

Non-Essential Activation of Rat Liver Porphobilinogen-Deaminase by Folic Acid*

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Dedicated to Professor Horst Senger on the occasion of his 60th birthday

Porphobilinogen-Deaminase, Cosynthetase, Uroporphyrinogen I, Folic Acid,
Nonessential Activation

This report demonstrates the ability of folic acid to activate rat liver porphobilinogen-deaminase (PBG–D). Lineweaver-Burk analysis revealed an increase in V_{\max} (38%) without affecting the K_m . In the concentration range assayed, secondary replots of $1/\Delta$ slope and $1/\Delta$ intersect versus $1/[\text{folic acid}]$ yielded straight lines, indicating the binding of a single molecule of activator to the enzyme PBG–D, with a $K_A = 1.66$ mM. Results presented here show that folic acid acts as a non-essential activator ($\alpha = 1$; $\beta = 1.6$).

The activating effect of folic acid has been observed employing the 35–70% ammonium sulphate precipitated fraction, desalted by dialysis or gel filtration, whereas no action was detected when other partially purified PBG–D preparations were utilized as the enzyme source, suggesting either the presence of sites saturated for the activator, or the existence of a different structural protein conformation, or both.

Introduction

Folates are a family of compounds, having in their structure a pteridine ring linked to *p*-aminobenzoate, coupled to an oligoglutamic acid chain, containing between one and seven glutamic residues. Its main physiological function is the transport and transference of one-carbon groups at different degrees of oxidation, for nucleotide synthesis. The pteroylpolyglutamate derivatives are known to be coenzymes for various enzymes.

In 1984 a reduced pteroylpolyglutamate was found to be associated to rat hepatic uroporphyrinogen III synthase (cosynthetase; EC 4.2.1.75), suggesting that it might play the role of a coenzyme in uroporphyrinogen III biosynthesis [1]. However, Hart and Battersby [2] and Smythe and Williams [3] did not detect any folate derivatives or any other reversible bound cofactors in purified cosynthetase preparations obtained from *Euglena gracilis* and rat liver, respectively.

Abbreviations: Porphobilinogen-deaminase, PBG–D; porphobilinogen, PBG.

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We should, however, recall that many years ago Juknat *et al.* [4] had reported the isolation of an endogenous factor which stimulated the synthesis of porphyrinogens from porphobilinogen (PBG) in homogenate and supernatant fractions of etiolated *Euglena gracilis* Z. Its activation properties could be replaced by $0.1 \mu\text{M}$ folic acid. More recent data suggested a 6-biopterin structure for this purified *Euglena* factor [5, 6].

Evidence given independently by Christenson *et al.* [7] and Kotler *et al.* [8] support the hypothesis of the probable existence of a binding site for folate on the enzyme porphobilinogen-deaminase (PBG–D; EC 4.3.1.8), which condenses four molecules of PBG to form the linear unrearranged tetrapyrrole hydroxymethylbilane (HMB), which then spontaneously cyclizes to uroporphyrinogen I.

The present paper shows the effect of folic acid on rat liver PBG–D activity and rises the question of whether folate is necessary for enzyme activity or is a so-called non-essential effector.

Materials and Methods

Materials

Porphobilinogen was enzymatically prepared according to the method of Sancovich *et al.* [9]. Folic acid was obtained from Sigma Chemical Co.,

USA and Sephadex gel was purchased from Pharmacia Fine Chemicals.

All other reagents were of the highest purity available from different commercial sources.

Partial purification of porphobilinogen-deaminase from rat liver

All procedures were carried out at 4 °C. Male rats (Chbb Thom strain, 200–300 g) were killed under ether anaesthesia and the livers were perfused *in situ* with ice cold 0.9% NaCl. The homogenate (10% w/v) was prepared in 50 mM TRIS–HCl buffer, pH 8.2, by using a Potter–Elvehjem glass homogenizer equipped with a motor driven Teflonpestle (30 sec). After centrifugation at $24000 \times g$ for 20 min, the resulting supernatant was centrifuged at $105000 \times g$ for 1 h in a Sorvall OTD 65B ultracentrifuge. The hepatic cytosol fraction was kept at 70 °C for 10 min and centrifuged at $24000 \times g$ for 20 min. This supernatant was treated with solid ammonium sulphate. The fraction precipitating between 35–70% saturation was collected and desalted through a Sephadex G-25 column (1.7 × 26.5 cm).

