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# Role and Reactivity of Sulfhydryl Groups in Firefly Luciferase*

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ABSTRACT: The chloromethyl ketone derivative of *N*-tosyl-L-phenylalanine is an effective inhibitor of firefly luciferase. The inhibition of enzymatic activity is accompanied by a loss of approximately two sulfhydryl groups. The chloromethyl ketone derivative of *N*-tosyl-L-lysine is without effect under similar conditions. Kinetically, *N*-tosyl-L-phenylalanine chloromethyl ketone inhibition is competitive with respect to one of the substrates, luciferin, and noncompetitive with respect to the other, adenosine 5′-triphosphate. The hydrophobic character of *N*-tosyl-L-phenylalanine chloromethyl ketone appears to be the major factor for its binding to the active site of luciferase. The fact that *N*-tosyl-L-phenylalanine is a competitive inhibitor also with a $K_i$ of the same order of magnitude as *N*-tosyl-L-phenylalanine chloromethyl ketone supports this conclusion. It appears, therefore, that *N*-tosyl-L-phenylalanine chloromethyl ketone inhibition of luciferase involves two phases: first, a reversible binding of *N*-tosyl-L-phenylalanine chloromethyl ketone molecules at the luciferin binding sites (demonstrable kinetically as competitive inhibition), and second, the reaction of the chloromethyl ketone group of *N*-tosyl-L-phenylalanine chloromethyl ketone with the SH groups at or near these sites. Inactivation by *N*-tosyl-L-phenylalanine chloromethyl ketone is pH dependent; inactivation–pH curve corresponds very closely to the luciferase activity–pH curve. It is suggested that two sulfhydryl groups are located at or near the binding site of luciferin, and that they are required in the luciferase-catalyzed production of yellow-green light.

Firefly luciferase catalyzes the following reactions (Rhodes and McElroy, 1958)

$$\text{enzyme} + \text{luciferin} + \text{ATP} \rightleftarrows \text{enzyme–luciferin–AMP} + \text{PP} \quad (1)$$

$$\text{enzyme–luciferin–AMP} + O_2 \longrightarrow \text{light} + \text{products} \quad (2)$$

$$\text{enzyme} + \text{dehydroluciferin} + \text{ATP} \rightleftarrows \text{enzyme–dehydroluciferin–AMP} + \text{PP} \quad (3)$$

DeLuca *et al.* (1964) have shown that two sulfhydryl groups on the enzyme are necessary for maintaining catalytic activity; the remaining six sulfhydryl groups could be modified without affecting enzymatic function. The amino acid sequence surrounding the two essential sulfhydryl groups has been determined (Travis and McElroy, 1966).

Since luciferase undergoes a large conformational change in the process of enzyme–dehydroluciferyl-adenylate complex formation (DeLuca and Marsh, 1967), it is not possible to determine whether the two essential sulfhydryl groups are at the catalytic site or are simply necessary for maintaining the structural changes that occur during the activation reaction.

Recently we found that TPCK,[1] an aromatic inhibitor of chymotrypsin, also inhibits luciferase activity. The results of these inhibition studies suggest that there are essential sulfhydryl groups present at the luciferin binding site.

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[1] Abbreviations used: TPCK, the chloromethyl ketone derivative of *N*-tosyl-L-phenylalanine; TLCK, the chloromethyl ketone derivative of *N*-tosyl-L-lysine.

## Materials and Experimental Procedures

Four-times-crystallized luciferase was prepared from firefly tails (*Photinus pyralis*) according to the method of Green and McElroy (1956). D-Luciferin and dehydroluciferin were synthesized according to White *et al.* (1961). The concentrations of luciferin and dehydroluciferin were determined by measuring the absorbance at 327 mμ (ε 18,000) and 350 mμ (ε 24,000) respectively, at a neutral pH.

TPCK, TLCK, and *N*-tosylphenylalanine were obtained from Cyclo Chemical Corp., *p*-mercuribenzoate and γ-chymotrypsin from Sigma Chemical Co., *N*-ethylmaleimide from Schwartz BioResearch, Inc., and dithiobis(2-nitrobenzoic acid) from Aldrich Chemical Co., Inc. Guanidine hydrochloride was prepared by adding an equivalent amount of HCl to a guanidine carbonate solution. The resulting solution was treated with Norit and the solid guanidine hydrochloride obtained upon evaporation was recrystallized once from methanol.

