of glycerokinase subunit. This was surprising in view of the MgATP-dependent elution of glycerokinase from immobilized Procion Blue MX-3G.

INTRODUCTION

Enzymes have been found to bind immobilized triazine dyes, which can therefore be used to purify them by the use of triazine dye affinity chromatography (Thompson *et al.*, 1975; Atkinson *et al.*, 1981; Lowe *et al.*, 1981a; Lowe & Pearson, 1983). The triazine dyes tend to bind preferentially to the active sites of globular proteins (Glazer, 1970), though these may not be catalytic centres (Dean & Watson, 1979). These dyes have been shown to be reversible inhibitors of enzyme catalysis (Lowe *et al.*, 1981a; Dean & Watson, 1979). For many enzymes, particularly dehydrogenases and phosphokinases, competitive inhibition has been observed with respect to the nucleotide substrates (Lowe *et al.*, 1981b). The accumulated evidence indicates that the polysulphonated aromatic chromophores of the triazine dyes mimic naturally occurring heterocyclic biological molecules such as adenosine phosphates, NAD^+ , NADP^+ , acetyl-CoA, folate and flavins (Lowe *et al.*, 1981a; Edwards & Woody, 1979; Johnson & Stevenson, 1980; Small *et al.*, 1981; Clonis *et al.*, 1981). The dyes do not bind solely to proteins with a 'dinucleotide fold', as originally suggested (Thompson *et al.*, 1975); for example the elution characteristics of aminoacyl-tRNA synthetases from triazine dye affinity-chromatography columns indicate that some dyes can mimic amino acids and others the tRNA substrates (Atkinson & Bruton, 1979; McArdell *et al.*, 1982).

The reactive dye binds to the protein with its triazine ring free in solution and in such an orientation that the reactive dye can interact with the nucleophilic amino acids of the protein. In direct contrast, in triazine dye affinity chromatography the dye is immobilized to the matrix via the triazine ring and thus the protein may interact with the dye and bind to it in a different orientation. However, free dyes have been successfully used as affinity probes for nucleotide-dependent enzymes

(Clonis & Lowe, 1980; Clonis *et al.*, 1981). Procion Green H-4G has been shown to inactivate yeast hexokinase specifically, and this inactivation can be prevented by the presence of substrates (Clonis & Lowe, 1980); Procion Blue MX-R has been shown to bind to the nucleotide-binding domain of horse liver alcohol dehydrogenase (Small *et al.*, 1982).

The main aim of the work described in the present paper was to use a dichlorotriazinyl dye, Procion Blue MX-3G, as a probe to determine which binding sites of the glucokinase (ATP: α -D-glucose 6-phosphotransferase, EC 2.7.1.2) and glycerokinase (ATP:glycerol 3-phosphotransferase, EC 2.7.1.30) from *Bacillus stearothermophilus* N.C.I.B. 11270 are attacked by the dye. This information could then be used as the basis for an affinity probe of the enzymes. Both glycerokinase and glucokinase are known to bind Procion Blue MX-3G–Sephacrose-4B, and both can be eluted with MgATP, and, in the case of glucokinase, by ATP only.

MATERIALS AND METHODS

Materials

The triazine dye Procion Blue MX-3G was a gift from Dr. C. V. Stead (I.C.I., Blackley, Manchester, U.K.). Hepes, ATP and α -D-glucose were obtained from Sigma Chemical Co. Glycerol, aq. NH_3 and NaOH were obtained from BDH Chemicals. Kieselgel 60 preparative t.l.c. plates (20 cm $\times$ 20 cm with 2 mm gel depth and without a fluorescent indicator) were obtained from Merck. Sephadex G-15 was obtained from Pharmacia. Homogeneous glycerokinase (specific activity 120 units/mg of protein) was isolated as described by Scawen *et al.* (1983), and homogeneous glucokinase (330 units/mg of protein) was prepared as described by Goward *et al.* (1986). The source of both enzymes was *Bacillus stearothermophilus* N.C.I.B. 11270.

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Glucokinase and glycerokinase activity

Glycerokinase activity was measured by the method of Garland & Randle (1962). Glucokinase activity was assayed by a method similar to that described for glycerokinase, except that glycerol was replaced by glucose in the assay system (Goward *et al.*, 1986). One unit of activity is defined as the amount of enzyme that catalyses the phosphorylation of 1 μ mol of substrate/min.