Enzyme preparations at this stage of purification were found to be stable for several months when stored at –20 °C, but a considerable lost activity was observed when the fractions were successively frozen and thawed.

A PBG–D fraction (15-fold purified) with a specific activity of 0.45 nmol uroporphyrinogen I h^{–1} mg^{–1} protein was used throughout the present study.

Assay of porphobilinogen-deaminase activity

Liver PBG–D activity was measured according to the method of Batlle *et al.* [10]. The standard incubation system contained 1 ml of enzyme fraction (usually 2 mg protein), 0.4 ml of a NaCl–MgCl₂ mixture (containing 0.6 M NaCl:0.12 M MgCl₂, 1:1, v:v), 50 mM TRIS–HCl buffer, pH 8.2, and 30 µg of PBG to give a final volume of 2 ml. Incubations were carried out aerobically in the dark at 37 °C for 2 h. Blanks were run with PBG, but without enzyme. Porphyrins formed were determined in acidic solution by a spectrophotometric method [11].

Porphyrins were esterified and extracted following the method of Seubert and Seubert [12]. Identifi-

cation, quantitative determination [13] and determination of the isomeric composition of uroporphyrin fractions (Polo *et al.*; unpublished results) were performed by HPLC.

Protein concentration was determined by the method of Lowry *et al.* [14].

Results and Discussion

Optimization of assay conditions for PBG–D from rat liver

Preliminary studies were performed to establish the optimum conditions for measuring PBG–D activity, using the desalted 35–70% ammonium sulphate precipitate fraction as enzyme source. Experiments were carried out varying the PBG concentration between 4.5 and 100 µM. Uroporphyrinogen I formation showed a hyperbolic substrate saturation curve, reaching the plateau at 66 µM PBG (data not shown).

When PBG–D activity was investigated as a function of incubation time and protein concentration, it was found that uroporphyrinogen I synthesis increased linearly over a range up to 150 min and 2.0 mg protein/ml, respectively (data not shown).

Effect of folic acid on partially purified PBG–D fractions

Piper and van Lier [15] first found that 100 µM folic acid produced a slight, but significant stimulation of rat liver PBG–D activity; however in a later report by the same group [7] no changes in uroporphyrinogen I formation were observed employing 1 mM pteroylglutamate.

Figure 1a shows the effect of folic acid on partially purified enzyme preparations from rat liver, using a concentration of 2 mM folic acid, that was found to be activating during preliminary experiments. It could also be observed that the effector failed to enhance uroporphyrinogen I synthesis when both fractions containing cosynthetase activity (H, S and Su) and a PBG–D preparation (supernatant of heat-treatment: ø) were used. For explanation of the abbreviations see legend of figure 1. However, experiments carried out employing the 35–70% ammonium sulphate precipitated fraction desalted by Sephadex G-25 chromatography or overnight dialysis, showed an increase in PBG–D activity of 34% and 43%, respectively,

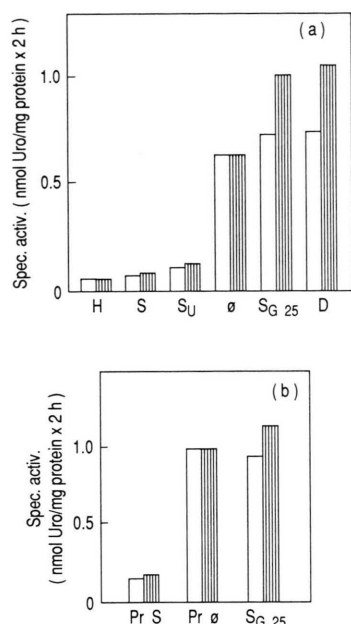


Fig. 1. Folic acid effect on different partially purified PBG-D fractions. Activity was measured using the standard PBG-D assay employing $44 \mu\text{M}$ PBG in the absence (\square) or presence (▨) of 2.0 mM folic acid. Each bar represents the mean value of three experiments run in duplicate. Other experimental conditions were as described in methods and in the text. a) H: homogenate; S: $24000 \times g$ supernatant; Su: $105000 \times g$ supernatant; ø: heat-treatment supernatant; S-G 25 and D: 35–70% ammonium sulphate fraction desalted by Sephadex G-25 chromatography and by dialysis respectively; b) PrS and Prø: S and ø fractions filtered through Sephadex G-25 column.

when these enzyme preparations were incubated with 2 mM folic acid.

