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INTERACTION OF FIREFLY LUCIFERASE WITH TRIAZINE DYES

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SUMMARY

Interaction of firefly luciferase with triazine dyes immobilised on Sepharose 4B has been studied by affinity elution chromatography. Sepharose-immobilised Procion dyes were subjected to chemical modifications in order to change their functional groups or parts. An attempt was made to assess the significance of specific parts of the dye chromophore in the dye–luciferase interactions. These modifications in the dye structure were checked simultaneously by a ratio-recording spectrophotometer. Any change in the polycyclic nature of the dye, *e.g.* cleavage of the azo linkage resulting in the removal of sulphonated parts, was found to result in weakened dye–enzyme interactions. Azo bonds, together with the polysulphonated groups, were found to contribute in some way to the specificity of the binding reaction. A case of stereospecific ionic interaction is proposed between luciferase and triazine dyes.

INTRODUCTION

Reactive triazine dyes are currently becoming extremely popular in both preparative and analytical fields, especially for those enzymes dependent on nicotinamide–adenine dinucleotide (NAD), adenosine triphosphate (ATP) and other polynucleotides[1,2]. It is hence not surprising that a number of studies have been initiated to establish the intricate mechanism of the action of these dyes[3,4]. It was suggested initially that the polysulphonated aromatic chromophores of the triazine dyes mimic certain heterocyclic nucleotide phosphates. However further work along these lines proved contradictory for it was found that only part of these chromophores actually mimic the specific co-factors, coenzymes or substrates with the complementary enzyme[5]. Following this, hydrophobic, ionic and charge-transfer interactions were attributed to explain the affinity of biological macromolecules towards aromatic dyes[6].

It was reported briefly by Lowe[7] that reduction of certain triazine dyes with sodium dithionite in alkaline solution is accompanied by marked structural changes in the dye and an alteration in the chromatographic properties towards yeast–glucose-6-phosphate dehydrogenase. This was followed by the work of Clonis[8] who conducted a series of experiments to give information on the functional groups or parts of the dye structure which appear to be important in the binding of the enzyme. Triazine dyes, immobilised on Sepharose, were then chemically modified to study their specificity.

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The purpose of the present study was to examine the different interactions between the mono- and dichlorotriazinyl sulphonated dyes and luciferase and to study the effect of post-immobilisation chemical modification of some of these dyes on luciferase binding.

The theory and application of luciferase-catalysed bioluminescent reactions are well documented[9]. The purification of luciferase by affinity elution chromatography on Sepharose Blue Dextran, a semi-specific high-molecular-weight compound substituted with the monochlorotriazinyl dye, Cibacron blue F-3GA (the o-isomer of Procion blue), has already been reported[10]. It was thus interesting to study the mode of binding of luciferase to Procion blue and other related triazine dyes which could help in an improved method of purification. A study of this nature was also intended to explain the less-known aspects of the luciferase–triazine dye interaction, such as the specificity of the functional groups of the dye structure in the binding of enzymes.

EXPERIMENTAL

Materials

Firefly lanterns (*Photinus pyralis*) and ATP were purchased from Sigma, Sepharose 4B from Pharmacia and 1,4-dithiothreitol (DTT) and 3-N-morpholinopropanesulphonic acid (MOPS) from E. Merck.

The triazine dyes were a much appreciated gift from Dr. C.V.Stead (ICI Organics Division, Blackley, Manchester, U.K.) Dyes are referred to in this paper by their commercial names. Those designated "HE" are monochlorotriazinyl dyes while "Mm" are dichlorotriazinyl dyes.

Luciferase extraction. Extraction of luciferase from the firefly abdomen was carried out according to the method published elsewhere[10].

