Velick, S. F., Baggott, J. P., and Sturtevant, J. M. (1971), *Biochemistry 10*, 779.
von Ellenrieder, G., Kirschner, K., and Schuster, I. (1972), *Eur. J. Biochem. 26*, 220.
Weber, G., and Anderson, S. R. (1965), *Biochemistry 4*, 1942.

Kinetics of Product Inhibition during Firefly Luciferase Luminescence†

John J. Lemasters* and Charles R. Hackenbrock

ABSTRACT: A theoretical and experimental analysis is made of the kinetics of product inhibition during firefly luciferase luminescence. Equations for competitive, noncompetitive, and uncompetitive inhibition are derived which are useful in determining inhibitory mechanism when the product inhibitor, or its concentration, is unknown and not subject to direct experimental manipulation. Comparisons of experimental data with predictions based upon the three inhibitory models show that product inhibition during luciferase luminescence is noncompetitive with respect to both luciferin and ATP as substrates. The competitive and uncompetitive models are inconsistent with experimental data. These findings provide the basis for using luminescence to measure ATP concentration continuously in in vitro biological systems such as isolated mitochondria.

The bioluminescent enzyme, firefly luciferase, catalyzes the following reactions (cf. McElroy and DeLuca, 1973):

$$E + LH_2 + Mg^{2+} + ATP \leftrightarrow E \cdot LH_2AMP + Mg^{2+} + PP_i \quad (1)$$

$$E \cdot LH_2AMP + O_2 \rightarrow E + CO_2 + AMP + \text{product} + \text{light} \quad (2)$$

$$E + L + Mg^{2+} + ATP \leftrightarrow E \cdot LAMP + Mg^{2+} + PP_i \quad (3)$$

The initial activation step (reaction 1) is the formation of enzyme-bound luciferyl adenylate (LH_2AMP).[1] This enzyme complex reacts with molecular oxygen (reaction 2) to produce light, AMP, CO_2, and a product of luciferin now identified as the decarboxyketo derivative, oxyluciferin (Suzuki and Goto, 1971). Luciferase also catalyzes the activation of dehydroluciferin (L) to form enzyme-bound dehydroluciferyl adenylate (LAMP) which cannot subsequently react to produce light (reaction 3). Dehydroluciferin is a competitive inhibitor of luciferin in reaction 1.

Firefly luciferase luminescence may be used for the accurate and sensitive measurement of ATP (cf. Strehler, 1968). Addition of ATP to a solution containing firefly luciferase, luciferin, oxygen, and divalent cation results in light production whose intensity maximum is proportional to ATP concentration in accordance with the Michaelis–Menten equation. The intensity maximum, or flash height, lasts only a few seconds and is followed by an exponential decay that may last for several hours. This decay does not result from the consumption of substrate but is due to specific inhibition by a product of the luminescence reaction, presumably oxyluciferin (McElroy and Seliger, 1961). Several derivatives of luciferin have been shown to be competitive inhibitors of luciferin and noncompetitive inhibitors of ATP in the luminescence reaction (Denburg et al., 1969). More recent studies by Goto et al. (1973) indicate that oxyluciferin is also a competitive inhibitor of luciferin. However, Gates and DeLuca (1975) have questioned the simple competitive role of oxyluciferin in luminescence decay since excess amounts of luciferin do little to impede the rapid decrease of luminescence during the ongoing reaction. Since our original interest was in measuring ATP continuously in suspensions of metabolically active mitochondria (Lemasters and Hackenbrock, 1973), it became necessary to investigate the continuous kinetics of luminescence, especially the mechanism and kinetics of product inhibition. In this communication we extend our previous findings to show that product inhibition during the ongoing luminescence reaction is noncompetitive with respect to both luciferin and ATP, and that such inhibition is inconsistent with predictions based on either the competitive or uncompetitive inhibitory models.

Methods

Synthetic luciferin (LH_2) and purified luciferase were obtained from Du Pont Corp., Instrument Division. One unit of enzyme equaled 1.6–1.9 μg of protein (Lowry et al., 1951). Luciferase luminescence was detected by a Brice-Phoenix light scattering photometer, Model 2000-D, from a conventional 1-cm light-path cuvette holding 2–3 ml of constantly stirred reaction medium. Light tight additions were made with microliter syringes through a rubber stopper in the lid of the photometer compartment. The luminescence signal was recorded on a strip chart recorder. Statistical estimates of kinetic constants, K_s and V_{max}, obtained from flash height data were calculated by the method of Wilkinson (1961) for each series of experiments. Reaction velocities were expressed in arbitrary light units equaling the millivolt output of the photometer. This arbitrary light unit was not equivalent for different experimental series because of variations in photomultiplier tube voltage from one series of experiments to the next. The sub-

†From the Department of Cell Biology, The University of Texas Health Science Center at Dallas, Southwestern Medical School, Dallas, Texas 75235. *Received January 20, 1975.* This work was supported by Research Grant BMS 75-20141 from the National Science Foundation and Research Grants CA 17065-01 and RR 05426-13 from the National Institutes of Health.

