Modification of hydroxymethylbilane synthase (porphobilinogen deaminase) by pyridoxal 5'-phosphate

Demonstration of an essential lysine residue*

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(Received 1 February 1984/Accepted 1 May 1984)

When hydroxymethylbilane synthase (porphobilinogen deaminase) from *Euglena gracilis* is incubated with pyridoxal 5'-phosphate at pH 7.0 and 0°C, it rapidly loses part of its activity. The proportion of activity that remains decreases as the concentration of the modifier increases up to approx. 2 mM, above which no further significant inactivation occurs. Dialysis of the partly inactivated enzyme restores its activity, whereas reduction with NaBH₄ makes the inactivation permanent. The maximum inactivation achievable from one cycle of the treatment with pyridoxal 5'-phosphate, then with borohydride, is 53 ± 5%; taking this modified enzyme through second and third cycles causes further loss of activity. The enzyme from *Rhodospseudomonas spheroides* behaves similarly, but there are quantitative differences. Spectroscopic evidence indicates that the inactivation procedure modifies lysine residues, and labelling studies show that ϵ -N-pyridoxyl-L-lysine is a product when permanently inactivated enzyme is completely hydrolysed. Several lysine residues per molecule of the *E. gracilis* enzyme are modified by the treatment with pyridoxal 5'-phosphate and borohydride, but only one appears to be essential for enzymic activity, since porphobilinogen protects the enzyme against inactivation and then one fewer lysine residue per molecule of enzyme is affected. It is suggested that, during the biosynthesis of hydroxymethylbilane, the first porphobilinogen unit is covalently bound to the enzyme through the ϵ -amino group of the essential lysine.

Porphobilinogen (PBG) deaminase (hydroxymethylbilane synthase; EC 4.3.1.8), together with co-synthetase (uroporphyrinogen III synthase; EC 4.2.1.75), catalyse the transformation of PBG (1) into uroporphyrinogen III (5) (Battersby *et al.*, 1980). This macrocycle is the precursor of the family of vitally important pigments including haem, chlorophyll and vitamin B-12. Some of the properties of PBG deaminase have been investigated (Jordan & Shemin, 1973; Battersby & McDonald, 1975; Williams *et al.*, 1981; Battersby *et al.*, 1983a,b), and its role in the biosynthesis of uroporphyrinogen III has been well documented (Battersby *et al.*, 1982a,b, 1983a,b; cf. Burton *et al.*, 1979).

Abbreviations used: PBG, porphobilinogen.

* This paper is considered as part 23 of the series 'Biosynthesis of Porphyrins and Related Macrocycles'. Part 22 is Battersby & Seo (1983).

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Scheme 1 summarizes the available knowledge. Hydroxymethylbilane (4), the product from deaminase, is assembled stepwise from four PBG units, and the first unit is bound covalently to the enzyme from *Euglena gracilis* (see 3) (Battersby *et al.*, 1983a). Similar covalent binding has been demonstrated for PBG deaminase from *Rhodospseudomonas spheroides* (Jordan & Berry, 1981). Under normal conditions, no intermediates between PBG (1) and the hydroxymethylbilane (4) are liberated into the solution, and the different forms of PBG deaminase carrying partly built pyrrolic chains of 1, 2 and 3 pyrrole units can be separated chromatographically (Anderson & Desnick, 1980) or electrophoretically (Berry *et al.*, 1981). Further experiments involved loading the enzyme with these covalently bound intermediates in ¹³C-labelled form by using [11-¹³C]PBG followed by degradation of the enzyme to a family of small PBG-containing peptides of *M*_r approx. 800;

Scheme 1

^{13}C -n.m.r. analysis of these peptides (Battersby *et al.*, 1983b) showed a set of signals assigned to $-\text{CH}_2-\text{NH}-^{13}\text{CH}_2-\text{pyrrole}$. The tentative conclusion drawn was that it is a lysine residue in PBG deaminase that is responsible for the covalent binding of the first PBG unit.

Lysine residues have been implicated in the catalytic mechanism of a number of NAD(P)-linked dehydrogenases (Chen & Engel, 1975b, and references cited therein) and for several enzymes of glucose metabolism (Colombo & Marcus, 1974,

and references cited therein). In these cases, an essential role for lysine residues has been suggested because modification of the enzymes with pyridoxal 5'-phosphate, specific for lysine among the amino acids normally found in proteins, led to loss of enzymic activity.

