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PORPHYRIN BIOSYNTHESIS IN SOYBEAN CALLUS. V. THE PORPHOBILINOGEN DEAMINASE-UROPORPHYRINOGEN COSYNTHETASE SYSTEM*. KINETIC STUDIES

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SUMMARY

Kinetic studies were carried out using purified porphobilinogenase and deaminase preparations in the presence and absence of ammonium ions.

It has been found in plots of v *versus* 'S' that a deviation from the Michaelis-Menten hyperbola occurs with both enzymes; double-reciprocal plots were concave downward; R_s values were greater than 81; and in some cases the Hill coefficient was less than 1, indicating negative homotropic kinetics.

Evidence also suggested that porphobilinogenase contains at least two substrate-binding sites per molecule of enzyme.

It has also been found that ammonium ions act competitively on the first reaction of the porphobilinogenase.

INTRODUCTION

Two enzymes are required for the conversion of porphobilinogen into uroporphyrinogen III: Porphobilinogen deaminase and uroporphyrinogen cosynthetase. Deaminase alone forms uroporphyrinogen I from porphobilinogen, while cosynthetase alone has no action on either porphobilinogen or uroporphyrinogen I. These two enzymes have been separated and partially purified from several sources (see Ref. 1 for bibliography).

The sigmoid response of bovine liver porphobilinogenase[2] has prompted SANCOVICH *et al.*[1,3] to include this enzyme in the growing list of allosteric enzymes, indicating that porphobilinogenase is highly adapted for regulation by its substrate. Data described in this report are in agreement with the above assumption; preliminary evidence has already indicated that the reaction mechanism involved in this reaction is complicated and the hypothesis was offered that the enzyme contains two or more

* The trivial name "porphobilinogenase" suggested by LOCKWOOD AND RIMINGTON[2] is used to designate the porphobilinogen deaminase uroporphyrinogen cosynthetase system.

interacting sites[1]. Therefore we have undertaken a kinetic investigation to attempt to gather further evidence on the reaction mechanism.

Direct studies were carried out on purified porphobilinogenase and deaminase preparations in the presence and absence of ammonium ions, which at certain concentrations are known to inhibit these enzymes[1,3,4]. Direct studies on the cosynthetase have not been done because one of its substrates, probably the pyrrolic compound described by LLAMBÍAS AND BATLLE[5,6], has not yet been identified; the other substrate is porphobilinogen.

MATERIALS AND METHODS

Porphobilinogen was biosynthetically obtained[7].

Measurement of enzyme activity. The standard incubation system contained the enzyme preparation (usually 8 ml) together with 0.1 M Tris-HCl buffer (pH 7.2) and porphobilinogen with or without the addition of NH_4^+ in a final volume of 10 ml (pH 7.2). Incubations were carried out anaerobically in Thunberg tubes in the dark with mechanical shaking at 38°. Blanks were always run with porphobilinogen and without enzyme. After incubation, trichloroacetic acid was added to precipitate the protein (final concentration 5%, w/v), the mixture was then exposed to 50 ft-candles

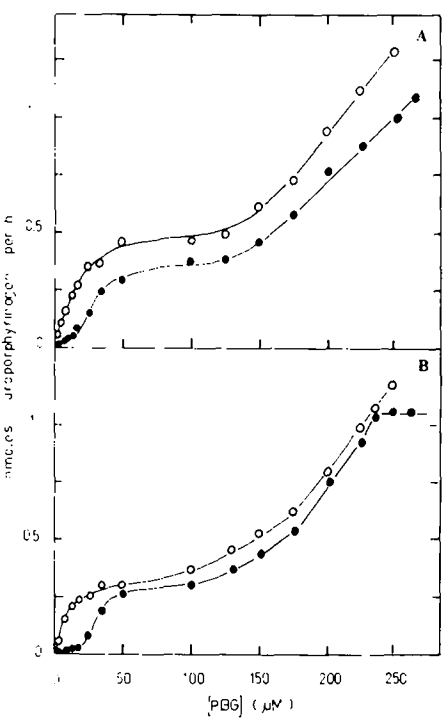

Fig. 1. The effect of porphobilinogen concentration on the rate of uroporphyrinogen formation catalysed by (A) 36-fold purified deaminase and (B) 30-fold purified porphobilinogenase from soybean callus in the absence (○ -○) and the presence (● —●) of 0.1 M ammonium. Assay conditions were as described. PBG, porphobilinogen.

