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Evidence that the pyrromethane cofactor of hydroxymethylbilane synthase (porphobilinogen deaminase) is bound through the sulphur atom of a cysteine residue

Graham J. HART,* Andrew D. MILLER and Alan R. BATTERSBY
University of Cambridge Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, U.K.

Hydroxymethylbilane synthase (porphobilinogen deaminase) from *Escherichia coli* uses a novel pyrromethane cofactor to bind the growing pyrrolic chain for hydroxymethylbilane biosynthesis [Hart, Miller, Leeper & Battersby (1987) J. Chem. Soc. Chem. Commun. 1762–1765]. We show that this cofactor is bound to the protein through the sulphur atom of a cysteine residue.

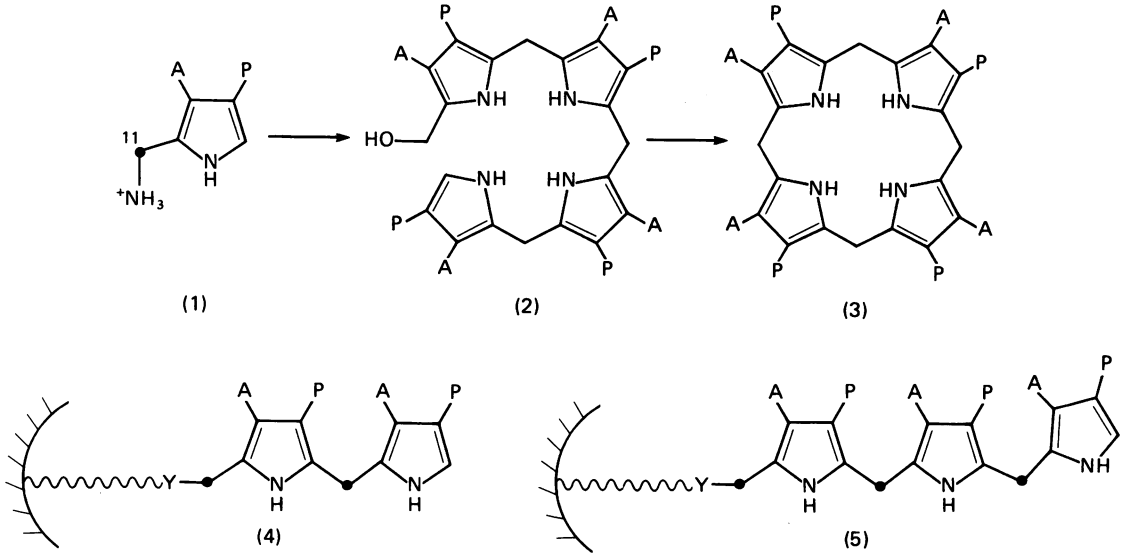
INTRODUCTION

Hydroxymethylbilane synthase [HMBS, EC 4.3.1.8; usually called 'porphobilinogen (PBG) deaminase'], together with uroporphyrinogen III synthase (EC 4.2.1.75), catalyses the conversion of PBG (1) into uroporphyrinogen III (3), the precursor for haem, chlorophyll and vitamin B-12 (Battersby *et al.*, 1980; Leeper, 1985*a*,*b*) (Scheme 1). HMBS is the assembly enzyme which builds hydroxymethylbilane (2) from PBG (1) (Battersby *et al.*, 1982), and the growing oligopyrrolic chain is covalently attached to HMBS through some group X of the enzyme (Jordan & Berry, 1981; Battersby *et al.*, 1982, 1983). Our recent work (Hart *et al.*, 1987) has established that (i) X is a pyrromethane residue of structure (4a) bound to the protein through a group Y, and (ii) the growing oligopyrrolic chain is attached to the α-free position of the terminal ring of the pyrromethane unit. A subsequent independent report (Jordan & Warren, 1987) supported the presence of a pyrromethane in HMBS but gave no evidence for its function or as to whether it was group X or not. We now show that, after treatment of HMBS with acid to cleave the pyrromethane residue from Y, the pyrromethane can be reconstructed, so regenerating enzymic activity. By using [11-^{13}C]PBG, we have identified the nature of the amino acid side chain carrying the Y group to which is bound the pyrromethane cofactor.

