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Correlation of cofactor binding and the quaternary structure of pyruvate decarboxylase as revealed by ^{31}P NMR spectroscopy

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The pH dependence of the quaternary structure of pyruvate decarboxylase (EC 4.1.1.1) has recently been discovered [(1990) FEBS Lett. 266, 17–20; (1992) Biochemistry (in press)]. In the present study we have investigated the change in quaternary structure by observing the binding of the cofactor, thiamine pyrophosphate, using ^{31}P NMR spectroscopy. The dissociation of the native tetramers into dimers when increasing the pH coincides with a weaker binding of the cofactor and loss of enzyme activity. The results provide further evidence that thiamine pyrophosphate is bound primarily via the β-phosphate moiety. In addition, a phosphoserine has been discovered in two of the four subunits.

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

1. INTRODUCTION

Pyruvate decarboxylase (PDC; EC 4.1.1.1) catalyzes the decarboxylation of 2-oxoacids to the corresponding aldehydes using thiamine pyrophosphate (TPP) and Mg^{2+} ions as cofactors. The enzyme from yeast constitutes an $\alpha_2\beta_2$ tetramer [3], with a molecular mass of 240 kDa [4]. At physiological conditions (around pH 6) four TPP and an equal amount of Mg^{2+} ions are bound to the tetrameric protein in a quasi-irreversible manner. X-Ray solution scattering studies have revealed changes in quaternary structure from tetramer to dimer when increasing the pH [1,2]. In the present study we have investigated the cofactor binding during pH-dependent changes of quaternary structure of PDC as inferred by ^{31}P NMR experiments.

2. MATERIALS AND METHODS

PDC was isolated from brewer's yeast obtained from the Wernesgrün brewery and purified according previously published procedures [3]. The enzyme, with a specific activity of 40–50 U/mg protein, is homogeneous, as indicated by SDS-PAGE. The activity was monitored spectroscopically at 340 nm using the alcohol dehydrogenase/NADH system (Serva) [5]. The concentration of PDC was determined at 280 nm using a molar extinction coefficient of 281 000 $M^{-1}\cdot cm^{-1}$. For the removal of excess cofactors from the enzyme PDC was gel filtrated using a Biogel P2 column (0.8 × 20 cm) equilibrated with 130 mM HEPES buffer, pH 6.0. The final concentration of PDC was 40 mg/ml. The different pH values of the enzyme solution were prepared by precipitation of the protein with ammonium sulfate followed by redissolving it in HEPES-buffer adjusted to the desired pH. Alkaline phosphatase with a specific activity of 2,800 U/mg was obtained from Sigma. Sample volumes were 2.36 ml in 10-mm diameter tubes containing 13% D_2O as the field/frequency lock. A concentric 2 mm NMR tube containing 1.65 mM methylendiphosphoric acid was used as the internal standard. ^{31}P NMR measurements were performed at 300 MHz on a Bruker AM 300 SWB superconducting spectrometer. All experiments were recorded at 8°C with broadband decoupling (0.5 W), 8 K spectral width in 32 K data points with a 90° pulse angle. An exponential line broadening of 10 Hz was used prior to Fourier transformation. Positive chemical shifts in ppm represent downfield changes relative to 85% phosphoric acid. Enzyme activity measured in the presence of 5 mM TPP and 1 mM Mg^{2+} ions in 0.1 M HEPES buffer, pH 6.0, did not change during the NMR experiment.

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3. RESULTS AND DISCUSSION

The doublets of the ^{31}P NMR spectrum of PDC around −10.5 and −6.2 ppm were assigned to the α- and β-phosphorus nuclei of the cofactor, TPP, respectively (Fig. 1). The observed pH dependence of the chemical shift of TPP in PDC is similar to that obtained for free TPP in the presence of Mg^{2+} ions [6]. Unfortunately the pH range for the experiments with protein is restricted to the pH stability range of the PDC enzyme. In addition to the TPP signals, another signal around 4 ppm was found in the ^{31}P NMR spectra of PDC shifting downfield with increasing pH. This result is in agreement with earlier observations [7]. From the pH dependence of the chemical shift of this signal a pK of 5.8 can be calculated by iterative computer analysis [8]. Treatment of PDC with alkaline phosphatase eliminates this signal but also the TPP signals concomitant with the appearance of a new signal at 2.7 ppm assigned to free inorganic phosphate (last spectrum of Fig. 1). Therefore, the singlet around 4 ppm represents the signal of a phosphoserine residue which seems to be an integral part of PDC.