of white light for 20 min to oxidize porphyrinogens, the protein precipitate filtered off, and total porphyrins estimated in the resulting solution. Reaction velocity is expressed as nmoles of uroporphyrinogen formed in 1 h. The purification procedures for porphobilinogenase and deaminase, as well as all other methods and materials not specified here, were those described in Ref. 6.

RESULTS

Saturation curves

It was pointed out that the reaction mechanism of the enzymic conversion of porphobilinogen into uroporphyrinogens must be a complicated one, possibly involving a multivalent enzyme with at least two interacting catalytic sites.

With the soybean callus enzymes, it has been found that the saturation curves of both porphobilinogenase and deaminase were quite unusual (Fig. 1); also double-reciprocal plots were rather complex (Fig. 2).

In the absence of ammonium ions, saturation kinetics of porphobilinogenase and deaminase were much alike (Fig. 1); at low substrate concentration a Michaelis Menten type portion was observed with a plateau from 50 to 125 μM for porphobilinogenase and from 30 to 100 μM for deaminase; increasing porphobilinogen concentration resulted in the addition of a second curve on top of the first. It is also noted

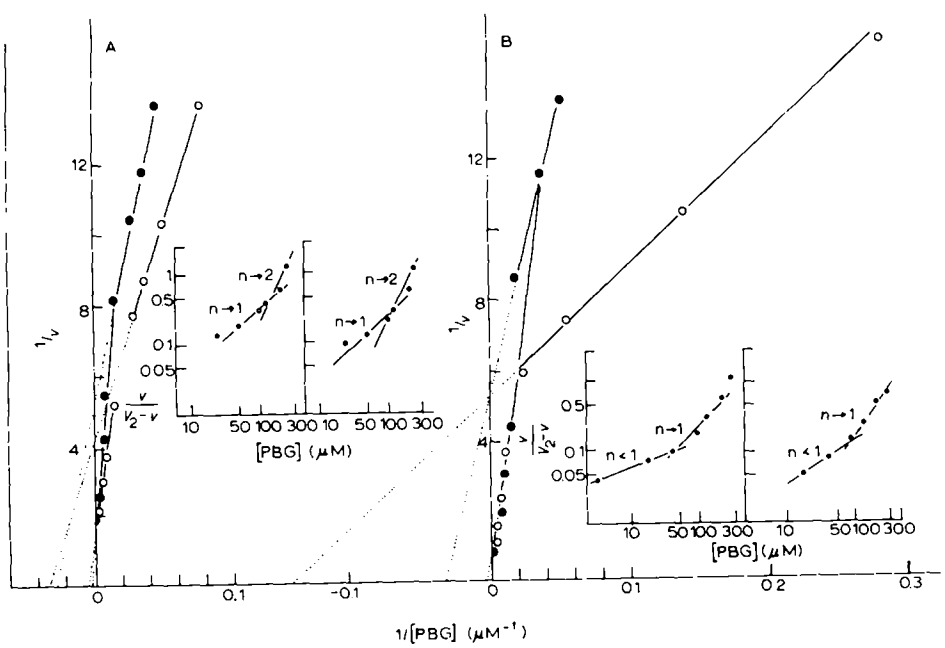

Fig. 2. Double-reciprocal plots of the rate of uroporphyrinogen formation with respect to porphobilinogen concentration catalysed by (A) 30-fold purified deaminase and (B) 30-fold purified porphobilinogenase from soybean callus in the absence (○ - ○) and presence (●—●) of 0.1 M ammonium. Assay conditions were as described. The inserts in these figures show the Hill's plots. V_2 values were taken from Table I. When n values were calculated in relation to V_1 they were the same as those shown here for low concentrations of substrate. PBG, porphobilinogen.

that even at high concentrations of substrate, the enzymes did not become saturated with it, since velocity continued to increase. In the presence of ammonium ion which behaved as an allosteric inhibitor, the rate–concentration plot, hyperbolic in its absence, becomes sigmoidal in its presence, so far as the first reaction is concerned. The effect on the second reaction seems to be different; thus porphobilinogenase appears to reach a second plateau at high concentrations of porphobilinogen in the presence of inhibitor (Fig. 1 B).