[1]Abbreviations used: LH_2, luciferin; L, dehydroluciferin; LH_2AMP, luciferyl adenylate; LAMP, dehydroluciferyl adenylate; E, luciferase.

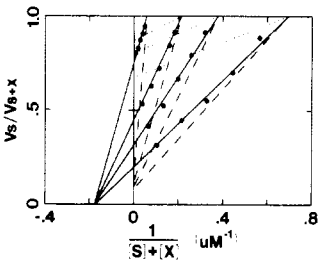

FIGURE 1: Product inhibition with respect to luciferin as substrate. V_s/V_{s+x} is plotted vs. $1/([S] + [X])$. The straight lines represent the theoretical predictions of the competitive (- - -), noncompetitive (—), and uncompetitive (· · ·) models. K_s and V_{max} for luciferin were 5.8 μM and 5.9 arbitrary light units as determined from flash height data. Data points were generated by adding [X], an aliquot of luciferin, 1 min after the initiation of luminescence with ATP. [S] is the initial luciferin concentration. For each line [S] and V_s were constant and [X] was varied. Reaction medium was 1 unit/ml luciferase, 244 μM ATP, 5 mM $MgCl_2$, 10 mM $NaPO_4$ buffer, pH 7.4, 23 °C.

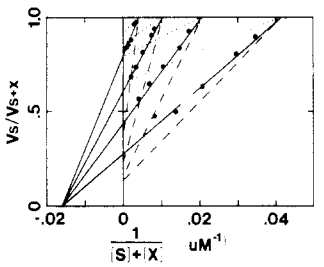

FIGURE 2: Product inhibition with respect to ATP as substrate. V_s/V_{s+x} is plotted vs. $1/([S] + [X])$. The straight lines represent the theoretical predictions of the competitive (- - -), noncompetitive (—), and uncompetitive (· · ·) models. Experimental details are as Figure 1 with [S] and [X] referring to ATP concentration. K_s and V_{max} for ATP were 62 μM and 18.8 arbitrary light units as determined from flash height data. Reaction medium was 5 units/ml luciferase, 55 μM luciferin, 5 mM $MgCl_2$, 11 mM $NaPO_4$ buffer, pH 7.4, 23 °C.

strates, ATP and luciferin, were kept at near saturation (80% or greater) except for the one being varied.

Results and Discussion

The paradigm experiment for studying product inhibition during the ongoing luminescence reaction is as follows: ATP is added to a mixture of luciferase and luciferin. The resulting rapid rise in light production reaches a maximal value, the flash height, which is employed to determine kinetic constants, K_s and V_{max}, as luciferin or ATP is varied. Following the flash height is a rapid decay of luminescence. However, the luminescence signal remains sensitive to substrate concentrations and increases sharply after addition of a second aliquot of ATP or luciferin. In the following kinetic analysis, [S] is the concentration of one or the other substrate when luminescence is first initiated. [X] represents substrate added some time later. The reaction velocity just prior to the addition of [X] is termed V_s. The reaction velocity just after the addition of [X] is termed V_{s+x}. K_s and V_{max} are the Michaelis constant and maximal velocity, respectively, of the uninhibited reaction.

We will consider three inhibitory models: the competitive, noncompetitive, and uncompetitive models. We assume that luciferase is sufficiently low in concentration or activity that its enzymatic action will not significantly alter ATP or luciferin concentrations during the time span of our experiments.