Luciferase assay. Luciferase activity was estimated by a Nucleotimeter (Interbio, France). The reaction mixture containing 100 μl of 10^5 ρg/ml ATP in 0.01 *M* MOPS (pH 7.4) containing 10 m*M* magnesium sulphate, 50 μl of luciferin (0.168 μg/ml) and 100 μl of luciferase sample, was introduced in a special cuvette into the Nucleotimeter at 18–20°C and the maximum intensity of light at 562 nm was recorded in mV. The unit of activity is the maximum intensity of light recorded in mV per pg of ATP per mg of protein at 562 nm.

Chromatographic procedures

Preparation of matrices. Triazine dyes were coupled to Sepharose by the procedure of Dudman and Bishop[11] as modified by Baird *et al.*[12]. Immobilised dye concentrations were determined by acid hydrolysis of the gels[4,13]. Moist gels (30 mg) were 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. Dye concentrations were determined spectrophotometrically at λ_{max}. The amounts of the dyes fixed on the Sepharose, expressed as μmol of dye per gram of moist weight gel, were as follows: Brown Mx-5BR, 6.6; Orange MxG, 6.3; Red HE 3B, 5.6; Blue Mx2G, 4.1; Green HE 4BD, 2.3.

Elution. All chromatographic elutions were performed at 4°C. A sample of the dialysed luciferase was applied to a column of Sepharose-bound triazine dye, 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

same buffer (Eluent 1), two volumes of buffer containing 0.5 m*M* ATP (Eluent 2) and finally two volumes of the buffer (Eluent 1) containing 1 *M* sodium chloride (Eluent 3), all the eluents being at the same chosen pH. Fractions of 1 ml were collected at a rate of 17 ml/h, dialysed against 0.02 *M* Tris acetate (pH 7.8) with 1 m*M* EDTA for 4 h and checked for protein concentration and luciferase activity.

Chemical modification of triazine dyes immobilised to Sepharose

Triazine dyes immobilised to Sepharose were chemically modified by the method of Clonis[8]. All reactions were carried out in a nitrogen atmosphere to avoid any partial oxidation due to air.

Elution. The chemically modified dye resins were packed into the required columns and elution was carried out in the same manner as for the native dyes bound to Sepharose. Fractions of 1 ml were collected at a rate of 17 ml/h and dialysed against 0.02 *M* Tris acetate (pH 7.8) with 1 m*M* EDTA for 4 h before checking for protein concentration and luciferase activity.

Spectrophotometric studies

These studies were performed with a "Solid phase spectrophotometer" (Beckman). Native or chemically modified immobilised dyes were exhaustively washed with acetone and dried under vacuum under identical conditions. Pellets of the dyes were made with magnesium oxide and introduced into the sample chamber of the spectrophotometer, with Sepharose (washed and dried identically) acting as the control. The absorbance was recorded in the scanning range 700–280 nm.

RESULTS AND DISCUSSION

Studies with small columns of Sepharose 4B dye (native) conjugates demonstrated variations both in the capacity of the different dyes to bind luciferase and in the subsequent elution of the enzyme by the substrate ATP, as shown in Table I. The percentage of recovery of protein and enzyme activity differed distinctly from dye to dye with both different eluents and different pHs.

In the case of Procion blue Mx 2G and Procion red HE 3B, the optimum pH for elution was found to be 6.9. In the case of Procion green HE 4BD, the maximum binding of proteins was observed at pH 8.2. However, pH 7.4 was found to be the optimum for luciferase binding in a specific way, as we could have a significant increase in the specific activity when eluted by the substrate ATP. Nevertheless, the interaction seems more electrostatic since *ca.* 50% of the total recovered activity is eluted with sodium chloride. The differences in the sulphonate groups in these dyes (see Table I) could probably explain the change in the optimum pH. The low ligand concentration in the case of Procion green HE 4B should, however, not be ignored.

Triazine dye–enzyme interactions have been suggested to be ionic and/or hydrophobic in nature. In the case of luciferase, systematic studies to evaluate any hydrophobic nature in its interaction with triazine dyes have not so far been conducted. From our studies, it seems quite appropriate to suggest a case of ionicity in luciferase–dye interactions especially in those cases (see Table I) where the addition of sodium chloride is successful in stripping off the enzyme from the dye matrix.

