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STUDIES ON THE INTERACTION OF FIREFLY LUCIFERASE WITH TRIAZINE DYES

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SUMMARY

The interaction of firefly luciferase with several triazine dyes has been investigated. Of those tested, the anthraquinone dye, Procion blue MX-R, displayed relatively high affinity [dissociation constant (K_D) = 5 μM] and irreversibly inactivated the enzyme in a time dependent fashion. The substrates, Mg–ATP (ATP is adenosine triphosphate) and luciferin, protected the enzyme against inactivation with Procion blue MX-R and following quantitative inactivation, incorporation of 1 mol dye per mol enzyme [molecular weight (M_r) = 50 000] was observed. It is suggested that Procion blue MX-R binding is active site-directed and that the dye binding traverses both Mg–ATP and luciferin binding sites. These data are exploited to evolve an improved purification protocol for firefly luciferase.

INTRODUCTION

Ever since Thompson *et al.*[1] suggested in 1975 that the chromophore of blue dextran, the triazine dye, Cibacron blue F3G-A, functioned as a nucleotide or coenzyme analogue, imitating the overall shape, aromaticity and charge distribution of natural nucleotide substrates, much progress has been made in the exploitation of these dyes[2–4]. Thus, immobilised Cibacron blue F3G-A and other reactive chlorotriazine dyes have been exploited as "group specific" adsorbents for the purification of proteins by affinity chromatography[5–9], for high-performance liquid affinity chromatography[10,11], and in analytical protein studies[12,13]. Recently, they have also been used as effective irreversible affinity labels for nucleotide-dependent enzymes[14–16]. Thus several reactive dichlorotriazinyl dyes were reported to specifically and irreversibly inactivate pig heart lactate dehydrogenase, glucose-6-phosphate dehydrogenase and yeast hexokinase at sites competitive with nicotinamide–adenine dinucleotide (NAD^+), nicotinamide–adenine dinucleotide phosphate ($NADP^+$) and adenosine triphosphate (ATP) respectively[14]. In addition, the aminated derivative of

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Procion brown MX-5BR has been shown to be a competitive inhibitor of the tryptophanyl-tRNA synthetase[17].

The objective of the present work was to study the affinity of various triazine dyes for luciferase in order to establish the nature of the dye enzyme interaction and thereby establish a better method for separating luciferase from other contaminating enzymes found in the crude extract by using such data to devise new affinity adsorbents.

Firefly luciferase has gained much popularity and importance in recent times because of the extreme specificity of the enzyme for ATP. It has been widely exploited for the measurement of ATP in a variety of samples. The essence of the reaction is represented by the following two equations:

$$LH_2 + ATP + E \overset{Mg^{2+}}{\rightleftharpoons} E\text{–}LH_2\text{–}AMP + PP \quad (1)$$

$$E\text{–}LH_2\text{–}AMP + O_2 \rightarrow E + Pr + CO_2 + AMP + h\nu \quad (2)$$

where E is the enzyme, LH_2 is luciferin, E–LH_2–AMP the luciferyl adenylate complex, Pr the product (oxyluciferin), AMP is adenosine 5′-monophosphate and P is phosphate.

Firefly luciferase was purified by affinity elution chromatography on blue dextran columns presumably due to the attraction of the blue chromophore, Cibacron blue F3G-A to the ATP binding site of the enzyme[18]. Following this, studies were undertaken to establish the mode of binding of luciferase to Procion blue H-B and other related triazine dyes such as Procion brown MX-5BR, Orange MX-G, Red HE-3B, Blue MX-2G and Green HE-4BD[19]. The effect of post-immobilisation chemical modification of these dyes was also investigated in order to determine the significance of some of the functional groups of the dye structure in the binding of the enzyme. As a result of these studies a model of stereospecific ionic interaction was proposed between luciferase and triazine dyes[19].

This paper deals with some affinity labelling studies of luciferase with triazine dyes such as Procion blue MX-R, Procion green HE-4BD, Procion blue HE-RD, Procion red HE-3B and Procion red H-3B.