EXPERIMENTAL

Materials

Urea (PrimaR grade) was from Fisons Chemicals, Loughborough, Leics., U.K. 4-(Dimethylamino)benzaldehyde (Ehrlich's reagent), 5,5′-dithiobis-(2-nitrobenzoic



Scheme 1. Biosynthesis of uroporphyrinogen III

Series (a) ● = ^{12}C; series (b) ● = ^{13}C. A = CH_2CO_2H; P = $CH_2CH_2CO_2H$.

Abbreviations used: HMBS, hydroxymethylbilane synthase; PBG, porphobilinogen.

* To whom correspondence should be addressed.

acid), ampicillin and isopropyl β-D-thiogalactopyranoside were from Sigma Chemical Co., Poole, Dorset, U.K. [11-^{13}C]PBG lactam methyl ester (90 atom%) was synthesized as described by Miller (1987). This was hydrolysed overnight at 30 °C under N_2 in 2 M-KOH (0.1 ml/mg) and was diluted 9-fold in 0.2 M-sodium phosphate buffer, pH 8.0, containing 0.6 mM-EDTA and adjusted to approx. pH 8 with 1 M-H_3PO_4 before use. Other chemicals were from the sources previously given (Hart *et al.*, 1986).

Enzyme assays

HMBS activity was determined as described by Hart *et al.* (1984). Protein concentrations for purified samples of HMBS were determined by A_{280} measurements, by using a value for $A^{1\%}_{1\,cm}$ of 4.73, determined from dry-weight measurements. The M_r for HMBS was taken to be 34245, being that derived from the amino acid sequence (Thomas & Jordan, 1986; P. R. Alefounder, unpublished work) plus 1 molecule of pyrromethane (Hart *et al.* 1987).

Buffer solutions, spectra and growth of cells

Buffer solutions were made, u.v.–visible absorption spectra were recorded and organisms were grown as described previously (Hart *et al.*, 1986), except that all growth media contained ampicillin (0.2 g/l) and isopropyl β-D-thiogalactopyranoside (12.5 mg/l).

Purification of HMBS

HMBS was purified from *Escherichia coli* strain TG1 *rec* O/pAT *tac hemC*, a strain that produces approx. 200 times the wild-type content of HMBS. This strain was provided by Dr. P. R. Alefounder and Dr. C. Abell of this Laboratory. The purification procedure was essentially as described previously (Hart *et al.*, 1986), but the f.p.l.c. at pH 6.0 was carried out on a Mono Q 10/10 column because of the larger amount of enzyme being purified. The gradient used was 0–0.4 M-NaCl in 110 ml of the buffer used previously at a flow rate of 2.75 ml/min. HMBS was eluted at an NaCl concentration of approx. 0.2 M, and was apparently homogeneous as judged by SDS/polyacrylamide-gel electrophoresis.

Treatment of HMBS with Ehrlich's reagent

A modified Ehrlich's reagent was prepared as 2% (w/v) 4-(dimethylamino)benzaldehyde in 98% formic acid/2 M-HCl (7:3, v/v). Equal volumes of enzyme solution and the Ehrlich's reagent were mixed, and spectra were recorded at intervals. HMBS was at all times completely soluble in this modified Ehrlich's solution.

