RELATIVE ALKALI STABILITY OF SOME PEPTIDE *o*-PHOSPHOSERINE AND *o*-PHOSPHOTHREONINE ESTERS

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1. Introduction

Protein phosphorylation is new recognized as an important form of covalent modification of proteins involved in the regulation of numerous biological processes [1]. The most widely studied protein phosphorylation reactions have involved the phosphorylation of serine and threonine residues in proteins and peptides [1] although phosphorylation of other residues is known to occur (reviewed in [2]).

In 1906 Plimmer and Bayliss reported that protein-bound phosphate in caseins was particularly labile to alkali [3]. Subsequently this chemical reaction has become a diagnostic test for the presence of serine and threonine phosphate monoesters in phosphoproteins [4]. In the presence of alkali the phosphorylated residue is believed to undergo a β-elimination reaction yielding dehydroalanine (2-amino-2-propenoic acid) and 2-amino-2-butenoic acid from *o*-phosphoserine and *o*-phosphothreonine, respectively (reviewed in [4,5]).

During the course of studying the phosphorylation of synthetic peptides by a number of protein kinases the alkali lability of the phosphorylated peptide has been tested as part of the chemical characterization of the phosphorylated product. It became apparent that there were striking differences in the alkali lability of some peptide serine and threonine monophosphate esters. The results obtained with several of these peptides are reported here and demonstrated that under conditions routinely used some peptide phosphoserine and phosphothreonine would not be quantitatively eliminated in alkali.

2. Methods

Peptides were synthesized by the Merrifield solid phase synthesis procedure [5] and purified by ion-exchange and gel chromatography as in [6]. Purity of the synthetic peptides was assessed by quantitative amino acid analysis, high-voltage electrophoresis, thin-layer chromatography and HPLC using reverse phase chromatography (results to be published elsewhere).

Synthetic peptide phosphorylation was carried out essentially as in [7] using the catalytic subunit of the cAMP-dependent protein kinase isolated from bovine heart muscle [8,9]. The reaction mixture contained 1.8 mM [γ-^{32}P]ATP (50–100 cpm/pmol), 20 mM Mes buffer (pH 6.8), 10 mM magnesium acetate, 0.125 mM EGTA, bovine serum albumin (1 mg/ml) and protein kinase catalytic subunit 12 μg/ml. Depending on the kinetics of phosphorylation of the particular synthetic peptide lower levels of protein kinase were sometimes employed. Peptide was 0.7 mM. The extent of the phosphorylation reaction was followed by removing aliquots (10 μl) at intervals and determining the incorporated ^{32}P as in [6]. The [^{32}P]phosphorylated peptide was separated from [γ-^{32}P]ATP by anion-exchange chromatography (BioRad AGI X 8 resin) in the presence of 5.5 M acetic acid as in [10]. The [^{32}P]phosphorylated peptide was recoverd from acetic acid by rotary evaporation and stored frozen in H_2O. The presence of [^{32}P]phosphoserine or [^{32}P]phosphothreonine was verified by partial acid hydrolysis (5.7 M HCl at 105°C, 4 h) and high-voltage paper electrophoresis at pH 1.9 (3000, V, 90 min). Authentic *o*-phosphoserine and *o*-phosphothreonine (Sigma) reference markers were applied to the electrophoretogram. Following partial acid hydrolysis only ~30% of the ^{32}P radioactivity applied to the electrophoretogram was associated with the phosphorylated amino acid

marker. The remainder electrophoresed with $^{32}P_i$ and unhydrolysed phosphopeptide.

Enzymic dephosphorylation of synthetic [^{32}P]-phosphopeptides was carried out essentially as in [11] for alkaline phosphatase and [12] for acid phosphatase except that in the latter case the reaction buffer was 50 mM Mes (pH 5.8). The specific activity of the commercial preparations of both phosphatases were measured using *p*-nitrophenol phosphate under the conditions used to measure [^{32}P]phosphopeptide dephosphorylation.

Acid phosphatase and alkaline phosphatase were used at 0.2 and 1.52 units/100 μl reaction vol., respectively. Aliquots (20 μl) were withdrawn at 10, 20 and 30 min and added to tubes containing 0.5 ml of 5.5 M acetic acid to terminate the reaction. The released $^{32}P_i$ was separated from the [^{32}P]phosphopeptide by chromatography on small columns of AG 50W × 8 resin (BioRad) equilibrated and eluted with 5.5 M acetic acid. The [^{32}P]phosphopeptide was bound whereas the $^{32}P_i$ eluted into scintillation vials and was counted by Cerenkov radiation as in [10].

