Interaction of analogues of porphobilinogen with porphobilinogen deaminase[1]

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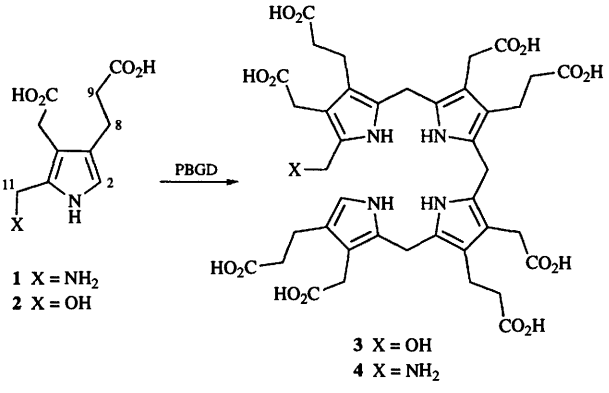
2-Methylporphobilinogen 5 and 8,9-didehydroporphobilinogen 7 are only weak inhibitors of porphobilinogen deaminase (hydroxymethylbilane synthase). The phosphonate analogue 8 and 9-fluoroporphobilinogen 9 are good inhibitors, however, and also act as slow substrates (the first unnatural substrates known for this enzyme). The particularly strong inhibition shown by the fluoro analogue 9 is shown to be due to the slow regeneration of free enzyme once the compound has become covalently bound to it. This results in a sigmoidal dependence of rate *vs.* [substrate].

Porphobilinogen deaminase (PBGD, hydroxymethylbilane synthase, EC 4.3.1.8) is a remarkable enzyme which catalyses the tetramerisation of porphobilinogen (PBG) **1** to give hydroxymethylbilane **3**, the precursor of all natural tetrapyrroles including haems, chlorophylls and vitamin B_{12} (Scheme 1).[2,3] During the course of the mechanism each successive



Scheme 1 The reaction catalysed by PBG deaminase

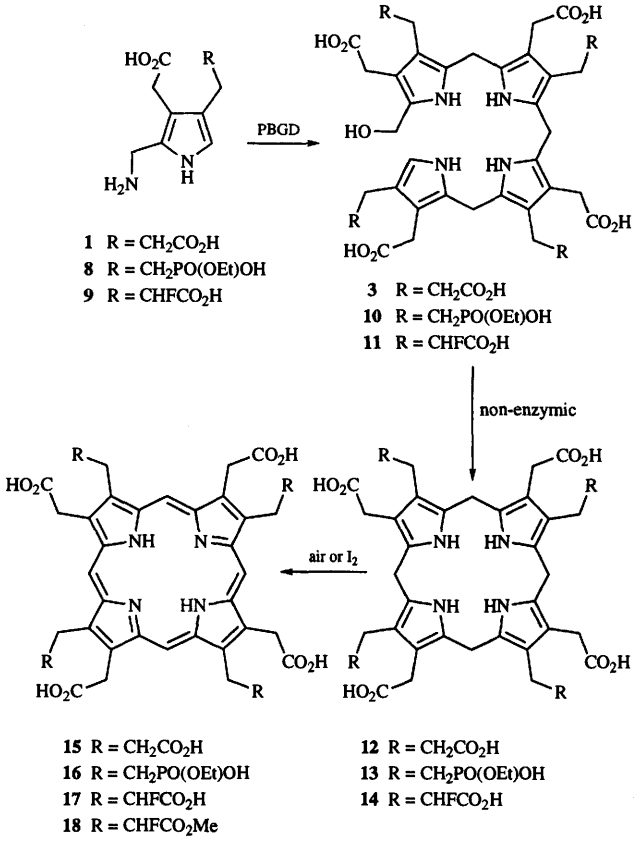
pyrrole ring becomes covalently bound to the dipyrromethane cofactor, giving complexes ES, ES_2, ES_3 and ES_4, and release of product occurs only once four pyrroles have been attached (see Scheme 1 of the preceding paper[4]). The free enzyme and ES, ES_2 and ES_3 can all be separated by polyacrylamide gel electrophoresis[5] and on a preparative scale by fast protein liquid chromatography (FPLC).[6] Recently crystal structures of the enzyme with an oxidised cofactor[7] and the natural reduced cofactor[8] have been obtained, which have confirmed the previous conclusions and given a great deal of detail about the structure of the enzyme and its active site. However, it is still uncertain how the substrate molecules are orientated in the active site and this is one reason why substrate analogues may be of interest.

A number of analogues of PBG have previously been made and tested as inhibitors or partial substrates of PBGD.[4] Also it has been shown that the aminomethylbilane **4** and other isomeric aminomethylbilanes are converted into the corresponding hydroxymethylbilanes (*e.g.* **3**) by the enzyme.[9] To date, however, the only compounds that are known to be accepted as substrates for the complete enzymic reaction are PBG **1** and the corresponding hydroxymethylpyrrole **2**, both of which give the same hydroxymethylbilane (HMB) (preuroporphyrinogen) **3** as the product.[10]

In this paper we detail experiments to test the interaction between PBG deaminase and some simple analogues of PBG **1**, whose synthesis was reported in the preceding paper.[4] Part of this work has been briefly reported in a communication.[1]