Lineweaver Burk plots

Diagnostic double-reciprocal plots (Fig. 2) were nonlinear, and in classical kinetic terms, consisted of highly complex functions. As can be seen, by a judicious selection of concentrations reciprocal plots of reaction rate appear to consist of two portions with different slopes.

In the presence or absence of ammonium ions Lineweaver–Burk plots showed a bimodal character that yielded two apparent K_m values at the extremes of por-

TABLE I

KINETIC CONSTANTS OF SOYBEAN CALLUS PORPHOBILINOGENASE AND DEAMINASE

Activities were assayed as described in MATERIALS AND METHODS. 35–40-fold purified deaminase and 30–35-fold purified porphobilinogenase preparations were used. Michaelis constants and maximum velocities were calculated both from Lineweaver–Burk plots and/or direct saturation curves and they are average values of 6 determinations.

Enzymes and addition	*Kinetic constants*			
	K_{m_1}	K_{m_2}	V_1	V_2
	(M)	*(M)*	*(nmoles urogen/h)*	
I. Deaminase				
None	$3.2 \cdot 10^{-5}$	$1.9 \cdot 10^{-4}$	0.32	1.25
0.1 M NH_4^+	$3.8 \cdot 10^{-5}$	$5.0 \cdot 10^{-4}$	0.20	0.90
II. Porphobilinogenase				
None	$6.5 \cdot 10^{-6}$	$5.0 \cdot 10^{-4}$	0.20	1.75
0.1 M NH_4^+	$3.0 \cdot 10^{-5}$	$5.0 \cdot 10^{-4}$	0.20	1.65

phobilinogen concentration (see Table I for kinetic constants). The high concentration constants are designated K_{m_2}, while those for low concentrations are referred to as K_{m_1}.

Fig. 2 B illustrates the results of typical experiments performed with porphobilinogenase preparations. The data obtained clearly indicated two apparent K_m values; Michaelis constants for the second reaction ($K_{m_2} = 5 \cdot 10^{-4}$ M) were the same in the absence and presence of inhibitor, but it can be seen that ammonium has the effect of changing K_{m_1} from $6.5 \cdot 10^{-6}$ to $3 \cdot 10^{-5}$ M while maximum velocity was unchanged, showing clear competitive inhibition.

With deaminase preparations the double-reciprocal plots shown in Fig. 2A were obtained. K_{m_1} and K_{m_2} were practically the same both in the presence and absence of inhibitor, although the values estimated differ slightly, probably due to the difficulty of determining kinetic constants from the nonlinear plots; in addition, V_1 and V_2 slightly decreased.

It is important to add, that saturation curves and double-reciprocal plots for porphobilinogenase and deaminase both in the absence and presence of ammonium ions, when velocity was measured in terms of nmoles of porphobilinogen consumed per h, also showed a complex pattern but essentially of the same kind as that described above, where reaction rate was measured as nmoles of uroporphyrinogen formed per h; in addition K_m values were of the same magnitude.

Apparent order of reaction with respect to porphobilinogen

As it has been shown, the kinetic behaviour of soybean callus porphobilinogenase and deaminase is rather unusual and strikingly different to that of the bovine liver enzymes[1,3]. Although it has been emphasized that the slope of the Hill plot[8] is a function not only of the number of binding sites but also of the strength of interaction between them, the empirical Hill equation $\log v/(V - v) = n \cdot \log S - \log K$ is frequently used to determine the number of interacting ligand-binding sites. When such manipulations were carried out, Hill plots for deaminase either in the absence or presence of ammonium ions resulted in a slope approaching 1 at very low porphobilinogen concentrations, being almost 2 at high concentrations of substrate (Fig. 2A, inset); however Hill coefficients for porphobilinogenase were about 1 at high concentrations of porphobilinogen but less than 1 at low substrate concentrations (Fig. 2B, inset). SANCOVICH *et al.*[1,3] have reported n values of about 2 for the porphobilinogenase, but either the addition of high concentrations of ammonium, heat treatment or dialysis appreciably changed the slope of the Hill plot to $n = 1$, which was the value obtained for the deaminase.