Luciferase concentrations were calculated using the absorbance at 278 mμ and the extinction coefficient of 0.75 for 1 mg of protein/ml of solution. A molecular weight of 100,000 was used in calculating the molar concentration of the enzyme (Green and McElroy, 1956). Luciferase activity was assayed by measuring the intensity of the initial flash of light emitted as described by McElroy and Seliger (1961). The reaction mixture for a normal assay contained 2.0 ml of 0.025 M sodium glycylglycine buffer at pH 7.9, 0.1 ml of 0.1 M $MgSO_4$, 0.1 ml of $6 \times 10^{-4}$ M luciferin, and 5–10 μg of luciferase. The reaction was initiated by rapid injection of 0.2 ml of 0.02 M ATP (pH7.5) with a hypodermic syringe. The maximum light intensity which occurs within a few seconds was taken as a measure of initial velocity and expressed in an arbitrary intensity unit. All assays were carried out in triplicate. For the determination of luciferase activity in the presence of dehydroluciferin and ATP, the assay was modified in the following way. The reaction mixture was similar to a normal assay mixture, except 0.2 ml of ATP replaced the luciferin solution. After the addition of enzyme–dehydroluciferin–AMP solution 10 μl of coenzyme A–cysteine solution ($6.25 \times 10^{-3}$ M in coenzyme A and 0.5 M in L-cysteine) was introduced to remove the dehydroluciferin which is bound to the enzyme as dehydroluciferin–AMP (Airth *et al.*, 1958). After a few minutes the light reaction was initiated by the injection of 0.2 ml of $3 \times 18^{-4}$ M luciferin solution.

The number of sulfhydryl groups in luciferase was determined by the method of Ellman (1959). To facilitate the reaction of dithiobis(2-nitrobenzoic acid) with sulfhydryl groups of luciferase, the reaction was carried out in 5 M guanidine hydrochloride at pH 8. The reaction mixture which was placed in a cuvet contained 3–8 mμmoles of luciferase in 1.2 ml of 5 M guanidine hydrochloride in 0.05 M sodium phosphate buffer at pH 8 and 0.02 ml of 0.01 M DTNB in 0.05 M sodium phosphate buffer at pH 7. Absorbance at 412 mμ was measured with a Beckman DU spectrophotometer.

Performic acid oxidation was carried out according to the method of Hirs (1967). Preformed performic acid reagent (1 ml) was used per 12 mg of protein.

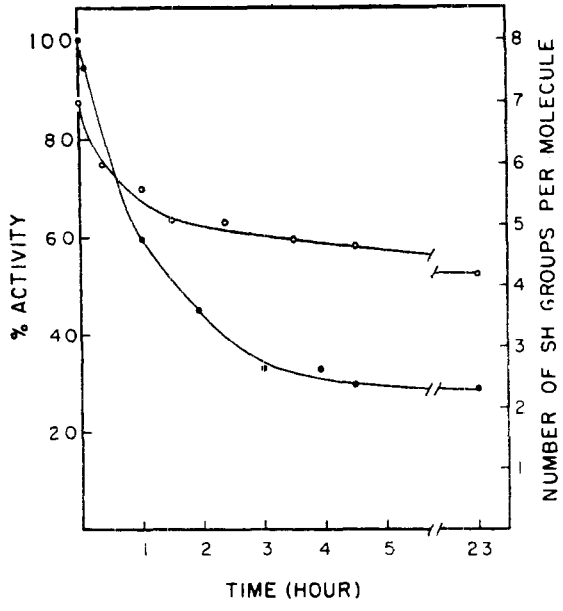


FIGURE 1: Inhibition of firefly luciferase by TPCK. A luciferase solution containing $2.8 \times 10^{-2}$ μmole of the enzyme in 2 ml of 0.05 M sodium phosphate buffer at pH 8.0 was treated with 10 μl of 4 mg/ml of TPCK solution in methanol. TPCK was present in 4 molar excess of luciferase. (●) Loss of luciferase activity; (○) loss of SH groups.

In a typical TPCK inhibition study, the reaction mixture contained $2.8 \times 10^{-2}$ μmole of luciferase in 2.0 ml of 0.05 M sodium phosphate buffer at pH 7.8–8.0 and 10 μl of $1.14 \times 10^{-2}$ M TPCK in methanol. Addition of 10 μl of methanol to a control tube did not cause any inactivation of luciferase for at least 24 hr at room temperature.