Results

The five PBG analogues, **5**–**9**,[4] were all tested as inhibitors of PBGD from *Escherichia coli*,[11] which was generously supplied by Drs G. J. Hart and A. Hädener. The assay procedure used for these experiments was a previously reported one.[11] This assay relies on the spontaneous cyclisation of the product, hydroxymethylbilane **3**, to give uroporphyrinogen (uro'gen) I **12**, which occurs with a half-life of about 4 min at pH 8 (Scheme 2).[12] This means that there is a lag in the formation of uro'gen I but it approaches a steady-state rate after about 5 min. Hence the concentration of uro'gen is measured after 5 and 10 min by oxidation to uroporphyrin I **15** using iodine and measurement



Scheme 2 Alternative substrates for PBG deaminase

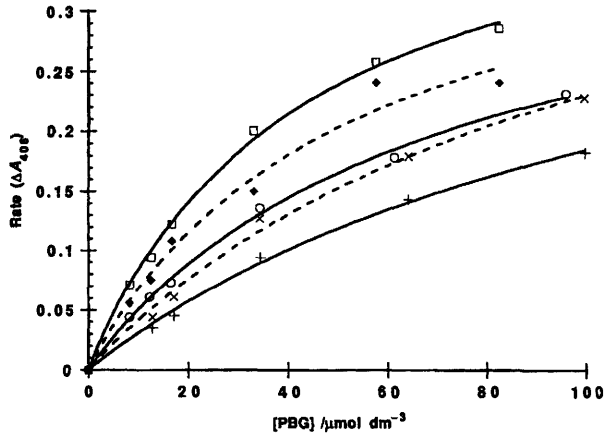


Fig. 1 Inhibition of PBG deaminase by PBG analogues **5**, **6**, **8** and **9**. The rate of reaction is expressed as the change in absorption at 406 nm between aliquots taken at 5 and 10 min treated as described in the Experimental section. —□—, no inhibitor; – –◆– –, **5** (300 μmol dm^{-3}); —○—, **6** (1.13 mmol dm^{-3}); —+—, **8** (50 μmol dm^{-3}); – –×– –, **9** (7 μmol dm^{-3}). The curves are the best fit of the Michaelis–Menten equation to the data.

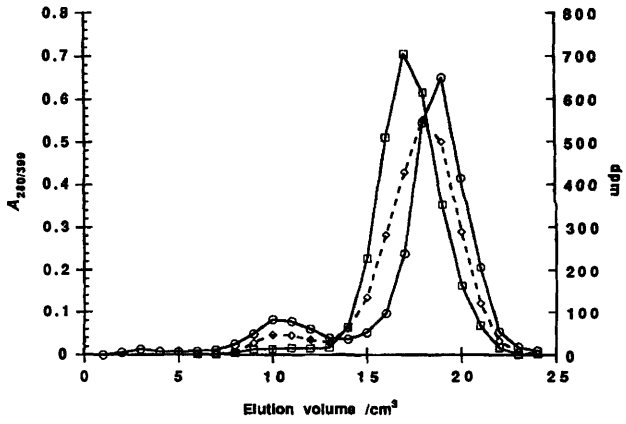
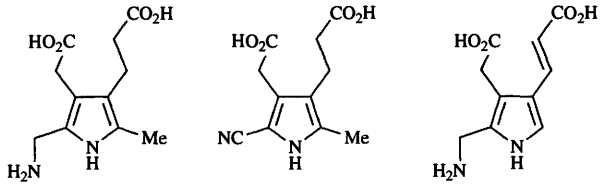


Fig. 2 Gel filtration of PBG deaminase after treatment with [8-^{3}H]PPBG. Each fraction (1 cm^3) was measured for absorption at 280 nm (—○—) and 399 nm (—□—) and for total radioactivity in dpm (– –◇– –). The peak at fraction 17 absorbing at 399 nm is probably due to porphyrin **16** whereas the peak at fraction 19 absorbing at 280 nm is probably due to unreacted PPBG. PBGD activity eluted in fractions 8–13.

of the absorbance at 406 nm (A_{406}). The difference between these two measurements is taken as the enzymic rate. The K_M value for *E. coli* PBGD has been reported[13,14] to be about 20 μmol dm^{-3} but in the present studies the value obtained was consistently higher, averaging about 41 μmol dm^{-3}. This value of K_M is the one used for calculating K_I values.

When the 2-methyl analogues of PBG,† **5** and **6**, were tested as inhibitors both showed competitive inhibition with K_I values around 1 mmol dm^{-3} (see Fig. 1). No inactivation of PBGD by 2-methylPBG **5** (82 μmol dm^{-3}) was observed over a period of



5 **6** **7**

35 min. The inhibition shown by didehydroPBG **7** was tested by Dr G. J. Hart.[15] At 205 μmol dm^{-3} **7** had a negligible effect on the normal assay but at higher concentrations and longer assay times a small effect was seen: 20% loss of activity was observed for PBGD after 16 min with **7** at 1.2 mmol dm^{-3} and 35% loss of activity after 30 min at 680 μmol dm^{-3}. The source of this inhibition was not investigated further but it seems possible that **7** was very slowly binding covalently to the enzyme in the place of PBG and thus inactivating the enzyme. No formation of any tetrapyrrolic product could be detected, however, when **7** alone (815 μmol dm^{-3}) was incubated with PBGD (0.1 μmol dm^{-3}).[15]

The phosphonate analogue **8** of PBG (PPBG) and 9-fluoroPBG **9** (FPBG) were much more promising as inhibitors than **7**. Assays with varying concentrations of PBG in the presence of PPBG (50 μmol dm^{-3}) or FPBG (7 μmol dm^{-3}) gave data consistent with competitive inhibition with K_I values of about 22 and 6 μmol dm^{-3} respectively (see Fig. 1). This is considerably stronger inhibition than had been observed for any previous competitive inhibitor and indeed the apparent affinity of both PPBG and FPBG for PBGD was greater than that of the natural substrate. This was a surprising result and we were interested to find out the reason for it. One possibility was that covalent attachment of the inhibitors to the enzyme might be occurring. Tests for time-dependent inactivation of PBGD were therefore undertaken.