Removal of pyrromethane and restoration of HMBS activity by the use of [11-^{13}C]PBG

Many different methods were tried for restoration of the activity of acid-treated HMBS. The method detailed below reproducibly gave the best results. Dithiothreitol was added at 2 mg/ml to HMBS (63 mg in 36 ml of the Bistris/HCl buffer, pH 6.0, used for the f.p.l.c.) followed by conc. HCl to a final concentration of 1 M. Protein was immediately precipitated, and after 25 h in the dark at room temperature the pyrromethane had been cleaved from Y and the supernatant solution was pink in colour. Protein was then collected by centrifugation (20000 *g* for 10 min), dissolved in 45 ml of 50 mM-sodium phosphate buffer, pH 7.2, containing 0.6 mM-EDTA, 1 mM-dithiothreitol, 0.1 M-NaCl and 0.1 M-urea, and dialysed against the same buffer for 1.5 h at room temperature. Dialysis was continued at 5 °C against the same buffer but without urea for 24 h (stirring was not used during this dialysis), and then overnight against 50 mM-sodium phosphate buffer, pH 7.4, containing 0.6 mM-EDTA and 0.2 mM-dithiothreitol.

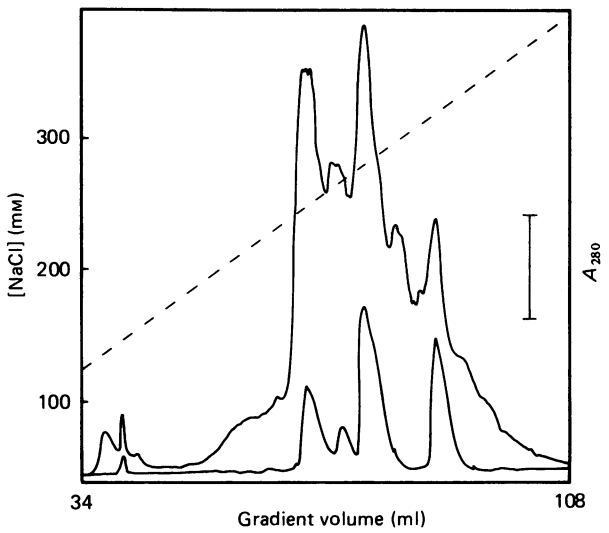


Fig. 1. Elution of reconstituted HMBS–PBG complexes from Mono Q at pH 8.3

The upper trace is an elution profile (measured by A_{280}) for reconstituted HMBS–PBG complexes (29 mg total protein) from Mono Q 10/10 at pH 8.3 carried out as described in the text. The broken line shows the NaCl gradient. The vertical bar is 0.1 A_{280}. For comparison, the lower trace shows an elution profile for native HMBS–PBG complexes (8 mg of protein) run under identical conditions. The four main peaks from left to right are holoenzyme, E, followed by E–PBG_1, E–PBG_2 and E–PBG_3.

A solution of [11-^{13}C]PBG was then added dropwise to the protein solution, which was stirred at 5 °C such that the final PBG concentration was equivalent to 2.7 mol of PGB/mol of HMBS. After 4 h at 5 °C, this solution was dialysed overnight on ice against 15 mM-Tris/HCl buffer, pH 8.3, containing 0.6 mM-EDTA and 0.2 mM-dithiothreitol.

HMBS–PBG complexes were separated by f.p.l.c. on a Mono Q 10/10 column with a gradient of 0–0.4 M-NaCl in 110 ml of the last-mentioned buffer. Two column runs were needed to process all the material from 63 mg of HMBS.

Three peaks were collected from the f.p.l.c. corresponding (Hart *et al.*, 1987) to (i) enzyme with the pyrromethane system rebuilt (4b), hereafter called holoenzyme, now in ^{13}C-labelled form, (ii) enzyme–PBG_1 mono-complex (5b) and (iii) the analogous enzyme–PBG_2 di-complex. These were combined, concentrated to 3–4 ml by ultrafiltration (Amicon ultrafiltration cell, PM-10 membrane) and dialysed overnight on ice against two changes of N_2-saturated 15 mM-sodium phosphate buffer, pH 12, conditions that have been shown (Hart *et al.*, 1987) to stabilize HMBS–PBG complexes. The pH 12-dialysed solution was further concentrated to approx. 0.5 ml by use of an Amicon Centricon 10 microconcentrator, and 0.1 ml of ^2H_2O and 1.6 mg of sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$]propionate