MATERIALS AND METHODS

Chemicals

The Procion dyes used in this study were a generous and much appreciated gift from Dr. C. V. Stead, (ICI organics Division, Blackley, Manchester, U.K.). Luciferase from *Photinus pyralis* was purchased from Boehringer. ATP, luciferin, N-tris(hydroxymethyl)methylglycine (tricine), 1,4-dithiotreitol (DTT) and N-ethylmaleimide were from Sigma. Sepharose 4B was from Pharmacia; buffer from Merck. All other chemicals were obtained from BDH. Dyes are referred to in this paper by their commercial ICI names.

Enzyme assay using a liquid scintillation spectrophotometer

The method is based on a quantum counter which measures the number of photons produced over a definite time interval after the addition of the enzyme.

Enzyme activity was assayed at 24°C using a PW-4700 liquid scintillation counter, previously programmed for luciferase assays. The experimental parameters were: lower limit LL, 1.0; upper limit UL, 500; pre-set time PT, 3; background BKG, 1.0; pre-set count PC, 90 000; CNT count, 1. The reaction mixture comprised 2.12 ml total volume: tricine buffer, 0.1 *M* (pH 7.8) containing 10 m*M* magnesium sulphate; luuciferin, $1.4 \cdot 10^{-6}$ *M*; ATP, $2.12 \cdot 10^{-6}$ *M*; DTT, 0.05 mmol and enzyme $3.77 \cdot 10^{-3}$ units. Luciferase activity was estimated by this method for all affinity labelling studies.

Enzyme assay using a nucleotimeter

In this case, the intensity of the initial light flash is measured by a photomultiplier. The reaction mixture containing 50 μl of 10^5 pg/ml ATP in 0.01 *M* MOPS (pH 7.4) with 10 m*M* magnesium sulphate, 50 μl of luciferin (0.168 μg/ml) and 50 μl of luciferase sample was introduced in a special cuvette in the nucleotimeter (Interbio, France) at 18–20°C and the intensity of light in mV during the first 2 s of emission was recorded at 562 nm.

This method was followed for all chromatographic studies reported here.

Purification of triazine dyes

The triazine dyes used in this work were purified by ascending preparative thin-layer chromatography on Kieselgel 60 silica-glass plates (20 × 20 × 2 cm) using a solvent system consisting of butan-1-ol–propan-2-ol–ethyl acetate–water (2:4:1:3, v/v). Crude dye was dissolved in distilled water (30–60 mg/ml), applied as a narrow continuous streak and chromatographed overnight at room temperature. The plates were dried and the principal dye band scraped off, eluted in methanol in a Buchner funnel and lyophilysed.

Dye concentrations were determined spectrophotometrically at λ_{max} using the following molar absorption coefficient valucs, determined in distilled water: Procion blue MX-R 4200 l mol^{-1} cm^{-1}; Procion green HE-4BD 20832 l mol^{-1} cm^{-1}; Procion blue HE-RD 7734 l mol^{-1} cm^{-1}; Procion red HE-3B 30103 l mol^{-1} cm^{-1}; Procion red H-3B 15050 l mol^{-1} cm^{-1}.

Inactivation of luciferase by triazine dyes

Firefly luciferase was inactivated as described below in 0.1 *M* tricine, (pH 7.8) containing 10 m*M* magnesium sulphate. The reaction vial contained in a total volume of 1.0 ml: enzyme (50 μg, $5 \cdot 10^5$ arbitary units or 0.4 units), triazine dye as mentioned in individual cases and DTT (50 μM).

The rate of inactivation of the enzyme at 25°C was followed by periodically removing samples (20 μl) and assaying for enzyme activity. In addition, the inactivation of luciferase was performed in the presence of Mg–ATP, ATP, luciferin and Mg^{2+}. Initial rates of inactivation were deduced from plots of $\log_{10}$ (% of activity remaining) *versus* time (min) for several dye concentrations and the slopes and intercepts of secondary double reciprocal plots were calculated. A sample without the dye served as the control.