3. Results

3.1. *The alkaline dephosphorylation of phosphopeptides*

During the characterization of the serine and threonine containing octa peptides

Arg–Arg–Arg–Arg–Pro–Thr(Ser)–Pro–Ala.NH_2

corresponding to the local phosphorylation site sequence in the phosphoprotein phosphatase inhibitor-1 [13] and heptapeptides

Leu–Arg–Arg–Ala–Ser(Thr)–Leu–Gly

corresponding in part to the phosphorylation site sequence in pig liver pyruvate kinase [13] it was observed that phosphate esterified to threonine was significantly more stable than that esterified to serine (fig.1A,B). A comparison of the alkali lability of the serine and threonine containing phospho-octapeptides in the presence of 3 M NaOH over 3 h is given in fig.1A. Less vigorous conditions (1 M NaOH, 37°C, 18 h) are usually employed to test the alkali lability of phosphoproteins [2] however, here higher temperature and NaOH concentrations were employed to facilitate comparison of the kinetics of dephosphorylation between peptides. Release of $^{32}P_i$ from the phosphopeptide was assessed in 3 ways:

(1) The [^{32}P]phosphate was extracted as the phosphomolybdate complex with butyl acetate [15] from acidified digests. The [^{32}P]phosphate released from the peptide migrated with authentic P_i on a high-voltage paper electrophoretogram ran at pH 1.9.

(2) There was a stoichiometric relationship between the [^{32}P]phosphate released as determined by high-voltage electrophoresis and the residual

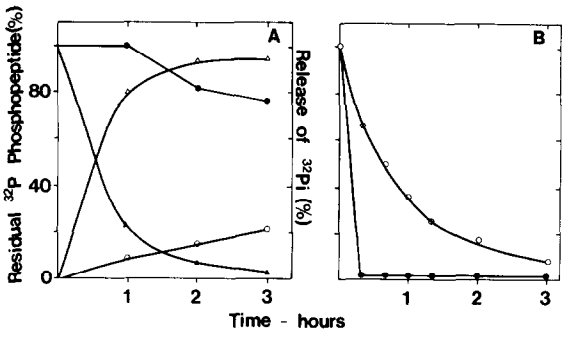

Fig.1.(A) Comparison of the effect of alkali on serine and threonine containing [^{32}P]phosphopeptides. The peptides Arg–Arg–Arg–Arg–Pro–Thr(P)–Pro–Ala amide (●, ○) and Arg–Arg–Arg–Arg–Pro–Ser(P)–Pro–Ala amide (▲, △) were incubated at 50°C in presence of 3 M NaOH at 59 μM respectively. The closed symbols refer to the residual [^{32}P]phosphopeptide remaining and the open symbols to $^{32}P_i$ released from the [^{32}P]phosphopeptide. Residual [^{32}P]-phosphopeptide was determined by anion exchange chromatography. Aliquots (10 μl) were removed at intervals and added to tubes containing 0.5 ml 5.5 M acetic acid the residual [^{32}P]phosphopeptide recovered by ion-exchange chromatography on BioRad AG1 × 8 resin equilibrated with 5.5 M acetic acid. The $^{32}P_i$ released from the phosphopeptide was bound to the column while the residual [^{32}P]phosphopeptide eluted into a liquid scintillation counting vial. The concentration of NaOH was determined by titration against a standard H_2SO_4 solution. $^{32}P_i$ was determined by high-voltage paper electrophoresis (pH 1.9, 2500 V, 1 h) of aliquots (10 μl) of the incubation mixture. The position of authentic P_i was located on the electrophoretogram by staining and quantitated by liquid scintillation counting.

Fig.1.(B) Comparison of effect of alkali on serine and threonine containing [^{32}P]phosphopeptides. The peptides Leu–Arg–Arg–Ala–Thr(P)–Leu–Gly (○) and Leu–Arg–Arg–Ala–Ser(P)–Leu–Gly (●) were incubated at 50°C in presence of 1 M NaOH at 0.156 mM and 0.143 mM, respectively. Residual [^{32}P]phosphopeptide was determined as in (A).