(Merck, Sharp and Dohme Canada Ltd., Montreal, Que., Canada) were added. Immediately before n.m.r. spectroscopic analysis, the pH was adjusted to pH 14 by addition of KOH from an N_2-saturated 12.5 M stock solution, and the n.m.r. tube was flushed with N_2 before being capped.

N.m.r. spectroscopic analyses

N.m.r. spectra were recorded at 100.6 MHz on a 9.3980 T Brüker AM-400 spectrometer fitted with a Brüker dual $^1H/^{13}C$ 5 mm probe head in 5 mm tubes with a spectral width of approx. 24000 Hz. The ^1H-decoupled (17 H Waltz) ^{13}C-n.m.r. spectra, which were directly referenced to sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$]-propionate ($\delta = 0.0$), were acquired at 10 °C with pulse width 45°, acquisition time 0.0865 and data table 8 K. The accumulated free induction decays were processed by exponential multiplication with a line broadening of 10 Hz, then Fourier-transformed. Spectra were obtained approximately every hour, after each 42000 transients.

Determination of thiol residues with 5,5′-dithiobis-(2-nitrobenzoate)

HMBS used in these experiments was dialysed against several changes of N_2-saturated 50 mM-sodium phosphate buffer, pH 8.0, containing only 0.6 mM-EDTA, to remove residual dithiothreitol. 5,5′-Dithiobis-2-(nitrobenzoate) was added, to a final concentration of 0.25 mM, from a 10 mM stock solution in the above buffer, to samples of HMBS solution containing either 1% (w/v) SDS or 5 M-guanidine hydrochloride. A_{412} was monitored and the final steady values were recorded. Blanks containing no HMBS were similarly treated, and the background A_{412} was subtracted before calculation of the amount of thiol groups modified by using ϵ_{412} 13700 $M^{-1} \cdot cm^{-1}$ (Riddles *et al.*, 1983). This value was confirmed, to within 4%, with GSH (Sigma Chemical Co.) as standard. All measurements were made at least in duplicate, and full duplicate experiments were performed that gave almost identical results.

RESULTS AND DISCUSSION

The protein obtained by treating HMBS overnight at room temperature with 1 M-HCl to cleave the pyrromethane from group Y can be resolubilized at around neutral pH by dissolving the precipitated protein in buffer containing 6 M-urea followed by slow removal of urea by dialysis at 5 °C. If the urea is removed rapidly, e.g. by shaking the flask containing the dialysis bag and buffer at 30 °C, the protein is precipitated.

The resolubilized protein, referred to below as apoenzyme, gave no reaction with Ehrlich's reagent, in marked contrast with the striking spectroscopic changes that were observed when the native enzyme was so treated (Hart *et al.*, 1987). This demonstrates that the acidic treatment has completely cleaved the bound pyrromethane from HMBS (4a) to leave a –YH group; protein yield (by A_{280}) at this stage was 85–90%. Standard assays for HMBS activity on the resolubilized protein showed not more than 1.7% of the initial activity.

Incubation of the apoenzyme with PBG (2.7 mol/mol of protein) for 4 h at 5 °C gave enzyme that in the standard assay showed 43 ± 3% of the starting activity, and this value was not increased by further incubation with PBG.