PBG deaminase was preincubated with PPBG or FPBG and after various intervals aliquots were removed and assayed. Initially these experiments were performed in air and a continual loss of activity was observed leading to almost complete loss of activity after 24 h. By contrast the enzyme was very stable (loss of activity, 10% or less) if the inhibitor was omitted. However, a further experiment revealed that PBGD preincubated with its natural substrate PBG also lost some activity (although not so much) over time under these conditions. We, therefore, suspected that PBGD–substrate complexes may be unstable to oxidation by air. On repeating the experiment with degassed solutions, the deactivation by PBG (200 μmol dm^{-3}) was greatly reduced whereas both PPBG and FPBG at 200 μmol dm^{-3} produced a rapid loss of *ca.* 20% of the activity followed by little further loss over 7 h.

In order to investigate whether the loss of activity caused by PPBG is due to covalent attachment to the enzyme, it was decided next to undertake experiments with radioactively labelled inhibitor. [8-^{3}H]PPBG was synthesised as for PPBG **8** from the [*formyl*-^{3}H]-labelled 1*H*-pyrrolo[2,3-*c*]pyridine (**14** in the preceding paper). The label was introduced into the formyl-1*H*-pyrrolo[2,3-*c*]pyridine by a previously reported method,[16] which involves tosylation on the indole nitrogen atom followed by conversion of the aldehyde into its morpholinonitrile derivative, deprotonation with butyllithium, quenching with [^{3}H]TFA and then deprotection.

In two separate experiments, the [8-^{3}H]PPBG was incubated with PBG deaminase in air for 1 h and 24 h, which according to separate experiments with unlabelled PPBG should give *ca.* 20% and 90% inactivation respectively. The mixture was then separated by gel filtration chromatography. The fractions were each tested for their absorption at 280 nm and 399 nm and for their radioactivity (see Fig. 2). The first peak absorbing at 280 nm (fractions 8–13) contained the enzyme (in a separate experiment all the enzymic activity was associated with this peak) and there was also a coincident peak in the radioactivity measurements. The amount of radioactivity indicated that the enzyme had an average of 0.21 and 1.38 mol of [8-^{3}H]PPBG attached per mol of enzyme after the 1 h and 24 h incubations respectively. Next the cofactor was removed by treatment of the enzyme with dilute hydrochloric acid[11] followed by extensive

† The numbering for the atoms of PBG, given in Scheme 1, is that used by, among others, J. Lascelles in *Tetrapyrrole Biosynthesis and its Regulation*, W. A. Benjamin Inc., New York, 1964, p. 42 and by R. B. Frydman, B. Frydman and A. Valasinas in *The Porphyrins*, ed. D. Dolphin, Academic Press, New York, 1979, vol. 6, p. 23.

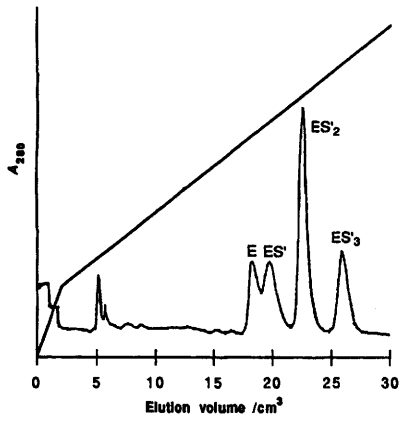


Fig. 3 Elution profile of PBGD–FPBG complexes on FPLC. Detection was by absorbance at 280 nm; the oblique line represents the increasing concentration of NaCl (0–350 mmol dm^{-3}).

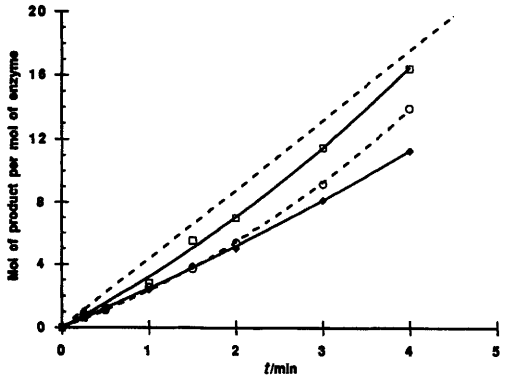


Fig. 4 Reactivation of PBGD–FPBG complexes with PBG. The tetrapyrrole produced is expressed as mol per mol of PBGD. The straight line (– – –) represents the average rate of production measured for native PBGD and the PBGD–PBG complexes ES_2 and ES_3, which were all the same within experimental error and showed no detectable lag; PBGD–PBG complexes: – – ○ – –, ES'; —□—, ES'$_2$; —◆—, ES'$_3$.

