The use of mass spectrometry to examine the formation and hydrolysis of the phosphorylated form of phosphoglycerate mutase

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Abstract Electrospray mass spectrometry has been used to study the formation and hydrolysis of the phopshorylated forms of two phosphoglycerate mutases. The half-life of the enzyme from *Saccharomyces cerevisiae* was 35 min at 20°C in 10 mM ammonium bicarbonate, pH 8.0. Addition of 1 mM 2-phosphoglycollate reduced this value by at least 100-fold. The phosphorylated form of the enzyme from *Schizosaccharomyces pombe* was much less stable with a half-life of less than 1 min. The results are discussed in terms of the kinetic properties of the enzymes. Mass spectrometry would appear to be a powerful method to study the formation and breakdown of phosphorylated proteins, processes which are of widespread significance in regulatory mechanisms.

Key words: Electrospray mass spectrometry; Protein phosphorylation; Phosphoglycerate mutase

1. Introduction

The advent of electrospray mass spectrometry with its ability to measure molecular masses with a precision of ±0.01% has made it much easier to detect and characterise both post-translational and chemical modifications of proteins [1–3]. The introduction of the phospho group ($-OPO_3^{2-}$) in place of -H would lead to a mass increase of 78 units and thus be readily detectable. This approach has been used, for instance, in the delineation of the sites of phosphorylation in glycogen synthase [4] after separation of the modified peptides. However, examination of an intact phosphorylated protein by mass spectrometry does not appear to have been widely studied. In this paper we describe the use of electrospray mass spectrometry to monitor the formation of the phosphorylated forms of two phosphoglycerate mutases (PGAMs) and to examine the stability of these phosphorylated enzymes towards hydrolysis.

The catalytic cycle of phosphoglycerate mutase is thought to proceed via an enzyme-substitution pathway involving the enzyme phosphorylated on a histidine side chain (His-8 in the case of *S. cerevisiae* PGAM) [5]. The phosphorylated PGAM is slightly unstable towards hydroysis leading to a low level of phosphatase activity (approx. 0.002% that of the mutase activity in the case of the *S. cerevisiae* enzyme). It has been suggested [5] that the flexible C-terminal segment (14 amino acids) of this enzyme may be important in preventing access of water to the active site and thus maintain a high level of mutase to phosphatase activity. Recently the monomeric PGAM from the fission yeast *Schizosaccharomyces pombe* has been shown to lack this flexible C-terminal tail sequence [6]. The kinetic properties of the *S. pombe* enzyme have not yet been explored in detail.

2. Experimental

PGAM from an overexpressing strain of *S. cerevisiae* was isolated as described previously [7], with the addition of a final FPLC Superose-12 gel-filtration step. The concentration of the enzyme was determined spectrophotometrically assuming a value of 1.45 for the A_{280} of a 1 mg/ml solution [8].

PGAM from *S. pombe* was produced using the PGK-based vector pMA91 [9] for the high level expression of recombinant GPM^{sp} in a transformed null mutant strain of *S. cerevisiae* (*S150-gpm::HIS3*) [7]. The overexpressed *S. pombe* PGAM was purified in a similar manner to overexpressed *S. cerevisiae* PGAM [7]; routinely 10–15 mg of enzyme of at least 95% purity on SDS-PAGE [10] could be obtained per litre of cells. Full details of the expression system and enzyme purification will be published elsewhere (Nairn, Fothergill-Gilmore and Price, in preparation).

The concentrations of the *S. pombe* enzyme were determined by a Coomassie blue binding method [11] using bovine serum albumin as a standard, or spectrophotometrically using a value for the A_{280} (1.40 for a 1 mg/ml solution) calculated from the aromatic amino acid content of the enzyme [6,12]; the values agreed to within 5%.

The assays of mutase, phosphatase and synthase activities were performed as described previously [7].

The phosphorylated forms of the PGAMs from *S. cerevisiae* and *S. pombe* were prepared by mixing the enzymes with 2,3-bisphosphoglycerate (BPG) in 10 mM Tris/HCl, pH 8.0, followed by rapid gel-filtration on NAP 5 columns (Pharmacia) equilibrated with 10 mM ammonium bicarbonate, pH 8.0, to remove free mono- and bisphosphoglycerates. This procedure allowed samples to be studied by mass spectrometry within 3 min of the mixing.

