Volume 233, number 2, 379–382 FEB 05978 June 1988

^{31}P NMR investigations on free and enzyme bound thiamine pyrophosphate

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Received 12 April 1988

Pyruvate decarboxylase (PDC) contains thiamine pyrophosphate (TPP) and Mg^{2+} as cofactors. ^{31}P NMR studies with PDC in the presence of added Mn^{2+} reveal the pyrophosphate moiety of TPP to be a nonaccessible area for the external Mn^{2+} and thus proving the Mg-P-complex (taking part in the binding of the coenzyme to the protein) to be a nonaccessible area for the medium. Glyoxylic acid, acting as an inhibitor of PDC by forming a noncleavable bond with the catalytic center of TPP causes a steric immobilization of the coenzyme indicated by a line broadening of the pyrophosphate moiety.

Thiamine pyrophosphate mechanism; Pyruvate decarboxylase; ^{31}P NMR

1. INTRODUCTION

The development of Fourier-Transform-NMR has made ^{31}P NMR investigations with enzyme bound coenzymes and the comparison with the corresponding parameters of the solved coenzymes into an efficient tool for the characterization of active sites structure and mobility [1–3].

PDC, containing TPP and Mg^{2+} as cofactors, consists of four subunits each pair being identical ($\alpha_2\beta_2$-structure). Depending on the conditions of enzyme preparation, 2–4 molecules of TPP and Mg^{2+} per molecule enzyme (240 kDa) are bound.

In accordance with the following equation

$$\text{PDC} \underset{\text{pH 8.4}}{\overset{\text{pH 6.0}}{\rightleftharpoons}} \text{apo-PDC} + n\text{TPP} + n\text{Mg}^{2+} \quad (1)$$

cofactors are eliminated from the holoenzyme at pH values >8.4. At pH 6.0, the apoenzyme recombines with added cofactors (TPP and Mg^{2+} or Mn^{2+}, respectively) to yield active enzyme.

Enzyme activity requires essentially the presence of bivalent metal ions which are in the native state Mg^{2+} but can be replaced, for example, by Mn^{2+} without any loss of the catalytic power [4,5].

TPP in solution was characterized at length with ^{1}H, ^{13}C and ^{31}P NMR [6–8]. On the other hand, ^{31}P NMR studies with enzyme bound TPP are not published as yet.

In this paper, ^{31}P NMR data obtained with PDC-bound TPP and TPP in aqueous solution are compared with respect to the influence of paramagnetic ions such as Mn^{2+} and of bound substrate analogs on the resonance of the pyrophosphate moiety. First conclusions could be drawn concerning the binding mechanism of TPP within the active center.

2. MATERIALS AND METHODS

PDC was obtained from dried brewer's yeast according to Ullrich [9] and purified by chromatography via CM Sephadex [10]. Preparations used showed generally a specific activity of more than 40 U/mg.

The NMR measurements were performed in D_2O. The enzyme solution contained ammonium sulfate to stabilize the

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Abbreviations: PDC, pyruvate decarboxylase (EC 4.1.1.1); TPP, thiamine pyrophosphate

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

native enzyme structure. A Brucker NMR spectrometer WP 200 was used with 81.3 MHz for ^{31}P. The probe temperature was kept constant at 10°C during the measurements. Enzyme activity was checked before and after NMR measurements and showed no substantial difference (3%). Chemical shifts refer to H_3PO_4 as the external reference.

3. RESULTS

3.1. *^{31}P NMR spectrum of PDC (Mg^{2+})*

A ^{31}P spectrum of a PDC solution (10^{-3} M, p*D* 6.0) shows the following peaks of the pyrophosphate moiety: β-phosphate at -5.35 ppm, α-phosphate at -9.2 ppm. Additionally, an undefined monophosphate peak appears at 3.0 ppm (fig.1). The ^{31}P resonances of free TPP (not shown) are: -9.5 ppm (β-phosphate) and -10.8 ppm (α-phosphate).

3.2. *^{31}P NMR spectrum of (Mg^{2+})-PDC in the presence of Mn^{2+}*

Addition of Mn^{2+} to the enzyme solution (5×10^{-3} M $MnSO_4$) results in a slight shift of the pyrophosphate peaks (fig.2). The β-resonance appears at -4.6 ppm, α-phosphate at -9.96 ppm. Both signals show no line broadening. The monophosphate signal disappears under these conditions. An aqueous solution of TPP (10^{-3} M in D_2O) with added Mn^{2+} (5×10^{-3} M) gives no signal in the ^{31}P NMR spectrum because of extreme line broadening by the paramagnetic metal ions.

3.3. *^{31}P NMR spectrum of Mn^{2+}-PDC*

A PDC preparation in which the native Mg^{2+} were exchanged by Mn^{2+} yielded no signals in the