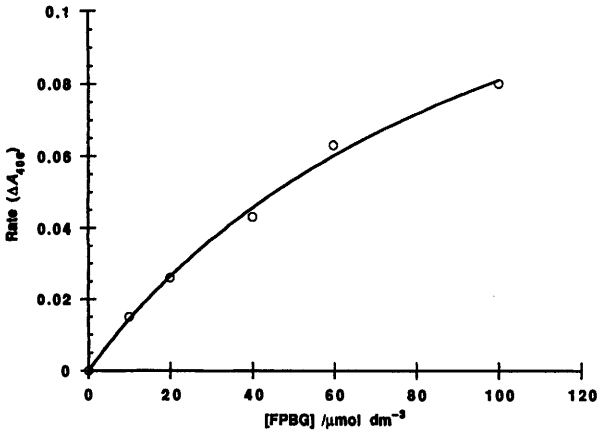


Fig. 5 Kinetic characterisation of FPBG **9** as a substrate for PBG deaminase

dialysis and gel filtration. This process removed 93 and 85% respectively of the radioactivity attached to the enzyme in the two samples. Thus it seems very likely that the enzyme has been inhibited primarily by binding the [8-^{3}H]PPBG to the cofactor in the same manner as the true substrate PBG binds.

For FPBG, a different approach was used to demonstrate the covalent attachment to the enzyme. It has previously been shown[6] that PBGD and its ES, ES_2 and ES_3 complexes can all be separated by FPLC. Therefore FPBG was incubated with PBGD for 15 min and then chromatographed. The elution profile, shown in Fig. 3, is very similar to that obtained with PBG and PBGD, with peaks for native enzyme and the ES', ES'$_2$ and ES'$_3$ complexes (S' denotes a substrate analogue). The spacing of the peaks and their elution volumes are almost identical for the experiments with PBG and FPBG and the only significant difference is that the peak for native enzyme is smaller in the incubation with FPBG.

It is apparent from the results above that PPBG and FPBG can both form covalent complexes with deaminase. As the enzyme is not inactivated by either inhibitor (in the absence of oxygen) to more than *ca.* 20%, it must be that, on incubation with PBG, the bound inhibitor is eventually released again probably as part of a modified hydroxymethylbilane molecule. To demonstrate this reactivation of the enzyme the three enzyme–FPBG complexes (ES', ES'$_2$ and ES'$_3$) were separated by FPLC and each was assayed for activity.

A different assay was required, however, to show the time-course of the reactivation as the lag assay described above requires a minimum of 10 min. The new assay (a modification of a previous one[17]) involved quenching aliquots of the reaction with trichloroacetic acid at different time intervals, which could be as little as 15 s. The acid not only stops the reaction but also causes rapid cyclisation of the HMB **3** produced to give uro'gen I **12**. Iodine is subsequently added to oxidise uro'gen I to uroporphyrin I **15**, which is detected spectrophotometrically, as before.

Using this 'acid-quench' assay, the time-course for formation of products on incubation of the PBGD–FPBG complexes with PBG is shown in Fig. 4. For each of the complexes there is a delay before the maximum rate of product formation is attained. For ES' and ES'$_2$ the final rate of product formation is more or less equal to that of native PBGD (for ES'$_3$ it is rather less, possibly due to some inactivation, *e.g.* by oxidation, or to an error in determining the amount of enzyme added). Native PBGD or the PBGD–PBG complexes ES_2 and ES_3, on the other hand, showed no such lag in the same experiment.

Finally, FPBG and PPBG were tested as substrates for PBGD on their own. Both showed slow formation of a product with an absorption spectrum almost identical to that of uroporphyrin I **15**. For PPBG the reaction was very slow (*ca.* 500 times slower than for PBG) and the absorption spectrum contained an additional peak at 485 nm, probably due to a dipyrromethene chromophore. No further purification or identification of this spectroscopically detected porphyrin was attempted due to the small amount produced but it is presumed to be the tetraphosphonate **16**, produced *via* hydroxymethylbilane **10** and porphyrinogen **13**.

FPBG **9** reacted with deaminase rather faster than PPBG **8**, allowing a study of its kinetics, albeit with an assay time extended from 10 min to 5.5 h. The graph of rate *vs.* [FPBG] is shown in Fig. 5. Curve-fitting gives K_M and V_{max} values of 108 μmol dm^{-3} and 0.168 respectively. The k_{cat} calculated from this V_{max} is 9.576×10^{-4} s^{-1}, approximately 100 times slower than that for PBG. The final product from this reaction is presumably tetrafluorouroporphyrin I **17**, produced by cyclisation of hydroxymethylbilane **11** and oxidation of the resulting porphyrinogen **14**. To confirm its structure, this porphyrin was methylated with methanol–trimethyl orthoformate–sulfuric acid to give a compound with the same fluoresence and R_f value on TLC as authentic uroporphyrin III octamethyl ester. After purification by TLC, field desorption mass spectrometry gave a molecular ion at *m/z* 1022, which is correct for the expected tetrafluorouroporphyrin I octamethyl ester **18** containing eight deuterium atoms (two on each acetate side-chain, from the hydrolysis of FPBG lactam in alkaline D_2O[4]).

Discussion

2-MethylPBG **5** is a relatively poor competitive inhibitor of PBGD (when compared to the affinity for substrate) and two

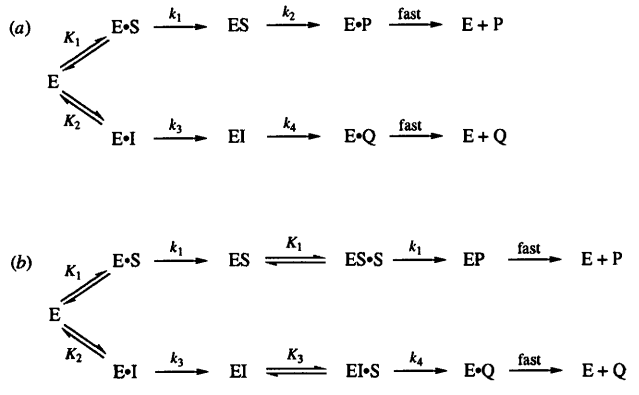
pieces of evidence suggest that it does not bind covalently to the enzyme in the way that PBG does. Firstly, the cyano analogue **6** could not bind in the same manner and yet it has a similar K_I value; secondly, covalent binding would be expected to lead to inactivation because release of a monopyrrole once it has covalently bound to the enzyme is known to be slow, at least for PBG.