Mass spectrometry was performed on a VG Platform quadrupole mass spectrometer (2–3000 amu range) fitted with a pneumatically assisted electrospray (ionspray) source and controlled via the VG MassLynx software (VG Biotech. Ltd, Altrincham, Cheshire, UK). Carrier solvent (1:1 (v:v) acetonitrile/water) infusion was controlled at 10 μl/min using a Harvard syringe pump (Harvard Apparatus, South Natic, MA, USA). Capillary voltages were between 2.8 and 3.2 kV, extraction cone voltages 20–30 V, and the focussing cone voltage offset by +10 V. The source temperature was set at 65°C, the nebulising gas flow at 10 l/h, and the drying gas flow at 250 l/h. Lens stack voltages were adjusted to give maximum ion currents. The M_r range 700–1500, which contained >95% of the signal intensity for both unmodified and phosphorylated forms of PGAM, was scanned with a sweep time of 5 s. The instrument was calibrated over this M_r range immediately before use with horse heart myoglobin (Sigma). Samples for analysis were

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Abbreviations: PGAM, phosphoglycerate mutase; BPG, 2,3-bisphosphoglycerate.

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diluted with an equal volume of 4% (v:v) formic acid in acetonitrile and 10–20 μl aliquots injected directly into the carrier stream. The MaxEnt deconvolution procedure [13] was applied for quantitative analysis of the raw data using 1.0 Da peak width and 1 Da/channel resolution.

3. Results and discussion

3.1. S. cerevisiae *PGAM*

The specific activities of the *S. cerevisiae* enzyme in the mutase and the phosphatase assays (970 and 0.020 μmol/min/mg, respectively) and the effect of 1 mM substrate analogue 2-phosphoglycollate on the latter activity (18-fold stimulation) were very similar to those described previously [7].

The mass spectrum of the *S. cerevisiae* enzyme shows a single peak of M_r 27478.9 ± 1.0 consistent with the subunit M_r (27,477) calculated from the cDNA-derived sequence of the enzyme [14]. After addition of 0.8 molar equivalents (expressed per active site) of BPG, followed by rapid gel-filtration, the mass spectrum clearly shows the formation of the (mono)phosphorylated enzyme, with a mass increase of 79 amu (Fig. 1a). Under these conditions, 60% of the total enzyme was present in the phosphorylated form. There was little or no (≤0.05 molar equivalents per active site) BPG or monophosphoglycerates either free or enzyme bound after the gel-filtration. By increasing the molar ratio of BPG to 10-fold, more than 95% of the enzyme could be isolated in the phosphorylated form (data not shown). When the enzyme which had been prepared by reaction with the sub-stoichiometric amount of BPG and then gel-filtered was subsequently incubated at 20°C, there was a slow loss of the phospho group from the enzyme. Fig. 1b shows the mass spectrum of the sample taken after 18.5 min incubation. The data from three independent experiments expressed as a semi-logarithmic plot are shown in Fig. 1c; in each case the proportion of the enzyme in the phosphorylated form is expressed relative to the initial proportion as 100%. From the semi-logarithmic plot the rate constant for the hydrolysis of the phosphorylated enzyme is 0.02 min^{-1}, corresponding to a half-life of approximately 35 min. This direct estimate half-life of the phosphorylated form of *S. cerevisiae* PGAM is rather longer than the value (1–2 min) quoted by Britton et al. [15] on the basis of unpublished work. It is, however, clear from preliminary work that the measured phosphatase activity is markedly influenced by factors such as ionic strength and the presence of phosphorylated substrates and analogues, and this may well account for at least some of the observed differences in stability of the phosphorylated enzyme, i.e. the phosphorylated enzyme would appear to be much less stable when the enzyme is turning over.

When the mass spectrometry experiment was repeated with 1 mM 2-phosphoglycollate added to the phosphorylated enzyme immediately after gel-filtration, it was found that within 1 min, less than 5% of the enzyme remained in the phosphorylated form (data not shown). This result indicates that in the presence of 2-phosphoglycollate the half-life of the phosphorylated enzyme is less than 20 s (i.e. the rate of the dephosphorylation reaction is accelerated at least 100-fold in the presence of this substrate analogue; a somewhat greater effect than the 18-fold stimulation of phosphatase activity).

In the presence of acetonitrile (50% (v:v)), the phosphorylated enzyme showed no detectable breakdown after 70 min incubation (data not shown). Since this concentration of acetonitrile leads to a considerable loss of secondary structure (as shown by far UV CD measurements), it can be concluded that the denatured phosphorylated enzyme is considerably more stable towards hydrolysis than is the native phosphorylated enzyme. This conclusion would be consistent with earlier work in which negligible breakdown had been shown to occur over 120 min in the presence of 1.5% (w/v) SDS [16].