Accordingly, the effect of pyridoxal 5'-phosphate on the activity of PBG deaminase has now been investigated, and evidence is provided that there is a single lysine residue per enzyme molecule that is essential for its activity.

Experimental

Materials

PBG deaminase from *Euglena gracilis* was prepared essentially according to the method of Battersby *et al.* (1983a), but the chromatography on DE-52 DEAE-cellulose was performed twice in order to obtain enzyme that appeared to be greater than 95% pure (only one very faintly staining minor band was seen in addition to the very strongly staining PBG deaminase band) by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by the method of Weber & Osborn (1969). This enzyme had a specific activity of 22000 units/mg when assayed as described by Williams *et al.* (1981), the protein concentration being determined by the method of Bradford (1976), with bovine serum albumin (Sigma Chemical Co.) as standard. The concentration of bovine serum albumin in solution was determined by using a value of $A_{1\text{cm}, 279}^1$ of 6.67 (Janatova *et al.*, 1968). Where indicated, some experiments were performed with 'deaminase/co-synthetase', and this was prepared by the method of Battersby *et al.* (1982a). Co-synthetase was prepared by a procedure involving $(\text{NH}_4)_2\text{SO}_4$ fractionation, gel filtration on Sephadex G-75, ion-exchange chromatography on Whatman DE-52 DEAE-cellulose and pH-gradient chromatography on DE-52 DEAE-cellulose (G. J. Hart, unpublished work). PBG deaminase from *Rhodospseudomonas spheroides* was partially purified by heat treatment and $(\text{NH}_4)_2\text{SO}_4$ fractionation according to the method of Jordan & Shemin (1973) followed by gel filtration on Sephadex G-75. Enzymes were dialysed before use against 0.1 M-sodium phosphate buffer, pH 7.0, containing 0.3 mM-EDTA and 0.1 mM-dithiothreitol, except where otherwise stated.

Pyridoxal 5'-phosphate and α -N-acetyl-L-lysine were obtained from Sigma Chemical Co., Poole, Dorset, U.K. ϵ -N-Pyridoxyl-L-lysine was synthesized from pyridoxal 5'-phosphate and α -N-acetyl-L-lysine with NaBH_4 by a modification of the method of Miles *et al.* (1972). The phosphate and N-acetyl groups were hydrolysed in 6M-HCl heated under reflux for 16 h. The product was purified by ion-exchange chromatography and recrystallization as described below for tritiated material. $\text{NaB}^3\text{H}_3\text{H}_1$ was obtained from Amersham International, Amersham, Bucks., U.K. Other chemicals were analytical-reagent grade whenever available, or the purest available grade, and obtained from Fisons Chemicals, Loughborough, Leics., U.K., or BDH Chemicals, Poole, Dorset, U.K. Deionized distilled water was used throughout.

Inactivation experiments

PBG deaminase (0.05 mg/ml) was incubated on ice with various concentrations of pyridoxal 5'-phosphate in 0.1 M-sodium phosphate buffer, pH 7.0. (The carry-over of EDTA and dithiothreitol into the mixtures resulted in their being present at final concentrations of approx. 0.03 mM and 0.01 mM respectively.) The incubation mixtures were protected from light with metal foil, and at intervals 25 μ l samples were withdrawn and assayed for enzymic activity in a standard 3 ml assay.

Assays were carried out by using the incubation conditions of Williams *et al.* (1981). At intervals, between 4 min and 12 min, portions (0.75 ml) of the incubation mixture were removed and added to 0.4 ml of 0.5% I_2 in 1% KI to oxidize porphyrinogens to porphyrins. After a minimum of 3 min, 0.1 ml of freshly prepared 1% sodium metabisulphite solution was added to destroy excess of I_2 , and 1.75 ml of 7% (w/v) trichloroacetic acid was added. The porphyrin produced was determined from the absorbance at 406 nm to give the linear rate of porphyrin production. An assay for modified enzyme of twice the normal volume was carried out in which the mixture contained sufficient added co-synthetase to abolish the 2–3 min lag seen above in the PBG deaminase assays in its absence. Samples assayed at intervals up to 23 min showed that porphyrin production was linear. Thus there is no slow conversion of inactive into active enzyme (by breakdown of Schiff base) during the time course of an assay, and the rate measured between 4 min and 12 min is a sound reflection of the activity of each sample of enzyme.