The poor inhibition by 2-methylPBG is surprising because while the work described here was in progress 2-bromoPBG was reported [18,19] to inactivate *E. coli* PBGD within 5 min at a concentration of only 25 μmol dm^{-3}. The van der Waals surface of a C–Br group is rather similar to that of a C–Me group and so it is unlikely that there is any steric reason why 2-methylPBG does not bind covalently to PBGD. Possibly the difference lies in the different electronic properties of Me and Br.

The poor inhibition by dehydroPBG **7** is, perhaps, easier to understand. The C–CH_2–CH_2–C unit of the propionate side-chain of PBG, when bound to the enzyme, is likely to be in the planar s-*trans* conformation (similar to the *trans* double bond of **7**) as this is the most stable conformation and it is the one observed for the propionate side-chains of the cofactor in the crystal structure of the enzyme.[7] However, the plane of this unit would be roughly perpendicular to that of the pyrrole ring (to avoid steric interactions), whereas for dehydroPBG conjugation would cause the C=C bond to prefer a conformation in which it is coplanar with the pyrrole. As a result, dehydroPBG probably exists predominantly in the wrong conformation for binding to the enzyme.

In contrast to the above analogues, PPBG **8** and FPBG **9** are surprisingly good competitive inhibitors with apparent K_I values (22 and 6 μmol dm^{-3} respectively) lower than for any previously reported competitive inhibitor[4] and lower than the K_M value for PBG (41 μmol dm^{-3}). It is demonstrated above, for PPBG by radioactive labelling and for FPBG by isolation of the enzyme–FPBG complexes, that both inhibitors bind covalently to PBGD. It is also shown that both are slowly reacting substrates.

Normally when an inhibitor is also a slow substrate the K_I value as a competitive inhibitor is equal to the K_M value as a substrate. Thus, for example, it can be shown mathematically that for the kinetic scheme in Scheme 3(*a*), in which the



Scheme 3 Kinetic schemes used as models for the inhibition of PBG deaminase by FPBG

inhibitor binds irreversibly to the enzyme and then requires a further step before a product is formed and free enzyme is regenerated, the formation of product P is competitively inhibited by I with an inhibition constant K_I equal to the K_M for I as the sole substrate, $K_I = K_{M(I)} = K_2k_4/(k_3 + k_4)$.

The apparent K_I value for FPBG (6 μmol dm^{-3}) is much smaller than the K_M value for FPBG as substrate (108 μmol dm^{-3}), which needs explanation. In the case of PBGD the kinetic scheme is much more complicated than that in Scheme 3(*a*) with four cycles of PBG binding non-covalently and then being covalently attached to the enzyme, followed by a final step of release of the product. An inhibitor could replace PBG in any or all of the four cycles, leading to an extremely complex reaction scheme with 16 branches. However, the important factor seems to be that the covalent enzyme–inhibitor complex needs to react with a further molecule of substrate in order to regenerate the free enzyme again. A very much simplified model incorporating this factor is shown in Scheme 3(*b*). This model is the same as in Scheme 3(*a*) but with two added substrate binding steps between the intermediates ES and EI and the products P and Q. Additionally the two dissociation constants, K_1, for substrate binding in the formation of P have been made equal and so have the two rate constants, k_1, because previous work has shown that each of the four cycles of binding of PBG to deaminase occurs at approximately the same rate.[20,21] The rate of formation of product P in Scheme 3(*b*) can be calculated in terms of the individual rate and equilibrium constants and is given in eqn. (1). If $k_3 = 0$, then the last term in the

$$\text{Rate} = \frac{k_1[E_0][S]}{2K_1 + 2[S] + [I]K_1/K_2 + k_3[I]K_1(K_3 + [S])/k_4[S]K_2} \quad (1)$$

denominator of eqn. (1), $k_3[I]K_1(K_3 + [S])/k_4[S]K_2$, disappears and a normal equation for competitive inhibition results with $K_I = 2K_2$. If $k_3 \neq 0$ but $K_3 \ll [S]$, then eqn. (1) can be

$$\text{Rate} \approx \frac{k_1[E_0][S]}{2K_1 + 2[S] + [I]K_1(k_3 + k_4)/K_2k_4} \quad (2)$$

simplified to eqn. (2). Eqn. (2) is again an equation for simple competitive inhibition, now with

$$K_I = 2K_2k_4/(k_3 + k_4).$$

Using the kinetic scheme in Scheme 3(*b*) as a model for the inhibition by FPBG, the explanation why the apparent K_I is less than the K_M for FPBG as a substrate seems to be that having covalently attached one molecule of FPBG to the enzyme in place of PBG, the binding of the next molecule of PBG (or FPBG) is weakened (*i.e.* the dissociation constant is raised). Hence K_3 is not small, as assumed in the derivation of eqn. (2), but larger than K_1, giving greater inhibition than would be indicated by eqn. (2). Similarly, if FPBG is used as the only substrate, then the second molecule would bind more weakly than the first, *i.e.* it would have a greater dissociation constant and this would result in a greater K_M value for the overall reaction.