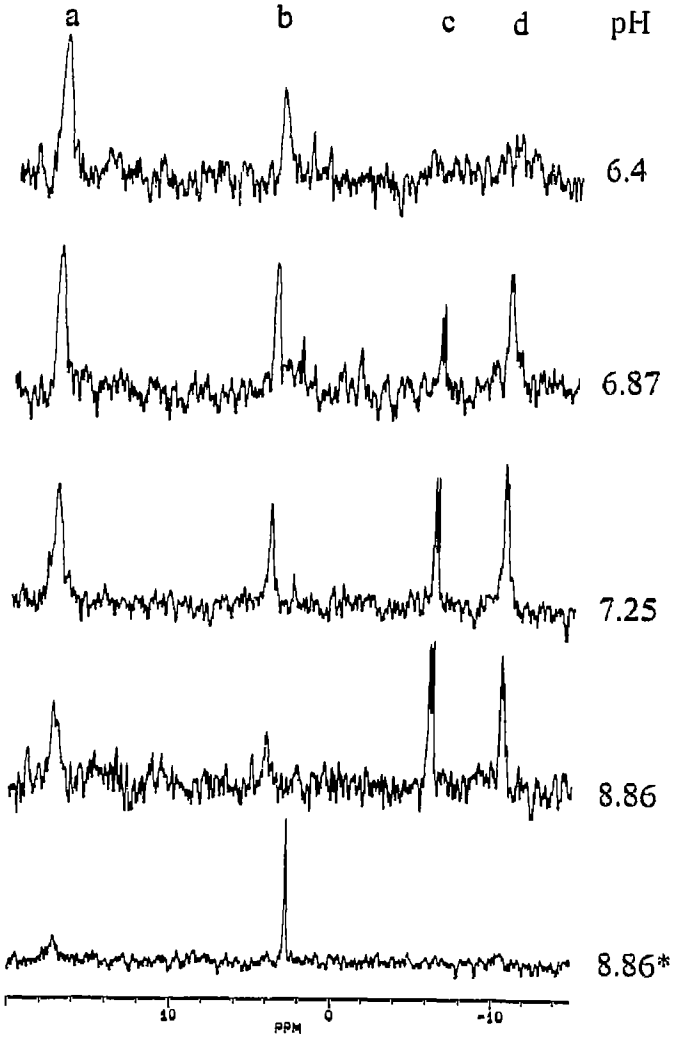

Fig. 1. ^{31}P NMR spectra of 34 μM PDC in 0.15 M HEPES or 0.15 M Tris-HCl containing 0.5 M ammonium sulfate at the indicated pH values and at 8°C. a, methylenediphosphonate; b, inorganic phosphate or phosphoserine; c and d, β-phosphate and α-phosphate of TPP, respectively. *After treatment with 4,000 U alkaline phosphatase.

The ratio of the integral of the peak areas of all phosphorus signals of PDC relative to that of the standard, methylendiphosphoric acid, as well as the pH dependence of the chemical shifts are given in Table I. The calculated integrals correspond to two phosphoserines per PDC supporting evidence for the phosphorylation of only two of the four subunits. After recombining dephosphorylated PDC with TPP and Mg^{2+} ions the sigmoid v/S profile is identical with that of native enzyme (data not shown). This finding clearly indicates that the phosphorylation of PDC influences neither the catalytic process nor its regulation. The ratio of the integrals of the phosphoserine and the standard signal remains unchanged within the investigated pH range, whereas the relative integrals of the cofactor signals exhibit a strong pH dependence. These signals are undetectable below pH 6.4, and above that pH their intensity increases with pH.

Interestingly, the integrals for α- and β-phosphate groups of TPP differ significantly up to pH 8.86. This difference between the α- and β-phosphate signals becomes smaller with higher pH's and disappears at pH 8.86. X-Ray solution scattering studies on PDC have shown that the active tetramer of PDC dissociates into dimers [2]. At pH 6.0, only tetramers are present. Increasing the pH leads to a shift of the equilibrium to dimers reaching 100% dimer above pH 8.5.

Considering the above facts the ^{31}P NMR spectra of PDC can be interpreted as follows: the cofactor, TPP, is tightly bound in the tetramer formation having no self mobility, i.e. the correlation time of the cofactor is determined by the correlation time of the whole protein resulting in a very fast relaxation. The resulting very broad signals are no longer detectable. The dissociation into dimers occuring with increasing pH results in a loosening of cofactor binding and even a partial build-up of free TPP. Therefore, the ^{31}P NMR spectrum shows both TPP still bound to the dimer and free TPP. The latter fraction is characterized by sharp doublets due to their high mobility. The chemical shift of both signals is identical to free TPP at the respective pH's. In contrast, the fraction of TPP still bound to the dimer shows a broad signal for the α-phosphate and no signal

Table I

The pH dependence of the chemical shifts and integrals of the ^{31}P NMR signals of PDC

pH	Relative ratios of the integrals of different signals of PDC								
	α	ppm	β	ppm	ratio α/β	phosphoserine	ppm	phosphate	ppm
5.48	0		0			0.5	2.6	0	
5.98	0		0			0.52	3.1	0	
6.40	0.2	-10.7	0			0.54	3.6	0	
6.87	0.5	-10.6	0.16	-6.5	3.1	0.47	3.9	0	
7.25	0.74	-10.5	0.42	-6.4	1.8	0.53	4.0	0	
8.86	0.97	-10.4	0.92	-6.1	1.05	0.44	4.1	0	
8.86*	0		0			0		2.53	2.7

*After treatment with alkaline phosphatase

for the β-phosphate, resulting in the observed differences of the integrals for these signals (see Table I). This interpretation would indicate a binding of the cofactor to the dimer primarily via the β-phosphate and is the reason for their fast relaxation. Bound β-phosphate gives a very broad non-resolvable signal. The α-phosphate, however, is not directly involved in the interaction with the protein and the signal is therefore more flexible and sharper.

The identity of the integrals of both signals, as well as the sharp doublets at higher pH values (pH 8.86), prove the complete dissociation of TPP from PDC. Furthermore, a comparison of the integral values of the cofactor with that of the standard clearly shows that exactly four TPP correspond to one PDC tetramer.

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