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Identification of a cysteine residue as the binding site for the dipyrromethane cofactor at the active site of *Escherichia coli* porphobilinogen deaminase

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The dipyrromethane cofactor of *Escherichia coli* porphobilinogen deaminase was specifically labelled with ^{13}C by growth of the bacteria in the presence of 5-amino[5-^{13}C]levulinic acid. Using ^{13}C-NMR spectroscopy, the structure of the cofactor was confirmed as a dipyrromethane made up of two linked pyrrole rings each derived from porphobilinogen. The chemical shift data indicate that one of the pyrrole rings of the cofactor is covalently linked to the deaminase enzyme through a cysteine residue. Evidence from protein chemistry studies suggest that cysteine-242 is the covalent binding site for the cofactor.

Porphobilinogen deaminase; Dipyrromethane cofactor; Cysteine; ^{13}C-NMR; *(E. coli)*

1. INTRODUCTION

Porphobilinogen deaminase (EC 4.3.1.8), the third enzyme of the tetrapyrrole biosynthesis pathway, catalyses the polymerization of four molecules of porphobilinogen **(1)** to give the highly unstable hydroxymethylbilane intermediate, preuroporphyrinogen **(2)** [1,2]. This intermediate acts as the substrate for the next enzyme of the pathway, uroporphyrinogen III synthase, resulting in its isomerisation and cyclization to yield uroporphyrinogen III **(3)** [3], the essential precursor for the biosynthesis of all tetrapyrroles. In the absence of the synthase, preuroporphyrinogen is transformed into uroporphyrinogen I **(4)** in a non-enzymic reaction [3] (scheme 1).

It is well established that the deaminase enzyme catalyses the synthesis of the tetrapyrrole by a stepwise mechanism and enzyme-bound intermediate complexes with one, two, three and four pyrrole rings [4–6] attached covalently [7] to the enzyme active site have been isolated and characterised. The nature of the enzymic group which is responsible for the covalent binding of the enzyme-bound intermediates was originally proposed as being lysine on the basis of ^{13}C-NMR experiments [8] and from studies with inhibitors [9]. Very recently, however, the active site of porphobilinogen deaminase isolated from a genetically engineered strain of *E. coli* [6] has been shown to contain a novel cofactor, identified as a dipyrromethane made up from two porphobilinogen molecules linked together [10]. The enzyme-bound cofactor reacts with Ehrlich's reagent to give a reaction characteristic of a dipyrromethane. On binding two molecules of the substrate, the enzyme exhibits an Ehrlich's reaction typical of a tetrapyrromethane, establishing that the cofactor interacts covalently with the substrate. Growth of this *E. coli* strain in the presence of 5-amino[5-^{14}C]-levulinic acid, the direct precursor for porpho-

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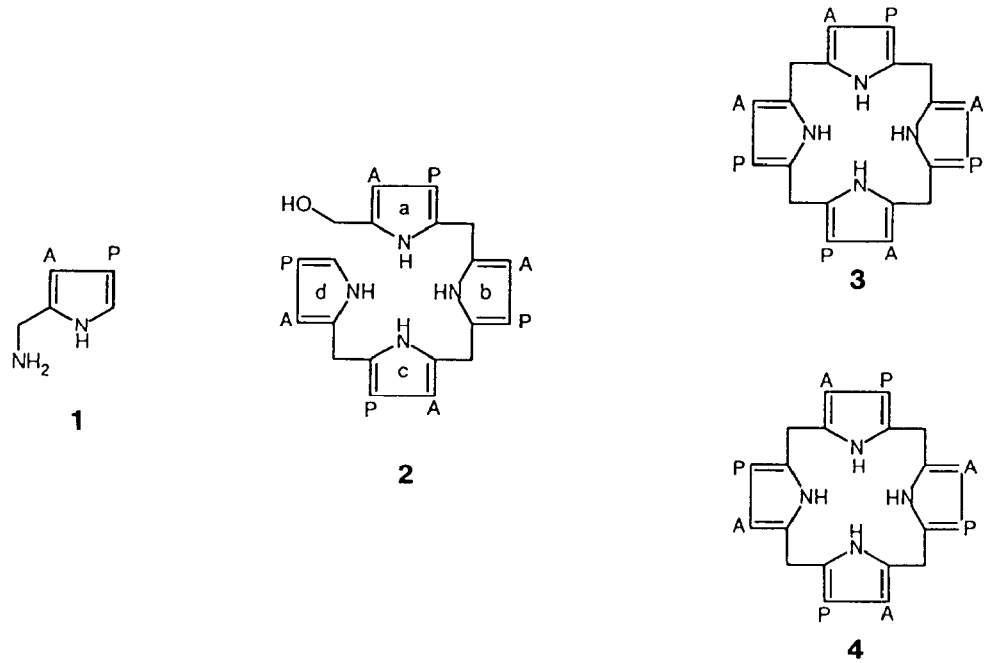