## Results

TPCK was found to be an effective inhibitor of luciferase, causing 50% inactivation in 1 hr and 70–90% inhibition in 5–7 hr with only 4 molar excess of the reagent over luciferase. This 70–90% inactivation was accompanied by a loss of 1.7–2.2 moles of sulfhydryl groups/mole of luciferase. When the reaction mixture was left overnight, there was usually no further loss of activity though a further small decrease in SH groups was noted (Figure 1). TLCK, the lysine analog of TPCK, did not inhibit luciferase under the same conditions, and the loss of sulfhydryl groups was negligible. When TPCK was used in 40-fold excess, the decrease in activity was very fast, resulting in 50% inactivation in 10 min and in a complete inactivation in 4 hr, at which time all but one sulfhydryl group had reacted.

When luciferase was treated first with dehydroluciferin and ATP to form the enzyme–dehydroluciferin–AMP complex, TPCK (4 molar excess) could no longer inhibit the enzyme. Activi y assayed after addition of coenzyme A and cysteine remained constant throughout the experiment, although approximately 1 mole of sulfhydryl group was lost per mole of luciferase.

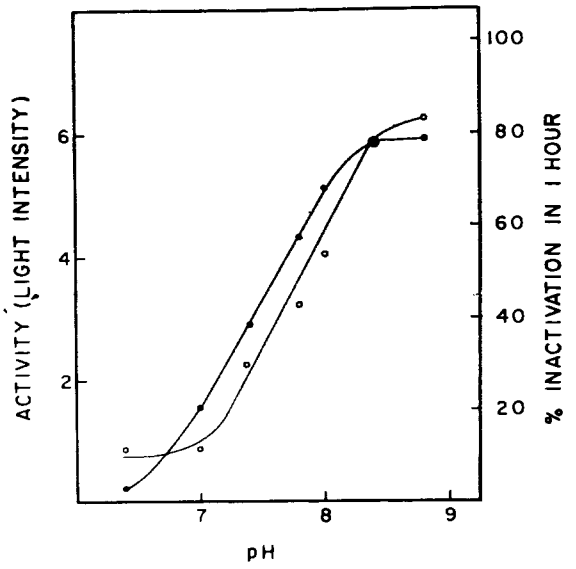


FIGURE 2: Effect of pH on the rate of luciferase-catalyzed light production and the rate of inactivation of luciferase by TPCK. Both light production and TPCK inactivation were carried out in 0.05 M sodium phosphate buffers. Activity of TPCK-inhibited luciferase was measured under the standard assay conditions in 0.025 M glycylglycine buffer at pH 7.9. (●) Luciferase activity in an arbitrary light intensity unit; (○) rate of TPCK inactivation.

The number of TPCK groups incorporated per molecule of luciferase during TPCK inhibition was estimated according to the method of Whitaker and Perez-Villasenor (1968). Luciferase was inactivated with a 4-fold molar excess of TPCK in a usual manner for 7.5 hr, at which time 78% of activity and 1.9 moles of SH groups had been lost. A control solution had full activity and no obvious loss of SH groups. Both solutions were dialyzed twice against 400 ml of 0.05 M sodium phosphate buffer at pH 7.6 and the absorbance difference of the two solutions at 227 mμ was determined. With an extinction coefficient of $8.9 \times 10^3$ $M^{-1}$ $cm^{-1}$ at 227 mμ for TPCK, the number of TPCK groups bound to luciferase was calculated to be 1.8/molecule.

In another experiment, TPCK–luciferase was isolated in the following manner. Luciferase was treated with a 5.6 molar excess of TPCK for 32 hr at room temperature, at which time 84% of activity and 2.7 moles of SH groups had been lost. The luciferase solution was made to 6 M with urea and dialyzed extensively against $10^{-2}$ M acetic acid in cold. A slightly cloudy solution was freeze dried. γ-Chymotrypsin (0.5 μmole) in 25 ml of 0.05 M sodium phosphate buffer (pH 7) was treated with 10-fold molar excess of TPCK for 4 hr at room temperature. It was then dialyzed extensively against distilled water in the cold and freeze dried. Amino acid analysis of performic acid oxidized TPCK–γ-chymotrypsin produced 0.9 mole of 3-carboxymethylhistidine/mole as expected (Stevenson and Smillie, 1965), whereas neither 1-carboxymethylhistidine nor 3-carboxymethylhisitidine was observed from oxidized TPCK–luciferase. The amino acid composition of oxidized luciferase was in good agreement with previously published results (De Luca *et al.*, 1964) except for lower serine and threonine content. This was also true for oxidized TPCK–luciferase. The loss of these two amino acids could have occurred during the oxidation step. One notable change in amino acid composition between oxidized luciferase and oxidized TPCK–luciferase was a decrease of approximately 2.7 moles of cysteic acid in oxidized TPCK–luciferase (see Table I).

