^{13}C NMR AS A PROBE FOR THE STUDY OF ENZYME-CATALYSED REACTIONS

Mechanism of action of 5-aminolevulinic acid dehydratase

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1. Introduction

The advantage of ^{13}C NMR as a probe for the investigation of biochemical problems over a broad sphere has been highlighted by several reports in which the technique has been utilized to show the existence of labile or transient species or for studies in systems where a non-invasive approach is obligatory. The visualisation of the acyl-enzyme intermediate of chymotrypsin [1], the discovery of preuroporphyrinogen the unstable substrate for uroporphyrinogen III cosynthetase in porphyrin biosynthesis [2,3] and the direct observation of metabolic pathways in intact bacterial [4,5] and mammalian cells [6] serve to illustrate the utility of the technique. The exquisite sensitivity of the ^{13}C nucleus to its environment also makes it a powerful probe for the illucidation of pathways and mechanism in the biosynthesis of natural products from ^{13}C enriched precursors [7]. This paper describes the application of ^{13}C NMR to the study of the mechanism of action of 5-aminolevulinic acid dehydratase, the second enzyme in the porphyrin biosynthetic pathway, and the value of the technique for the study of enzyme reactions of this type in which more than one molecule of the same substrate is involved.

2. Materials and methods

Dithioerythritol was obtained from Sigma, London. All other laboratory reagents were purchased from BDH, Poole, Dorset. 5-Amino[5-^{13}C]-levulinic 90% enriched with ^{13}C was a generous gift from Professor A. I. Scott.

5-Aminolevulinic acid dehydratase was purified from bovine liver [8]. The purified enzyme was stored as an ammonium sulfate pellet, spec. act. 38 units/mg. One unit of enzyme catalyses the formation of 1 μmol porphobilinogen/h. Prior to use, the enzyme was dissolved in 0.1 M potassium phosphate buffer (pH 6.8) containing 10 mM dithioerythritol and was dialyzed against the same buffer.

[2,11-$^{13}C_2$]Porphobilinogen was prepared by incubating 0.25 ml 5-aminolevulinic acid dehydratase (5.7 nmol)* with 5-amino [5-^{13}C]levulinic acid (1.43 μmol) in 0.5 ml potassium phosphate buffer (pH 6.8) containing 10 mM dithioerythritol. The mixture was incubated at 37°C for 30 min to ensure complete conversion of substrate into porphobilinogen. The sample thus prepared was used as a reference for the chemical shifts of C-2 (δ = 117 ppm) and C-11 (δ = 36.5) in porphobilinogen [9] (scheme 1, fig.1).

Single turnover experiments were carried out by mixing 0.3 ml 5-aminolevulinic acid dehydratase (0.49 μmol)* with 0.3 ml 5-amino[5-^{13}C]levulinic acid (0.45 μmol) in a rapid mixing device such that the enzyme and ^{13}C substrate interacted with each other for 100 ms before being quenched into 0.3 ml unlabelled 5-aminolevulinic acid (8.4 μmol). The mixture was incubated at 37°C for 10 min to allow quantitative conversion into porphobilinogen (see fig.2).

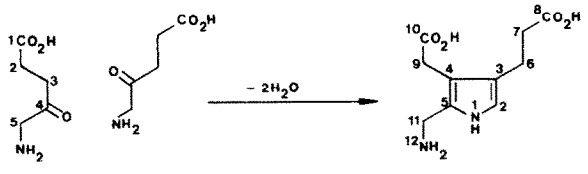
Proton decoupled ^{13}C NMR spectra were measured in phosphate buffer (900 μl) in a 10 mm NMR tube fitted with a PTFE spacer spinning at 20 rev./s at 26°C using a Varian XL-100 FT spectrophotometer at

* In all cases μmol enzyme relates to the subunit mol. wt 35 000. The native enzyme exists as an octamer mol. wt 280 000 [11]

25.2 MHz. Data were accumulated for 75 000 pulses (acquisition time 0.8 s) using a spectral width of 5000 Hz and a pulse width of 20. ^{19}F was the external lock and chemical shifts were related to trimethylsilane (δ = 0 ppm).

