Journal of Chromatography, 315 (1984) 175–184
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,167

METAL-ION MEDIATED INTERACTION OF LUCIFERASE WITH TETRAIODOFLUORESCEIN

SUNANDA RAJGOPAL* and M. A. VIJAYALAKSHMI
Laboratoire de Technologie des Séparations, Département de Génie Biologie, Université de Technologie de Compiègne, B.P. 233, 60206 Compiegne Cedex (France)
(Received July 17th, 1984)

SUMMARY

Tetraiodofluorescein binds to luciferase in an apparently competitive manner, according to kinetic inactivation studies. Among the metal ions studied, Cu^{2+} and Ni^{2+} are the only two of the first row transition series that enhance enzyme inactivation by the dye. Mg^{2+}, Co^{2+} and Mn^{2+} seem to have a protective effect whereas Zn^{2+} prevents enzyme inactivation and even enhances its activity. Luciferase bound to tetraiodofluorescien–Sepharose via a metal ion can subsequently be eluted with appropriate ligands. Luciferase was found to be sensitive to the spacer geometry of immobilized tetraiodofluorescein.

INTRODUCTION

Firefly luciferase reaction, or the bioluminiscent reaction as it is popularly called, has given rise to a whole technology for ATP determination. This is exemplified by a volume of *Methods in Enzymology*[1], an excellent review on ATP determination by Leach[2] and extensive work by De Luca and McElroy[3].

A number of techniques have been used to study the binding of substrates to luciferase, and some have detected substrate-induced conformational changes that were postulated to be important in the reaction mechanism[4].

Recently, the dye, 2′,4′,5′,7′-tetraiodofluorescein (TIF) has been used to probe the nature of the nucleotide binding site of several enzymes, namely lactate dehydrogenase[5], aspartate transcarbamylase[6], creatine kinase[7] and yeast hexokinase[8]. X-ray crystallographic studies of lactate dehydrogenase have shown unambiguously that the dye binds at a site coincident with that of the co-factor NAD in a competitive manner. It was also reported that TIF activates the allosteric enzyme aspartate transcarbamylase, normally activated in the same manner as the natural effector, ATP.

The interaction of TIF with creatine kinase (a member of another general class of nucleotide binding proteins) was undertaken by Somerville and Quiocho[7], to see whether the dye would mimic the adenosine of substrates ATP and/or ADP. Tucker *et al.*[9] studied the chromatographic behaviour on immobilized TIF of a heterogeneous protein mixture and of a series of homogeneous proteins, among which were

0021-9673/84/$03.00

rabbit muscle lactate dehydrogenase, bovine heart lactate dehydrogenase, pig heart malate hydrogenase, yeast glucose 6-phosphate dehydrogenase (all with NAD domains), rabbit muscle adenylate kinase, yeast phosphoglycerate kinase (with Mg-ATP domains) and pig heart citrate synthetase, *E. coli* aspartate transcarbamylase (with ATP binding sites). Moreover, because the dye is influenced by the environment of its binding site, the specific interaction of the dye with the enzyme might prove useful in judging the polarity of the adenosine binding site.

In this communication, we report the interaction of TIF with firefly luciferase because the xanthene ring of TIF was reported to bind to the adenine pocket of NAD in lactate dehydrogenase in a manner analogous to the binding of the anthraquinone ring of Cibacron blue F3G-A (a triazine dye) to the adenine pocket of NAD in alcohol dehydrogenase[9]. This study also deals with the effect of spacer geometry with regard to immobilized TIF, and with the influence of multivalent metal ions on TIF–luciferase interaction.

EXPERIMENTAL

Materials

Luciferase was obtained from Boerhinger, Mannheim, France; ATP and luciferin from Sigma; Sepharose 4B and aminohexyl (AH)–Sepharose 4B were purchased from Pharmacia. TIF was a product of Eastman Kodak and exhibited essentially one spot on thin-layer chromatography. All other reagents were of analytical grade. TIF concentrations were determined spectrophotometrically at 523 nm using an extinction coefficient of $8.4 \cdot 10^4\ M^{-1}\ cm^{-1}$.