Procion brown Mx 5BR, which is known to possess four sulphonate groups and

TABLE I

EFFECT OF pH ON THE BINDING OF LUCIFERASE TO NATIVE TRIAZINE DYE–SEPHAROSE CONJUGATES

P = Protein; LA = luciferase activity. Eluent 1 = 0.01 *M* MOPS+10 m*M* magnesium sulphate+ 1 m*M* DTT (pH 7.4); Eluent 2 = Eluent 1+ 0.5 m*M* ATP (pH 7.4); Eluent 3 = Eluent 1+ 1 *M* sodium chloride (pH 7.4). See text for details.

Dye	*No of sulphonate groups*	*Ligand concentration (μm dye per g of moist gel)*	*pH Optimum for protein binding*	*Recovery (%)*					
				Eluent 1		*Eluent 2*		*Eluent 3*	
				P	*LA*	*P*	*LA*	*P*	*LA*
Procion blue, Mx 2G	2	4.1	6.9	8	13	102	52	0	0
Procion brown, Mx 5BR	4	6.6	no optimum pH	55	5	20	10	22	0
Procion orange, Mx G	2	6.3	6.9	22	8	70	220	0	0
Procion green, HE 4BD	*	2.3	8.2	32	11	89	110	0	0
Procion red, HE 3B	2	5.6	6.9	70	27	30	247	0	0
Sepharose (underivatized)	0	0	/	100	0	0	0	0	0

* Procion green HE 4BD is known to contain several polysulphonated moieties.

the metal Cr^{2+} in its structure, revealed no difference in binding pattern within the pH range studied; no activity was recovered in any of the three eluent fractions (data not shown). This could probably be due to the strong binding or multiple point attachment of the enzyme to the ligand. The effect of Cr^{2+} in the binding reaction and on the catalytic activity of luciferase should also be considered.

In spite of more or less similar concentrations of ligand, these dyes do show differences in their enzyme adsorption profiles, in accordance with the results of Lowe and co-workers[13–16]. Procion blue was not found to be a specific dye for luciferase although studies with blue dextran proved otherwise. Blue dextran substituted with Cibacron blue, the *o*-isomer, was found to be specific for luciferase[10] whereas Procion blue HB, which is an *m–p* mixture, was not. Procion red HE 3B at a lower pH (6.9) is an interesting dye ligand for luciferase when coupled to Sepharose 4B. A recovery of 247 % of the initial enzyme activity is obtained along with a gain in specific activity of fifteenfold, thereby suggesting a strong specificity for the substrate (ATP) binding site of the enzyme.

Procion orange Mx G also showed similar specificity. The increase in activity after elution could also be explained by the removal of foreign proteins and possibly luciferase inhibitors, as observed by Filippova and Ugaravova[17].

Post-immobilisation chemical modifications of triazine dyes have already been reported[7,8]. The different chemical modifications used in our studies and their consequences in the chromophore structure have been described by Clonis[8]. Sodium dithionite, in accordance with its known properties, does seem to cleave parts of the dye structure by reducing the azo linkage[3,16]. When the sodium dithionite-treated dyes were allowed to react with acetic acid and sodium nitrite to convert the arylamino groups

into diazonium groups, these in turn being treated with alkali, leading to the replacement of the arylamino groups with phenolic hydroxyl groups, changes in colour did occur (data not shown). Treatment of dye gels with sodium borohydride is known to lead to hydrogenation of the azo bonds to form the corresponding hydrazine analogues.

The changes in the functional groups or parts of the dye structure after chemical modification were checked by spectral studies. The ratio-recording spectrophotometer allowed us to read the spectra of native and chemically modified dye–Sepharose conjugates in the solid phase, thereby avoiding the problem of suspended dye–Sepharose particles in solution, which would have greatly affected normal spectrophotometric studies.