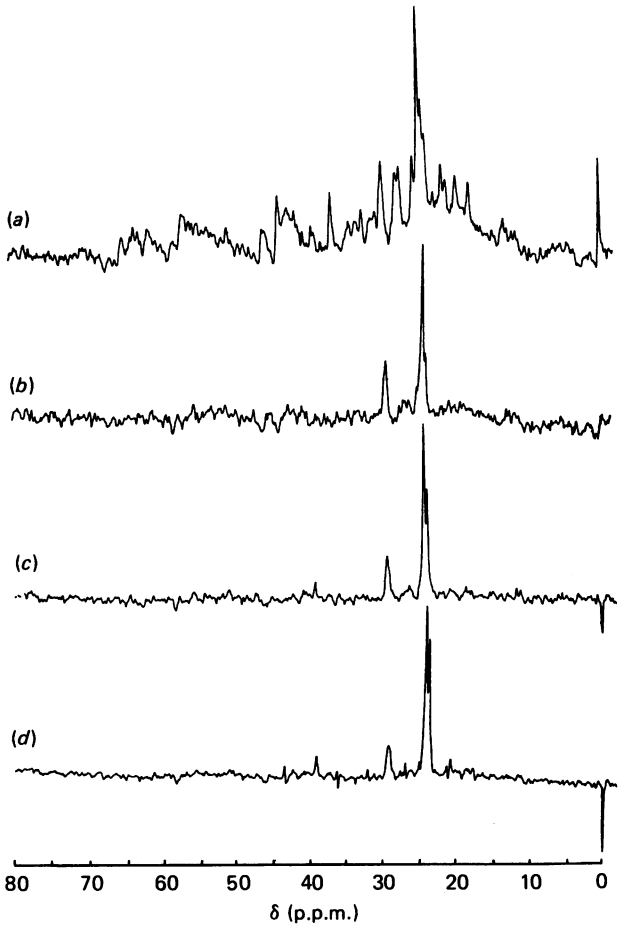


Fig. 2. ^1H-decoupled ^{13}C-n.m.r. spectra of reconstituted HMBS/HMBS–PBG complexes

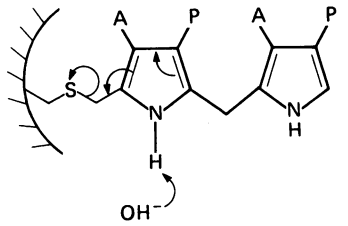
^1H-decoupled ^{13}C-n.m.r. spectra were obtained as described in the text. (*a*) Spectrum of HMBS/HMBS–PBG complexes obtained after 42000 transients. (*b*), (*c*) and (*d*). Difference spectra (reconstituted [^{13}C]enzyme minus native [^{12}C]enzyme) obtained after 42000, 168000 and 336000 transients.

F.p.l.c. of the re-activated material showed the characteristic peaks for holoenzyme, enzyme–PBG_1, enzyme–PBG_2 and enzyme–PBG_3 complexes plus several smaller peaks not normally seen when separating HMBS–PBG complexes (Fig. 1). These unusual peaks presumably result from protein that has not refolded in quite the normal way; they have not been investigated further.

The peaks corresponding to holoenzyme, mono-complex and di-complex were used for further study and together contained 36% of the starting protein.

Reconstituted holoenzyme is indistinguishable from native enzyme as regards its u.v. spectrum, its behaviour towards Ehrlich's reagent, its elution properties from Mono Q and its K_m for PBG (18 μM) determined in 0.2 M-sodium phosphate buffer, pH 8.0 (results not shown). The best specific activity of reconstituted enzyme so far obtained has been 7300 units/mg, or 50–60% of typical native enzyme values. The native enzyme treated with 6 M-urea and then dialysed as above recovered 89% of its starting activity.

Combined holoenzyme, mono-complex and di-com-



Scheme 2. Proposed mechanism for the base-catalysed breakdown of the cysteinyl-S–CH_2–pyrromethane linkage

A = $CH_2CO_2^-$; P = $CH_2CH_2CO_2^-$.

plex also gave a normal value for K_m for PBG at pH 8 with a specific activity identical with that of reconstructed holoenzyme. Also, the behaviour of the mixture towards Ehrlich's reagent was essentially identical with that seen (G. J. Hart, unpublished work) for mixed HMBS–PBG complexes.