Enzyme extraction and assay

A crude extract of luciferase was prepared as described elsewhere[10] and dialysed against 0.02 *M* Tris acetate containing 1 m*M* EDTA (pH 7.8) for 24 h with three changes of the buffer. This dialysed and partially purified extract was used in our chromatographic studies.

Luciferase activity was assayed by a Nucleotimeter (Interbio, France). The reaction mixture containing 100 μl of 10^5 pg/ml ATP in 0.01 *M* 3-morpholinopropanesulphonic acid (MOPS) (pH 7.4) containing 10 m*M* magnesium sulphate, 100 μl of luciferin (0.168 μg/ml) and 50 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 millivolts. The unit of activity is the maximum intensity of light recorded in millivolts per picogram of ATP per milligram of protein at 562 nm.

Synthesis of immobilized TIF

TIF was coupled to Sepharose by the procedure of Tucker *et al.*[9]. Sepharose 4B or AH–Sepharose 4B (5 g) was washed with 1 l of 0.5 *M* sodium chloride and then suspended in 30 ml of water. The dye, 0.67 g (2 m*M*) in 30 ml dioxane, was added, mixed and the pH adjusted to 5.0 with 1 *N* hydrochloric acid. Carbodiimide (EDAC) (0.83 g) was added slowly and the pH was maintained at 5.0 for 20 h with continual stirring. No magnetic stirrer was used. The gel was washed with dioxane to remove any unreacted dye, then with 2% SDS and 1 *M* potassium chloride. TIF immobilized gel was stored in 0.05% sodium azide in the dark. TIF immobilized to

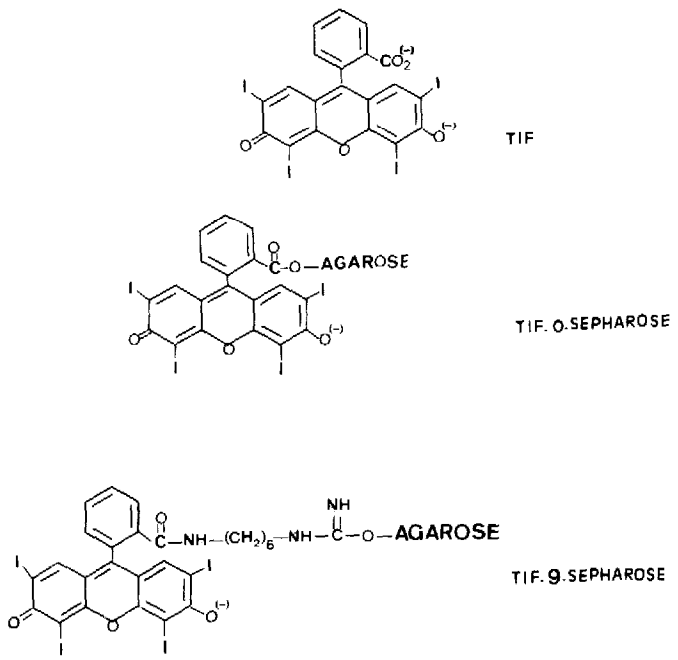

Fig. 1. The structures of free TIF, TIF immobilized to Sepharose 4B (TIF-0-Sepharose) and TIF immobilized to AH-Sepharose 4B (TIF-9-Sepharose).

Sepharose 4B was termed TIF-0-Sepharose and that immobilized to AH-Sepharose 4B was termed TIF-9-Sepharose[9] (Fig. 1 shows the relevant molecular structures).

Enzyme inactivation studies

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, 50 μg of enzyme ($5 \cdot 10^{-5}$ arbitrary units or 0.4 units), dye as mentioned in individual cases and 50 μM DTT. The metal ions Co^{2+}, Ni^{2+}, Mn^{2+}, Cu^{2+} and Zn^{2+} (2 μmol) were added where appropriate (Fig. 5). The rate of inactivation of the enzyme at 25°C was followed by periodically removing samples (50 μl) and assaying for enzyme activity. Initial rates of inactivation were deduced from plots of $\log_{10}$ (percentage of activity remaining) *versus* time (min) for several dye concentrations, and the slopes and intercepts of secondary double reciprocal plots were calculated as described by Clonis *et al.*[11]. A sample without the dye served as the control. In addition, the inactivation of luciferase was performed in the presence of Mg-ATP and K_{ID} values were determined.