Permanent inactivation and repetitive treatments

Deaminase (0.56 mg/ml) or deaminase/co-synthetase (2.14 mg/ml) was partly inactivated as above, and after 5 min freshly prepared ice-cold 80 mM- NaBH_4 in 0.1 M-phosphate buffer, pH 7.0, was slowly added until the incubation mixture was just decolorized. After a further 20–30 min at 0°C, the samples were dialysed overnight against several changes of 0.1 M-phosphate buffer, pH 7.0, containing 0.3 mM-EDTA and 0.1 mM-dithiothreitol. Since control samples containing pure unmodified PBG deaminase lost up to 8% of their initial activity during overnight dialysis, a correction was made to the activity of the samples treated with pyridoxal 5'-phosphate. At the higher protein concentrations for the experiments with deaminase/co-synthetase, there was negligible loss of activity in the control samples. After overnight dialysis and assay, the samples were incubated as before with pyridoxal 5'-phosphate followed by reduction with NaBH_4 .

Stoichiometry

Absorption spectra of samples of enzyme that had been inactivated, reduced and extensively dialysed as described above were recorded with a Pye-Unicam SP.8-100 recording spectrophotometer. The extent of incorporation of pyridoxal 5'-phosphate was calculated by using a molar absorption coefficient of $1.07 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 325 nm for the reduced Schiff base derived from pyridoxal 5'-phosphate and the ϵ -amino group of lysine (Fischer *et al.*, 1963). In using this value it is assumed that all ϵ -N-phosphopyridoxyl-L-lysine residues have the same molar absorption coefficient. If the slightly lower estimates of the molar absorption coefficient for ϵ -N-phosphopyridoxyl-L-lysine that are given by Forrey *et al.* (1971) and Means & Feeney (1971) are used, then the apparent incorporations of pyridoxal 5'-phosphate are slightly increased (by up to about 10%) but the conclusions drawn are unaffected. The concentration of PBG deaminase was determined by using the method of Bradford (1976) and the method of Lowry *et al.* (1951), with bovine serum albumin as standard; the two methods gave almost identical results. The M_r of PBG deaminase was taken to be 40000 (Williams *et al.*, 1981).

Kinetic measurements

Kinetic measurements were made in 0.2 M-phosphate buffer, pH 8.0, containing 0.6 mM-EDTA with several concentrations of PBG. Excess of co-synthetase was added to assays to ensure that the hydroxymethylbilane was cyclized immediately it was formed. As discussed by Battersby *et al.* (1983b), it is only when the concentration of hydroxymethylbilane is essentially zero that the true K_m for PBG can be determined. It was taken that excess of co-synthetase was present when increasing its concentration had no effect on the determined K_m for PBG.

Identification of ϵ -N-pyridoxyl-L-lysine

PBG deaminase (1.25 mg) was inactivated and reduced as described above except that NaBH_3H_3 was used for the reduction. After extensive dialysis, initially against 0.1 M-phosphate buffer, pH 7.0, and then against deionized distilled water, the sample was evaporated to dryness under reduced pressure, redissolved in 2 ml of 6 M-HCl and hydrolysed at 110°C for 26 h under N_2 . The hydrolysate (which contained 1.1×10^6 d.p.m.) was evaporated to dryness under reduced pressure, and the residue dissolved in 3 ml of water was again evaporated to dryness.

Synthetic ϵ -N-pyridoxyl-L-lysine (37 mg) in water (5 ml) was added to the above residue, and the solution was applied to a column of Dowex 50-

X8 (20–50 mesh; H^+ form), which was eluted with water, then aq. 1 M-pyridine and then aq. 1 M- NH_3 . The aqueous- NH_3 fraction was evaporated to dryness under reduced pressure, redissolved in water and applied to a column of Dowex 50W-X8 (100–200 mesh; NH_4^+ form), which was eluted with aq. 0.1 M- NH_3 . The fractions containing ϵ -N-pyridoxyl-L-lysine were detected by their fluorescence when spotted on to a silica-gel t.l.c. plate (ϵ -N-pyridoxyl-L-lysine was eluted quickly whereas lysine was eluted much more slowly). These fractions were combined, evaporated to dryness under reduced pressure, redissolved in water and adjusted to pH 6.9 with HCl. Further synthetic ϵ -N-pyridoxyl-L-lysine (13 mg) was added, and the solution was evaporated to dryness. The residue was recrystallized from hot aqueous ethanol, with the addition of ethyl acetate, to give radioactive ϵ -N-pyridoxyl-L-lysine hydrochloride (29 mg). Two further recrystallizations gave constant radioactivity, m.p. 181–184°C; (Found: C, 47.45; H, 7.3; N, 11.8; Cl, 9.95%. $\text{C}_{14}\text{H}_{23}\text{N}_3\text{O}_4 \cdot \text{HCl} \cdot \text{H}_2\text{O}$ requires: C, 47.79; H, 7.45; N, 11.94; Cl, 10.08%); R_F 0.3 (SiO_2 ; chloroform/methanol/conc. NH_3 , 2:2:1, by vol.); δ ($^2\text{H}_2\text{O}$, 400 MHz), 7.44 (1H, s), 4.51 (2H, s), 4.20 (2H, s), 3.63 (1H, brt), 2.96 (2H, t, 7 Hz), 2.28 (3H, s) 1.80 (2H, m), 1.68 (2H, quintet, 7 Hz), and 1.41 (2H, m).

