PSEUDO-ALLOSTERIC BEHAVIOR OF FIREFLY LUCIFERASE*

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Received April 1, 1968

Under certain conditions the reversal of the activation of dehydroluciferin (L) by firefly luciferase (reaction 1a) shows typical sigmoidal kinetics.

$$L + ATP\text{-}Mg^{++} + Enzyme \underset{1a}{\overset{1}{\rightleftharpoons}} Enzyme \bullet L\text{-}AMP + Mg^{++}\text{-}PP$$

The results presented below indicate that at low Mg^{++} concentrations all of the inorganic pyrophosphate (PP) added to the reaction mixture does not exist as the active species, $[Mg^{++}\text{-}PP]$. This results in sigmoidal type kinetics which is analogous in behavior to enzymes that are classified as allosteric. That this catalytic behavior in the case of luciferase is not allosteric is shown by the fact that when the velocity of the reaction is plotted against substrate concentration one obtains normal Michaelis-Menten kinetics at high Mg^{++} concentrations where all the PP exists in the active form.

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* This research was supported in part by the U.S. Atomic Energy Commission, The National Science Foundation, and The National Institutes of Health. Contribution No. 524, from The McCollum-Pratt Institute.

** Research Career Development Awardee of the National Institutes of Health.

Furthermore, the addition of citrate or EDTA can change the normal Michaelis-Menten type kinetics to sigmoidal in a manner analogous to "allosteric effectors". In those systems which require metal ions and have substrates or effectors that are chelating agents (i.e., ATP, ADP, PP, etc), it is important to exclude the phenomenon described before attributing allosteric behavior to the enzyme.

Materials and Methods

Firefly luciferase was isolated and crystallized according to the method of Green and McElroy (1956). Dehydroluciferyl-adenylate (L-AMP) was synthesized as described by Rhodes and McElroy (1958) and purified by elution from a Sephadex G-25 column (R. Morton, personal comm.). The concentration was determined by $OD_{353}m\mu$ using the molar extinction coefficient of 1.5×10^4.

The assay for the activation of L is based on the fact that the fluorescence of free dehydroluciferin is much greater than the fluorescence of L-AMP. The rate of production of L from L-AMP was followed by the increase in fluorescence emission at 548 mμ while exciting at 348 mμ (Rhodes and McElroy, 1958). Fluorescence was measured on the Aminco-Bowman Spectrophotofluorometer.

All assays were run in 0.5 M Tris pH 7.5, at 25°C. The typical reaction mixture contained 6μg luciferase, 3-5 mμ moles L-AMP, and varying concentrations of $MgSO_4$. Reaction 1a was started by the addition of inorganic pyrophosphate (PP). Since the initial velocity was too rapid to measure at high PP concentrations it was necessary to use PP concentrations below the K_m.

The light emitting activity of the enzyme was assayed by the flash height technique (DeLuca et al, 1964). These reactions are shown in the following equations:

(2) $\text{Enzyme} + LH_2 + ATP + Mg^{++} \rightleftharpoons \text{Enzyme} \bullet LH_2\text{-AMP} + Mg^{++}\text{-PP}$

(3) $\text{Enzyme} \bullet LH_2\text{-AMP} \xrightarrow{O_2} \text{Light} + AMP + CO_2 + \text{Products}$

Results

The velocity of the hydrolysis of L-AMP as a function of pyrophosphate concentration is shown in Fig. 1b. In these experiments the ratio of Mg^{++} to pyrophosphate was maintained

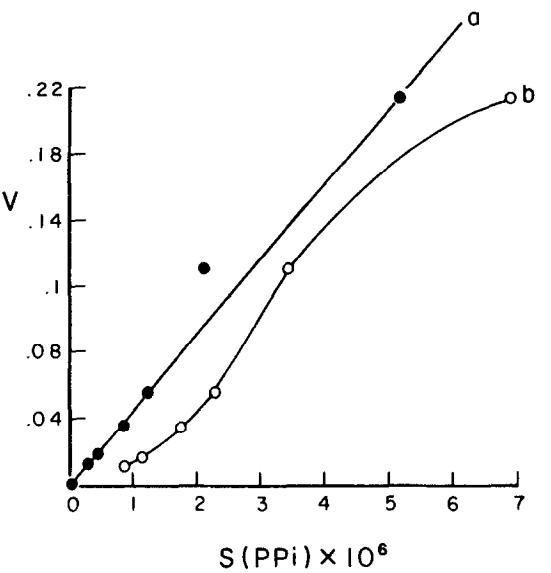

Fig. 1 The effect of concentration of pyrophosphate on the rate of hydrolysis of E-LAMP. The reaction mixture contained 6 μmoles LAMP, 0.3 mμ moles enzyme and a constant ratio of Mg^{++}/PP of 2.5. Curve b represents observed experimental data. In curve a) plotted on the abscissa is the theoretically calculated concentration of the Mg-PP complex (See Discussion).

at 2.5/1. A plot of the same data is linear when 1/V is plotted against $1/S^2$ (Fig. 2). An initial interpretation of the data is that more than one molecule of PP is involved in the reaction. This would imply two binding sites with different dissociation constants.