TABLE I: Amino Acid Composition[a] of Oxidized Luciferase Compared with Oxidized TPCK-Inhibited Luciferase.

| Amino Acid | Oxidized Luciferase | Oxidized TPCK–Luciferase |
|---|---|---|
| Lysine | 61 | 63 |
| Histidine | 22 | 24 |
| Arginine | 29 | 31 |
| Cysteic acid | 7.4 | 4.7 |
| Aspartic acid | 77 | 82 |
| Threonine | 24 | 34 |
| Serine | 12 | 26 |
| Glutamic acid | 75 | 77 |
| Proline | 45 | 43 |
| Glycine | 67 | 69 |
| Alanine | 65 | 66 |
| Valine | 71 | 67 |
| Methionine[b] | 25 | 24 |
| Isoleucine | 56 | 56 |
| Leucine | 83 | 79 |
| Tyrosine | 26 | 25 |
| Phenylalanine | 47 | 46 |

[a] Residues per mole, assuming a molecular weight of 100,000. Not corrected for the loss during oxidation and acid hydrolysis. [b] Sum of methionine and methionine sulfone.

The rate of inactivation of luciferase by TPCK was studied at different pH values and compared with the rate of the light reaction. The results are presented in Figure 2. The rate of inactivation of luciferase at different pH values resembles remarkably the catalytic activity at these pH values.

Inhibition of luciferase by TPCK was found to be of a competitive nature with respect to one of the substrates, luciferin, but noncompetitive with respect to ATP. This is shown both by Lineweaver–Burk and by Eadie–Hofstee-type plots (Figure 3). In order to minimize the irreversible inactivation caused by TPCK, activity assays for such kinetic studies were done as fast as possible after the addition of luciferase to the assay mixture containing TPCK. The time required from the mixing to the completion of assay was estimated to be 15–25 sec. In this time period, approximately 1% of luciferase could have been inactivated irreversibly. When *p*-mercuribenzoate (*ca.* $3 \times 10^{-6}$ M) was used as the inhibitor in the kinetic study with varying luciferin concentration, data showed the inhibition to be neither com-

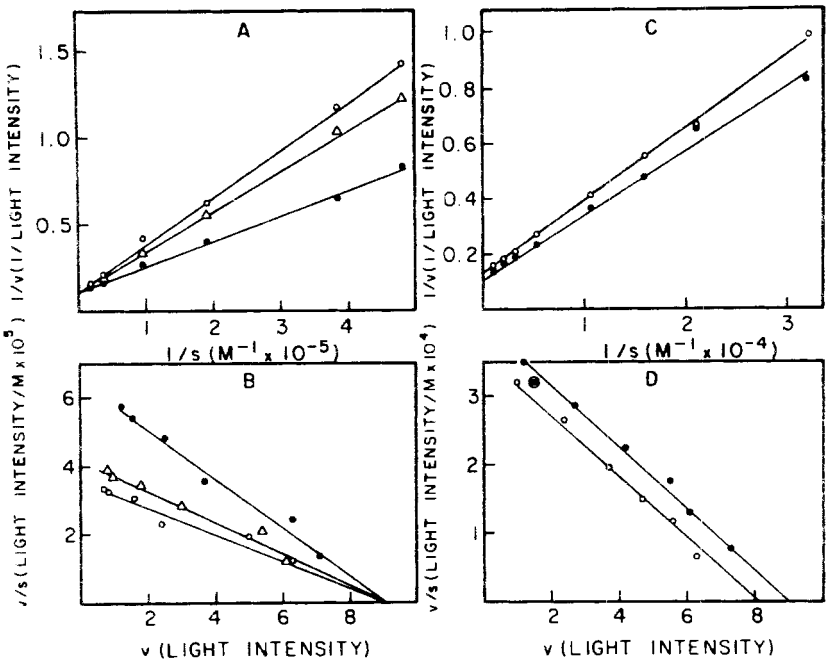


FIGURE 3: Kinetic studies of TPCK inhibition. The experiments were conducted in 0.025 M glycylglycine buffer (pH 7.9) with 0.14 mμmole of luciferase either at a constant ATP concentration of $1.9 \times 10^{-3}$ M or a constant luciferin concentration of $5.2 \times 10^{-6}$ M. (●) No TPCK added; (○) in the presence of $2.6 \times 10^{-4}$ M TPCK; (△) in the presence of $1.3 \times 10^{-4}$ M TPCK. (A) Lineweaver–Burk plot of data obtained under constant ATP and variable luciferin concentrations. (B) The same set of data as A plotted according to the method of Eadie and Hofstee. (C) Lineweaver–Burk plot of data obtained under constant dehydroluciferin and variable ATP concentrations. (D) Data as C plotted according to the method of Eadie and Hofstee.