Determination of the pK of the essential lysine residue

Inactivation experiments were carried out as above by incubating PBG deaminase/co-synthetase (1700 units) with pyridoxal 5'-phosphate (3.3 mM) over the pH range 6.0–8.5; the enzyme solutions were dialysed against buffer of the required pH before use. Over the range pH 6.0–pH 8.0, 0.1 M-sodium phosphate buffers were used, and at pH 8.5 the buffer used was 10 mM-sodium pyrophosphate/0.09 M- Na_2HPO_4 . All dialysis buffers contained 0.3 mM-EDTA and 0.1 mM-dithiothreitol. Samples (20 μ l) were withdrawn at intervals for assay. Control experiments showed that pyridoxal 5'-phosphate at 3.3 mM concentration was saturating over the entire pH range used.

Results and discussion

Incubation of PBG deaminase with pyridoxal 5'-phosphate in 0.1 M-phosphate buffer, pH 7.0, at 0°C caused a rapid decline in enzymic activity. A time course of inactivation with a saturating concentration of pyridoxal 5'-phosphate is shown in Fig. 1(a). Other concentrations of modifier were also studied, and, for all, the enzymic activity fell to its final steady value within 3 min; the size of this value depended on the concentration of modifier used. Fig. 1(b) shows that a modifier concentration of 2–3 mM produced the maximum effect, which

Fig. 1. Time course of inactivation of PBG deaminase by pyridoxal 5'-phosphate and effect of pyridoxal 5'-phosphate concentration on the final residual activity at equilibrium

(a) PBG deaminase (0.05 mg/ml, ●) or deaminase/co-synthetase (2500 units/ml, ■ and □) were incubated at 0°C with pyridoxal 5'-phosphate (3.3 mM, ● and ■, or 6.6 mM, □) in 0.1 M-sodium phosphate buffer, pH 7.0. Samples were removed at intervals and assayed for activity of PBG deaminase as described in the text. Also shown are results from two experiments in which PBG deaminase was incubated with PBG (Δ, 6.8 μM; ▲, 55 μM) for 5 min at 0°C before the addition of pyridoxal 5'-phosphate. (b) PBG deaminase (0.05 mg/ml) was incubated at 0°C with various concentrations of pyridoxal 5'-phosphate in 0.1 M-sodium phosphate buffer, pH 7.0. Each point represents the activity obtained at equilibrium in such an incubation, and in most cases the measurements plotted were made after 5 min of incubation.

corresponded to 65% of the initial activity being retained. Similar results were obtained when preparations of PBG deaminase/co-synthetase were used, and these are also shown in Fig. 1.

When the mixture of partially inactivated enzyme and pyridoxal 5'-phosphate was dialysed overnight against several changes of 0.1 M-phosphate buffer, the enzymic activity was fully restored. However, treatment of the mixture with NaBH₄ before the dialysis step prevented the reactivation. NaBH₄ at the concentrations used in these experiments was shown not to affect the activity of PBG deaminase. Higher concentrations of borohydride did cause an apparent decrease in

activity, which was concentration-dependent. This effect was eliminated by dialysis of the enzyme/borohydride mixture and is probably due to the larger amounts of borohydride interfering with the assay.