If [S] is less than K_3 then the rate of reaction as given by eqn. (1) is considerably less than the estimate given by eqn. (2). This effect is greatest as [S] approaches zero, resulting in a sigmoid curve for rate *vs.* [S], rather than the usual hyperbolic type of curve. Close inspection of the data points for inhibition by FPBG and PPBG in Fig. 1 shows that the rate is indeed lower than expected at low values of [PBG]. Fig. 6(*a*) replots the same data with curves calculated using eqn. (1). These curves are a much better fit to the data than can be obtained using the standard Michaelis–Menten equation. The deviation from Michaelis–Menten kinetics is much more obvious in an Eadie–Hofstee plot, Fig. 6(*b*), which is linear for PBG in the absence of inhibitor, as expected, but distinctly curved in the presence of FPBG and PPBG. The values of the parameters used in calculating the curves in Fig. 6 are given in the caption. Curves which fitted the data almost as well could be obtained with substantially different sets of values for the parameters and so these values should not be taken as accurate values for the PBGD reaction. The close fit of the calculated curves to the experimental data does demonstrate, however, that a sigmoid curve is the natural result of this type of inhibition.

Finally, some comment should be made on the inactivation

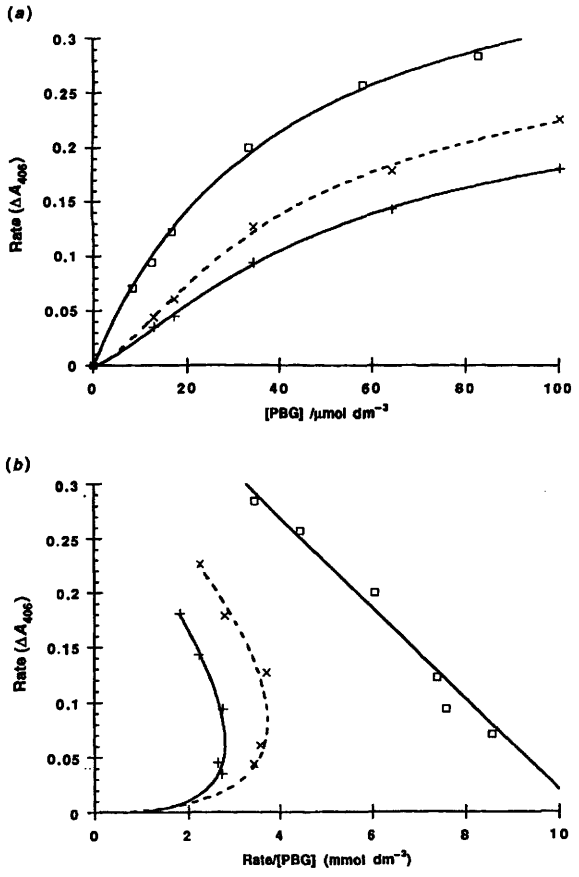


Fig. 6 Inhibition of PBG deaminase by PPBG **8** and FPBG **9**. (*a*) The data are the same as in Fig. 1: —□—, no inhibitor; —+—, **8** (50 μmol dm^{-3}); – – × – –, **9** (7 μmol dm^{-3}). The curves are calculated using eqn. (1) and the following parameters: for no inhibitor, K_1 = 41.4, k_1 = 0.87; for PPBG **8**, K_1 = 41.4, k_1 = 0.60, K_2 = 85, K_3 = 88, k_3 = 0.0525, k_4 = 0.0753; for FPBG **9**, K_1 = 41.4, k_1 = 0.68, K_2 = 122, K_3 = 122, k_3 = 0.090, k_4 = 0.025. (*b*) Eadie–Hofstee plot of the same data and curves as in (*a*).

of PBGD observed with FPBG and PPBG (and to a lesser extent PBG) under aerobic conditions. It has been reported that, whereas native PBGD is relatively insensitive to inactivation by thiol-directed reagents, the PBGD–PBG complexes become increasingly more susceptible the more PBG molecules are bound.[19,22] The thiol involved has been identified as belonging to Cys-134, which is located at the interface between domains 2 and 3 of the protein.[23] It has been proposed that on forming the enzyme–PBG complexes, a conformational change occurs in which these two domains move apart, exposing Cys-134 to alkylating agents. In view of this, it seems likely that the inactivation observed here is due to attachment of FPBG or PPBG to the enzyme, which causes the change in conformation and allows slow oxidation of the exposed thiol. The effect is less with PBG because it is used up more rapidly than either of the two analogues and, once this has happened, there will be a smaller proportion of enzyme present as a substrate complex.

In summary, we report here two analogues of PBG, PPBG **8** and FPBG **9**, which are not only the best competitive inhibitors of PBGD discovered so far, but also the first substrates for the enzyme to yield products other than HMB **3**. This has lead to the production of novel porphyrins, **16** and **17**, which were partially characterised. Formation of these products was slow due to the requirement that four successive analogue molecules become attached to the enzyme. Inhibition, on the other hand, results from the attachment of only one analogue molecule and is therefore much more effective. With PBG present, the inhibited enzyme turns over, presumably releasing a tetrapyrrole modified in one ring only. If this occurs *in vivo*, the modified tetrapyrrole may then inhibit the next enzyme or pass on to inhibit still later enzymes in the biosynthetic pathway. For example, porphyrinogens modified in the propionate side-chain would be most likely to inhibit coproporphyrinogen oxidase, which catalyses the oxidative decarboxylation of two of the propionate side-chains to give vinyl groups.[3] The accumulation of porphyrins resulting from inhibition of protoporphyrinogen oxidase, one of the later enzymes in the pathway, is known to be damaging to cells due to photo-oxidation and this is the mode of action of a number of commercial herbicides.[24] Therefore, the type of substrate analogue described here may have interesting cytotoxic properties.