3. Results and discussion

5-Aminolevulinic acid dehydratase (EC 4.2.1.24) catalyses the condensation of 2 molecules of 5-aminolevulinic acid (1) to give the pyrrole porphobilinogen (2) a Knorr condensation in which the C-5 carbon atom of 5-aminolevulinic acid gives rise to C-2 and C-11 in (2). The dehydratase from *Rhodopseudomonas spheroides* has been the subject of a comprehensive investigation [11] and the participation of a Schiff base linkage between the substrate and an amino group on the enzyme has been established [11]. In the mechanism proposed for the *Rhodopseudomonas spheroides* enzyme, the substrate molecule initially bound to the enzyme through a Schiff base is the one which gives rise to the 'acetic acid side' of the porphobilinogen (atoms 4,5,9–12) the second substrate molecule reacting with the enzyme giving rise to the propionic acid side the molecule (atoms 1,2,3,6–8). Although the mechanism has been accepted for over a decade and has been extended to include the bovine liver enzyme no direct supporting experimental evidence has been obtained for either bacterial or mammalian enzymes. Implicit in our understanding of the catalytic events which occur at the active site of 5-aminolevulinic dehydratase is a knowledge of the order in which the 2 molecules of 5-aminolevulinic acid bind to the enzyme prior to the condensation. Since the steady state kinetic approach is not readily applicable to the study of enzyme reactions involving $\geqslant$2 molecules of the same substrate, we required a method by which the 2 substrate molecules giving rise to porphobilinogen could be distinguished from one another in their ultimate positions in the product. By virtue of the fact that the ^{13}C chemical shifts of the C-2 and C-11 positions in porphobilinogen differ appreciably from one another, an evaluation of the ^{13}C spectrum of porphobilinogen enzymically synthesised from 5-amino[5-^{13}C]levulinic acid can be used to distinguish between the two 'sides' of the product.



Scheme 1

Incubation of 5-amino[5-^{13}C]levulinic acid (chemical shift of enriched carbon atom, δ = 48 ppm) with 5-aminolevulinic acid dehydratase from bovine liver (5-aminolevulinic acid is 250 × molar excess over enzyme) gave porphobilinogen. The NMR spectrum of which, as expected, showed two signals of equal intensity (fig.1), one in the aromatic region (δ = 117) corresponding to C-2 and the other at δ = 36.5 ppm arising for the aminomethyl carbon C-11 (2). The absence of any signal at δ = 48 ppm indicated a total conversion of the labelled aminolevulinic acid into product had occurred. The use of a molar excess of substrate over enzyme however, yields no information regarding the order in which the 2 molecules of 5-aminolevulinic acid are incorporated into the pyrrole or details of the enzyme mechanism.

The problem was approached by use of a single turnover enzyme reaction in which equimolar amounts of the enzyme and [^{13}C]substrate were initially mixed, in anticipation that one of the two substrate binding sites would be preferentially occupied by the substrate. Addition of excess non-labelled substrate would carry the bound labelled substrate into the product which should consequently be labelled at either C-2 or C-11, the position of label

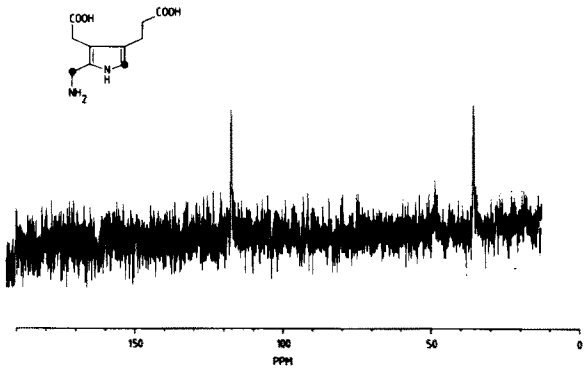


Fig.1. ^{13}C NMR spectrum of [2,11-^{13}C]porphobilinogen biosynthesised from 5-amino[5-^{13}C]levulinic acid using 5-aminolevulinic acid dehydratase. See section 2 for details.