petitive nor noncompetitive. *N*-Tosylphenylalanine, which has a carboxylic acid group instead of the chloromethyl ketone group of TPCK, and hence is not a chemically reactive compound, also inhibited luciferase competitively with respect to luciferin. The inhibition constant, $K_i$, for TPCK was $2.5 \times 10^{-4}$ M. The $K_i$ for *N*-tosylphenylalanine was $7.3 \times 10^{-4}$ M.

TPCK inhibition of luciferase was also carried out in the presence of one of its substrates. The presence of luciferin concentration equivalent to its $K_m$ ($3 \times 10^{-5}$ M) did not change the TPCK inactivation rate by any appreciable extent. However, a tenfold increase in luciferin concentration decreased the inactivation rate by threefold. ATP–$Mg^{2+}$ concentration ($2 \times 10^{-3}$ M in ATP) equivalent to ten times its $K_m$ was without effect on the inactivation rate.

Initial velocities of light production were also measured with different concentrations of TPCK in order to determine the number of inhibitor molecules per enzyme molecule that is necessary for inactivation. The plot according to the method of Johnson *et al.* (1942) is shown in Figure 4. The slopes obtained in two sets of experiments were 0.87 and 1.19, indicating that the inhibition was caused by one molecule of TPCK.

Earlier studies from this laboratory showed that *p*-mercuribenzoate and *N*-ethylmaleimide both inhibited luciferase (DeLuca *et al.*, 1964; Travis and McElroy, 1966). Two other sulfhydryl reagents, iodoacetamide and ethylenimine, also inhibited luciferase, though much higher concentrations of the reagents were needed. Thus 90–100% inhibition was obtained with 0.4 M ethylenimine and 0.02 M iodoacetamide. These conditions were however still mild enough so that iodoacetamide was not expected to react with other amino acids such as histidine, methionine, and lysine (Gurd, 1967). The following study was undertaken to investigate the relationship between reactivity of various sulfhydryl reagents and their ability to inactivate luciferase. A yellow solution

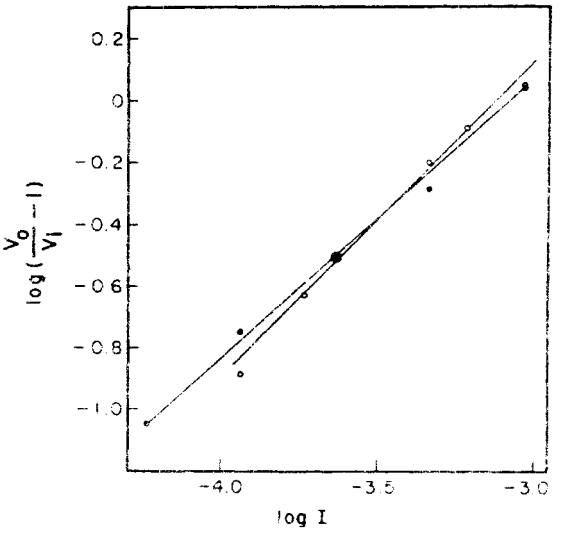


FIGURE 4: The effect of various concentrations of TPCK on luciferase activity. Plots according to Johnson *et al.* (1942). $V_0$ and $V_i$ are initial velocities of luciferase-catalyzed light production in the absence and the presence, respectively, of TPCK; I, concentration of TPCK. Light production was measured under the standard conditions.