Purification of Procion Blue MX-3G and preparation of dye derivatives

Reactive Procion Blue MX-3G was purified on silica-gel preparative t.l.c. plates. The solvent system used was butan-2-ol/propan-1-ol/ethyl acetate/water (2:4:1:3, by vol.). The major reactive dye band was scraped from the plate, and the dye was eluted from the silica with water. It was then filtered and freeze-dried. The dye was subsequently rechromatographed in the same solvent system to show homogeneity. Hydroxylated dye was prepared by dissolving 1 mmol of pure dye in 10 ml of water, adjusted to pH 10.5 with NaOH and incubated at 65 °C for 48 h, and finally freeze-dried. Aminated dye was prepared by reacting 1 mmol of pure dye dissolved in 10 ml of 1 M-NH₃ at 65 °C for 3 h. Ar was passed over the solution to remove excess NH₃, and the aminated dye was then freeze-dried.

Inactivation and protection of the enzymes

Glucokinase (150 pmol) was inactivated at 25 °C in a total volume of 1 ml in 20 mM-Hepes/NaOH buffer, pH 8.0, containing 10–100 nmol of pure reactive dye. The reaction mixture contained 250 mM-KCl to prevent stacking of the dye molecules at the higher concentrations. The rate of inactivation was followed by removing 25 μ l samples for assay at various time intervals.

Glycerokinase (100 pmol) was investigated in a similar manner but the dye concentration was varied between 50 and 5000 nmol/ml.

Determination of K_i for substituted dye

Inhibitor constants for both hydroxylated and aminated Procion Blue MX-3G were determined with respect to the substrates of glucokinase and glycerokinase. The assay mixture was in a total volume of 1 ml and contained 0–10 nmol of dye. MgATP at 1.9 mM was the saturating fixed-concentration substrate for glucokinase. The dye concentration was varied with a series of glucose concentrations around the apparent K_m . Glucose was also used as the saturating fixed-concentration substrate at 2.5 mM when the MgATP concentration was varied. Glycerokinase was treated in a similar fashion, with glycerol replacing glucose at a fixed saturating concentration of 2.4 mM.

Fig. 1. Structure of Procion Blue MX-3G

Fig. 2. Determination of K_d of pure reactive Procion Blue MX-3G

A double-reciprocal plot of the rates of inactivation of 150 pmol of glucokinase by increasing dye concentrations is shown. K_d was determined from the intercept on the abscissa.

Stoichiometry

The amount of dye covalently bound to the enzyme was determined quantitatively by treating 3.7 nmol of glucokinase and 0.43 nmol of glycerokinase with 500 nmol of dye each for 2 h in 20 mM-Hepes/NaOH buffer, pH 8.0. The dye-enzyme conjugate was isolated by gel filtration on Sephadex G-15 equilibrated with 20 mM-Hepes/NaOH buffer, pH 8.0. The protein concentration of the eluate was determined by the Coomassie-Brilliant-Blue-binding method of Bradford (1976), with bovine γ -globulin as standard. The dye-conjugated protein was made 8 M with respect to urea, and the dye concentration was determined from the absorbance at 616 nm. The molar absorption coefficient of Procion Blue MX-3G was determined to be 14700 M⁻¹·cm⁻¹. Substrates were also added to the reaction mixture at a concentration of 5 mM. In these instances the gel-filtration column was equilibrated in and eluted with buffer containing an identical concentration of the appropriate substrate. Addition of urea or the binding of dye to denatured protein did not affect the absorbance of the dye.

RESULTS AND DISCUSSION

The structure of Procion Blue MX-3G is shown in Fig. 1. The reactive chlorine atoms on the triazine ring were replaced with hydroxy or amine groups by substitution.

The reaction between an active-site-directed reactive dye (D) and an enzyme (E) may be described by the following equation:

where $E \cdot D$ is the enzyme-dye conjugate, ED' is the irreversibly inactivated enzyme and k_{+2} is a rate constant for the rate-limiting step (Clonis & Lowe, 1980). A steady-state treatment of the process produces the equation:

$$\frac{1}{k_{\text{obs}}} = \frac{1}{k_{+2}} + \frac{K_d}{k_{+2}} \cdot \frac{1}{[D]}$$