The deviation from a Michaelis-Menten pattern can also be seen by calculating the R_s values[9], that is, the ratio of the 90 10% saturation velocities; both for deaminase and porphobilinogenase R_s values greater than 81 were obtained.

DISCUSSION

The series of kinetic experiments shown in this paper demonstrate that the soybean enzymes have a kinetic behaviour and parameters different to those of the same enzymes obtained from other sources[1,3] but are consistent with the suggestion that the enzyme system contains at least two interacting active sites, although the possibility of there being more than two should not be excluded.

It has been shown that when the concentration of porphobilinogen was varied over a wide range, the Lineweaver Burk plots showed bimodality. Several possibilities may be considered to explain these nonlinear plots.

The details of the reactions by which porphobilinogen is converted into uroporphyrinogens are still subjects of speculation; it has been already suggested that the reaction could occur through the formation of an intermediate polypyrrol[10], but no such intermediate has been identified so far. However we have reported very recently[5,6] the formation and isolation of a pyrrolic compound formed by the action of porphobilinogenase on porphobilinogen; this compound acted as the substrate of porphobilinogen in the formation of uroporphyrinogen III by purified cosynthetase preparations. The possibility that the bimodality of the Lineweaver-Burk plots is due to the accumulation of the intermediate polypyrrole can be discarded by the fact that the same type of kinetic behaviour and K_m values are obtained whether the

appearance of uroporphyrinogen or the disappearance of porphobilinogen is used as a measure of rate.

There are several examples in the literature of the kind of kinetics exhibited by soybean callus porphobilinogenase and deaminase[11,22], and it has been noted by LEVITZKY AND KOSHLAND[22], that the sequential model for subunit interactions[9,23,24] is ideally suited for the complex phenomena observed for these enzymes. It has been observed by the same authors[22] that these results are easily understood if we consider the types of curves that one would expect from various types of cooperativity; when these criteria are applied to soybean porphobilinogenase and deaminase, many of the characteristics of negative cooperativity are seen to be present, that is, high R_s values, biphasic double reciprocal plots and in some cases Hill coefficient less than 1. LEVITZKY AND KOSHLAND[22] have concluded that negative cooperativity is apparently not a feature of a few odd enzymes but is rather a pervasive pattern in many, and we think it can well explain the accompanying kinetic behaviour, although it is also possible that a combination of negative and positive cooperativity in k_{cat}[24] and negative cooperativity plus Michaelis Menten binding can lead to leveling in the plot of v *versus* $[S]$. Some support for the occurrence of negative interactions between two or more identical active centers is provided by the linearity of Lineweaver-Burk plots in the two regions, and the sharp break between the two linear portions[25,26].

Other possibilities remain that can explain the kinetic behaviour shown here, but they seem to be less probable. FRIEDEN[27] has discussed the interpretation of kinetic data for enzyme systems in which reciprocal plots are nonlinear, in essence, the interpretation based on the graphical analysis of the data was in terms of changes in kinetic parameters and applicable to single substrate systems, but under specific conditions, could be extended to other systems which involve two or three substrates as reactants. Consideration of Fig. 1 and 2 suggested to us that the data could fit an equation derived by FRIEDEN[28], applicable to single substrate systems having at least two binding sites per molecule of enzyme where:

$$v = \frac{V_1 + V_2 \frac{[S]}{K_{m_2}}}{1 + \frac{K_{m_1}}{[S]} + \frac{[S]}{K_{m_2}}} \qquad (1)$$

In our case, K_{m_1} and K_{m_2} are the Michaelis constants at the binding site with the highest affinity for porphobilinogen and the site with the lowest affinity for porphobilinogen, respectively. V_1 refers to the maximum velocity when the first active site is filled and V_2 when both sites are filled; substrate (S) is porphobilinogen.