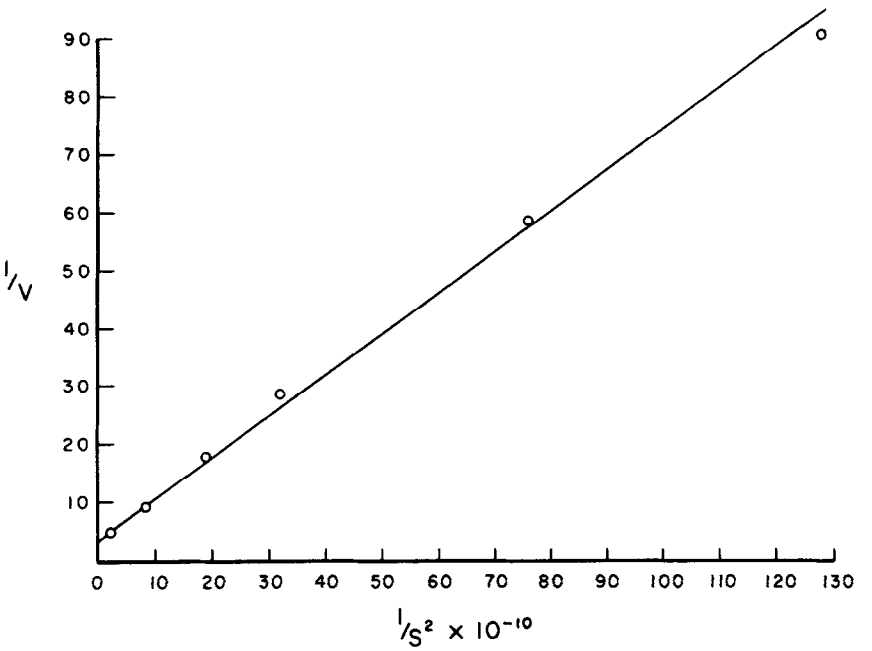

Fig. 2 The data from Fig. 1b plotted in the form of $\frac{1}{V}$ with respect to $1/PP^2$.

Slight variation of the reaction conditions, in which excess Mg^{++} was added, gives the results in Fig. 3. These are typical Michaelis-Menten kinetics with a Km for pyrophosphate in the reverse reaction of 1×10^{-5}M. A study of the inhibition of the light reaction by PP (see Methods) gives a K_i of 1.4×10^{-5} M, indicating that PP is acting at the same site in both cases.

A Hill plot of the data in Fig. 3 gives an interaction

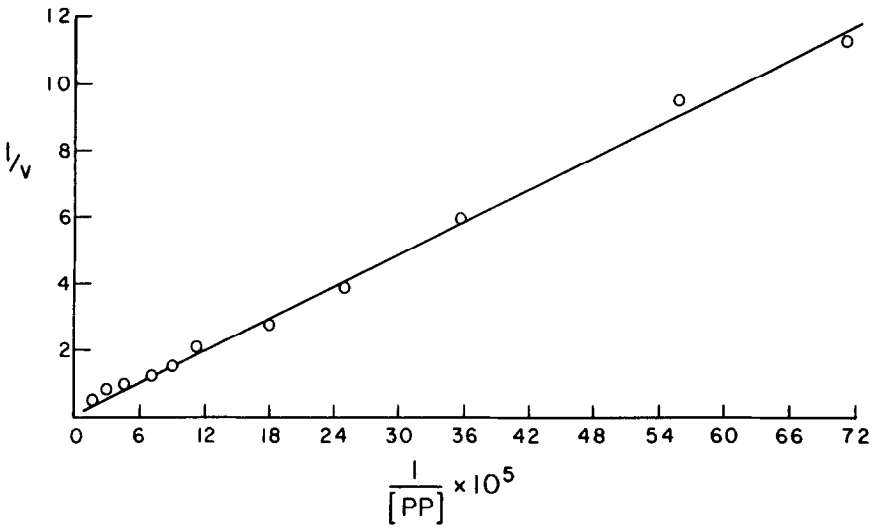

Fig. 3 The effect of concentration of pyrophosphate on the rate of hydrolysis of E-LAMP in the presence of a constant and saturating concentration of Mg^{++} (3×10^{-3}M).