Fig. 3. Protection of glucokinase from inactivation by Procion Blue MX-3G

A 150 pmol portion of glucokinase was incubated with 100 nmol of pure reactive dye in the presence of a control containing no dye (○), a control containing no protector (■), 5 mM-ATP (●), 5 mM-glucose (▲), 5 mM-Mg²⁺ (△), 5 mM-ATP + 5 mM-glucose (□), 5 mM-ATP + 5 mM-Mg²⁺ (⊙), 5 mM-Mg²⁺ + 5 mM-glucose (△) or 5 mM-ATP + 5 mM-Mg²⁺ + 5 mM-glucose (⊞). The enzyme activity was then assayed at various time intervals after addition of the dye. Further experimental details are included in the text.

where k_{obs} is the observed rate of enzyme inactivation at a given concentration of the dye, D , k_{+2} is the maximum rate of inactivation (min^{-1}) and K_d is the apparent dissociation constant, k_{-1}/k_{+1} , of the enzyme-dye complex (Kitz & Wilson, 1962; Clonis & Lowe, 1980; Witt & Roskoski, 1980; McArdell *et al.*, 1982).

Glucokinase was covalently inactivated by the triazine dye Procion Blue MX-3G. The rate of inactivation of glucokinase by reactive dye increased as the amount of dye was increased, but with amounts of dye greater than 100 nmol inactivation was decreased. The K_d of pure reactive dye with glucokinase was determined to be $16 \mu\text{M}$ and the maximum rate of inactivation for the reaction, k_{+2} , was 0.645 min^{-1} (Fig. 2). The K_d is considerably lower than the apparent K_m values for MgATP ($70 \mu\text{M}$) or glucose ($210 \mu\text{M}$). ATP and MgATP appeared to exclude the dye from the active centre, indicating that the dye was recognized by the effector-binding site (Fig. 3). Glucose and Mg²⁺ did not protect the enzyme from attack by the dye, and therefore the binding sites for these substrates were not involved in the binding of dye. Glucose with MgATP is an active mixture for glucokinase; ATP is converted into ADP, and therefore the protective effect of ATP is removed. An accelerated rate of inactivation is thus observed as substrates are utilized.

The rate of inactivation of glycerokinase by reactive Procion Blue MX-3G was not rapid enough to allow accurate quantitative experiments. The situation was further complicated by the fact that an apparent transient initial activation of the glycerokinase molecule was observed before inactivation occurred, implying that dye binding beneficially altered the conformation of the

Fig. 4. Time course for the inactivation of glycerokinase by Procion Blue MX-3G

A 100 pmol portion of glycerokinase was incubated with a control containing no dye (●) or 50 nmol (▲), 100 nmol (■), 500 nmol (△) or 5000 nmol (□) of pure reactive dye. Further experimental details are included in the text.

enzyme. A $5 \mu\text{mol}$ amount of dye was required to inactivate 50% of the enzyme in 120 min in the 1 ml reaction volume (Fig. 4).

In the absence of dye, the apparent K_m values of glucokinase for MgATP and glucose were $70 \mu\text{M}$ and $210 \mu\text{M}$ respectively (Fig. 5). The apparent K_m values of glycerokinase for MgATP and glycerol were $38 \mu\text{M}$ and $13 \mu\text{M}$ respectively (Fig. 6). These results were in approximate agreement with values obtained with different assay systems (Comer *et al.*, 1979; Goward *et al.*, 1986).

The inhibition characteristics of the two enzymes by the substituted dyes, deduced from the results shown in Figs. 5 and 6, are presented in Table 1. The hydroxylated dye showed uncompetitive kinetics with glucokinase, suggesting that the dye bound to the enzyme together with MgATP to form an inhibitory ternary complex, in which the dye interacts with the enzyme at a site separate from the catalytic ATP-binding site. In contrast, the catalytic ATP-binding site was accessible to the aminated dye. Space-filling models of the aminated and the hydroxylated dye have been constructed and indicate that the two derivatives have different conformations, which may affect binding. Derivatives of Procion Brown MX-5BR also showed different inhibition characteristics with tryptophyl-tRNA synthetase, where the aminated dye mimicked tryptophan to bind the enzyme more specifically than did the hydroxy and methoxy derivatives (McArdell *et al.*, 1982). The lack of involvement of the glucose-binding site of glucokinase was confirmed by the

