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A SECOND-ORDER KINETIC REACTION CATALYZED BY RIBOFLAVIN SYNTHETASE FROM A HIGH-RIBOFLAVINOGENIC *EREMOTHECIUM ASHBYII*

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SUMMARY

The initial reaction velocity of the reaction catalyzed by riboflavin synthetase from a high-riboflavinogenic *Eremothecium ashbyii* shows a second-order dependency on 6,7-dimethyl-8-ribityllumazine concentration in the range of $1.5 \cdot 10^{-4}$–$6.25 \cdot 10^{-6}$ M, suggesting that binding of the two molecules of the lumazine to the enzyme occurs with affinities of similar orders of magnitude. Binding of one lumazine molecule to the enzyme increases the affinity of the second molecule for the enzyme.

Riboflavin synthetase catalyzes the conversion of two molecules of 6,7-dimethyl-8-ribityllumazine to one molecule of riboflavin and one molecule of 4-ribitylamino-5-aminouracil. HARVEY AND PLAUT[1] have proposed the existence of two substrate-binding sites on the enzyme. One site (donor site) binds the lumazine in such a way that it functions as donor of the 4-C moiety to be transferred in the reaction, and the other site (acceptor site) binds the lumazine that serves as acceptor of the 4-C fragment. The rate equation derived from such a mechanism contains a second-order term with respect to the lumazine concentration, indicating a possible non-linear relationship between the reciprocals of the reaction velocity and the lumazine concentration. Such a relationship, however, has not been discovered yet; the reactions catalyzed by the purified enzymes from yeast[1], spinach[2] and *Ashbya gossypii*[3] have been recognized to proceed by zero- to first-order kinetics. In contrast to these observations, the present communication demonstrates that the catalytic reaction by riboflavin synthetase from a high-riboflavinogenic *Eremothecium ashbyii* shows a second-order dependency of the reaction rate on the lumazine concentration.

The reaction mechanism catalyzed by riboflavin synthetase is formulated as follows:

$$E + S \rightleftharpoons ES_a \qquad (K_a) \qquad (1)$$

$$E + S \rightleftharpoons ES_d \qquad (K_d) \qquad (2)$$

$$ES_a + S \rightleftharpoons ES_aS_d \qquad (K_d') \qquad (3)$$

$$ES_d + S \rightleftharpoons ES_aS_d \qquad (K_a') \qquad (4)$$

$$ES_aS_d \rightarrow E + \text{Products} \qquad (k) \qquad (5)$$

where E, S, S_a and S_d denote the enzyme, 6,7-dimethyl-8-ribityllumazine, the lumazine bound to the acceptor site and the lumazine bound to the donor site, respectively, and K_a, K_d, K_d' and K_a' are the respective dissociation constants of Eqns. 1–4, and $K_a'K_d = K_aK_d'$. Assuming that Reactions 1–4 are in rapid equilibrium compared to Reaction 5, the following formulation can be derived:

$$\frac{1}{v} = \frac{1}{V}\left[1 + \frac{K_a'}{[S]} + \frac{K_d'}{[S]} + \frac{K_a'K_d}{[S]^2}\right] \tag{6}$$

where v and V are the initial and maximal initial velocities, respectively. When binding of the first and second molecules of the lumazine occurs with very significantly different affinities, *i.e.* $K_a \ll K_d'$ or $K_d \ll K_a'$, and K_a or $K_d \ll [S]$, $K_a'K_d/[S]^2$ of Eqn. 6 becomes negligible, and under these conditions plots of $1/v$ against $1/[S]$ (Lineweaver–Burk plot) result in a straight line, giving an apparent Michaelis constant (K_m) of $K_a' + K_d'$. When the affinities of the lumazine binding sites have similar orders of magnitude, the Lineweaver–Burk plot gives a curve instead of a straight line because of the contribution of the second-order term. The logarithmic formulation of Eqn. 6 is

$$\log \frac{v}{V - v} = 2 \log [S] - \log [[S] (K_a' + K_d') + K_a'K_d] \tag{7}$$

This can be reduced to simple approximations when the lumazine concentration is made small or large, namely

$$\log \frac{v}{V - v} = 2 \log [S] - \log K_a'K_d \tag{8}$$

or

$$\log \frac{v}{V - v} = \log [S] - \log (K_a' + K_d') \tag{9}$$

On plotting $\log v/(V - v)$ against $\log [S]$ (Hill plot), a curve with two asymptotic straight lines with a slope $= 2$ and a slope $= 1$ is obtained. The former and latter asymptotes cut the horizontal axis ($\log [S]$ axis) at $\log \sqrt{K_a'K_d}$ ($= \log \sqrt{K_aK_d'}$) and $\log (K_a' + K_d')$, respectively. When $\log \sqrt{K_a'K_d} \geqq \log (K_a' + K_d')$, the following relation must hold:

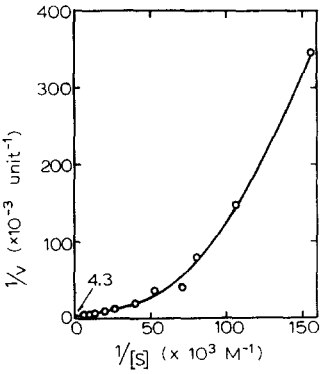
$$K_a' \leqq K_a/4 \quad \text{or} \quad K_d' \leq K_d/4 \tag{10}$$

This means that the binding of the first lumazine molecule to the enzyme increases the affinity of the second lumazine molecule binding to the enzyme.