The rate equation for competitive inhibition (cf. Webb, 1963) is:

$$V_s = \frac{[S]V_{max}}{[S] + K_s(1 + [I]/K_i)} \quad (4)$$

where [I] is the inhibitory product concentration, and K_i is its inhibition constant. We solve for the inhibition term:

$$1 + [I]/K_i = \frac{[S](V_{max} - V_s)}{V_s K_s} \quad (5)$$

After addition of more substrate, [X], rate eq 4 becomes:

$$V_{s+x} = \frac{([S] + [X])V_{max}}{[S] + [X] + K_s(1 + [I]/K_i)} \quad (6)$$

This expression reflects both the change in substrate concentration and the change in the velocity of the reaction. Since the inhibition term, $1 + [I]/K_i$, should be the same immediately before as immediately after the addition of [X], we substitute eq 5 into eq 6. After rearranging, we obtain:

Competitive

$$\frac{V_s}{V_{s+x}} = \frac{[S]}{[S] + [X]}\left(1 - \frac{V_s}{V_{max}}\right) + \frac{V_s}{V_{max}} \quad (7)$$

The corresponding expression for noncompetitive inhibition, as shown previously (Lemasters and Hackenbrock, 1973), is:

Noncompetitive

$$\frac{V_s}{V_{s+x}} = \frac{1}{[S] + [X]}\left(\frac{K_s[S]}{K_s + [S]}\right) + \frac{[S]}{K_s + [S]} \quad (8)$$

In a similar fashion, the following expression for uncompetitive inhibition may be derived:

Uncompetitive

$$\frac{V_s}{V_{s+x}} = \frac{1}{[S] + [X]}\left(\frac{K_s V_s}{V_{max}}\right) - \frac{K_s V_s}{[S]V_{max}} + 1 \quad (9)$$

When experimental data are plotted as $1/[S] + [X]$ vs. V_s/V_{s+x}, eq 7-9 may be employed to predict families of straight lines upon which data points will fall for specific constant values of [S] and V_s. K_s and V_{max} are estimated from independent flash height determinations. Figures 1 and 2 show such predictions applied to either luciferin or ATP as substrate. The data points are generated by adding [X], an aliquot of ATP or luciferin, 1 min after the initiation of luminescence. V_s is essentially constant for a particular value of [S]. The data show a good fit to the noncompetitive model for both luciferin (Figure 1) and ATP (Figure 2) as substrate. The data fit neither the competitive nor uncompetitive models.

Since there is evidence which indicates that oxyluciferin spontaneously degrades or polymerizes to other products (Plant et al., 1968; McElroy et al., 1969), it was important to ascertain whether noncompetitive inhibition persists after longer incubation periods. Equations 7-9 may be used to predict the ratio V_s/V_{s+x}. If [S] and [X] are the same and approximately equal to K_s, then the noncompetitive model predicts that V_s/V_{s+x} will be about 0.75 irrespective of the magnitude of V_s. By contrast, the competitive and uncompetitive models predict that V_s/V_{s+x} will approach 0.5 and 1, respectively, as V_s decreases to zero. Experimentally, V_s may be allowed to fall to low levels by simply increasing the time interval between the initiation of the reaction and the addition of [X]. From experimental values of V_s after various time intervals, V_s/V_{s+x} may be predicted according to the various inhibitory models. These are compared with experimental values of V_s/V_{s+x} (Figure 3). The latter values correspond closely to the pre-

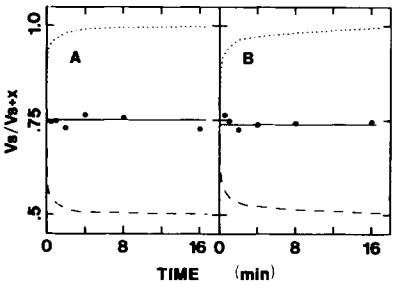

FIGURE 3: Product inhibition as a function of time. V_s/V_{s+x} is plotted vs. duration of the luminescence reaction. The lines represent the predicted values of V_s/V_{s+x} based on the competitive (- - -), noncompetitive (—), and uncompetitive (· · ·) models and experimental values for V_s after various time intervals after the initiation of luminescence. In A, experimental values for V_s/V_{s+x} were generated by adding 8.6 μM luciferin 0.5 to 16 min after the initiation of luminescence with 730 μM ATP in a reaction medium containing 1 unit/ml luciferase, 8.6 μM luciferin, 5 mM $MgCl_2$, 10 mM $NaPO_4$ buffer, pH 7.4, 23 °C. K_s and V_{max} for luciferin were 8.5 μM and 9.8 arbitrary light units. In B, aliquots of 49 μM ATP were added 0.5 to 16 min after the initiation of luminescence with 49 μM ATP in the same reaction medium as A excepting luciferin concentration which was 55 μM. K_s and V_{max} for ATP were 53 μM and 6.9 arbitrary light units.

dictions of the noncompetitive model, and there is no indication of deviation even after 16 min of incubation at which time the reaction is inhibited by greater than 99%.

In noncompetitive inhibition, the apparent V_{max} declines in proportion to the amount of inhibition, while K_s remains unchanged. The constancy of V_s/V_{s+x} implies, therefore, that K_s remains the same over the time course of the reaction. Equation 8 may be rearranged and solved for K_s. K_s may then be estimated following two successive additions of substrate to an unreacted luciferase solution. Values for K_s based on such calculations agree closely with those derived from flash height determinations.