Fig. 5. Inhibition of glucokinase by substituted dyes

Double-reciprocal plots for the determination of inhibitor constants from a series of dye concentrations: no dye (●), 25 μM (○), 50 μM (▲), 75 μM (△) and 100 μM (■). (a) and (c) Hydroxylated Procion Blue MX-3G; (b) and (d) aminated Procion Blue MX-3G.

non-competitive nature of the kinetic data. The kinetic data for glycerokinase indicated that the glycerol-binding site interacted with the dye, and the presence of MgATP uncovered further dye-binding sites by inducing a conformational change of the enzyme. This was supported by the elution of glycerokinase from Procion Blue MX-3G-Sepharose-4B by either MgATP (Scawen *et al.*, 1983) or glycerol (C. R. Goward, M. D. Scawen & T. Atkinson, unpublished work). Although it was likely the dye interacted with the glycerol-binding site, MgATP was actually the most effective eluting agent, implying the presence of coupled binding sites in which MgATP induces a conformational change in the enzyme. The stoichiometry of 4 mol of covalently bound dye/mol of glycerokinase subunit implied the presence of multiple binding sites for the reactive dye and that the reaction was not specific to the glycerol-binding site.

Quantitative studies showed that 1 mol of dye bound/mol of glucokinase subunit of M_r 33000. The binding

stoichiometry implies a specific and selective interaction between dye and protein. Similar binding stoichiometry was observed between Procion Green H-4G and yeast hexokinase (Jörnvall, 1970) and between Procion Blue MX-R and horse liver alcohol dehydrogenase (Small *et al.*, 1982). Predictably the binding stoichiometry was not altered by the presence of glucose. However, the addition of MgATP, under identical conditions, appears to induce a conformational change that reveals two further sites accessible to attack by the dye. The dye therefore possesses a good affinity for the enzyme, and the enzyme is protected from inactivation by the effector, has a stoichiometry of 1 mol of dye/mol of binding site and is a competitive inhibitor of the ATP-binding site. The ATP-binding site of glucokinase may therefore be probed as described for horse liver alcohol dehydrogenase and Procion Blue MX-R (Small *et al.*, 1982). In the case of glycerokinase, 4 mol of dye bound/mol of enzyme subunit of M_r 58000. This stoichiometry implied

Fig. 6. Inhibition of glycerokinase by substituted dyes

Double-reciprocal plots for the determination of inhibitor constants from a series of dye concentrations: no dye (●), 25 μM (○), 50 μM (▲), 75 μM (△) and 100 μM (■). (a) and (c) Hydroxylated Procion Blue MX-3G; (b) and (d) aminated Procion Blue MX-3G.

Table 1. Inhibition of glucokinase and glycerokinase by derivatives of Procion Blue MX-3G

Inhibitor constants were determined from double-reciprocal plots made at a series of inhibitor concentrations. The intercepts on the ordinate from these curves were plotted against the inhibitor concentrations and the K_i values were determined directly for uncompetitive and non-competitive inhibition. For competitive inhibition the K_i value was determined from the slopes of curves plotted against the inhibitor concentration.

Enzyme	Substituent of Procion Blue MX-3G	Substrate whose concentration was varied	Inhibition	K_i (μM)
Glucokinase	Hydroxy	MgATP	Uncompetitive	180
Glucokinase	Amino	MgATP	Competitive	47
Glucokinase	Hydroxy	Glucose	Non-competitive	100
Glucokinase	Amino	Glucose	Non-competitive	130
Glycerokinase	Hydroxy	MgATP	Uncompetitive	210
Glycerokinase	Amino	MgATP	Uncompetitive	280
Glycerokinase	Hydroxy	Glycerol	Competitive	36
Glycerokinase	Amino	Glycerol	Competitive	110

the presence of multiple binding sites for the reactive dye and that the reaction was not specific to the glycerol-binding site. When MgATP or glycerol was present no binding of dye to the enzyme was observed. The Procion Blue MX-3G was therefore unlikely to be a useful affinity label for a specific binding site on the glycerokinase molecule, whereas with glucokinase the reaction of the dye was specific for the ATP-binding site. The presence of either MgATP or glycerol protected glycerokinase to the extent that reactive dye did not covalently bind to the enzyme, which supports the hypothesis of coupled binding sites. The glycerokinase had high affinity for MgATP (apparent K_m 38 μM) and glycerol (apparent K_m 13 μM) and apparently low affinity for reactive dye in the catalytic sites, and thus the binding sites were made unavailable by interaction with the natural substrates.

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