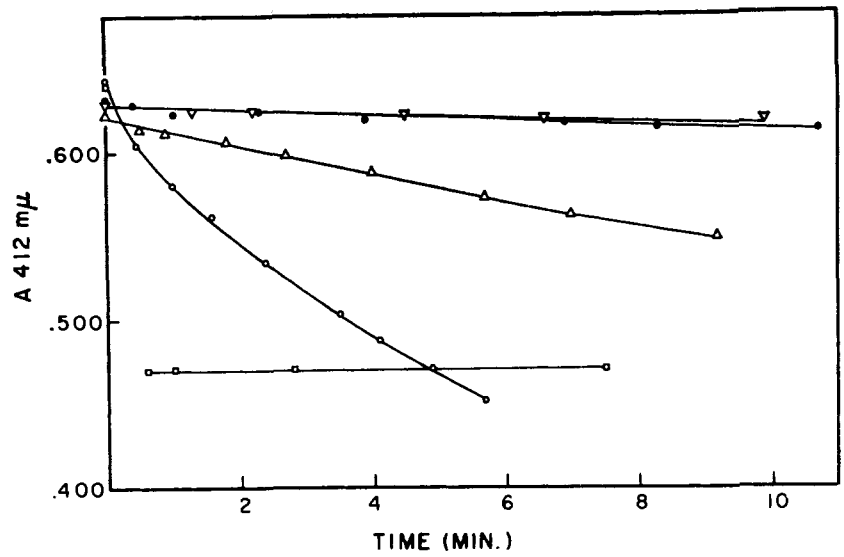


FIGURE 5: Rate of reaction of various SH reagents with 2-nitro-5-thiolbenzoic acid dianion. The thiolbenzoic acid dianion was produced in a cuvet (1.2 ml) by treating a constant amount of L-cysteine (0.05 μmole) with excess 5,5'-dithiobis(2-nitrobenzoic acid) reagent (0.1 M, 0.02 ml) in 0.05 M sodium phosphate buffer at pH 8. After the absorbance determination at 412 mμ, 0.1 μmole of one of the following SH reagents was added and absorbance determination continued: (●) TPCK, (○) *N*-ethylmaleimide, (△) iodoacetamide, and (▽) ethylenimine. Only 0.01 μmole of *p*-mercuribenzoate (□) was used.

of 2-nitro-5-thiolbenzoic acid dianion was formed according to the method of Ellman by treating excess dithiobis(2-nitrobenzoic acid) with 0.05 μmole of L-cysteine in 0.05 M sodium phosphate buffer. To this was added 0.1 μmole of one of the following sulfhydryl reagents: *p*-mercuribenzoate, *N*-ethylmaleimide, TPCK, iodoacetamide, and ethylenimine. The yellow color diminished as these reagents reacted with the thiolbenzoic acid dianion. The rate of this reaction was determined by following the decrease in absorbance at 412 mμ immediately after mixing one of the reagents with the yellow solution in a cuvet. The addition of 0.1 μmole of *p*-mercuribenzoate to the yellow solution caused an immediate drop of absorbance to the background level. When only 0.01 μmole of *p*-mercuribenzoate was added, the drop of absorbance was much less, though still instantaneous (see Figure 5). These results indicate that the reaction of *p*-mercuribenzoate with thiolbenzoic acid dianion is very fast, indeed. All of the other reagents reacted slow enough so that a reaction rate could be measured. Note in Figure 5 that TPCK and ethylenimine reacted very slowly and that the reaction of iodoacetamide was several times faster than that of TPCK. Figure 6 shows the activity loss of luciferase when an equal amount of the above five reagents was treated with luciferase. It is obvious that TPCK caused much more inhibition of luciferase than that expected from its reactivity toward 2-nitro-5-thiolbenzoic acid dianion.

## Discussion

It has not been possible to decide whether the two essential sulfhydryl groups for luciferase action are actually at the catalytic site or are essential merely for the conformational changes and may therefore be located elsewhere in the protein.

In our present studies with TPCK, it was found that the inhibitor competed with luciferin and not with ATP. This suggests to us that TPCK is most likely to be reacting at the catalytic site. If the inhibitor were reacting at a different site, thus preventing conformational changes, then one might expect TPCK to be competitive with both luciferin and ATP rather than just for the one (luciferin).

In the original studies of TPCK inhibition of chymotrypsin (Schoellmann and Shaw, 1963) and TLCK inhibition of trypsin (Shaw *et al.*, 1965), the only site of reaction of these enzymes with TPCK or TLCK was at a histidine residue. More recently, several groups have reported the TPCK and TLCK inactivation of sulfhydryl peptidases, such as papain (Whitaker and Perez-Villasenor, 1968) and ficin (Stein and Liener, 1967). In both cases a single SH group of the enzyme was the only site of reaction. Involvement of an SH group was also suggested for the inactivation of stem bromelain (Murachi and Kato, 1967).

In our present study with luciferase, TPCK also appeared to react only with SH groups. This conclusion is supported by (i) the stoichiometry between the number of SH groups lost and the number of TPCK groups incorporated into luciferase, (ii) decrease in cysteic acid content in oxidized TPCK–luciferase equivalent to the amount of SH groups lost, and (iii) absence of either 1-carboxymethyl- or 3-carboxymethylhistidine in the hydrolysate of performic acid oxidized TPCK–luciferase.