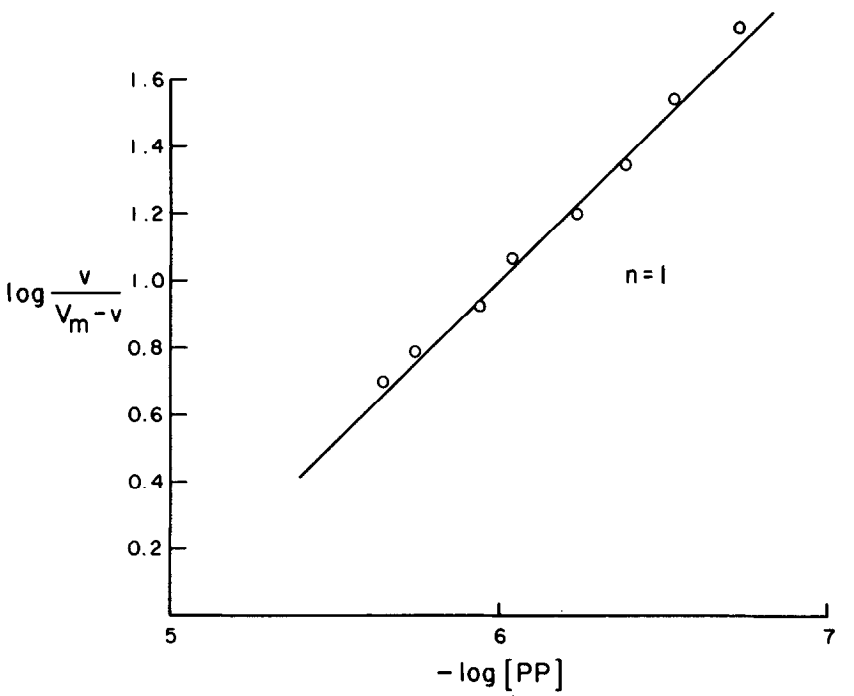

Fig. 4 A Hill plot using the data from Fig. 3.

constant of 1, suggesting only one pyrophosphate binding site. (Fig. 4).

It seemed probable that other compounds might elicit sigmoidal type kinetics by competing with PP for Mg^{++}. Two such "effectors" were tried, citrate and EDTA. As expected, sigmoidal kinetics were obtained with both compounds under conditions in which controls (absence of effectors) gave normal kinetics. This is shown by the non-linear reciprocal plots in Fig. 5.

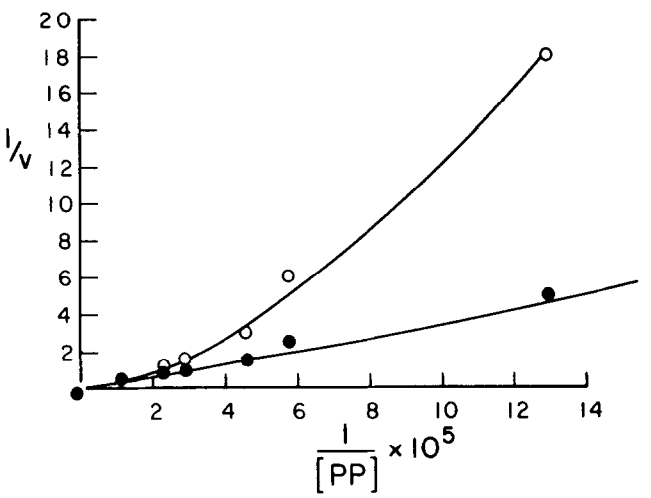

Fig. 5 Double reciprocal plots of velocity against PP concentration in the absence (●—●) and the presence (○—○) of effectors 1 x 10^{-3} M Citrate and 3 x 10^{-4} M EDTA.

Discussion

In the case of luciferase we are able to obtain sigmoidal type kinetics simply by maintaining a constant Mg^{++} to PP ratio of 2.5/1. The sigmoidal kinetics are accounted for by a decrease in the concentration of the $[Mg^{++} - PP]$ complex at low total concentrations

of Mg^{++}. This is the predicted result from the equilibrium equation

$$(4)\quad [Mg^{++}\text{-}PP] = \frac{\left(K_D + [Mg^{++}]_T + [PP]_T\right) \pm \sqrt{\left(K_D + [Mg^{++}]_T + [PP]_T\right)^2 - 4[Mg]_T[PP]_T}}{2}$$

in which $[Mg]^{++}_T$ and $[PP]_T$ represent the total initial concentration of Mg^{++} and PP. K_D is the dissociation constant of the $[Mg^{++} - PP]$ complex and is equal to 4×10^{-6} (Gosselin and Coghlan, 1953). Equation (4) was used to calculate the concentration of $[Mg^{++} - PP]$ in Fig. 1 for each corresponding concentration of total pyrophosphate. The velocities when plotted against the true $[Mg^{++} - PP]$ concentration are shown in Fig. 1a. This straight line is expected of an enzyme obeying Michaelis-Menten kinetics at low substrate concentrations.

The sigmoidal kinetics are not interpreted in terms of two binding sites since high concentrations of Mg^{++} result in normal kinetics and the data from the Hill plot give n = 1. Furthermore, the sigmoidal kinetics obtained with unrelated compounds, such as citrate and EDTA, are to be expected because of their ability to combine with Mg^{++} and thus lower its concentration. It is obvious that any compounds which form a complex with Mg^{++} will exhibit similar effects on this and other Mg^{++} requiring enzymes.

Frequently the observation of sigmoidal kinetics upon the addition of various "effector" molecules has been interpreted in terms of allosteric interactions. As originally defined (Monod et. al, 1963) the effector molecules bind at specific sites other than the "active site". It is clear from the present results that more information than just sigmoidal kinetics is needed before one can attribute allosteric properties to an enzyme.

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