The product from PBG deaminase and pyridoxal 5'-phosphate after reduction and extensive dialysis showed two absorption maxima, the normal absorbance at 280 nm for protein together with a band at 325 nm that strongly indicated the presence of ε-N-phosphopyridoxyl-L-lysine residues (Fischer *et al.*, 1963); native enzyme shows negligible absorbance at 325 nm. Confirmation was obtained by using NaB³H₃ in the reduction step followed by complete acidic hydrolysis of the permanently modified enzyme. The hydrolysis products were mixed with synthetic ε-N-pyridoxyl-L-lysine, which was re-isolated by ion-exchange chromatography and recrystallized to constant radioactivity as its monohydrochloride. At this stage, the recovery of ε-N-pyridoxyl-L-lysine was 58%, and it showed 38.5% of the ³H radioactivity carried by the modified enzyme; the latter was thus proved to contain ε-N-pyridoxyl-L-lysine residues.

There are two explanations of the partial loss of activity shown in Fig. 1. Either (a) the modified enzyme is still active but less so, or (b) it is inactive but formed incompletely. This same issue has arisen in studies of the modification of several NAD(P)-linked dehydrogenases by pyridoxal 5'-phosphate (Chen & Engel, 1975a,b,c,d,e). In these cases, an inactive covalently modified form of the enzyme is produced in equilibrium with a non-covalent complex of enzyme and pyridoxal 5'-phosphate (cf. Scheme 2); the latter can rapidly dissociate and appears as active enzyme in the assay.

If a similar situation exists for the modification of PBG deaminase by pyridoxal 5'-phosphate [i.e., explanation (b) above], then enzyme that has in part been permanently inactivated by reduction should undergo a further loss of activity by repeating the de-activation cycle (cf. Chen & Engel, 1975a). This was found to be true, and the results of successive cycles are shown in Table 1 together with the stoichiometry for incorporation of pyridoxal 5'-phosphate. The proportion of activity lost in each successive cycle is the same, within experimental error, despite the finding (Table 1) that more than one lysine residue/molecule of enzyme is reacting with the modifier; this result is in keeping with only one lysine residue being essential for enzymic activity.

Further support for explanation (b) above came from kinetic studies on PBG deaminase that had been permanently inactivated to the extent of 52%. The results (Fig. 2) gave a value of K_m for PBG of $105 \pm 5 \mu\text{M}$, which is the same as for native PBG

Table 1. *Inactivating effect of successive cycles of pyridoxal 5'-phosphate treatment, borohydride reduction and dialysis on PBG deaminase and deaminase/co-synthetase from E. gracilis*

Deaminase (initially 0.56 mg/ml) and deaminase/co-synthetase (initially 2.14 mg/ml) were each incubated on ice in 0.1 M-phosphate buffer, pH 7.0, with 3.3 mM-pyridoxal 5'-phosphate until the activity had reached a steady value (5 min). The samples were then reduced with NaBH₄, and extensively dialysed. After dialysis, the same procedures were repeated. After each cycle, the specific activities of the samples were measured, and the stoichiometry of incorporation of pyridoxal 5'-phosphate into deaminase was determined from the absorption at 325 nm.

PBG deaminase

Cycle no.	Residual activity (%)	Incorporation of pyridoxal 5'-phosphate into PBG deaminase (mol/mol)
First	50	1.6
Second	23	2.6

Deaminase/co-synthetase

First	44
Second	20
Third	7

deaminase operating in the presence of an excess of co-synthetase (Battersby *et al.*, 1979, 1983b). Thus the remaining 48% of activity is due to 48% of the total enzyme behaving normally, the rest being inactivated.

Reference was made in the introduction to experiments that indicated that the first PBG unit covalently binds to PBG deaminase through the ϵ -amino group of a lysine residue. If this is true, then it is probable that the same lysine residue is the essential one modified by pyridoxal 5'-phosphate, and therefore PBG should protect the enzyme against this modifier. In fact, Fig. 1(a) shows that PBG deaminase pretreated with PBG is not appreciably inactivated by pyridoxal 5'-phosphate. The apparent small loss of activity in Fig. 1(a) may be within experimental error, but if it is real some small decrease due to modification of lysine residues away from the PBG-binding site would not be surprising.