SH groups are apparently reactive enough so that almost all the SH groups of luciferase react with TPCK if an excess of TPCK (40-fold over luciferase concentration or 5-fold over SH concentration) was used. Two essential SH groups of luciferase react more rapidly than other SH groups. This could be due to (i) higher reactivity of these two groups, (ii) more accessibility of the reagent to these two groups, or (iii) preferential bind-

ing of TPCK to the site at or near the SH groups. While *p*-mercuribenzoate, *N*-ethylmaleimide, and TPCK all show various degrees of preference for the two essential SH groups, causing 60–80% activity loss per two SH groups reacted (DeLuca *et al.*, 1964; Travis and McElroy, 1966), preliminary experiments with iodoacetamide and ethylenimine showed that the loss of activity is almost a linear function of the loss of SH groups. Though this result does not completely eliminate possibilities i and ii, a preferential binding of TPCK at or near the two SH groups is definitely a factor in the TPCK inactivation of luciferase, as will be discussed below.

A comparison of the inactivation of luciferase by several reagents of different reactivity shows that the ability of TPCK to inactivate luciferase far exceeds that expected from its chemical reactivity, suggesting that something more than chemical reactivity is operating in the TPCK inactivation of luciferase. Since *N*-tosylphenylalanine itself can inhibit luciferase competitively, and TLCK does not inhibit luciferase under the comparable conditions used for TPCK, it appears that the hydrophobic nature of TPCK brings this compound to the binding site of luciferin (or dehydroluciferin), thus achieving an effect of affinity labeling. It should be mentioned in this respect that luciferin also is a hydrophobic compound containing a benzthiazole ring and a thiazolene ring, and that a number of aromatic compounds have been found to compete with luciferin. $K_i$'s for TPCK and *N*-tosylphenylalanine were of the same order of magnitude, again suggesting the tosylphenylalanine portion to be the major determinant for the competitive character of the inhibition. In contrast, the *p*-mercuribenzoate inhibition was not strictly competitive, having $V_{max}$ lower than that in the absence of inhibitor. This is to be expected, since the reaction of *p*-mercuribenzoate with sulfhydryl groups is very much faster so that a measurable portion of luciferase is expected to be irreversibly inhibitied between the time of mixing and the completion of the assay.

Dehydroluciferin–AMP successfully protected the enzyme from TPCK inactivation. This is to be expected, since in such a reaction mixture, both ATP and dehydroluciferin were present in excess of luciferase, and with the known equilibrium constant of $2.5 \times 10^5$ for the enzyme–dehydroluciferin–AMP formation (eq 3) (Rhodes and McElroy, 1958), the concentration of free enzyme in the reaction mixture was estimated to be negligibly small. More significant is the fact that luciferin (or dehydroluciferin) could alone retard the TPCK inactivation rate considerably when present in the reaction mixture at a concentration equivalent to $10 \times K_m$. ATP–$Mg^{2+}$ did not have a similar effect. Since neither dehydroluciferin nor ATP–$Mg^{2+}$ caused any significant conformational change in luciferase (DeLuca and Marsh, 1967), the above results must mean that luciferin (or dehydroluciferin) interferes with TPCK inactivation by competing for the same site.

The two substrates of luciferase, luciferin (or dehydroluciferin) and ATP, must be in close proximity to each other on the luciferase surface, since the carboxylic acid group of luciferin (or dehydroluciferin) must react with the AMP–PP bond of ATP. The fact that TPCK is strictly