The theoretical changes proposed by Lowe and Clonis during the modifications of triazine dyes immobilised on Sepharose were confirmed spectrally before undertaking the luciferase-binding studies of the various modified conjugates. A typical example is given in Fig. 1. Native green HE 4BD Sepharose conjugate exhibited two peaks at 384 and 520 nm. When the amino groups were replaced with phenol groups by treatment of sodium dithionite-reduced conjugates with acetic acid–sodium nitrite, the peak was shifted to 550 nm. Hydrogenation of the azo bonds to form the corresponding hydrazine analogues displaced the peak to 635 nm. These studies were also relevant in the case of Procion blue Mx 2G conjugates which were also allowed to react with the same various reagents as used with Procion green HE 4BD. In this case the spectral shift indicating the reduction of azo groups by sodium dithionite was not noticed. This can be explained by the fact that Procion blue Mx 2G has no azo bonds and hence no modification in its functional groups occurred (data not show).

Modification of the functional groups of triazine dyes coupled to Sepharose also alters its chromatographic properties with respect to luciferase. Table II illustrates the binding properties of luciferase to native and modified triazine–Sepharose dye gels. Under identical experimental conditions, the binding of luciferase to chemically modified dyes is markedly different from that occurring with native dyes. The red HE 3B

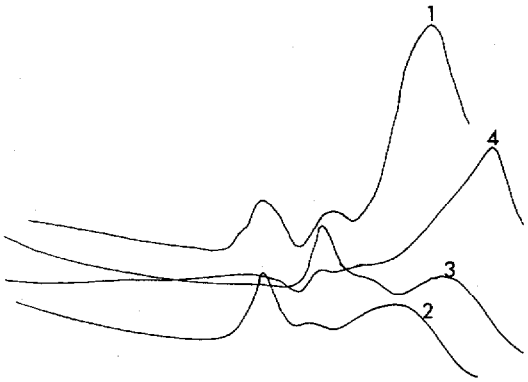

Fig. 1. Ratio-recording spectrophotometric study of post-immobilisation chemical modification of Procion green HE 4BD. 1 = Native Procion green coupled to Sepharose; the two peaks are at 384 and 520 nm. 2 = Sodium dithionite-reduced green after cleavage of azo linkages; the peaks are at 384 and 440 nm. 3 = Acetic acid–sodium nitrite-treated green HE 4BD, replacing the aryl amino groups by hydroxyl groups *via* diazonium formation; the peak is shifted to 550 nm. 4 = Reduction of azo linkages of immobilised green HE 4BD with sodium borohydride; the peak is displaced to 635 nm.

TABLE II

BINDING STRENGTHS OF LUCIFERASE TO NATIVE AND CHEMICALLY MODIFIED IMMOBILISED TRIAZINE DYES

All elutions were performed at pH 7.4. P = Protein; LA = luciferase activity. Eluents as detailed in Table I. See text for details.

Dye	*Eluent*	*Native (%)*		*Modified by sodium dithionite (%)*		*Modified by acetic acid–sodium nitrite (%)*		*Modified by sodium borohydride (%)*	
		P	*LA*	*P*	*LA*	*P*	*LA*	*P*	*LA*
Procion green, HE 4BD	1	54	11	93	4	101	79	20	16
	2	31	126	13	158	0	0	35	11
	3	21	180	0	0	0	0	47	0
Procion red, HE 3B	1	100	19	48	12	9	0	4	2
	2	0	0	59	6	0	0	0	0
	3	0	0	0	0	90	192	94	0

and green HE 4BD conjugates were chosen because of their obvious differences (Table I).