Scheme 1. Biosynthesis of uroporphyrinogen I and III from porphobilinogen.

bilinogen (scheme 2), has permitted the specific labelling of the cofactor with ^{14}C radioactivity [10]. Although made up from two porphobilinogen-derived units the cofactor, unlike the substrate, is not subject to catalytic turnover, since the ^{14}C label remains firmly attached to the deaminase even after prolonged reaction with substrate. Other workers have also suggested the existence of an active-site group based on a pyrromethane structure but have not been able to eliminate the possibility of a tri- or even a tetrapyrromethane structure [11].

The discovery that the dipyrromethane cofactor is involved with the binding of the substrate eliminates lysine or any other amino acid as the group responsible for the covalent attachment site of the substrate. Since we have demonstrated that the dipyrromethane cofactor is itself covalently linked to the enzyme active site a completely new question is raised regarding the identity of the amino acid responsible for the attachment of the cofactor. This paper describes ^{13}C-NMR experiments which not only confirm the structure of the dipyrromethane cofactor but which establish that it is attached to the deaminase enzyme through a sulphydryl group of a cysteine residue.

2. EXPERIMENTAL

5-Amino[5-^{13}C]levulinic acid (90 atom% ^{13}C) was prepared as described [12]. 5-Amino[5-^{14}C]levulinic acid was purchased from New England Nuclear (Southampton, England). Porphobilinogen deaminase was purified from *E. coli* strain ST1048 as described [6] and had a specific activity of 43 μmol/mg per h.

Enzyme with the cofactor labelled with ^{14}C was obtained as in [10]. Enzyme with the cofactor labelled specifically with ^{13}C was obtained by growth of the bacterium ST1048 on 5-amino[5-^{13}C]levulinic acid (5 mg/l) with ^{14}C-substrate to act as a tracer, The ^{13}C label was incorporated into the enzyme cofactor in at least 60% yield.

NMR experiments were conducted in 5-mm NMR tubes at 20°C with a Bruker AM-500 NMR spectrometer at an observation frequency of 125.7 MHz using a pulse width of 7 μs, a repetition time of 1.0 s and an acquisition time of 0.311 s. Exponential line broadening of 10 Hz was applied to the 16K FID prior to Fourier transformation. Samples of enzyme were dissolved in 10 mM sodium pyrophosphate buffer (pH 8.5) containing 10% $^{2}H_2O$ at 1 mM. A sample of non-labelled *E. coli* porphobilinogen deaminase was used in order to obtain $^{13}C - ^{12}C$ difference spectra. This was essential in order to remove resonances due to natural-abundance signals from the protein.

Determination of the enzyme cysteine residues was performed by titration of the enzyme (1 mg, 30 nmol) denatured with 0.1% SDS against a solution of 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in 0.1 M Tris-HCl buffer (pH 7). The absorbance of the liberated thionitrobenzoate was measured at 412 nm ($E_{412} = 12\,800$) [13].

Identification of the peptide to which the dipyrromethane cofactor is linked was carried out as follows. Porphobilinogen deaminase labelled with ^{14}C-cofactor (2 mg, 60 nmol, 0.25 μCi) [10] was subjected to chemical cleavage with 2 ml of 70% formic acid (v/v) for 30 h. After preliminary separation of the resulting peptides using Sephadex G-50, a single radioactive peptide was isolated by reverse-phase chromatography using a Pharmacia FPLC system fitted with a PepRPC column. The column was equilibrated in 0.1% trifluoroacetic acid, the peptide being eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid. The purified peptide was identified by polyacrylamide gel electrophoresis in the presence of SDS [6].

Radioactivity measurements were carried out as described in [6].

3. RESULTS AND DISCUSSION

Since growth of *E. coli* on 5-amino[5-^{14}C]-levulinic acid resulted in labelling of the dipyrromethane cofactor [10] at the active site of the deaminase enzyme, it follows that the enzyme isolated from *E. coli* grown in the presence of ^{13}C-precursor would be similarly labelled. The C-5 position of 5-aminolevulinic acid is incorporated into the C-2 and C-11 positions of porphobilinogen. Incorporation of two molecules of porphobilinogen into the dipyrromethane cofactor would thus result in the labelling pattern indicated in scheme 2.