Experimental

General directions

The concentration of PBGD was determined from the absorbance at 280 nm (A_{280}) using the known extinction coefficient (16.21 dm^3 mmol^{-1} cm^{-1}).[11] The stock solution of enzyme contained PBGD from *Escherichia coli* (2–3 mg cm^{-3}) dissolved in Bis/Tris buffer (15 mmol dm^{-3}; pH 6) containing ethylenediaminetetraacetic acid (EDTA) (0.6 mmol dm^{-3}), dithiothreitol (DTT) (0.1 mmol dm^{-3}), benzamidine hydrochloride (0.1 mmol dm^{-3}), phenylmethanesulfonyl fluoride (0.6 mmol dm^{-3}) and NaCl (0.2 mol dm^{-3}). Phosphate buffer refers to aq. sodium phosphate (0.2 mol dm^{-3}) at pH 8 containing EDTA (0.6 mmol dm^{-3}), unless stated otherwise.

To make up the stock solutions of the PBG analogues, a solution of the analogue in D_2O (0.5 cm^3) was mixed with phosphate buffer (0.5 cm^3), then aqueous phosphoric acid (1 mol dm^{-3}) was added to restore the pH to 8 and the volume was made up to 2 cm^3 with more buffer. To determine the concentrations of the α-free analogues, an aliquot (10 mm^3) of the solution was diluted with phosphate buffer (4.5 cm^3) and the solution was divided into three portions. Freshly prepared modified Ehrlich's reagent[25] (1.5 cm^3) was added to each portion and the absorbance at 555 nm (A_{555}) of each was then measured. This procedure was also performed on a PBG solution of known concentration for comparison. Typically, a solution of PBG of concentration 1 mg cm^{-3} (4.5 μmol dm^{-3}) gives an A_{555} of 1.6.

Gel filtration chromatography was performed on a column of Sephadex G75 (25 cm × 1 cm diam.) equilibrated with phosphate buffer (0.1 mol dm^{-3}; pH 8) containing EDTA (0.3 mmol dm^{-3}) and eluted with the same buffer at 0.3–0.4 cm^3 min^{-1} using a slight pressure of nitrogen. Fractions (1 cm^3) were collected and their A_{280} (for detection of protein and pyrroles) and A_{399} (detection of porphyrins) values were recorded.

Dialysis was performed in a Visking tubing bag against Tris buffer (20 mmol dm^{-3}; pH 7.4; 500 cm^3) containing EDTA (0.6 mmol dm^{-3}) overnight at 4 °C. Ultrafiltration was carried out using an Amicon Centricon microconcentrator.

Assay procedures

Assay (*a*). PBGD solution was added to a solution of PBG in phosphate buffer (30 μmol dm^{-3}; 3.0 cm^3) containing various concentrations of a PBG analogue at 37 °C to give an enzyme concentration of 0.1 μmol dm^{-3}. At certain times (commonly 5 and 10 min), aliquots (600 mm^3) were removed and treated with a solution of potassium iodide (0.5% w/v) and iodine (1% w/v) in water (400 mm^3) and then water (150 mm^3) and the mixture was left for 3 min. Freshly prepared aq. sodium metabisulfite (1% w/v; 100 mm^3) was added to remove excess iodine followed by aq. trichloroacetic acid (7% w/v; 1.75 cm^3) to give a final volume of 3 cm^3. The absorbance at 406 nm (A_{406}) was then measured and the difference between the readings at 5 and 10 min (ΔA_5) is taken as a measure of enzymic activity. The tetrafluorouroporphyrin I **17** produced from FPBG and the tetraphosphonate **16** produced from PPBG were assumed to

have the same extinction coefficient as that of uroporphyrin I (541 dm^3 $mmol^{-1}$ cm^{-1}).[26]

Assay (*b*). At certain times up to 3 min, aliquots (0.75 cm^3) from the incubation mixture described above were added to aq. trichloroacetic acid (7% w/v; 1.75 cm^3). After 4 min, a solution of potassium iodide (0.5% w/v) and iodine (1% w/v) in water (0.4 cm^3) was added and the mixture was left for 3 min. Freshly prepared aq. sodium metabisulfite (1% w/v; 0.1 cm^3) was added and the A_{406} was taken as a measure of the amount of hydroxymethylbilane produced in the relevant time period.

Assay (*c*). When PPBG **8** or FPBG **9** were used as substrates, the same incubation mixture was used except that concentrations of enzyme up to 1 μmol dm^{-3} and substrate up to 0.8 mmol dm^{-3} were used. Aliquots of the incubation mixture were taken at intervals over 1 h (for FPBG) or 19 h (for PPBG) and treated as in (*a*). The A_{406} was taken as a measure of the amount of hydroxymethylbilane produced in the relevant time period. For the determination of its K_M value the incubation mixture contained FPBG (5–100 μmol dm^{-3}) and aliquots were taken after 5.5 h. The A_{406} value indicated that only 7–12% of the FPBG had been converted into porphyrin in this time.

