IS THE ACTIVE FORM OF PYRIDOXAL-P IN α-GLUCAN PHOSPHORYLASES A 5′-PHOSPHATE DIANION?

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1. Introduction

The essential role of pyridoxal-5′-phosphate for the activity of all α-glucan phosphorylases received strong support from recent studies with bacterial phosphorylases [1]. The advantage of utilizing these enzymes for the study of the role of pyridoxal-P is obvious since they neither depend on allosteric effectors or covalent modification for the expression of activity [1–6].

The 5′-phosphate group of pyridoxal-P is the sole phosphate moiety in catalytically active bacterial phosphorylases. Accordingly, ^{31}P NMR spectra of *Escherichia coli* maltodextrin phosphorylase exhibit an exceptionally simple pattern. When compared with the ^{31}P NMR spectra of rabbit skeletal muscle phosphorylase *a* and *b* [7] the resonances common to phosphorylase allow us to define the ionization state of the cofactor in the catalytically active enzyme.

2. Experimental

2.1. *Materials*

Morpholinoethane sulfonic acid (MES) and morpholinopropane sulfonic acid (MOPS) were obtained from Serva (Heidelberg). Buffer A is 50 mM MOPS, with 50 mM KCl, 2 mM EDTA, 10 mM 2-mercaptoethanol. Buffer B is 50 mM MES with the same additions. The buffers were adjusted to the desired pH with KOH. Dextrin type III was from Sigma (Taufkirchen); only the water soluble fraction was used.

To Professor Peter Karlson on the occasion of his 60th birthday

2.2. *Enzyme*

Maltodextrin phosphorylase was purified from *E. coli* K12 Hfr H 8000 and assayed as in [1]. The purified enzyme was homogenous based on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Molar concentrations of the enzyme were based on a monomer mol. wt 81×10^3 [1]. The phosphorylase (350 mg) was precipitated from a stock solution with 2.7 M $(NH_4)_2SO_4$, redissolved in a small volume of buffer A and chromatographed on a Sephadex G-25 column (2 × 25 cm) with the same buffer. Changes and additions were introduced by dialysis against several changes of the new solute.

2.3. ^{31}*P NMR*

Fourier transform ^{31}P NMR spectra were recorded at 72.86 MHz on a Bruker WH-180 wide bore superconducting spectrometer [7]. Enzyme samples (10–12 ml, $2.1–2.5 \times 10^{-4}$ M per monomer) in 20 mm tubes were used. A concentric 5 mm NMR tube containing ^2H_2O was used as field/frequency lock. Orthophosphoric acid (85%) enclosed in a capillary was used for chemical shift referencing. All spectra were recorded with broadband proton decoupling (0.4 W). The exponential line broadening before Fourier transformation was usually 10 Hz. The temperature was kept at 26–30°C. Scans ($18–78 \times 10^3$) were collected. For a series of experiments the enzyme sample was recovered after each run, centrifuged, readjusted to the original concentration and new conditions by dialysis and reutilized. Even after scanning for 12 h and despite some loss of protein at the lower pH values maltodextrin phosphorylase was recovered with constant specific activity.

3. Results

3.1. *The ionization state of pyridoxal-P in maltodextrin phosphorylase*

Typical ^{31}P NMR spectra at pH 6.7 in the absence and presence of substrate are shown in fig.1. They all show a single resonance with a chemical shift of −3.4 ppm and linewidths of 35 ± 4 Hz in the absence and 55 Hz in the presence of substrates. In fig.2 the pH dependence of the chemical shift of pyridoxal-P bound to maltodextrin phosphorylase is compared with that of pyridoxal-P in D-serine dehydratase [8] and with a model Schiff-base formed with pyridoxal-P and ε-aminocaproate [9]. With maltodextrin phosphorylase the measurements were not extended below pH 5.8 due to increasing loss of protein. Since the remainder of the enzyme preserves constant specific activity this effect does not interfere with the NMR measurements. As can be seen the pH dependence of the chemical shifts plots like a titration curve based on the Henderson-Hasselbalch equation. Superimposition with the corresponding titration curves for