These results are suggesting that depending on the enzyme fraction used, there are no free or there are free binding sites for folic acid to allow this factor to demonstrate its activating properties.

In order to determine, whether there might be any sites available for folic acid, S and ø fractions were filtered through a Sephadex G-25 column ($1.7 \times 26.5 \text{ cm}$). The eluted protein was pooled and designated as PrS and Prø, respectively. As can be seen in figure 1b, 2 mM folic acid had no action either on PrS and Prø activities.

From these findings it is likely that it is the high ionic strength which might influence the response of PBG-D to folic acid. It is, however, not yet elucidated if there exists a regulatory site on PBG-D

for folate, which would be provided [7] or induced (Kotler *et al.* unpublished results) by the urogen III cosynthetase. This, however, would verify our data using H, S, PrS and Su fractions. Moreover, results obtained employing ø and Prø preparations, which are free of cosynthetase, would suggest either the presence of sites saturated by folic acid or the existence of a structural molecular arrangement, in which the functional groups involved in the binding of the factor are not easily exposed, so that the modifier cannot reach a site on the enzyme to interact with.

Effect of folic acid on kinetic properties of PBG-D

To examine the effect of folic acid on the kinetic properties of PBG-D, Lineweaver-Burk analyses were performed at pH 8.2 in the presence of defined concentrations of the effector (0.5 – 2.0 mM) employing different substrate concentrations in the range of 14 – $50 \mu\text{M}$. Typical plots are shown in Fig. 2. As can be seen folic acid only produces 38% increase in V_{max} without altering the K_m -value ($12 \mu\text{M}$ PBG).

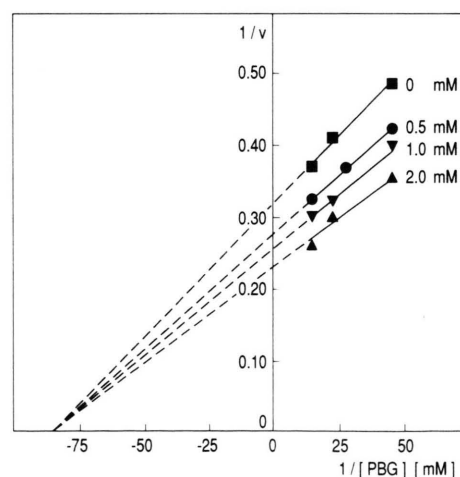
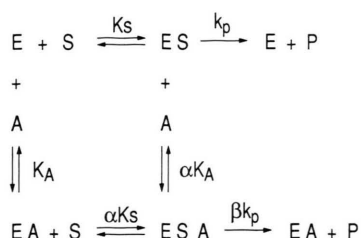


Fig. 2. Kinetic of activation of rat liver PBG-D activity by folic acid. Activity was measured using the standard PBG-D assay in the absence (\blacksquare) or presence of 0.5 mM (\bullet), 1.0 mM (\blacktriangledown) and 2.0 mM (\blacktriangle) folic acid. The desalted ammonium sulphate fraction (2 mg/ml) was used as enzyme source throughout the assay. Each point represents the mean value of three experiments run in duplicate. Lines were fitted by the standard least-squares method.

Since the PBG-D-catalyzed reaction also occurs in the absence of the effector, folic acid can be classified as a non-essential activator (Scheme 1) as proposed by Segel [16].



Scheme 1. Diagram for a non-essential activation as proposed by Segel [16].