Spectral studies of TIF–luciferase interaction

The spectral titration studies were conducted with the help of a double beam spectrophotometer (Jobin Yvon, France). The sample cuvette contained 0.8 units of luciferase in 1.0 ml of 0.1 *M* Tricine (pH 7.8), and the sample and reference cuvettes both contained a known amount of TIF. Both cuvettes had a light path of 10 mm. Spectra were recorded at room temperature in the wavelength range 330–600 nm.

Analytical chromatography

All chromatographic elutions were performed at 4°C. Disposable columns (Biorad laboratoires, France, 30 × 8 mm I.D.) containing 1.6-ml bed volume of Sepharose 4B or AH–Sepharose 4B immobilized TIF was equilibrated with 10 volumes of 0.01 *M* MOPS buffer (pH 7.4) containing 10 m*M* magnesium sulphate and 1 m*M* DTT. A sample of the dialysed extract (0.2 ml, corresponding to 1.68 mg of protein) was loaded on the column and sequentially washed with 10 volumes of equilibrating buffer, 10 volumes of buffer containing ATP and finally 10 volumes of buffer with 1 *M* sodium chloride. Fractions of 1.0 ml were collected at 35 ml/h, and 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 278 nm and luciferase activity.

The effects of added metal ions on the binding of luciferase to immobilized TIF were investigated by equilibrating the column with MOPS buffer containing 1.5 m*M* metal ion. The percentage of enzyme bound was determined by assaying the void volume eluate, and bound enzyme was subsequently eluted with buffer containing the specific ligand.

Stoichiometric studies

Luciferase (1 mg) was quantitatively inactivated by an excess of TIF. The enzyme activity was monitored for 30 min till more than 95% of the activity was lost. The enzyme–dye complex was diafiltered using an Amicon ultrafiltration unit with 0.1 *M* Tricine buffer (pH 7.8). Excess dye was thus washed off quantitatively and enzyme-bound dye complex recovered. The amount of dye bound to luciferase was determined at 523 nm using a molar absorption coefficient of $8.4 \cdot 10^4\ M^{-1}\ cm^{-1}$.

RESULTS

Binding of firefly luciferase to TIF

The reaction between an active-site directed reactive dye (D) and an enzyme (E) can be represented as follows[11]:

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

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

$$\frac{1}{K_{obs}} = \frac{1}{K_3} + \frac{K_D}{K_3}\left(\frac{1}{[D]}\right)$$

where K_{obs} is the observed rate of enzyme inactivation for a given concentration [D], K_3 is the maximum rate of inactivation (min^{-1}) and K_D is the dissociation constant (k_2/k_1) of the enzyme–dye complex.

Luciferase inactivation by TIF was found to be active-site directed; at low concentrations of the dye, increasing concentrations of the substrate ATP gave in-

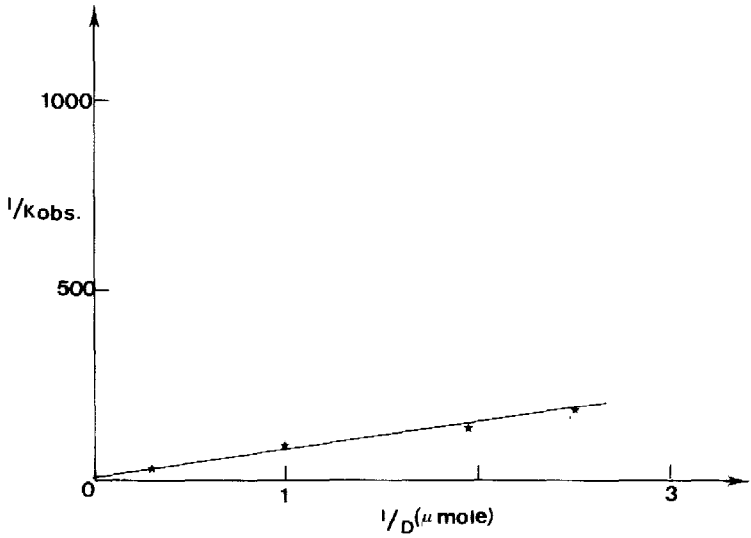

Fig. 2. Inactivation of luciferase by TIF represented as a double reciprocal plot of $1/K_{obs}$ *versus* 1/[D]. The assays were carried out as described in Experimental in the presence of TIF (0.37 2.22 μmol). The intercept of the abcissa of this plot is equivalent to the dissociation constant (K_D) for TIF.