Preparation of luciferase labelled with Procion blue MX-R

Firefly luciferase (1 mg) was inactivated with Procion blue MX-R essentially

as described above. The decrease in enzyme activity was monitored until it was reduced to less than 5% of the original. Enzyme thus inactivated was used for stoichiometric studies.

Stoichiometry of dye binding

The amount of dye covalently attached to the enzyme was determined as follows: Quantitatively inactivated enzyme was precipitated from free dye by the addition of 20:1 (v/v) ice cold ethanol (98%) drop by drop. The precipitate was carefully removed by centrifugation (10 min at 10 000 rpm) in a MSE S centrifuge, washed with ethanol and recentrifuged. The labelled protein was dissolved in 8 *M* urea (1 ml) and the absorbance was measured at 625 nm *versus* an 8 *M* urea blank. The concentration of the bound dye was determined using the molar extinction coefficient for Procion blue MX-R in 8 *M* urea *i.e.* 14 940 l mol^{-1} cm^{-1}. Protein concentration was determined by the method of Lowry *et al.*[20]. The stoichiometry was also determined in the presence of ATP and luciferin.

Inactivation of luciferase by a sulphydryl reagent

Luciferase was incubated at 0°C with an excess of N-ethylmaleimide (two fold molar excess) until all enzymatic activity was lost. Stoichiometry of dye binding was deduced as described above.

Preparation of crude extract of luciferase

A crude extract of luciferase was prepared as described elsewhere[18] and dialysed against 0.02 *M* Tris acetate buffer containing 1 m*M* ethylenediaminetetraacetic acid (EDTA) (pH 7.8) for 24 h with three changes of the buffer. This dialysed and partially purified preparation was used in all chromatographic studies.

Immobilisation of Procion blue MX-R

Procion blue MX-R was coupled to Sepharose 4B by the procedure of Baird *et al.*[21].

The immobilised dye concentration was determined by acid hydrolysis of the gels[7]. Moist gel (30 mg) was transferred to 5 *M* hydrochloric acid (0.6 ml), incubated at 37°C for 5 min and 2.5 *M* sodium phosphate buffer (2.4 ml) was added. The dye concentration was determined spectrophotometrically at 625 nm and calculated from the molar absorption coefficient cited above.

Chromatography of luciferase on a Sepharose-immobilised Procion blue MX-R column

The chromatographic elutions were performed at 4°C. A sample (0.3 ml) of the dialysed luciferase corresponding to 3.27 units of protein was applied to a column (1.5 × 16 cm I.D.) of Sepharose bound dye (6.5 μmol dye per g moist weight gel) equilibrated with 0.01 *M* MOPS (pH 7.4) containing 10 m*M* magnesium sulphate and 1 m*M* DTT (eluent 1). The column was then sequentially washed with two volumes of the equilibrating buffer (*i.e.* eluent 1), two volumes of the buffer containing appropriate concentrations of ATP or luciferin as the case may be (*i.e.* eluent 2) and finally two volumes of the equilibrating buffer containing 1 *M* sodium chloride (*i.e.* eluent 3). Fractions (1.2 ml) were collected at 17 ml h^{-1}, dialysed against 0.02 *M* Tris acetate (pH 7.8) with 1 m*M* EDTA for 4–8 h before checking for protein concentration at 280 nm and luciferase activity by nucleotimeter assay.

RESULTS

The chemical structures of two of the anthraquinone dyes used in this study are illustrated in Fig. 1. The structural similarity between the monochlorotriazinyl dye Cibacron blue F3G-A and the more reactive dichlorotriazinyl dye Procion blue MX-R is clearly evident.