Since all the labelled carbon atoms of the dipyrromethane are in unique environments each should exhibit a characteristic chemical shift. The two labelled carbon atoms at the free α-position and the substituted α-position of the pyrrole rings would be expected to show resonances in the aromatic region at around $\delta = 117$ and 124 ppm, respectively. The *meso*-carbon atom linking the two rings would be expected to show a resonance at about $\delta = 24$ ppm and to exhibit single-bond ^{13}C–^{13}C coupling to the substituted aromatic carbon at $\delta = 124$ ppm which in turn would be similarly affected. The resonance of the remaining labelled carbon would be dependent on the nature of the amino acid to which the cofactor is attached.

The ^{13}C-NMR spectra of the ^{13}C-labelled and non-labelled deaminase are shown in fig.1a and b, respectively. The ^{13}C – ^{12}C difference spectrum (fig.1c) revealed broad resonances representing labelled carbon atoms from the enzyme-bound cofactor. Signals at $\delta = 116$ and 128.5 ppm were assigned to the aromatic carbon atoms occupying

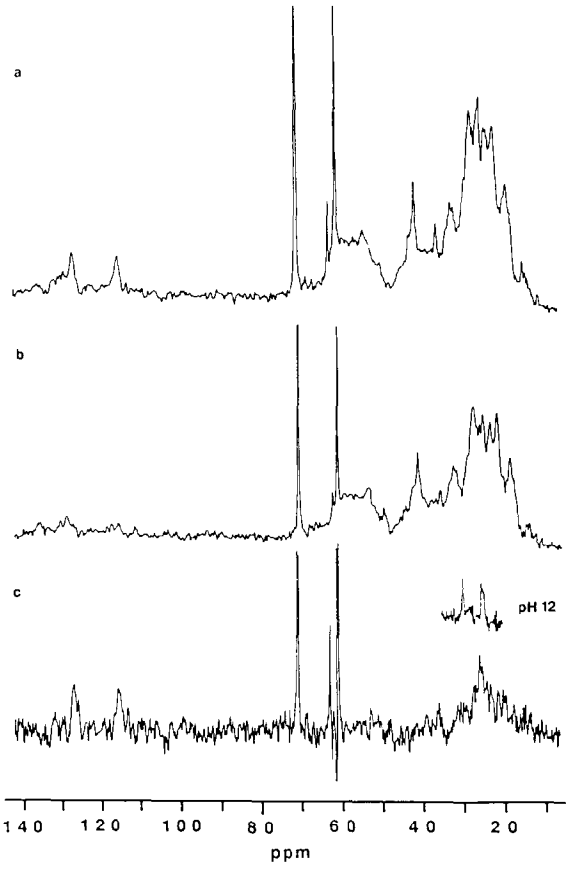


Fig.1. ^{13}C-NMR spectra of *E. coli* porphobilinogen deaminase at pH 8.5. (a) Enzyme isolated from bacteria grown on 5-amino[5-^{13}C]levulinic acid; (b) non-labelled enzyme; (c) difference spectrum of (a) – (b). Inset shows (c) at pH 12.0 (see section 2 for details of conditions). The sharp signals between 60 and 75 ppm are due to EDTA and residual Tris buffer.

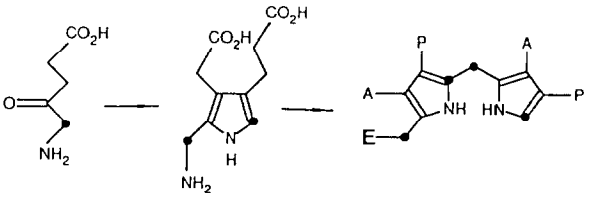
the free and substituted α-positions, respectively, in the cofactor. The complex signal at $\delta = 24$–26 ppm, which integrated to approximately two ^{13}C atoms, was made up of a resonance at $\delta = 24.7$ ppm with a shoulder at $\delta = 25.6$ ppm. The former signal was tentatively assigned to the *meso*-carbon atom of the cofactor and the latter fourth resonance to the carbon atom of the cofactor which is covalently attached to the enzyme.

In an attempt to resolve the fourth resonance more fully, both samples of deaminase were adjusted to pH 12 in order to cause protein unfolding. When the ^{13}C – ^{12}C-NMR difference spectrum was measured in the $\delta = 20$–35 ppm region (fig.1c, inset) two clearly defined broad resonances were evident, one at $\delta = 24.7$ ppm as

before and the fourth signal, now well resolved and centred at $\delta = 29.7$ ppm. The existence of four resonances, each of which integrates to one carbon atom, is consistent with the structure of the cofactor as shown in scheme 2. Further confirmation of this structure was obtained when it was found that the resonances at $\delta = 128.5$ and 24.7 ppm exhibited a ^{13}C–^{13}C single-bond coupling of 54 Hz consistent with the presence of two adjacent labelled carbon atoms. These data and more detailed NMR studies have been reported [14].