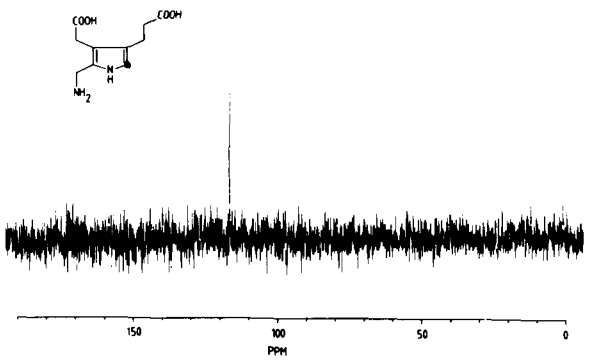
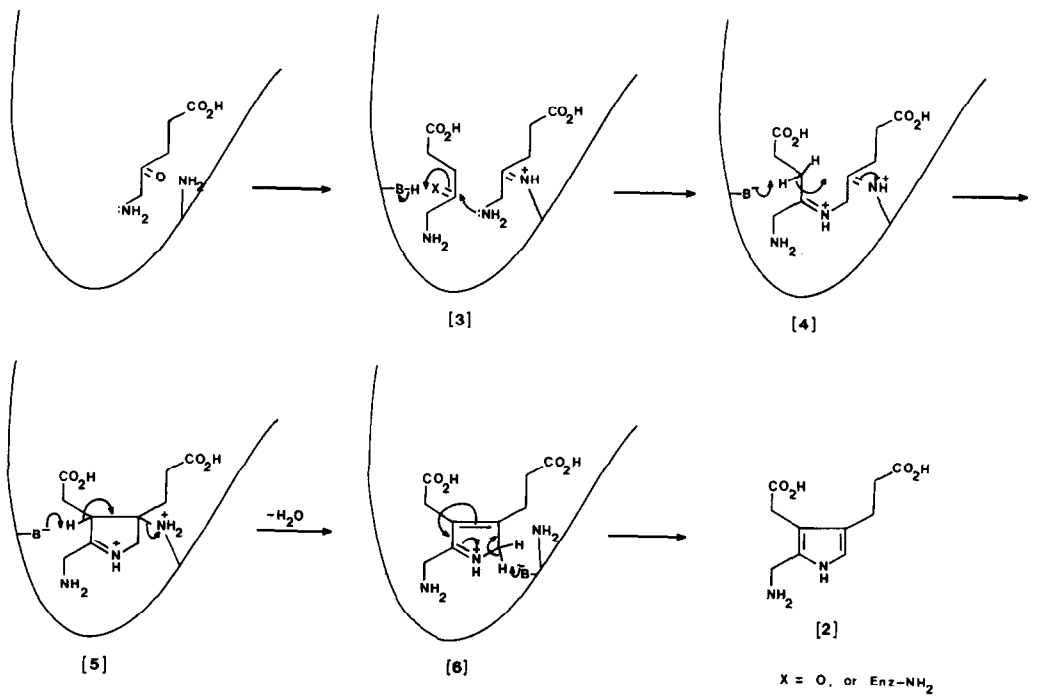


Fig.2. ^{13}C NMR spectrum of [2-^{13}C]porphobilinogen biosynthesised from 5-amino[5-^{13}C]levulinic acid using 5-aminolevulinic acid dehydratase in a single turnover experiment. See section 2 for details.

reflecting the substrate molecule originally bound to the enzyme active site. Fig.2 shows the ^{13}C spectrum of porphobilinogen obtained from a single turnover experiment in which 0.49 μmol enzyme and 0.45 μmol 5 amino[5-^{13}C]levulinic acid were initially mixed before the addition of 8.4 μmol ^{12}C 5-aminolevulinic acid. The appearance of only one signal, attributable to the aromatic carbon C-2 of porphobilinogen (δ = 117 ppm) and the complete absence of any signal for C-11 (δ = 36.5 ppm) establish that the first molecule of 5-aminolevulinic acid bound to the enzyme is the one giving rise to the propionic acid side of porphobilinogen (atoms 1,2,3,6–8 in (2)). The data also highlight the fact that the molecule of 5-aminolevulinic acid initially bound to the enzyme must have an affinity at least an order of magnitude greater than the 5-aminolevulinic acid molecule giving rise to the acetic acid side of porphobilinogen (atoms 4,5,9–12).

These findings allow the formulation of a mechanism for the enzymic formation of porphobilinogen by the bovine liver enzyme (scheme 2) in which initially bound 5-aminolevulinic acid molecule forms a Schiff base with the enzyme (3), followed by the reaction of the second molecule of substrate to yield the new Schiff base intermediate (4), the latter being converted into porphobilinogen through the sequence (4)→(5) and (5)→(6) (6)→(2). Results of single turnover experiments with the enzyme from *Rhodopseudomonas spheroides* were similar to those obtained with the bovine liver enzyme establishing that the bacterial enzyme follows the same sequence of binding the two substrate molecules as the dehydratase from mammalian sources.



Scheme 2

The single turnover approach can also be carried out using ^{14}C-labelled substrates and this technique has been employed to investigate the mechanism of biosynthesis of preuroporphyrinogen by the porphobilinogen deaminase system using [2,11-$^{14}C_2$]porphobilinogen [12,13]. However, the use of ^{13}C NMR by the virtue of the sensitivity of the ^{13}C chemical shifts enables the position of the label to be determined without degradation although in practice it is also preferable to have established the outcome by chemical degradation. This latter point is covered in [14].

Acknowledgements

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