The reaction between an active site-directed reactive dye (D) and an enzyme (E) may be formulated as described by Clonis and Lowe[14]:

$$E + D \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{–}D \xrightarrow{k_3} ED$$

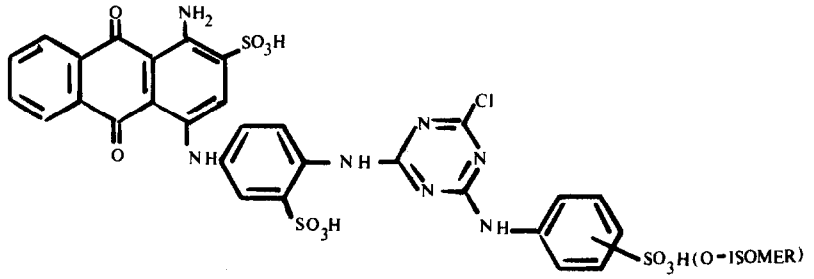
where E–D is the enzyme–dye complex, ED is the irreversibly inhibited enzyme and k_3 is the rate limiting step. A steady state treatment of the process yields the equation

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

where k_{obs} is the observed rate of enzyme inactivation for a given concentrationn of the dye D, k_3 is the maximal rate of inactivation (min^{-1}) and K_D is the dissociation constant (k_2/k_1) of the enzyme–dye complex.

A

CIBACRON BLUE F3G-A C.1.61211



B

PROCION BLUE MX-R C.1.61205

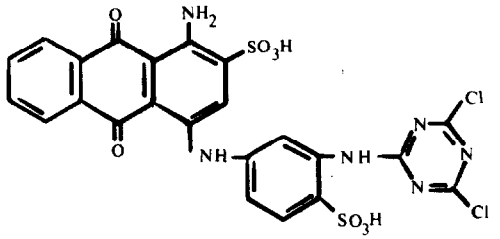


Fig. 1. The structures of (A) Cibacron blue F3G-A and (B) Procion blue MX-R.

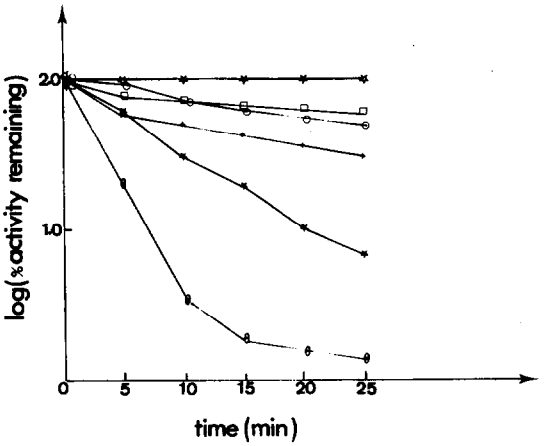


Fig. 2. Time course for the inactivation of firefly luciferase by several Triazine dyes at pH 7.8 and 25°C. See text for experimental details. Key to symbols: ☆, control; ○, Procion blue MX-R (4 nmol); □, Procion green HE-4BD (100 nmol); ★, Procion red HE-3B (1.2 μmol); ◆, Procion red H-3B (58 μmol); ⊖, Procion blue HE-RD (38.57 μmol).

The inactivation of luciferase by several triazine dyes is shown in Fig. 2. The range of dye concentrations varied from dye to dye as can be seen in the figure. Dissociation constants (K_D) of the dyes tested (Table I) suggest that the monochlorotriazynyl dyes of the designation "H" or "HE" do not significantly inactivate luciferase. In contrast, Procion blue MX-R, irreversibly inactivates luciferase with over 90% loss of the enzyme activity within 25 min at pH 7.8 and at 25°C. The dissociation constants (K_D) calculated as described above reveal that Procion blue MX-R (K_D = 5.3 μM) has the strongest affinity for luciferase.

In order to test the validity of the hypothesis that Procion blue MX-R is an active-site directed reagent, luciferase was incubated with the dye in the presence of substrates. Fig. 3 demonstrates that the rate of inactivation is significantly retarded in the presence of 0.5 or 0.75 mM luciferin and almost completely protected by 0.2 mM Mg–ATP. The protective effect of ATP without Mg^{2+} was also verified. An Mg–ATP complex was found to be slightly more efficient in protecting the enzyme from Procion blue MX-R although ATP alone did offer protection to a considerable

TABLE I

DISSOCIATION CONSTANTS (K_D) OF PROCION DYES FOR FIREFLY LUCIFERASE

The inactivation of luciferase by triazine dyes was performed in 0.1 M tricine buffer (pH 7.8) containing 10 mM magnesium sulphate at 25°C. See text for details.