The nature of the amino acid involved in the covalent link between the enzyme and the dipyrromethane cofactor may be deduced from the ^{13}C chemical shift of the fourth resonance exhibited by the enzyme-bound cofactor. For instance, direct bonding of a ^{13}C methylene carbon to a lysine ϵ-nitrogen or to a histidine ring nitrogen would give a chemical shift in the range $\delta = 40$–50 ppm, whereas a linkage with the OH group of serine would be reflected by a chemical shift of the order of $\delta = 60$–70 ppm. A methylene carbon attached to the SH group of cysteine would exhibit a chemical shift in the range $\delta = 25$–35 ppm [15]. The chemical shift exhibited by the fourth resonance at pH 8.5 ($\delta = 25.6$ ppm) is precisely in the range expected for a methylene carbon atom bonded directly to a cysteine residue. At pH 12 this resonance moves downfield to $\delta = 29.7$ ppm and remains at this value even after the pH is lowered back to 8.5. The change in chemical shift of this fourth resonance almost certainly reflects a permanent change in the environment of the cysteine residue which links the cofactor to the deaminase protein. The slightly sharper signals also suggest that in the inactivated enzyme at pH 12, there is a greater degree of flexibility in the protein in the environment of the cofactor. Lowering of the pH back to 8.5 does not restore enzymic activity and the fact that the fourth resonance remains at $\delta = 29.7$ ppm indicates that the native conformation of the enzyme has not re-formed.

In order to establish that the cofactor remains bound to the protein at pH 12 a parallel experiment was carried out in which the enzyme containing the cofactor labelled with ^{14}C radioactivity [10] was also adjusted to pH 12. No ^{14}C label was released under these conditions. It was not possible to recover enzyme activity on readjustment of the pH back to 8.5. The conclusions from these results



Scheme 2. Incorporation of ^{13}C label from 5-amino[5-^{14}C]-levulinic acid into the dipyrromethane cofactor.

suggest therefore that cysteine is the amino acid responsible for providing the covalent attachment site for the cofactor. To add further weight to this proposal, sulphur-porphobilinogen model compounds have now been synthesised [14] which exhibit resonances around $\delta = 29$ ppm for the methylene linked to the sulphur atom as expected. The fact that in the native enzyme the resonance is upfield at $\delta = 25.6$ ppm suggests that the cofactor may be in an unusual environment in the active enzyme.

Examination of the gene-derived primary protein sequence of *E. coli* porphobilinogen deaminase [16] reveals that four cysteine residues are present, only three of which react with thiol reagents such as DTNB under denaturing conditions. A comparison of the *E. coli* and human [17] DNA-derived protein sequences indicates that of these four cysteines only two are conserved in both the *E. coli* and human deaminases. Since the human porphobilinogen deaminase has also been shown to contain a dipyrromethane cofactor [18], which must also be bound to a cysteine, one of these two conserved cysteine residues is likely to be involved with the binding of the cofactor to the *E. coli* enzyme. The two cysteine residues are located at positions 99 and 242 and fall either side of the single aspartyl–proline bond at positions 103–104 which is cleaved specifically with formic acid [6,16].

In order to determine which of these cysteine residues is involved, enzyme containing the ^{14}C-labelled cofactor [10] was treated with formic acid as described in section 2. Over half of the ^{14}C label was converted into uroporphyrin [10] but sufficient radioactivity remained bound to permit the isolation of a peptide containing 20% of the initial ^{14}C label. The small formic acid peptide (M_r 11 000), contained negligible radioactivity. Isolation and purification of the labelled peptide show-

ed it to have an M_r value of 24000, establishing it as the fragment extending from amino acid residue 104 to the C-terminus. Since this ^{14}C-peptide includes cysteine-242, but not cysteine 99, we conclude that it is the former cysteine residue which provides the covalent binding site for the dipyrromethane cofactor in the gene-derived primary sequence of the *E. coli* deaminase [16]. Generation of modified *E. coli* porphobilinogen deaminases in which either cysteine-99 or 242 had been substituted by serine using site-specific mutagenesis supported this view. Deaminase containing serine-99 was catalytically active whereas the enzyme containing serine-242 was completely devoid of enzyme activity.

The finding that cysteine is involved in the attachment of the pyrrole rings of the dipyrromethane cofactor to the *E. coli* porphobilinogen deaminase enzyme is not surprising, since both cytochrome *c* and phytochrome utilize this amino acid in order to secure their prosthetic groups to the protein.

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