The binding constant for the interaction between PBG-D and folic acid (K_A) was calculated from secondary replots of $1/\Delta\text{slope}$ or $1/\Delta\text{intercept}$ versus $1/[\text{folic acid}]$ (Fig. 3). The Δ slope and the Δ intercept values were obtained from individual Lineweaver-Burk plots (Fig. 2). The replots are perfectly linear, providing kinetic evidence for the binding of one molecule of effector and also exhibit a positive slope, confirming the activating effect of folic acid. The constants α and β refer to as the α -fold change in K_m and V_{\max} , respectively, obtained in the presence of a non-essential activator. The values obtained for the constants are as follows: $\alpha = 1$; $\beta = 1.6$; $K_S = 12 \mu\text{M}$; $K_A = 1.66 \text{ mM}$. Because of α being 1, the binding of folic acid before or after the formation of the intermediate ES does not

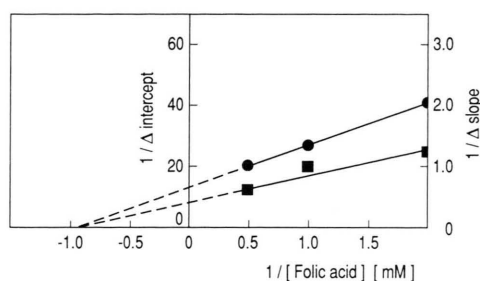


Fig. 3. Secondary replots of $1/\Delta\text{slope}$ (●) and $1/\Delta\text{intercept}$ (■) versus $1/[\text{folic acid}]$ for folic acid as a non-essential activator ($\alpha = 1$; $\beta = 1.6$).

affect the affinity for the reactants to synthesize E-folic-PBG or E-PBG-folic, respectively. However, it produces an enhancement in the rate of uroporphyrinogen I production since v is $k_p[\text{ES}] + \beta k_p[\text{ESA}]$.

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- [1] M. Kohashi, R. P. Clement, J. Tse, and W. N. Piper, *Biochem. J.* **220**, 755–765 (1984).
- [2] G. J. Hart and A. R. Battersby, *Biochem. J.* **232**, 151–160 (1985).
- [3] E. Smythe and D. C. Williams, *Biochem. J.* **253**, 275–279 (1988).
- [4] A. A. Juknat, M. V. Rossetti, and A. M. del C. Batlle, *Int. J. Biochem.* **13**, 343–353 (1981).
- [5] A. A. Juknat, D. Doernemann, and H. Senger, *Z. Naturforsch.* **43c**, 351–356 (1988).
- [6] A. A. Juknat, D. Doernemann, and H. Senger, *Z. Naturforsch.* **43c**, 357–362 (1988).
- [7] W. R. Christenson, V. R. Reddy, and W. N. Piper, *Life Sci.* **38**, 1679–1684 (1986).
- [8] M. L. Kotler, A. A. Juknat, S. R. Correa Garcia, F. G. Princ, and A. M. del C. Batlle, *Med. Sci. Res.* **16**, 983–984 (1988).
- [9] H. A. Sancovich, A. M. Ferramola, A. M. del C. Batlle, and M. Grinstein, in: *Methods in Enzymology* (H. Tabor and C. W. Tabor, eds.), **Vol. 17**, pp. 220–222, Academic Press, New York, London 1970.
- [10] A. M. del C. Batlle, E. A. Wider, and A. M. Stella, *Int. J. Biochem.* **9**, 871–875 (1978).
- [11] C. Rimington, *Biochem. J.* **75**, 620–623 (1960).
- [12] A. Seubert and S. Seubert, *Anal. Biochem.* **124**, 303–307 (1982).
- [13] C. Polo, A. M. Stella, E. A. Wider, A. M. del C. Batlle; J. M. Lenczner, L. Altuna, and P. H. Maguin, *Prensa Medica Argentina* **70**, 433–440 (1983).
- [14] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.* **193**, 265–275 (1951).
- [15] W. N. Piper and R. B. L. van Lier, *Mol. Pharmacol.* **13**, 1126–1135 (1977).
- [16] I. H. Segel, in: *Enzyme Kinetics*, pp. 227–272. Wiley Interscience, New York 1975.