Procion dye	*K_D (mM)*
Blue MX-R	0.005
Green HE-4BD	0.5
Blue HE-RD	67
Red HE-3B	125
Red H-3B	>1000

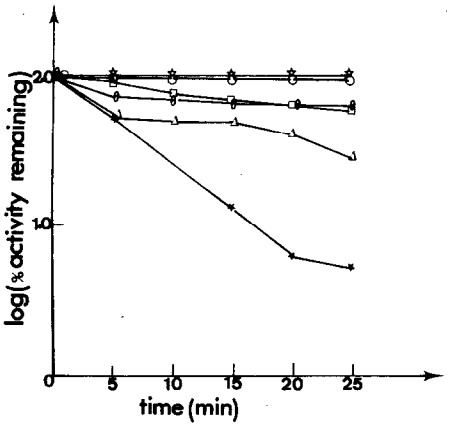


Fig. 3. Effect of competing substrates in the time course for the inactivation of firefly luciferase by Procion blue MX-R (5 nmol). Key to symbols: ☆, control; ★, no addition; □, 0.1 μmol Mg–ATP; ○, 0.2 μmol Mg–ATP; △, 0.5 μmol LH_2; ⊖, 0.75 μmol LH_2.

extent. Mg^{2+} alone, on the other hand, did not protect luciferase from inactivation by Procion blue MX-R. Interestingly, luciferase inactivation by the other dyes used in this study could not be protected by either ATP or luciferin.

Experiments on the stoichiometry of Procion blue MX-R binding to luciferase revealed that 1 mol dye was bound per mol enzyme [molecular weight (M_r) = 50 000] (Table II). In the presence of 1 m*M* Mg–ATP or 0.75 m*M* luciferin, there was complete inhibition of dyebinding. Luciferase inactivated with the sulphydryl reagent, N-ethylmaleimide, still bound the same amount of dye as the native enzyme. In addition, there was no effect of pH on the binding of Procion blue MX-R to luciferase, in the pH range 6–9.

The purification of luciferase on Sepharose-immobilised Procion blue MX-R is presented in Table III.

Elution of the bound enzyme with 0.5 m*M* ATP in the equilibration buffer leads to an enzyme preparation purified 3.6-fold. On the other hand, elution with 0.5 m*M* luciferin gives a markedly inferior purification. As is evident from Table III, the purification of luciferase by a Procion blue MX-R column could prove to be supe-

TABLE II

STOICHIOMETRY OF BINDING OF PROCION BLUE MX-R TO LUCIFERASE

Additions	*mol dye/mol enzyme subunit (M_r = 50 000)*
None	1.04
0.2 m*M* ATP	0
1 m*M* ATP	0
30 m*M* ATP	0
0.75 m*M* luciferin	0
N-Ethylmaleimide-reacted luciferase	1.06

TABLE III

PURIFICATION OF LUCIFERASE ON DYES COUPLED TO SEPHAROSE 4B

Dye	*Eluent*	*Activity regained (%)*	*Protein recovered (%)*	*Purification (fold)*
Blue dextran	0.5 m*M* ATP	180	63	2.9
Blue MX-R	0.5 m*M* ATP	200	52	3.6
Blue MX-R	0.5 m*M* LH_2	80	50	1.8

rior compared to that reported by blue dextran coupled to Sepharose. The ballast protein is stripped off by the equilibrating buffer and luciferase is specifically eluted by adding 0.5 m*M* ATP in the same buffer. The purified fractions did not exhibit any of the contaminating enzyme activity and there was no background noise when assayed with ATP.

DISCUSSION

Blue dextran, a semispecific, high-molecular-weight compound substituted with the monochlorotriazynyl dye Cibacron blue F3G-A is known to interact with firefly luciferase[18]. Our present studies indicate that Procion blue MX-R, a dichlorotriazinyl structural analogue of Cibacron blue F3G-A, also binds specifically to luciferase.