The above demonstration that apo-HMBS can catalyse the covalent binding of two PBG units to generate its own pyrromethane cofactor is very important to our understanding of how active HMBS, i.e. holoenzyme, is synthesized in the cell. It is not necessary to postulate the existence of a separate enzyme to catalyse this; apo-HMBS possesses all the required catalytic capability.

Having a method for reconstitution of holo-HMBS from the apoenzyme, it was possible to determine the nature of the amino acid residue to which is bound the pyrromethane cofactor. This involved using [11-^{13}C]PBG for the reconstitution to connect a ^{13}C atom directly to the Y group [see (4b)].

The ^{1}H-decoupled ^{13}C-n.m.r. spectrum from 22.5 mg of reconstituted HMBS, consisting approx. of 36% holoenzyme, 17% enzyme–PGB_1 mono-complex and 47% enzyme–PBG_2 di-complex, determined at pH 14 after 42000 transients is shown in Fig. 2(*a*). The difference spectrum for ^{13}C-reconstituted enzyme minus [^{12}C]HMBS (i.e. native enzyme) run under identical conditions is shown in Fig. 2(*b*). It was found necessary to record the spectra under strongly alkaline conditions to cause unwinding of the protein and so avoid relaxation problems seen at lower pH values.

In addition to the strong signal at δ 24.5 p.p.m., characteristic of pyrrole–$^{13}CH_2$–pyrrole groups (Battersby *et al.*, 1982), there are signals at δ 29.5 and δ 24.0 p.p.m. The latter signal is characteristic of the methylene bridge positions of uroporphyrinogen (Battersby *et al.*, 1982), while the δ 29.5 p.p.m. signal matches exactly that observed for the –S–CH_2–pyrrole methylene group in the synthetic model pyrrolylpeptide Gly-Cys(–S–PBG)-Phe (Miller, 1987). [The expected chemical-shift values for –NH–$^{13}CH_2$–pyrrole and –O–$^{13}CH_2$–pyrrole are respectively δ 45.1 and δ 57.2 p.p.m. (Miller, 1987; Battersby *et al.*, 1982).] This provides extremely strong evidence that the Y group of HMBS is sulphur, and so the amino acid residue that binds the pyrromethane cofactor is a cysteine residue. Difference spectra recorded after 168000 and 336000 transients (approx. 4 and 8 h) show an increased uroporphyrinogen signal at δ 24.0 p.p.m (Figs. 2*c* and 2*d*), while the signal from cysteinyl-$^{13}CH_2$–PBG (δ 29.5 p.p.m) becomes less sharp. This suggests that at pH 14 the cysteinyl-S–CH_2–pyrrole linkage is being cleaved, probably by the mechanism shown in Scheme 2, and is in keeping with the observation that significant breakdown of the above model Gly-Cys(–S–PBG)-Phe occurs under strongly alkaline conditions (Miller, 1987). In marked contrast, the corresponding lysylpyrrolylpeptide, Gly-Lys(–NH–PBG)-Phe, was remarkably stable under alkaline conditions.

Further (indirect) support for a cysteine residue binding the pyrromethane cofactor is provided by 5,5′-dithiobis-2-nitrobenzoate) titrations of native HMBS in the presence of a denaturant (SDS or guanidine hydrochloride). Only three (2.75 ± 0.09) free thiol groups were found, and this value was not increased by incubation of enzyme with 0.1 M-dithiothreitol at room temperature for 4 h, followed by dialysis against several changes of N_2-saturated buffer. The nucleotide sequence for the *hemC* gene (Thomas & Jordan, 1986; P. R. Alefounder, unpublished work) indicates that there are four cysteine residues in the polypeptide backbone. With one of these binding the pyrromethane cofactor, no more than three can be available for reaction with thiol-modifying reagents.

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