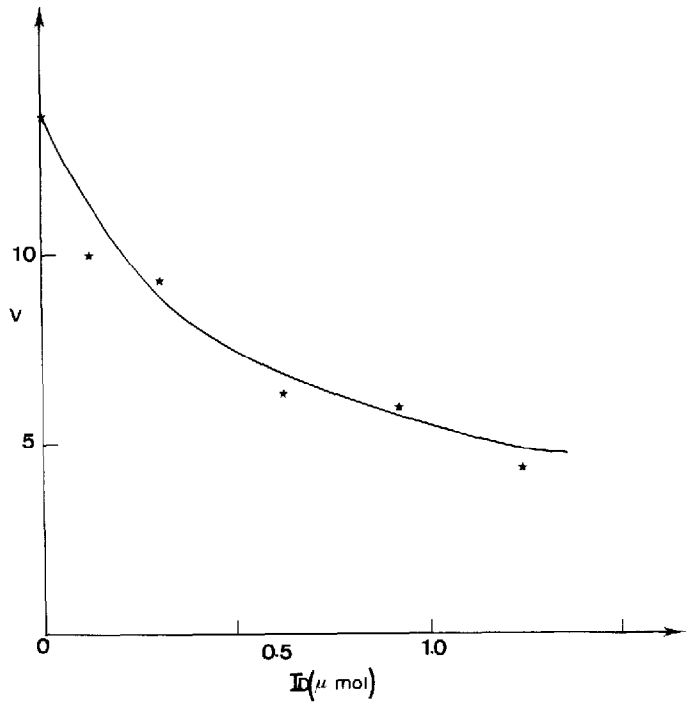

Fig.3. Kinetics of inhibition of luciferase by TIF. Dixon plot of *V versus* [ID], the concentration of the dye inhibitor. The assays were carried out as described in Experimental. The non-linear curve indicates that more than one molecule of the dye bind to the enzyme.

creasing protection to the enzyme in an apparently competitive manner. Although the secondary reciprocal plot of $1/K_{obs}$ *versus* 1/[D] (Fig. 2) was linear, the Dixon plot (Fig. 3) of V *versus* [ID] was not, where V is the rate of the reaction and [ID] the concentration of the competitive dye inhibitor. (Note that [ID] and [D] both represent the concentration of the dye that is the inhibitor, but for the sake of distinction between inhibition constant and dissociation constant, different symbols are used.)

A K_D value (dissociation constant of the enzyme–dye complex) of 16.6 μM (Fig. 2) and a K_{ID} value (factor of inhibition *vis-á-vis* its substrate ATP) of 400 μM were calculated from double reciprocal plots of $1/K_{obs}$ *versus* 1/[ID] and according to the following equation:

$$\frac{1}{K_{obs}} = \frac{1}{K_3} + \frac{K_D}{K_3}\left(\frac{1}{[D]}\right)\left(1 + \frac{[ID]}{K_{ID}}\right)$$

where K_{ID} is the inhibition constant of the dye.

Spectral studies

Binding of TIF to luciferase results in a slight redshift in the absorption maximum, at high concentrations. At low TIF concentrations, however, there seems to be no noticable shift in the absorption maximum. Addition of Mg-ATP does not completely abolish the visible absorption spectrum of TIF–enzyme complex. This indicates that TIF does not binds solely to the Mg-ATP site of the enzyme.

Chromatographic studies

The results of the chromatography of a crude extract of luciferase on TIF–0-Sepharose and TIF–9-Sepharose are given in Table I. It can be seen that the addition of Co^{2+} (1.5 mM) in the equilibrating buffer leads to a better retention of luciferase

TABLE I

CHROMATOGRAPHY OF LUCIFERASE ON TIF–SEPHAROSE

Disposable columns (30 × 8 I.D.) containing 1.6-ml bed volume of TIF–0-Sepharose or TIF–9-Sepharose was equilibrated with 10 volumes of 0.01 M MOPS containing 1 mM DTT (pH 7.4) and low concentrations of metal ions (1.5 mM) where mentioned. The enzyme sample (1.68 mg of protein) was applied, unbound protein eluted with 10 volumes of the equilibrating buffer and elution effected with 5 mM ATP in the same buffer.