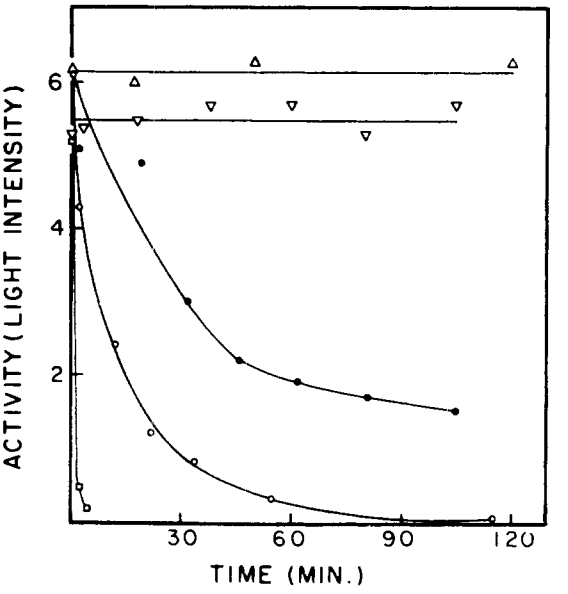


FIGURE 6: Rate of inactivation of luciferase by several SH reagents. Luciferase (2.3 mμmoles) in 0.2 ml of 0.05 M sodium phosphate buffer (pH 8) was treated with 20 mμmoles of the SH reagents. Activity was assayed under the standard conditions. (●) TPCK, (○) *N*-ethylmaleimide, (△) iodoacetamide, (▽) ethylenimine, and (□) *p*-mercuribenzoate.

noncompetitive with respect to ATP must mean, therefore, that TPCK is fairly specifically bound to the luciferin (or dehydroluciferin) site.

It has been shown that luciferase has one dehydroluciferin–AMP forming site per molecule (Rhodes and McElroy, 1958). Quite recently, however, DeLuca (1969) showed the existence of two binding sites on luciferase for naphthalene dyes which competed with luciferin binding. Determination of the binding of dehydroluciferin to luciferase by both equilibrium dialysis and fluorescence also showed the existence of two sites (J. Denburg, personal communication). In the TPCK inactivation study, two TPCK molecules were incorporated per molecule of luciferase, and this involved two SH groups, yet only one molecule of TPCK was required for inactivation of enzyme activity (Figure 4). These observations suggest that luciferase contains two luciferin binding sites, but only one site can function at any one time to form enzyme–dehydroluciferin–AMP or enzyme–luciferin–AMP. The possible significance of this observation is currently under investigation.

The plot of TPCK inactivation rate *vs.* pH closely resembles the activity–pH curve of luciferase. This does not prove, but strongly suggests, that the sulfhydryl anion is somehow required in the production of characteristic yellow-green light of the luciferase-catalyzed reaction. Luciferase can also catalyze the production of red light in addition to the typical yellow-green light (Seliger and McElroy, 1964). This can be achieved by using lower pH, higher temperature, or low concentration of urea. Low concentration of heavy metal ions, such as $Zn^{2+}$ and $Cd^{2+}$, also produced the red shift. Among the metals studied, $Hg^{2+}$ was the most effective, causing red shifts at concentrations 100 times less than that re-

quired for either $Zn^{2+}$ or $Cd^{2+}$. In the production of red light, the bulk of the luciferase active site is probably still intact, and the spectral shift is more likely to be caused by the change in the mode of luciferin binding to luciferase. Among the several groups in the protein which could interact with the heavy metal ions, sulfhydryl groups seem to be the most reactive, especially toward $Hg^{2+}$ (Gurd and Wilcox, 1956). Thus the sulfhydryl group would appear to play an important role in the binding of luciferin for the production of the typical yellow-green light.

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# Chromatography and Activity of Thiol-Subtilisin*

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ABSTRACT: Thiol-subtilisin, in which a serine side chain at the active site was replaced by a cysteine residue, was prepared from chromatographically purified subtilisin. The thiol derivative was separated from native subtilisin by ion-exchange chromatography. The unexpectedly marked difference in chromatographic behavior indicates an alteration in some of the physical properties of the protein molecule. Pure thiol-subtilisin exhibits a very low activity toward *p*-nitrophenyl *N*-benzyloxycarbonylglycinate which is a specific substrate for subtilisin. This low activity is interpreted in terms of a distortion of the active site of the thiol-enzyme rather than in terms of a difference in chemical reactivity between hydroxyl and sulfhydryl groups.

In previous papers (Polgar and Bender, 1966, 1967; Neet and Koshland, 1966) it was shown that the serine residue at the active site of subtilisin can be transformed into a cysteine side chain. This thiol derivative of subtilisin retains enzymatic activity when tested with substrates possessing a good leaving group such as nitrophenyl esters or *N-trans*-cinnamoylimidazole (Polgar and Bender, 1967). Quantitative values are only available for the hydrolysis of *p*-nitrophenyl acetate and *N-trans*-cinnamoylimidazole (Polgar and Bender, 1967) since some extraneous enzymatic activity has interfered with other substrates. The present paper was aimed at obtaining thiol-subtilisin of a high purity in order to study the hydrolysis of *p*-nitrophenyl *N*-benzyloxycarbonylglycinate, a more specific substrate.

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## Experimental Section

Subtilisin, Bacterial Proteinase Novo, was a produc