Procion blue MX-R inactivates luciferase in a time-dependent fashion. The hyperbolic dependence of inhibition on dye concentration, the protection against inhibition exhibited by the substrates ATP and luciferin and the equimolar ratio of bound dye to enzyme are characteristic of active-site directed irreversible inhibition[14–16,22]. The decrease in the rate of inactivation and the ultimate protection afforded by both ATP and luciferin suggest that the dye binding may traverse both the nucleotide and luciferin binding domains on the enzyme. A similar observation was made by Clonis *et al.*[15] in the case of the binding of Procion green H-4G to yeast hexokinase. It was suggested that the copper phthalocyanine dye was binding across the nucleotide and sugar binding sites of the enzyme. Dye occupation of both coenzyme and substrate binding sites has also been proposed with the interaction between Cibacron blue F3G-A and lactate dehydrogenase[5], 3-phosphoglycerate kinase[5] and adenylosuccinate synthetase[14].

Studies on the substrate binding properties of luciferase have shown that it is a dimer of 50 000 molecular weight subunits with the enzymatically active substrate, Mg–ATP, binding to only one site and luciferin and ATP to two sites per 100 000 molecular weight of the enzyme[23,24]. However, only one of the subunits in the 100 000 molecular weight aggregate is enzymatically active. Mg^{2+} was not found to bind to luciferase by itself which also explains why Mg^{2+} alone could not protect the enzyme from inactivation by the dye. It has also been suggested that the active subunit must contain both the sites for luciferin binding and one for Mg–ATP.

Binding studies of luciferase with naphthalene dyes (2,6-toluidinonaphthalene-sulphonate and 1,5-anilinonaphthalenesulfonate) revealed approximately 2 mol of dye binding per mol of enzyme[25].

These dyes were found to bind to the luciferin site in a very specific manner. Nevertheless, dye binding was not hindered when thiol groups found at the luciferin site were oxidized.

From the findings reported here and those found in the literature, it seems appropriate to suggest that Procion blue MX-R binds to luciferase in a manner reminiscent of the binding of Procion green H-4G to yeast hexokinase. It is quite conceivable that the dye binds across the two sites, Mg–ATP and luciferin, since both protect luciferase from inactivation by Procion blue MX-R. This explanation is supported by the proposals of McElroy *et al.*[26] which describe the action of luciferase. Since the carboxylic acid group of luciferin reacts with the AMP–PP bond of ATP, the two substrates must be in close proximity to each other on the surface of the enzyme.

Binding of substrates to the catalytic site of luciferase has been reported to result in remarkable conformational changes[26]. It is hence conceivable that the protective ability of Mg–ATP and/or luciferin against inactivation by Procion blue MX-R is due to the effect of conformational changes on the dye–enzyme interaction and occasioned by substrate binding. Lack of crystallographic data on luciferase prevents a more precise interpretation of the data.

Luciferase is known to contain eight thiol groups of which two are indispensible for catalytic activity[27]. These two thiol groups have been suggested to be at the luciferin binding site, at or near the position where the carboxylic moiety of luciferin is bound, perhaps even slightly buried[28]. The apparent non-involvement of thiol groups in the binding of the dye to luciferase again suggests that the dye molecule overlaps the Mg–ATP and luciferin sites but in such a way that it does not disturb the "buried" thiol group of the enzyme. The complete lack of pH effect on binding (over the range studied) indicates that the groups which ionize in this region, do not affect the binding site for the dye. DeLuca[25] observed the same phenomenon in the binding of naphthalene dyes to luciferase.

Clonis *et al.*[15] suggested that investigation of dye binding in solution could provide useful information on which to base new purification protocols. The specific, active site-directed binding of Procion blue MX-R to luciferase was thus exploited to evolve a superior procedure for its purification. The fact that both Mg–ATP and luciferin could elute luciferase from Sepharose 4B-bound Procion blue MX-R with enhanced specific activity supports the hypothesis that the dye is binding across both two sites.

This work confirms the previous indications by Lowe and co-workers[14–16] that reactive triazine dyes make ideal covalent labels for nucleotide-dependent enzymes.

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