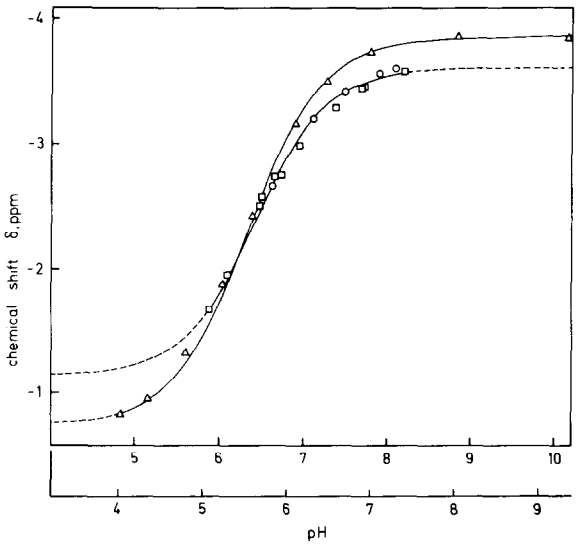

Fig.2. pH dependence of ^{31}P chemical shifts of pyridoxal-P bound to maltodextrin phosphorylase (○); D-serine dehydratase (□); Schiff base with ε-aminocaproate (△). The lower pH scale from 4–9 applies to maltodextrin phosphorylase only.

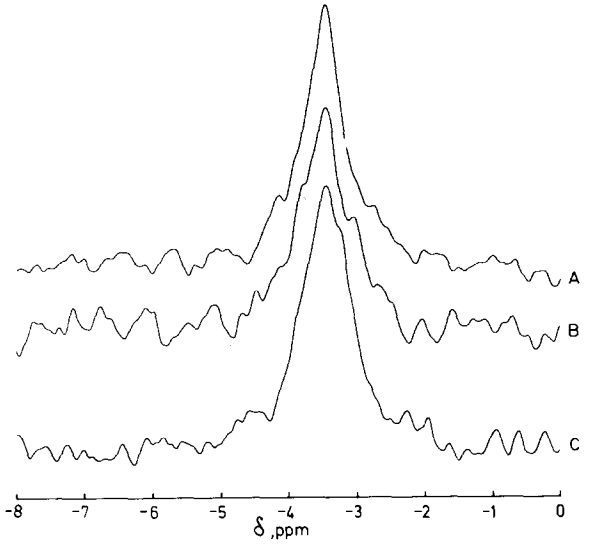

Fig.1. ^{31}P NMR spectra of pyridoxal-P bound to maltodextrin phosphorylase (2.1–2.5 × 10^{-4} M (pH 6.7)) (A) in the absence of substrates (B) in the presence of 2 × 10^{-4} M arsenate and (C) in the presence of dextrin (1.2 mg/ml) and residual arsenate. The chemical shifts are relative to external orthophosphate. The linewidths are (A) 40 Hz and (B,C) 55 Hz.

D-serine dehydratase and the model compound shows a nearly identical fit with a pH shift of about −0.8 pH units. Since in these two compounds the unprotonated form is the dianion of the 5′-phosphate group of pyridoxal-P [8,9] the close fit with the maltodextrin phosphorylase suggests that the pH dependence of the chemical shift is due to the conversion of the monoanionic to the dianionic form of pyridoxal-P bound as a Schiff base. The shift of the titration curve to lower pH values in the case of maltodextrin phosphorylase might indicate stabilization of the dianionic form in the enzyme against protonation. Formation of a salt bridge could influence the protonation equilibrium in favour of the dianionic form. In any event, the predominant ionization state of pyridoxal-P at the pH where activity is optimal (6.2–6.9) is the dianion.

Since no discrete and separate signals for the monoanionic and dianionic form were observed, proton exchange at equilibrium with the solvent or another proton accepting group must be fast. A comparison of the linewidths of the phosphoresonance signals in maltodextrin phosphorylase (35 ± 4 Hz) and rabbit skeletal muscle phosphorylase a (55–60 Hz) [7] leads

to a similar conclusion. Considering the difference in molecular weight [8] of the active dimeric enzymes a linewidth of 45 Hz would have been expected. The linewidth might be reduced because of a greater mobility of the phosphate residue in maltodextrin phosphorylase. The greater mobility could facilitate the fast proton exchange discussed above.