Not only does PBG protect PBG deaminase against loss of activity, it also decreases the amount of pyridoxal 5'-phosphate incorporated. Modification of the enzyme in the absence of PBG caused an activity loss of 50% with incorporation of 1.63 ± 0.13 mol of pyridoxal 5'-phosphate/mol of enzyme (determinations were made in triplicate). Enzyme protected with PBG and similarly treated lost only 6.7% of its initial activity, with 1.25 ± 0.12 mol of pyridoxal 5'-phosphate/mol being

Fig. 2. *Initial-rate measurements for the formation of uroporphyrinogen III by PBG deaminase in its native state and after permanent modification (pyridoxal 5'-phosphate then reduction), both in the presence of an excess of co-synthetase*

The initial rates were measured at 37°C in 0.2 M-phosphate buffer, pH 8.0, containing 0.6 mM-EDTA, with various concentrations of PBG, with native enzyme (●) and reduced partially modified enzyme (○). The modified enzyme showed 48% of the activity of the native enzyme in the standard assay.

incorporated. Thus PBG preserved the activity of 0.43 mol.prop. of enzyme and also protected approx. 0.38 mol of lysine/mol of enzyme. These results again point to there being just one essential lysine residue in a molecule of PBG deaminase. Whether the additional incorporation of pyridoxal 5'-phosphate is due to reaction with a small number of non-essential but exceptionally reactive lysine residues or to more-random reaction at many less reactive ones cannot be deduced from the present results.

Two further points require comment. The first concerns our observation that the measured loss in activity of PBG deaminase on treatment with pyridoxal 5'-phosphate is less when the assay is done before borohydride reduction ($35 \pm 3\%$) than

it is after reduction ($53 \pm 3\%$). A possible explanation is that the Schiff base (7) (Scheme 2) is partly hydrolysed, and a new equilibrium is set up, when the sample of modified enzyme is diluted into the PBG solution for the normal assay. This was tested by incubating the enzyme and pyridoxal 5'-phosphate for 4 min at the concentrations that they experience after dilution into the normal assay and then initiating the assay by addition of PBG without significant further dilution. The loss of activity (31%) was close to that found above ($35 \pm 3\%$) for more-concentrated incubation followed by dilution. Moreover, borohydride reduction of a second portion of the more-dilute incubation mixture caused a permanent loss of 37% of the activity, demonstrating that borohydride simply traps the available Schiff base (7). It is noteworthy that on dilution of a sample of modified enzyme into an assay, the new equilibrium is set up very rapidly; this follows from the fact (see above) that an assay to which had been added an excess of co-synthetase showed strictly linear production of porphyrinogen throughout the monitored period of 23 min. Hydrolysis of the Schiff base must therefore be rapid, and the value of k_{-2} significantly greater than the values seen in the interaction between pyridoxal 5'-phosphate and glutamate dehydrogenase and alcohol dehydrogenase, where (Chen & Engel, 1975a,c) half-times for re-activation due to hydrolysis of the Schiff base were found to be approx. 15 min and 4 min respectively. The existence of equilibria, as shown in Scheme 2, is further confirmed by the observation that, in the absence of borohydride, the apparent extent of inactivation is a function of the volume withdrawn for assay. It is therefore important to note that the time courses shown in Fig. 1, and those that gave rise to the plots shown in

Fig. 3 (see below), were constructed by withdrawing the same volume for assay at each time of sampling.

The second point relates to the speed of reaction between the essential lysine residue and pyridoxal 5'-phosphate, which, at saturating concentrations of the latter, is very fast even at 0°C and pH 7.0. At this pH, free lysine would have less than 1% of its ϵ -amino groups in the unprotonated form necessary for reaction with the aldehyde group of pyridoxal 5'-phosphate.

The essential lysine residue in PBG deaminase is more reactive towards the modifier than are the essential lysine residues of the dehydrogenases studied by Chen & Engel (1975a, b, c, d, e), where incubations of up to 40 min at 25°C (10°C for alcohol dehydrogenase) were required before the maximum inactivation was achieved. Similarly, for pyruvate kinase (Johnson & Deal, 1970), a 3–4 min incubation at 25°C was required.

The unusual reactivity of the essential lysine residue in glutamate dehydrogenase has been shown to be due to its perturbed pK value, which at 7.7–8.0 (Piszkiewicz & Smith, 1971; Veronese *et al.*, 1972) is approx. 2 pH units lower than the normal value for the ϵ -amino group of lysine. The analogous lysine residue in acetoacetate decarboxylase is a still weaker base, $pK_{5.9}$ (Schmidt & Westheimer, 1971).