Isolation of tetrafluorouroporphyrin I octamethyl ester

Incubation mixtures from the foregoing experiments with FPBG **9** as substrate which had turned pink after exposure to air were pooled and freeze-dried. The residue was stirred with 5% conc. sulfuric acid in methanol (1.5 cm^3) and trimethyl orthoformate (0.5 cm^3) for 48 h at room temperature in the dark. The mixture was then evaporated *in vacuo*, diluted with water (1 cm^3), neutralised to pH 7 with sodium hydrogen carbonate and extracted with dichloromethane (3 × 5 cm^3). The combined extracts were dried and evaporated *in vacuo* and the residue was purified by flash chromatography on silica gel, eluting with 3% methanol in dichloromethane, to give a small amount of tetrafluorouroporphyrin I octamethyl ester as a red solid. This product had the same characteristic UV–VIS spectrum, the same orange fluorescence under UV light and the same R_f value (R_f 0.60, 5% MeOH–CH_2Cl_2) as authentic uroporphyrin III octamethyl ester; *m/z* (FD) 1022 ($C_{48}H_{42}D_8N_4O_4F_4$).

Determination of inhibition constants

Assay (*a*) was performed using a range of concentrations of inhibitor to determine the concentration (IC_{50}) which gave approximately 50% inhibition. Assay (*a*) was then carried out using five or six concentrations of PBG (0–120 μmol dm^{-3}) with and without the analogue at approximately the IC_{50}. K_M (without inhibitor) and K_M' (with inhibitor) values were obtained from fitting the curve $v = V_{max}[S]/(K_M + [S])$ to the data and K_I values were calculated from the equation $K_M' = K_M(1 + [I]/K_I)$.

Inactivation of PBG deaminase

PBG deaminase (13–37.5 μmol dm^{-3}) was incubated at room temperature with the inhibitor (66–560 μmol dm^{-3}) in phosphate buffer (400 mm^3). After certain times, aliquots (20 mm^3) were removed and either assayed directly using procedure (*a*) or (for the higher concentrations of enzyme) diluted with buffer (20 mm^3) and an aliquot of this solution (20 mm^3) assayed. In each case a control experiment was set up using identical conditions but with no inhibitor. The residual activity is expressed as a percentage of the activity of the control.

Incubation of [8-^{3}H]PPBG with PBG deaminase

A solution of [8-^{3}H]PPBG (0.72 Ci mol^{-1};‡ 418 μmol dm^{-3}) in phosphate buffer was incubated with PBGD (37.5 μmol dm^{-3}) for 24 h. The inactivated enzyme was then isolated by gel filtration chromatography (fractions 8–13). The A_{280} values (ranging from 0.025 to 0.082) of the protein-containing fractions indicated a total of 15 nmol of enzyme and scintillation counting gave a total of 32 000 dpm,‡ equivalent to 20.7 nmol of [8-^{3}H]PPBG (1.38 mol of PPBG per mol of enzyme).

The cofactor was removed following the procedure of Hart *et al.*[11] and the protein solution was dissolved in phosphate buffer (50 mmol dm^{-3}; pH 7.6; 1.5 cm^3) containing DTT (1 mmol dm^{-3}), EDTA (0.6 mmol dm^{-3}) and urea (6 mol dm^{-3}) and dialysed against this buffer (50 cm^3) for 3 h at 4 °C, then three times against the same buffer without urea (200 cm^3) for 24 h. After gel filtration chromatography the fractions containing protein were pooled, dialysed overnight against Tris buffer (15 mmol dm^{-3}) containing EDTA (0.6 mmol dm^{-3}) and DTT (0.2 mmol dm^{-3}) and then concentrated by ultrafiltration to 0.3 cm^3. The A_{280} value indicated a total of 6.04 nmol of enzyme (50% recovery) and scintillation counting gave a total of 1905 dpm, equivalent to 1.23 nmol of [8-^{3}H]PPBG (0.21 mol of PPBG per mol of enzyme).

The foregoing procedure was repeated except that the [8-^{3}H]PPBG (418 μmol dm^{-3}) was incubated with PBG deaminase (200 μmol dm^{-3}) for just 1 h. Before the cofactor was removed, the enzyme (8.82 nmol) contained 0.21 mol of PPBG per mole of enzyme. After removal of the cofactor, the recovered protein (3.48 nmol) contained 0.015 moles of PPBG per mole of enzyme.

Isolation and assay of enzyme–FPBG complexes

A solution of PBGD (0.5 mg) and FPBG (3 mol per mol enzyme) in Tris buffer (15 mmol dm^{-3}; 5 cm^3) containing EDTA (0.6 mmol dm^{-3}) and DTT (0.1 mmol dm^{-3}) at pH 7.5 was allowed to stand for 20 min at room temperature and then filtered and passed down a Pharmacia Mono Q HR 5/5 FPLC column eluting with the same buffer (30 cm^3) containing a gradient of NaCl (0–350 mmol dm^{-3}) at a flowrate of 1 cm^3 min^{-1}. Elution of FPBG–enzyme complexes was monitored at 280 nm (see Fig. 3) and each separate complex was collected manually and stored on ice.

The volume of each fraction collected was calculated from the flowrate and the length of time of collection. The proportion of the total enzyme represented by each complex was estimated by measuring the areas of each of the peaks on the elution profile.

Aliquots (0.1 cm^3) of each fraction were mixed with phosphate buffer (3.2 cm^3) and aq. PBG (0.1 cm^3; 4.5 mmol dm^{-3}) at 37 °C. At certain time intervals aliquots (0.75 cm^3) were removed and analysed according to assay (*b*). The results are shown in Fig. 4.

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‡ 1 Ci = 3.7 × 10^{10} Bq; 1 Bq = 60 dpm.

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