When this equation was applied using the kinetic parameters shown in Table I for porphobilinogenase and deaminase, plots of Fig. 3 were obtained. It can be seen that the experimental and theoretical curves fitted quite well, describing the same type of kinetics, thus supporting the hypothesis that there are at least two porphobilinogen-binding sites per molecule of enzyme. It must be pointed out that the mere fact that the data fit Eqn. (1) does not necessarily indicate that the kinetic behaviour is due to substrate activation.

Ammonium acts competitively on the first reaction of the porphobilinogenase, indicating that there is some kind of competition between porphobilinogen and ammonium in the formation of the polypyrrolic intermediate. Perhaps ammonium

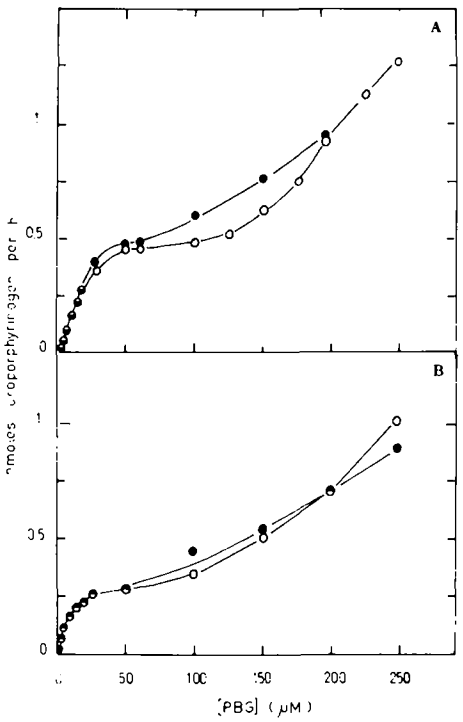

Fig. 3. The effect of porphobilinogen concentration on the rate of uroporphyrinogen formation catalysed by (A) deaminase and (B) porphobilinogenase from soybean callus. ○—○, experimental data; ●—●, theoretical data resulting from the application of Eqn. 1 (Ref. 28). PBG, porphobilinogen.

could inhibit by binding at the same site of porphobilinogen on the cosynthetase or it could still produce dissociation of the deaminase–cosynthetase complex; thus, it is likely that a varying degree of tightness among the different peptide chains of porphobilinogenase could be introduced by a change in the salt concentration of the enzymatic environment which should be expressed by a change in the enzyme rate. SANCOVICH *et al.*[1] have reported for the bovine liver enzymes that high ionic strength influenced the behaviour of cosynthetase on calibrated gel columns; we have also observed that both soybean deaminase and cosynthetase-purified preparations tend to aggregate under certain conditions (unpublished results), and it has become evident that there are many enzymes which undergo a reversible association dissociation reaction and that some of the kinetic properties of these enzymes may depend upon the particular molecular weight species. FRIEDEN[29] has proposed a somewhat different mechanism which is based on the possibility of dissociation of an enzyme to a form which has other properties, but his proposed mechanism might only be tested by performing velocity experiments as a function of ligand concentration over a much wider range of enzyme levels than is ordinarily used in enzyme kinetic work.

Although no changes in the kinetic constants of porphobilinogenase for the second reaction were obtained, in the presence and absence of ammonium ions, it can be seen from the saturation curves of Fig. 1B that while in the absence of inhibitor, porphobilinogenase did not appear to become saturated with porphobilinogen up to

the concentrations tested, in the presence of ammonium ions, however, it seemed to reach a second plateau at high levels of porphobilinogen. As suggested by BOGORAD[4], ammonium ions could produce accumulation of the intermediate polypyrrol, which, in turn, is also substrate for the second reaction; in the absence of inhibitor, the intermediate might be formed in limited amounts. So although porphobilinogen concentration is increased, there is not enough polypyrrol to finally produce uroporphyrinogen and higher levels of substrate are necessary to saturate the enzyme. However, in the presence of ammonium, these ions, although inhibiting the first reaction, could also produce accumulation of the intermediate polypyrrol, which could not then be at limiting concentrations, allowing saturation of the second reaction to be attained at lower levels of porphobilinogen.

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