In the case of Procion green HE 4BD, the chemical modification with sodium dithionite resulted in a more specific binding of the enzyme protein. The binding specificity increased on treating the dye with acetic acid–sodium nitrite to replace the amino groups by hydroxyl groups, because all the non-specific protein injected could be stripped off by Eluent 1, *i.e.* 0.01 *M* MOPS containing 10 m*M* magnesium sulphate and 1 m*M* DTT (pH 7.4) and luciferase was eluted specifically by the substrate (0.5 m*M* ATP). This suggests that any change in the azo linkage or of the azo group itself would change the binding pattern of luciferase to the dye Procion green HE 4BD. However when the dye was reduced by sodium borohydride, surprisingly only 20% of the protein injected could be eluted by Eluent 1 and at the same time there was a tremendous loss in luciferase activity, due probably to the strong binding observed in this case. These results are also in agreement with those of Thompson and Stellwagen[19] who report that binding of the enzyme with a nucleotide domain (in this case NAD) to Cibacron blue F3 GA requires a precise orientation of the ionic groups of the dye, (although Beissner and Rudolph[20] think otherwise).

Experiments of a similar nature with Procion red HE 3B gave different results (Table I). There was no binding at all with the native dye at pH 7.4. When the azo linkage was cleared there was an increase in binding which also destroyed the activity of the enzyme. Replacement of the aryl groups in Procion red HE 3B by hydroxyl groups increased the binding strength of the matrix, *i.e.* only 9% of the protein injected was recovered by Eluent 1. There was an activity gain of 192% on eluting the column with 1 *M* sodium chloride (Eluent 3) whereas no activity could be detected in the eluate obtained by addition of substrate to the buffer. This leads again to a case of ionic interaction in the case of native green HE 4BD and modified red HE 3B (modified by acetic acid–sodium nitrite) with luciferase. Thus reduction of the dye by acetic acid–sodium nitrite in this case increases the affinity of luciferase for the dye. Treatment of Procion red HE 3B with sodium borohydride results in strong binding of the protein and a very poor recovery of activity. The importance of the role played by the terminal

benzosulphonyl groups in luciferase–dye interactions again comes into question.

In the case of Procion green HE 4BD, our results agree with the interpretation of the interaction of triazine dyes by Clonis[8]. We agree that the dramatic changes occurring in the chromatographic behaviour with the Procion green HE 4BD chemically modified gel might be due to the elimination of polysulphonated polycyclic species from the native dye molecule rather than the simple replacement of amino groups by hydroxyl groups. Disruption of the dye structure evidently destroyed its specificity to luciferase. As suggested earlier for lactate dehydrogenase, perhaps also in the case of luciferase the reduction of the double bonds increases the flexibility of the dye, allowing orientation to a more favourable position for binding with the macromolecule. Our results are also in concordance with the hypothesis that cleaving of the dye to remove hydrophobic (aromatic) or ionic (sulphonic acid) species would result in weak dye–macromolecule interactions[7,8].

The binding of luciferase to dyes reduced by sodium borohydride was not exactly the same as that observed by Clonis with different enzymes, such as glucose-6-phosphate dehydrogenase, lactate dehydrogenase and yeast hexokinase. With both green HE 4BD and red HE 3B there was a stronger binding of the enzyme to the modified matrix, leading to a big loss in luciferase activity. It can thus be concluded that as long as the azo bonds are not modified and as long as the terminal polysulphonated groups are intact, the affinity of luciferase for the dye is not hindered.

The stereospecificity seems to play a role, as we have observed an interaction of luciferase with Cibacron blue (*o*-isomer) and not with Procion blue (the *m,p*-isomer). However we are carrying out some further investigations to confirm this.

In short, it can be said that the above findings, when exploited would give a superior method for purifying luciferase by triazine dye affinity chromatography. Although the interactions are not strictly "biospecific" in the manner of conventional affinity techniques, these synthetic media can prove useful because of their low cost, high binding capacity, regenerability (the gels can be effectively regenerated with 6 *M* urea in 0.5 *M* sodium hydroxide solution) and durability.

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