Our results are in apparent contradiction with those of Goto et al. (1973) who report that oxyluciferin is a competitive inhibitor of luciferin in the luminescence reaction. Such divergence could be explained if our findings reflect pseudoirreversible inhibition (mutual depletion zone C of Webb, 1963) which occurs when enzyme concentration is about equal to or larger than K_i. Under such circumstances, both competitive and noncompetitive inhibitors will behave identically and generally in agreement with the expectations of noncompetitive inhibitory kinetics. The boundary conditions for pseudoirreversible inhibition depend on the amount of inhibition. As inhibition increases, relatively greater excess of enzyme over K_i is required to obtain zone C or pseudoirreversible kinetics. In Figure 3A, after 16 min of reaction, inhibition exceeds 99%, and, for pseudoirreversible (zone C) kinetics to hold with reasonable accuracy, K_i would have to be more than 100-fold less than enzyme concentration; i.e., $K_i < 4 \times 10^{-10}$ M assuming a minimum molecular weight of 50 000 for luciferase and pure enzyme. This value is orders of magnitude smaller than any K_i reported for various luciferin derivatives (Denburg et al., 1969), or for oxyluciferin itself (Goto et al., 1973). Therefore, it appears unlikely that the noncompetitive pattern of inhibition which we observe can be explained on the basis of pseudoirreversible inhibition. DeLuca and Marsh (1967) have demonstrated by optical rotary dispersion and tritium-hydrogen exchange studies that a large conformational change in luciferase occurs after addition of substrates and initiation of the reaction. Such a conformational change may underlie the difference in the inhibitory mechanism observed by Goto and ourselves since Goto measured reaction velocity upon initiation of luminescence while we measured luminescence after one to several minutes. Alternatively, our results may indicate that oxyluciferin is not the active species in product inhibition of luminescence.

The expressions derived for competitive, noncompetitive, and uncompetitive inhibition provide a useful way of determining inhibitory mechanisms in situations where the inhibitor or its concentration is unknown and not subject to direct manipulation. However, K_i of the inhibitor cannot be estimated. We are able to determine that product inhibition of luciferase luminescence is noncompetitive with respect to both luciferin and ATP as substrates during the continuous reaction. With this information, it is then possible to use the continuous luminescence signal for quantitative measurement of ATP concentration in metabolic systems (Lemasters and Hackenbrock, 1973, 1976).

References

DeLuca, M., and Marsh, M. (1967), *Arch. Biochem. Biophys. 121,* 233–240.

Denburg, J. L., Lee, R. T., and McElroy, W. D. (1969), *Arch. Biochem. Biophys. 134,* 381–394.

Gates, B. J., and DeLuca, M. (1975), *Arch. Biochem. Biophys. 169,* 616–621.

Goto, T., Kubota, I., Suzuki, N., and Kishi, Y. (1973), in Chemiluminescence and Bioluminescence, Cormier, M. J., Hercules, D. M., and Lee, J., Ed., New York, N.Y., Plenum Press, pp 325–335.

Lemasters, J. J., and Hackenbrock, C. R. (1973), *Biochem. Biophys. Res. Commun. 55,* 1262–1270.

Lemasters, J. J., and Hackenbrock, C. R. (1976), *Eur. J. Biochem. 67,* 1–10.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265–275.

McElroy, W. D., and DeLuca, M. (1973), in Chemiluminescence and Bioluminescence, Cormier, M. J., Hercules, D. M., and Lee, J., Ed., New York, N.Y., Plenum Press, pp 285–308.

McElroy, W. D., and Seliger, H. H. (1961), in Light and Life, McElroy, W. D., and Glass, B., Ed., Baltimore, Md., Johns Hopkins Press, pp 219–254.

McElroy, W. D., Seliger, H. H., and White, E. H. (1969), *Photochem. Photobiol. 10,* 153–170.

Plant, P., McElroy, W. D., and White, E. H. (1968), *Biochem. Biophys. Res. Commun. 31,* 98–103.

Strehler, B. L. (1968), *Methods Biochem. Anal. 16,* 99–181.

Suzuki, N., and Goto, T. (1971), *Tetrahedron Lett.,* 2021.

Webb, J. L. (1963), Enzyme and Metabolic Inhibitors, Vol. 1, New York, N.Y., Academic Press.

Wilkinson, G. N. (1961), *Biochem. J. 80,* 324–332.