Measurement of the initial velocity of the reaction catalyzed by riboflavin synthetase from *E. ashbyii* showed second-order enzymic kinetics over a range of 6,7-dimethyl-8-ribityllumazine concentrations from $1.5 \cdot 10^{-4}$–$6.25 \cdot 10^{-6}$ M. As shown in Fig. 1, the Lineweaver–Burk plot of the initial velocity was found to be a curved plot and not a straight line. The Hill plot of the initial velocity consisted of two straight lines with a slope $= 2$ at substrate concentrations lower than $6 \cdot 10^{-5}$ M and with a slope $= 1$ at the levels beyond this point (Fig. 2). Values of $K_a'K_d$ (or K_aK_d') and $K_a' + K_d'$ were calculated to be $2.7 \cdot 10^{-9}$ M^2 and $4.5 \cdot 10^{-5}$ M, respectively, from the intercepts of the two straight lines on the horizontal axis, and a relationship of $\log \sqrt{K_a'K_d} > \log (K_a' + K_d')$ was obtained. These results are in good agreement with

the theoretical model described previously that two binding sites on riboflavin synthetase of *E. ashbyii* have similar affinities for the lumazine and that binding of the one lumazine molecule to one site on the enzyme increases the affinity of the other site for the second lumazine molecule.

The catalytic reactions by riboflavin synthetase from yeast[1], spinach[2] and *A. gossypii*[3] show zero- to first-order kinetics from $2 \cdot 10^{-4}$–$6 \cdot 10^{-7}$ M lumazine concentration, giving single apparent K_m values of $1.0 \cdot 10^{-5}$, $4.5 \cdot 10^{-5}$ and $2.9 \cdot 10^{-5}$ M, respectively. This implies that the two binding sites have widely dissimilar affinities for the lumazine, and $K_a'K_d$ in Eqn. 6 is restricted to values lower than $3.6 \cdot 10^{-13}$ M^2.



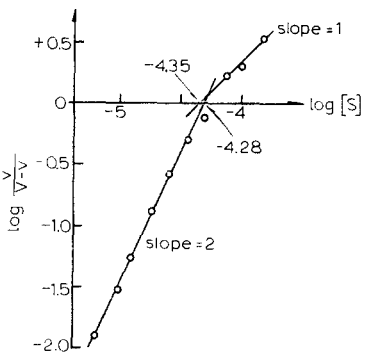


Fig. 1. Lineweaver–Burk plot of the initial velocity of riboflavin synthetase. v, initial velocity, $[S]$, concentration of 6,7-dimethyl-8-ribityllumazine. The reaction mixture (1.5 ml) contained 15 μmoles mercaptoethanol, 15 μmoles Na_2SO_3, 75 μmoles potassium phosphate (pH 7.0), 1.05 mg of enzyme and 6,7-dimethyl-8-ribityllumazine. The reaction proceeded for 15 min at 37°, and was stopped by adding 1.0 ml of 30% trichloroacetic acid and 0.5 ml of distilled water, followed by centrifugation. Riboflavin was determined by the photometric method[3]. 1 unit of enzyme activity was defined as 1 nmole of riboflavin formed in 1 h. The enzyme was isolated from the mycelia of *E. ashbyii* cultured for 2 days[4] by the procedures based on rupturing the cells with alumina, extracting with 0.1 M potassium phosphate (pH 7.0) involving 10 mM Na_2SO_3 and 10 mM mercaptoethanol, and fractionating with $(NH_4)_2SO_4$ (35–50% saturation).
Specific activity of the enzyme was 225 units/mg protein.

Fig. 2. Hill plot of the initial velocity (v) of the riboflavin synthetase reaction. V, maximal initial velocity.

The K_m value becomes equal to the sum of the dissociation constants of the donor and acceptor sites of the ternary enzyme–lumazine complex, *i.e.* $K_a' + K_d'$. Values of $K_a' + K_d'$ with *E. ashbyii* enzyme and with the enzyme from yeast, spinach and *A. gossypii* are quite similar, while the $K_a'K_d$ value in the case of the former enzyme is higher than those in the case of the latter enzymes by a factor of at least 7500. Recently, HARVEY AND PLAUT[1] have isolated a stable binary enzyme–lumazine complex in which the lumazine binds at the donor site on the yeast enzyme with a very small dissociation constant. These observations suggest that *E. ashbyii* enzyme differs markedly from the other enzymes in respect to its affinity for lumazine and the fundamental protein conformation at or around the donor site.

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REFERENCES

1 R. A. HARVEY AND G. W. E. PLAUT, *J. Biol. Chem.*, 241 (1966) 2120.
2 H. MITSUDA, F. KAWAI, Y. SUZUKI AND S. YOSHIMOTO, *J. Vitaminol.*, 16 (1970) 285.
3 G. W. E. PLAUT, *J. Biol. Chem.*, 238 (1963) 2225.
4 H. MITSUDA AND Y. SUZUKI, *J. Vitaminol.*, 16 (1970) 172.