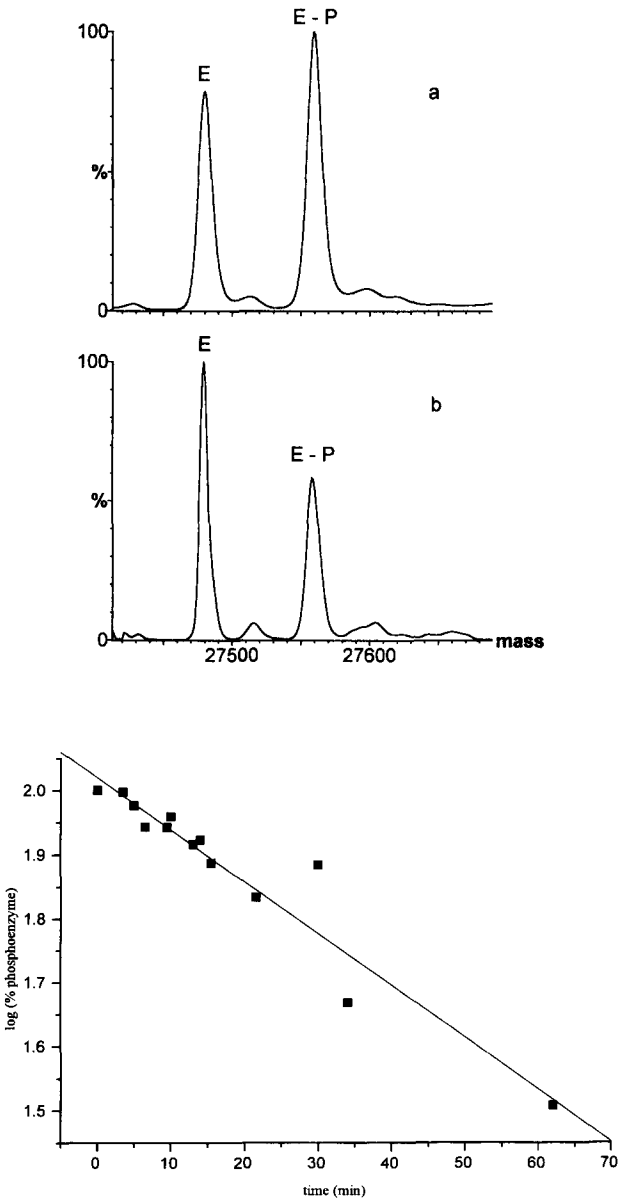

Fig. 1. Formation and hydrolysis of the phosphorylated form of *S. cerevisiae* PGAM monitored by mass spectrometry. The spectra over the M_r range shown (27,420–27,680) were obtained by applying the MaxEnt deconvolution procedure to the raw data. In each case the size of the major peak is set as 100%. The peaks labelled E and E-P represent the dephosphorylated and phosphorylated forms of the enzyme, respectively. (a) Mass spectrum of the sample immediately after gel-filtration. (b) Mass spectrum recorded after a further 18.5 min incubation. (c) Semi-logarithmic plot to show the conversion of the phosphorylated form to the dephosphorylated form of the enzyme. Data from three independent experiments are plotted; in each case the initial proportion of phosphoenzyme (immediately after gel-filtration) is scaled to 100%.

3.2. S. pombe *PGAM*

In the mutase assays, the specific activity of *S. pombe* PGAM (215 μmol/min/mg) is about 20% of that of the *S. cerevisiae* enzyme, whereas in the phosphatase assay it is some 2.5-fold higher (0.06 μmol/min/mg). Thus the ratio of the phosphatase/mutase assays is 12-fold higher for the *S. pombe* enzyme. The degree of stimulation of the phosphatase activity by 2-phosphoglycollate is considerably lower in the case of the *S. pombe* enzyme (4.2-fold).

The mass spectrum of *S. pombe* PGAM shows a peak with an M_r of 23679.4 ± 1.5, corresponding to that calculated from the published sequence [6], assuming that the initiating Met has been removed and the N-terminal threonine acetylated. On addition of 0.8 molar equivalents of BPG followed by gel-filtration (a process lasting 3 min), less than 5% of the enzyme was present in the phosphorylated form (data not shown). This was not due to an inability to form the phosphorylated enzyme since the mixture prior to gel-filtration showed that 60% of the enzyme was present in the phosphorylated form. From these results it could be concluded that the half-life of the phosphorylated form of *S. pombe* PGAM was less than 1 min (at least 35-fold shorter than that of the *S. cerevisiae* enzyme). This greater instability of the phosphorylated form of the *S. pombe* enzyme is in qualitative agreement with the higher ratio of phosphatase to mutase activities for this enzyme.

In conclusion, mass spectrometry should prove to be a very useful technique for monitoring the phosphorylation and dephosphorylation of a number of enzymes and proteins, a process which has been recognised to be a key regulatory mechanism for a large number of crucial biological processes [17]. The mass spectrometric technique avoids the necessity of using radioactive isotopes. Further refinements would include increasing the time resolution, allowing the rates of faster processes to be monitored accurately.

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