In order to check whether the pK value of the essential lysine residue in PBG deaminase is perturbed by its environment, the rate and the extent of modification of the enzyme by pyridoxal 5'-phosphate was determined over a range of different pH values. Progress curves of the type shown in Fig. 1 were determined at each pH value with a saturating concentration of pyridoxal 5'-phosphate; reduction of the equilibrium mixture

Scheme 2

Fig. 3. First-order kinetic analysis of inactivation of PBG deaminase/co-synthetase by pyridoxal 5'-phosphate at various pH values

The Figure shows plots of $\ln[(A_0 - A_e)/(A_t - A_e)]$ against time for the inactivation of PBG deaminase/co-synthetase by a saturating concentration of pyridoxal 5'-phosphate at various pH values. A_0 , A_e and A_t are respectively the activity of the enzyme at time zero, at equilibrium and at time t . The pH values used were: ●, 6.0; ○, 6.5; △ and ▽, 7.0; ▲, 7.5; ■, 8.0; □, 8.5. Also shown (▼) are the results from an experiment at pH 7.0 in which mixtures of enzyme and pyridoxal 5'-phosphate were reduced with NaBH_4 before assay of the remaining activity.

with borohydride allowed an accurate estimate of the maximum degree of modification. From each of the plots, the extent of progress towards equilibrium was calculated by plotting $\ln[(A_0 - A_e)/(A_t - A_e)]$ against time (Frost & Pearson, 1961), where A_0 , A_e and A_t are respectively the enzymic activity at time zero, at equilibrium and at time t . These first-order plots for the inactivation process are shown in Fig. 3, the slope of each plot being the sum of the two kinetically important opposing rate constants at each pH. These rate constants are taken to be k_{+2} and k_{-2} in Scheme 2, and the following discussion suggests that this is reasonable.

The formation of significant amounts of non-covalent complex (6) between pyridoxal 5'-phosphate and PBG deaminase had to be proposed in order to account for the observed saturation behaviour (Fig. 1); this means that the rate-limiting step at saturating concentration of pyridoxal 5'-phosphate is the conversion of the non-covalent complex into the Schiff base (7), i.e.

$k_{+2} \ll k_{+1}[\text{pyridoxal 5'-phosphate}]$. Considering the reverse reaction, breakdown of the Schiff base (7) involves formation of a new covalent bond (to water) and cleavage of another (between C and N), and so can be expected to be slower than the dissociation of a Michaelis complex (6). In support, dilution of the incubation mixture of enzyme and modifier into an assay medium that contained co-synthetase showed strictly linear production of uroporphyrinogen III; there was no detectable increase in rate with time, so the k_{-1} step is clearly not a slow one. For the interaction of glutamate dehydrogenase with pyridoxal 5'-phosphate (Chen & Engel, 1975a) it has been firmly established that $k_{-2} \ll k_{-1}$, and the assumption is made here that this holds true for PBG deaminase. After reduction of mixtures of pyridoxal 5'-phosphate and enzyme with borohydride, dissociation of the covalent complex is no longer possible. So, if it is taken that under these conditions the non-covalent complex (now enzyme and pyridoxal 5'-phosphate) dissociates completely on dilution,

Table 2. Extent of modification of PBG deaminase/co-synthetase by pyridoxal 5'-phosphate and values for k_{+2} at various pH values

PBG deaminase/co-synthetase (1700 units/ml) was incubated on ice with 3.3 mM-pyridoxal 5'-phosphate in buffers of various pH values (see the text for full details). After 6 min, sufficient NaBH_4 was added just to decolorize the solutions, and the residual activity was determined. Two separate experiments were carried out at pH 7.0, and in all cases the values quoted are the means of duplicate assays. Also tabulated are the values for k_{+2} (derived as described in the text) that were obtained at each of the pH values used.

Residual activity		
pH	(%)	$10^3 \times k_{+2} \text{ (s}^{-1}\text{)}$
6.0	87.5	4.3
6.5	73.5	8.9
7.0	50.0, 45.0	10.5, 13.2
7.5	40.0	15.0
8.0	40.0	17.1
8.5	29.5	18.3

then the activity of the permanently modified enzyme (given in Table 2) cannot have been decreased below $[k_{-2}/(k_{+2} + k_{-2})] \times \text{initial activity}$. Two equations in k_{+2} and k_{-2} are now available, that just developed from Table 2 and one from the slopes in Fig. 3; these may be solved in order to obtain values for k_{+2} , and the values so derived are listed in Table 2.