Support	*Metal ion in equilibrating buffer*	*Luciferase retained on the column (%)*	*Purification factor**
TIF–0-Sepharose	No metal ion	40	
	Cu^{2+}	60	
	Mn^{2+}	0	
	Mg^{2+}	10	
	Co^{2+}	73	
TIF–9-Sepharose	No metal ion	100	6.5
	Mg^{2+}	300	9.4

* Purification factor compared with the dialysed extract: luciferase eluted with 5 mM ATP and 1 mM DTT in 0.01 M MOPS (pH 7.4).

on TIF–0-Sepharose. Inclusion of Cu^{2+}, Mg^{2+} or Mn^{2+} does not promote the binding of the enzyme to the TIF–0-Sepharose column.

Luciferase is well retained on TIF–9-Sepharose in the presence of 10 m*M* Mg^{2+}, and elution can be effected with 5 m*M* ATP in the buffer, resulting in a purification of 9.4 fold, compared with the dialysed extract.

MOPS buffer containing EDTA alone could not strip off active luciferase from a TIF-immobilized column.

DISCUSSION

The affinity of halogenated polycyclic aromatic dyes of the triazine series for the ATP binding site of luciferase[10–13] prompted the examination of the interaction with the enzyme of a much smaller fluorescein dye, TIF. We found that TIF has a lower affinity for luciferase than many triazine dyes, with a K_D value of 16.66 μM for TIF and 2.13 μM and 5.26 μM for Cibacron Blue F3G-A and Procion Blue Mx-R (a monochloro- and dichlorotriazinyl dye), respectively. The most significant observation is that TIF caused the inactivation of luciferase in an apparently competitive manner at high concentrations. However, a K_{ID} value of 400 μM (or more) for the TIF–Mg-ATP–luciferase system does not represent sufficient specificity and affinity for the Mg-ATP binding site of the enzyme. [Luciferase possesses both Mg-ATP and ATP binding domains (one ATP-Mg site and two ATP sites per dimer) and TIF has been reported as binding to both.]

It is also prudent at this stage to look into the effect of the donor–acceptor properties of iodine atoms of TIF. Though iodine is known to be a bulky solvent molecule[14], compared with other halogens, it is the least powerful oxidant among them because of a more negative enthalpy of formation of the anion. This information could be important in the interpretation of the findings on the protein–TIF interaction.

It should be emphasized that 1-amino-4-(4′-aminophenylamino)anthraquinone 2,3′-disulphonic acid (ASSO), the essential chromophore of Cibacron Blue F3G-A and many other related triazinyl dyes, exhibits a much greater affinity and specificity for the Mg-ATP site of luciferase: the K_{ID} value for ASSO was 16.06 μM, compared with 400 μM for TIF[12].

The type of inhibition of TIF on luciferase was further elucidated by the method of Dixon. A plot of $1/V$ against [ID] yielded a series of intersecting straight lines at different ATP concentrations (Fig. 4), which suggests a competitive inhibition mechanism. A plot of V against [ID] (Fig. 3), however, tends to indicate that more than one molecule of TIF binds to the enzyme. Stoichiometric studies of TIF with luciferase revealed that when the enzyme was quantitatively inactivated in the presence of excess dye, two molecules of the dye were found to bind per enzyme subunit, thereby confirming that TIF binds to both the ATP and the Mg-ATP sites. This observation is in agreement with that reported by earlier workers[5,7]. TIF also binds to the ATP site of the non-catalytic subunit.

The inability to abolish completely the difference spectrum of the luciferase–TIF complex with Mg-ATP suggests that TIF is able to bind to the luciferase–Mg-ATP binary complex. This is consistent with the lack of strictly competitive type kinetics of inhibition of luciferase by TIF and the non-linear Dixon plots of inhibition data.