[^{32}P]phosphopeptide (fig.1A) measured by ion-exchange chromatography.

(3) The residual [^{32}P]phosphopeptide was separated from [^{32}P]phosphate by chromatography or either cation exchange resin (BioRad AG-50W X 8) or anion exchange resin (BioRad AG 1 X 8) eluted with 0.1 N HCl and 5.5 M acetic acid, respectively. In the case of the cation exchange resin the [^{32}P]phosphopeptide was bound to the resin and the $^{32}P_i$ eluted, whereas for the anion exchange resin the $^{32}P_i$ was bound and the residual phosphopeptide recovered by elution with 5.5 M acetic acid.

These results demonstrate that release of $^{32}P_i$ from the phosphopeptides was being measured.

The differential effect of alkali on threonine and serine containing phosphopeptides was also observed for the pyruvate kinase heptapeptide analogs Leu–Arg–Arg–Ala–Ser(Thr)–Leu–Gly (fig.1B). The $^{32}P_i$ was quantitatively released from the phosphoserine-containing heptapeptide in <1 h in 1 M NaOH at 50°C.

There were significant differences in the alkali lability of the phosphorylated residues in peptides with different sequence. Threonine phosphate in the octapeptide Arg–Arg–Arg–Arg–Pro–Thr–Pro–Ala.NH_2 was relatively stable in 3 M NaOH for 1 h at 50°C whereas the phosphate esterified to threonine in the heptapeptide Leu–Arg–Arg–Ala–Thr–Leu–Gly was essentially quantitatively released (fig.2A). Similar sequence-dependent effects on the susceptibility to alkali were observed for the corresponding phosphoserine containing analogs (fig.2B).

It has been suggested that substitution of the α-amino group of phosphoserine markedly influences its susceptibility to β-elimination in alkali [16]. Indeed peptide-bound phosphoserine is significantly more labile in alkali than phosphoserine [2]. The phosphopeptide analog of the alkali-labile pyruvate kinase heptapeptide was synthesized with Pro replacing Ala, Leu–Arg–Arg–Pro–Ser(P)–Leu–Gly. Although this peptide was found to be slightly more stable than the parent peptide Leu–Arg–Arg–Ala–Ser(P)–Leu–Gly it was nevertheless substantially more labile to alkali than the phospho-octapeptide, Arg–Arg–Arg–Arg–Pro–Ser–Pro–Ala.NH_2 (fig.2B) containing proline on both sides of the phosphoserine.

3.2. *Enzymic dephosphorylation of phosphopeptides*

Large differences in the rates of enzymic dephosphorylation of some of these phosphopeptides were observed using both alkaline and acid phosphatases (table 1). The threonine containing octapeptide Arg–Arg–Arg–Arg–Pro–Thr(P)–Pro–Ala.NH_2 was resistant to both acid and alkaline phosphatase. The serine containing octapeptide analog was slowly dephosphorylated (20%). The serine containing phosphoheptapeptide was >90% dephosphorylated by acid phosphatase under the same conditions. In general the susceptibility of the phosphopeptides to enzymic dephosphorylation correlated with their alkali lability properties.

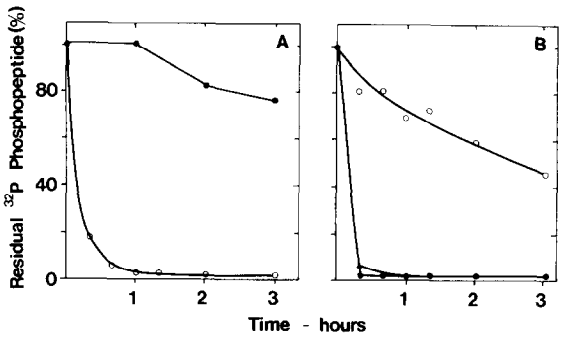

Fig.2.(A) The effect of neighbouring amino acids on the alkali lability of threonine containing phosphopeptides. The [^{32}P]phosphopeptides Arg–Arg–Arg–Arg–Pro–Thr(P)–Pro–Ala amide (•) and Leu–Arg–Arg–Ala–Thr(P)–Leu–Gly (○) were incubated at 50°C in presence of 3 M NaOH at 59 μM and 156 μM, respectively. Aliquots (10 μl) were removed at intervals and the residual [^{32}P]phosphopeptide determined as in fig.1(A). The data for the phosphopeptide Arg–Arg–Arg–Arg–Pro–Thr(P)–Pro–Ala amide is taken from fig.1(A).