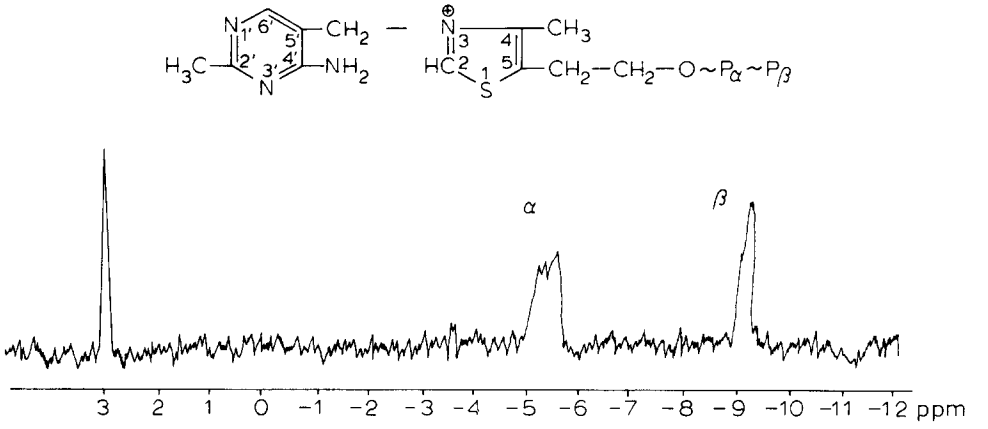

Fig.1. ^{31}P NMR spectrum of PDC (10^{-3} M) a 81.3 MHz, $T = 10$°C, p*D* 6.0. α and β indicate the resonance positions of the α- and β-phosphates of the enzyme.

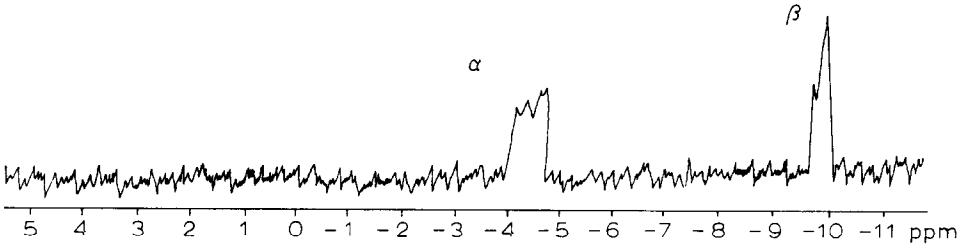

Fig.2. ^{31}P NMR spectrum of PDC (10^{-3} M) in the presence of $MnSO_4$ (5×10^{-3} M) at 81.3 MHz, $T = 10$°C, p*D* 6.0.

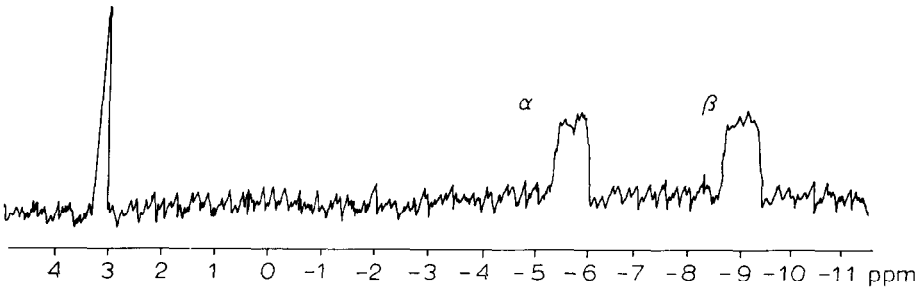

Fig.3. ^{31}P NMR spectrum of PDC (10^{-3} M) in the presence of glyoxylic acid (10^{-2} M) at 81.3 MHz, T = 10°C, pD 6.0.

^{31}P NMR spectrum as a consequence of the paramagnetic influence on the linewidth of the phosphate resonances.

3.4. *C-2 modification of enzyme-bound TPP by glyoxylic acid*

Experiments with ^{14}C-labeled glyoxylic acid have proved this substrate analog to be an irreversible blocker of the enzyme mechanism by forming a covalent bond to the catalytic C-2 position of TPP [11].

Addition of glyoxylic acid (pD 6.0) causes no change in the chemical shifts of the β- and α-phosphate peaks: β-phosphate appears at -5.75 ppm, α-phosphate at -8.91 ppm. The linewidth increases for the α-phosphate peak from 51 to 86 Hz whereas the linewidth of the β-phosphate peak remains unchanged.

4. DISCUSSION

Addition of paramagnetic ions such as Mn^{2+} to suitable ligands leads (due to shortening of the transversal relaxation time T_2) to line broadening of those resonances of which the nuclei are located near the environment of the metal ion. Therefore, addition of Mn^{2+} is often used to distinguish solvent accessible parts in molecules from those where solvent interactions can be excluded.

Our investigations have shown, that external Mn^{2+} cannot attain the environment of the enzyme bound pyrophosphate moiety of TPP. Therefore, the native cofactor Mg^{2+} is not replaced in the metal binding site by the Mn^{2+} added. On the other hand, the unspecifically bound monophosphate ion is accessible for Mn^{2+}. Its resonance disappears therefore due to the strong paramagnetic influence on the linewidth. It may be concluded that this monophosphate ion, perhaps an artefact of the preparation, is located on the surface area of the enzyme.

The measurements with PDC obtained from the apoenzyme in the presence of Mn^{2+} at pH 6.0 (according to eqn 1) show that the linewidth of the α- and β-phosphates are too broad to be detected in the spectrum. According to [12], this reveals the distance of the metal ion to the pyrophosphate moiety of TPP to be smaller than 10 Å.

Irreversible binding of glyoxylic acid to the C-2 of TPP causes, due to the formation of a noncleavable C-C-bond, an irreversible blocking of the catalytic mechanism. Thereby, the coenzyme experiences a steric fixation in the specific conformation of substrate binding. The addition of glyoxylic acid changes drastically the linewidth of the α-phosphate signal. This can be attributed to a restricted mobility of the α-phosphate residue in this case. The β-phosphate on the other hand, probably involved in the binding of the coenzyme to the apoenzyme, shows no change in the linewidth of its resonance.

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