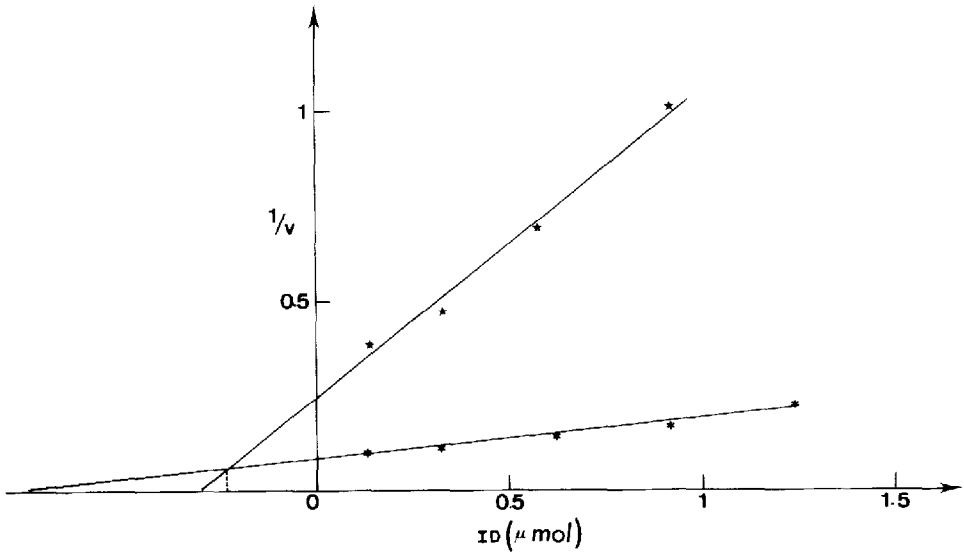

Fig. 4. Kinetics of inhibition of luciferase by TIF. Dixon plot of $1/V$ *versus* [ID], the concentration of the dye inhibitor. The assays were carried out as described in Experimental. (★) In the presence of 10^4 pg/ml ATP; (*) in the presence of 10^6 pg/ml ATP.

The results shown in Table I indicate that the longer spacer arm in TIF–9-Sepharose results in the apparently stronger binding of luciferase to the immobilized dye, as was observed by Tucker *et al.*[9]. Quantitative analysis of the TIF–luciferase interaction reveals that the affinity of the protein for the dye is diminished by immobilization and that the matrix can provide a significant steric barrier to complexation, because the inclusion of a nine-carbon spacer arm between the dye and the matrix results in a greater accessibility of the dye.

The interaction of luciferase and lactate dehydrogenase with TIF appears to be similar, since lactate dehydrogenase with a K_D of 1.0 μM is well retained by TIF–9-Sepharose and also TIF–6-Sepharose (a six-carbon spacer arm) but is not retained at all by TIF–0-Sepharose. Also, because the columns used are relatively highly substituted by dye, a multivalent attachment of the dye on the protein polypeptide chain seems likely. In the interaction of TIF with luciferase, we can say that the complexation of ligand weakens the binding of the dye sufficiently to facilitate protein elution, as demonstrated by Yon[15], and that it need not necessarily be solely an active-site process.

In conclusion it can be said that since very little movement of the protein may be necessary to accommodate the rather compactly structured TIF, it seems valid to interpret our observations in the light of "homosterism" and "induced-fit hypothesis" already suspected in the firefly luciferase bioluminiscent reaction[16]. The initial reaction of the enzyme with the substrates was attributed to the induced-fit theory of Koshland[17], which raised the possibility that the conformational changes accompanying substrate binding could result in an increase in the reactivity of the side-chain groups or open new sites on the enzyme surface, which would in turn bind more TIF molecules (when it is in excess).