Fig.2.(B) The effect of neighbouring amino acids on the alkali lability of serine containing phosphopeptides. The [^{32}P]phosphopeptides, Arg–Arg–Arg–Arg–Pro–Ser(P)–Pro–Ala amide (○), Leu–Arg–Arg–Pro–Ser(P)–Leu–Gly (▲) and Leu–Arg–Arg–Ala–Ser(P)–Leu–Gly (•) were incubated at 50°C in presence of 1 M NaOH at 58 μM, 86 μM and 143 μM, respectively. Residual [^{32}P]phosphopeptide was determined as in fig.1(A).

4. Discussions

These results have shown that the alkali lability of phosphate esterified to peptides may vary widely depending on the nature of the phosphorylated residue, serine or threonine, as well as the surrounding amino acid residues.

Table 1
Enzymic dephosphorylation of synthetic phosphopeptides

	[^{32}P]Phosphate released (%)		
	Conc. (μM)	Acid phosphatase	Alkaline phosphatase
Arg–Arg–Arg–Arg–Pro–Ser(P)–Pro–Ala amide	60	22	15
Arg–Arg–Arg–Arg–Pro–Thr(P)–Pro–Ala amide	69	1	1
Leu–Arg–Arg–Ala–Ser(P)–Leu–Gly	51	94	n.d.

n.d., not determined

Synthetic [^{32}P]phosphopeptides were incubated with acid and alkaline phosphatase as in section 2

The greater stability of peptide phosphothreonine compared with peptide phosphoserine may be due to the stereospecific requirements of the β-elimination reaction which is believed to proceed more readily if the eliminating groups are *trans* to each other [17]. In the case of phosphothreonine the β-methylene group may restrict the leaving groups (α-H and β-PO_4) from assuming the *trans*-orientation.

From a practical viewpoint the differences in the alkali lability of the phosphoserine and phosphothreonine present in the heptapeptide Leu–Arg–Arg–Ala–Ser(Thr)–Leu–Gly were not sufficiently large to be detected during the standard conditions used to test the alkali lability of phosphoproteins [2] 1 M NaOH room temp., 18 h. Nevertheless the phosphothreonine present in the octapeptide Arg–Arg–Arg–Arg–Pro–Thr(P)–Pro–Ala.NH_2 was capable of withstanding these conditions. These results have practical significance since this peptide corresponds to the local phosphorylation site sequence of a natural substrate of the cyclic AMP-dependent protein kinase, namely, the phosphoprotein phosphatase inhibitor – 1. The reason for the stability of the threonine containing phospho-octopeptide in alkali is not entirely clear. Inspection of space-filled CPK-models suggests that the presence of proline residues on both sides of the threonine results in strong steric hindrance. A single proline on the N-terminal side of the phosphorylated residue does not appear to have a strong influence on the alkali lability of the phosphopeptide. The peptide Leu–Arg–Arg–Pro–Ser(P)–Leu–Gly was almost as labile as the alanine containing analog, Leu–Arg–Arg–Ala–Ser(P)–Leu–Gly.

In a tryptic peptide derived from β-casein (residues 1–25), 1 of the 4 phosphorylated serine residues was moderately stable in alkali [18]. The phosphorylated residues contained in this peptide occur in the sequence Glu–Ser(P)–Leu–Ser(P)–Ser(P)–Ser(P)–Glu. Unfortunately the particular alkali stably phosphorylated residue has not been identified. However, inspection of the sequence would suggest that the reason for the apparent alkali stability in this instance differs from that for the proline containing octapeptides reported herein.

It is clear from the results reported here that the relative stability of [^{32}P]phosphorylated protein in alkali cannot alone be taken as evidence for the lack of *o*-phosphoserine or *o*-phosphothreonine. Moreover it follows that development of methods for detecting phosphorylated serine and threonine residues in proteins based on the β-elimination reaction, as have been developed for glycoproteins [19], would not be expected to be quantitative in all cases.

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