3.2. *Interactions with substrates*

Due to interference of the resonance signals, P_i or glucose-1-P could not be used for forming binary complexes with the enzyme. Therefore, arsenate was substituted for phosphate. Moreover, extended dialysis against arsenate also helped to remove enzyme-bound primer. NMR measurements with maltodextrin phosphorylase in the presence of arsenate at pH 6.7 are shown in fig.1. The chemical shift was not changed by the addition of arsenate and hence the anionic substrate does not affect the ionization equilibrium. The linewidth broadening from 35–55 Hz may reflect a reduced mobility of the phosphate residue or a reduced rate of proton exchange or both. This effect deserves further study because of its possible relevance to the catalytic mechanism. Arsenate was partially removed from the enzyme by dialysis and dextrin (1.2 mg/ml) was added. This led to the formation of the enzymatically active ternary complex, as shown by slow glucose formation. But again the chemical shift was not changed and the same linewidth broadening was observed as in the experiment with arsenate alone (fig.1).

3.3. *Fluorescence measurements*

Whereas ^{31}P NMR studies report on the ionization state of the phosphate moiety information about the functional state of the pyridinium ring moiety may be deducted from fluorescence (fig.3) and ultraviolet spectroscopic measurements [10]. The pH dependence of the fluorescence emission at 527 nm, characteristic for the pyridoxal-P Schiff base, remains unchanged over the whole stability range of the enzyme from pH 5–11 despite the changes seen in protein fluorescence (fig.3). This is consistent with a rigid and stable attachment of the pyridinium ring and the 3-OH group of pyridoxal-P to the hydrophobic cofactor binding site in maltodextrin phosphorylase. The results are also consistent with studies on mammalian glycogen phosphorylase [11]. Hence the

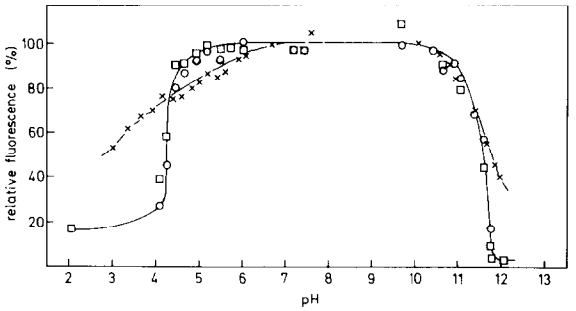

Fig.3. pH dependence of fluorescence of maltodextrin phosphorylase. Relative fluorescence at 527 nm, with excitation at 280 and 335 nm, respectively, for pyridoxal-P bound to the enzyme (□,○). Relative fluorescence at 342 nm, with excitation at 280 nm for intrinsic protein fluorescence of maltodextrin phosphorylase (×).

unresponsiveness of the pyridoxal Schiff base portion to pH makes it unlikely that this part of the cofactor will reflect catalytic changes. It also shows the limitations of fluorescence and uv spectroscopy which up to now were used exclusively to study the properties of enzyme bound pyridoxal-P [4,10,11].

4. Conclusion

The observation of only one ^{31}P NMR signal representing a rapid equilibrium between monoanionic and dianionic forms of pyridoxal-P both in the absence and presence of substrates most probably demonstrates that no other forms of the cofactor are relevant for the active state of the enzyme. In contrast pyridoxal-P bound to regulated mammalian skeletal muscle phosphorylase shows different ^{31}P NMR resonances depending on the functional state of the enzyme. Only the phosphate resonance which is generated on allosteric activation of phosphorylase *b* by 5′-AMP (AMP-S) and arsenate or on activation by phosphorylation with ATP-γ-S, Mg^{2+} and phosphorylase kinase [7,12,13] is identical with the signal found in active maltodextrin phosphorylase. Thus the active form of pyridoxal-P in all α-glucan phosphorylases appears to require a 5′-phosphate available for proton uptake. The ionization equilibrium can be shifted in individual phosphorylases by the formation of a salt bridge of the phosphate dia-

nion to neighbouring amino acids. In contrast to the phosphate group projecting into a polar environment, the pyridinium ring is rigidly bound to a hydrophobic cavity.

These findings and in addition the location of the pyridoxal-P in rabbit skeletal muscle phosphorylase near the site where the anionic substrates bind [14,15] make a catalytic role of pyridoxal-P in reactions catalyzed by α-glucan phosphorylase more likely. However, whether the pyridoxal-P dianion reacts directly with substrates in a general acid–base catalyzed reaction or merely positions a suitably charged amino acid sidechain which then functions as the actual proton donor–acceptor in the catalytic reaction can not yet be decided.

Acknowledgements

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