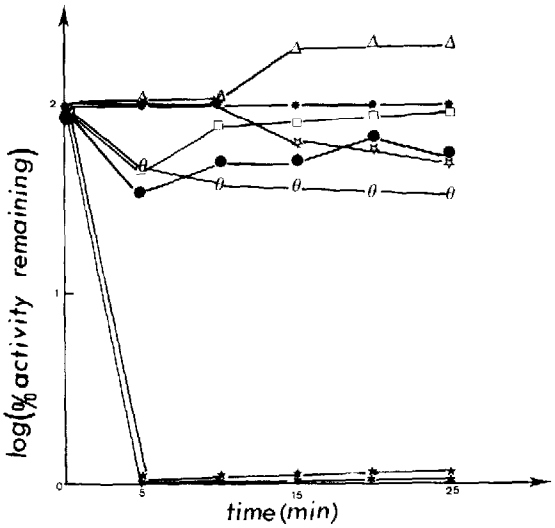

Fig. 5. Effect of metal ions on the inactivation of luciferase by TIF. Luciferase (0.4 units) was inactivated by TIF (0.62 μmol) in the presence of 2 μmol of metal ion in 0.1 *M* Tricine (pH 7.8). A sample without the dye served as a control. See text for experimental details. (✳) Control; (△) in the presence of Zn^{2+}; (□) in the presence of Mg^{2+}; (●) in the presence of Mn^{2+}; (☆) in the presence of Co^{2+}; (⊖) in the absence of metal ion; (★) in the presence of Ni^{2+}; (•) in the presence of Cu^{2+}.

It is not possible to present a thorough description of the TIF binding site of luciferase owing to the lack of data available (amino acid sequence, etc.) on this region of the protein.

These kinetic data and those of the spectral studies suggest that as the concentration of TIF increases, it begins to compete with Mg-ATP for the limited number of active sites on the enzyme surface. This sort of TIF interaction is observed with aspartate transcarbamylase[18]. Therefore, at least two classes of sites for TIF binding are proposed. The inactivating class of site, presumably located on the active α subunit of the enzyme coincident with the Mg-ATP site because significant loss of activity is seen only at high concentrations of TIF, and another site to which TIF binds at low concentrations.

TIF should prove to be an optical probe of ATP site conformation of luciferase once its interaction with the enzyme is fully understood. In order to learn more, we propose to study the interaction of TIF with the isolated regulatory and catalytic subunits, as well as with modified luciferase (modified with SH reagents etc.).

ACKNOWLEDGEMENT

The authors are grateful to Dr. J. F. Hervagault for his valuable comments and suggestions.

REFERENCES

1 M. De Luca, *Methods Enzymol.*, 57 (1978).
2 F. R. Leach, *J. Appl. Biochem.*, 3 (1981) 473.

3 M. De Luca and W. D. McElroy (Editors), *Bioluminiscence and Chemiluminiscence*, Academic Press, New York, 1981.
4 M. De Luca, *Advan. Enzymol.*, 44 (1978) 37.
5 P. M. Wasserman and P. J. Lentz, Jr., *J. Mol. Biol.*, 60 (1971) 509.
6 L. B. Jacobsberg, E. R. Kantrowitz and W. N. Lipscomb, *J. Biol. Chem.*, 250 (1975) 9238.
7 L. L. Somerville and F. A. Quiocho, *Biochem. Biophys. Acta*, 481 (1977) 493.
8 B. P. Yip and F. B. Rudolph, *J. Biol. Chem.*, 251 (1976) 7151.
9 R. F. Tucker, J. Babul and E. Stellwagen, *J. Biol. Chem.*, 256 (1981) 10993.
10 S. Rajgopal and M. A. Vijayalakshmi, *J. Chromatogr.*, 243 (1982) 164.
11 Y. D. Clonis, M. J. Goldfinch and C. R. Lowe, *Biochem. Biophys. Acta*, 659 (1981) 86.
12 S. Rajgopal and M. A. Vijayalakshmi, *J. Chromatogr.*, 280 (1983) 77.
13 S. Rajgopal and M. A. Vijayalakshmi, *Proceeding of the Third International Symposium on Analytical Applications of Bioluminiscence and Chemiluminiscence, Birmingham, U.K., 1984.*
14 A. G. Sharpe, *Inorganic Chemistry - The Halogens*, Longman Group Ltd., London, New York, 1981, p. 399.
15 R. J. Yon, *Biochem. J.*, 185 (1980) 211.
16 W. D. McElroy, M. De Luca and J. Travis, *Science*, 157 (1967) 150.
17 D. E. Koshland, Jr., in P. D. Boyer (Editor), *The Enzymes*, Vol. 1, Academic Press, New York, 3rd ed., 1970.
18 L. B. Jacobsberg, E. R. Kantrowitz, C. H. McMurray and W. N. Lipscomb, *Biochem. Biophys. Res. Commun.*, 55 (1973) 1255.