The above approach for determining k_{+2} will only be valid if the partial dissociation of the non-covalent enzyme-modifier complex (6) on dilution (see above) occurs to the same percentage in each assay used in the set of experiments at a particular pH. To test this, two approaches were made to determining k_{+2} for inactivation at pH 7.0. The first involved the usual removal of samples at timed intervals for assay from a mixture of enzyme and pyridoxal 5'-phosphate; for the second, equivalent samples of the mixture were reduced with borohydride after different times. After dialysis, these were assayed; no dissociation is possible in this second approach. The progress curves for inactivation allowed first-order plots to be constructed (Fig. 3), and the values for k_{+2} were $13.2 \times 10^{-3} \text{ s}^{-1}$ when the Schiff base was not reduced and $12.6 \times 10^{-3} \text{ s}^{-1}$ with reduction of the Schiff base. The difference is well within the experimental error, and shows the initial assumption to be valid at pH 7.0. If this is also true at the other pH values, then a plot of k_{+2} against pH (Fig. 4) gives a pK value of 6.7 for the essential lysine residue in the Michaelis complex (6) of enzyme with pyridoxal 5'-phosphate. Such a pK value for this lysine residue in the Michaelis complex of PBG and the enzyme would allow 70–80%

Fig. 4. Variation with pH of the rate of inactivation of deaminase/co-synthetase by pyridoxal 5'-phosphate

The rate constants for inactivation were obtained from the first-order plots shown in Fig. 3 together with the extent of modification after reduction of mixtures of enzyme and pyridoxal 5'-phosphate with NaBH_4 as described in the text. The drawn line was calculated on the assumption that only the unprotonated form of a group with pK 6.7 in the non-covalent complex of enzyme and pyridoxal 5'-phosphate reacts with pyridoxal 5'-phosphate with a rate constant of $18 \times 10^{-3} \text{ s}^{-1}$ when all the ϵ -amino group is in the unprotonated state.

of the essential lysine residues to be in the unprotonated form over the physiological pH range. It is this form that is required for nucleophilic attack on the methylenepyrrrolene (2) derived from PBG (1) to set up the covalently bound form of the first PBG unit (3) (see Scheme 1). This unit becomes ring A of the hydroxymethylbilane (4) and subsequently ring A of uroporphyrinogen III (5) (Battersby *et al.*, 1982b).

Effect of pyridoxal 5'-phosphate on PBG deaminase from *Rps. spheroides*

Limited experiments were carried out on PBG deaminase partially purified from *Rps. spheroides*. This enzyme showed essentially the same behaviour towards pyridoxal 5'-phosphate as the deaminase from *E. gracilis* when incubated under the same conditions. A saturating concentration (3.3 mM) of pyridoxal 5'-phosphate caused a decline in enzymic activity over a period of 20 min to a steady value that was 59% of the initial activity. Almost total protection against the effect of pyridoxal 5'-phosphate was afforded by PBG when used at a concentration of 40 μM . Other concentrations of modifier were also used and a final steady value was reached in all cases within 40 min. Such incubations showed that a 2.5–3 mM concentration of pyridoxal 5'-phosphate was required to saturate the enzyme. Repetitive treatment with pyridoxal 5'-phosphate (3.3 mM) then NaBH_4 , followed by

dialysis, decreased the activity to 58.6, 30.2 and 18.5% of the initial activity after one, two and three cycles respectively (means of duplicate experiments). This strongly suggests that the two PBG deaminases from the different sources follow the same basic mechanism, with the linear oligopyrrolic chain being built up covalently attached to the enzyme via a lysine residue (Scheme 1). It is noteworthy that, under identical conditions, the reaction between PBG deaminase from *Rps. spheroides* and pyridoxal 5'-phosphate is much slower (by an order of magnitude) than the corresponding reaction with the enzyme from *E. gracilis*. The two enzymes have very similar pH optima (Jordan & Shemin, 1973; Williams *et al.*, 1981), and the maximum rates of uroporphyrinogen III production are also very similar (Jordan & Shemin, 1973; Battersby *et al.*, 1983a). The different rates of reaction towards pyridoxal 5'-phosphate do not, therefore, appear to be reflected in significantly different behaviour towards PBG.

Conclusion

PBG deaminase is reversibly inactivated by pyridoxal 5'-phosphate. This inactivation, which can be made permanent by reduction of the enzyme-modifier complex with borohydride, is accompanied by the modification of lysine residues. PBG protects the enzyme against this inactivation, and there is a one-to-one relationship between protection of activity and decreased lysine modification. This and other evidence points to there being just one essential lysine residue in each molecule of enzyme for which a pK value of 6.7 is derived (in the enzyme-pyridoxal 5'-phosphate complex). It is suggested that the role of the essential lysine residue is to bind the first PBG unit covalently to the enzyme during biosynthesis of the hydroxymethylbilane (4) (see Scheme 1, with X now being NH).

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