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Rapid and selective modification of phosphoserine residues catalysed by Ba^{2+} ions for their detection during peptide microsequencing

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The β-elimination of phosphoserine residues by dilute alkali is catalysed by the presence of group II metal ions. The use of 0.1 M-Ba $(OH)_2$ catalysed the rate of β-elimination of phosphoserine by more than two orders of magnitude compared with the use of NaOH at the same OH^- ion concentration. Serine and threonine residues are unaffected by this treatment. Free thiol groups and disulphide bonds are labile to these conditions, but carboxymethylcysteine is stable. The rate of β-elimination of *O*-glycosidically linked moieties is not catalysed under these conditions, and the rate of reaction is thus two orders of magnitude slower than for phosphoserine. This specific catalysis was readily exploited in the rapid and selective modification of phosphoserine residues under mildly alkaline conditions with the nucleophile methylamine via the $\alpha\beta$-desaturated dehydroalanine intermediate to yield the β-methylaminoalanine residue. This modified residue could be easily detected on sequence analysis and in amino acid compositions.

INTRODUCTION

The reversible phosphorylation of serine residues in proteins is a common post-translational modification and is clearly of key importance in the regulation of diverse cellular processes [1].

Unambiguous identification of the sites of serine phosphorylation, however, is difficult during sequence analysis. For reasons that are not completely understood, the phenyl(thiocarbamoyl) derivatives of the phosphoester of serine (and threonine) are unstable to the Edman degradation and fail to produce an unambiguously identifiable phenylthiohydantoin derivative. The only unambiguously identifiable product of the Edman degradation of [^{32}P]phosphoserine (and [^{32}P]phosphothreonine) is inorganic [^{32}P]phosphate [2], the polarity of which precludes extraction in the apolar solvents used to extract the amino acid phenylthiohydantoin derivative. This phenomenon results in extremely poor yields of inorganic [^{32}P]phosphate from the phosphoserine residue.

An often-suggested alternative approach to this problem has been to exploit the sensitivity of the β-phosphate ester to β-elimination at high pH. The resultant $\alpha\beta$-desaturated residue is a Michael substrate, which is sensitive to nucleophilic addition. Addition of a suitable nucleophile in quantitative yield would then result in a novel amino acid residue specifically at the position occupied by the parent phosphoserine residue. Conditions for performing such chemistry have been described previously with aliphatic primary amines [3,4], sulphite [5] or aliphatic thiols [6] as convenient nucleophiles. More recently, minor modifications of the latter protocol have been reported [7,8]. In the present paper a novel method for the modification of phosphoserine residues exploiting the rapid, efficient and selective catalysis of the β-elimination reaction by Ba^{2+} ions in dilute alkali is described.

MATERIALS AND METHODS

Materials

The C-subunit of cyclic AMP-dependent protein kinase was from Dr. E. Krebs (University of Washington). Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) was from Peninsula Laboratories (Belmont, CA, U.S.A.), and the phosphopeptide Arg-Thr-Lys-Pro-[^{32}P]phosphoSer-Gly-Ser-Val-Tyr-Glu-Pro-Ile was supplied by Dr. D. K. Blumenthal (University of Washington). The threonine analogue of Kemptide (Leu-Arg-Arg-Ala-Thr-Leu-Gly) was a gift from Dr. D. Glass (Emory University, Atlanta, GA, U.S.A.). Methylamine [aq. 40% (v/v) solution] was from MCB Inc. (Lansing, MI, U.S.A.). $Ba(OH)_2$ was prepared by mixing approx. 4 M-NaOH solution with approximately 4 M-$BaCl_2$ solution. The resulting needle-shaped translucent crystals of the less soluble $Ba(OH)_2$ were filtered off and rapidly air-dried. The product was recrystallized from water, washed thoroughly with ice-cold ethanol and stored under vacuum desiccated over KOH pellets. Submaxillary-gland mucin, D-amino acid oxidase, scopoletin, Pronase, horseradish peroxidase and phosphotyrosine were from Sigma Chemical Co.

Phosphorylation of peptides

Synthetic peptides were phosphorylated in 50 mM-potassium phosphate buffer, pH 6.8, 18 mM-magnesium acetate, 10 mM-ATP, 2 mM-peptide and 5 units of cyclic AMP kinase C-subunit in a final volume of 250 μl. After 1 h (overnight for threonine phosphorylation) at 30 °C the mixture was applied to a 2 cm × 0.5 cm column of Dowex AG-2-X8 (acetate form) anion exchange-resin equilibrated with aq. 1 M-acetic acid. The column was eluted with this solution, and the breakthrough fraction (about 1 ml) was freeze-dried. The phosphopeptide was purified by reverse-phase h.p.l.c. on an Altex Ultrasphere C_{18} column. Completeness of the phosphorylation reaction could be readily assessed by the decreased retention time of the peptide on the h.p.l.c. column.

Modification of peptides

A saturated solution of $Ba(OH)_2$ was prepared fresh for each use and centrifuged to pellet $BaCO_3$ and excess $Ba(OH)_2$. Titration against standard 0.1 M-HCl solution gave a $Ba(OH)_2$ concentration of 0.155 M at room temperature (20 °C) for the saturated solution. Dried phosphopeptides were dissolved initially in water (100 μl), and then 200 μl of saturated $Ba(OH)_2$ solution was added. Final peptide concentrations were usually 0.5–20 μM. For modification to the final stable adduct in a single incubation, the appropriate nucleophile was added at 0.2–1.0 M

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final concentration. The final pH was around pH 12. The reaction mixture (routinely 350 μl) was vortex-mixed and incubated at 30 °C. The reaction was normally complete in about 90 min. It is advisable to overlay the solution with inert gas and seal the reaction tube to prevent loss of $Ba(OH)_2$ as $BaCO_3$ as a result of absorption of CO_2. The reaction was terminated by addition of sufficient aq. 1 M-acetic acid to lower the pH to about pH 4. The solution could be frozen for at least several weeks at this stage. The modified peptides were desalted by reverse-phase h.p.l.c. on an Altex Ultrasphere C_{18} column and freeze-dried before analysis.

Spectrophotometry

The formation and destruction of the dehydroalanine intermediate was monitored by its absorbance at 241 nm with a Beckman DU-6 spectrophotometer. Peptides were dissolved at about 50 μM in the appropriate concentration of group II metal hydroxide. The cuvette was sealed with Parafilm to minimize precipitation of the metal carbonate. Nucleophilic addition was monitored after passing a stream of CO_2 through the solution before centrifugation, thus removing the metal cation as the insoluble carbonate. The various nucleophiles were then added to the solution, maintaining the pH at approx. 12, if necessary, by addition of 0.1 M-NaOH solution.

Pronase digestion

The two species of modified Kemptide eluted from reverse-phase h.p.l.c. were dried, and approx. 10 nmol of each was digested with approx. 10 μg of Pronase overnight at room temperature in 50 mM-ammonium bicarbonate buffer, pH 7.8. The proteinase was then inactivated by boiling the digest for 10 min.

Detection of D-amino acids with D-amino acid oxidase

A fluorimetric D-amino acid oxidase assay [9] was used as a qualitative test for the stereochemically inverted amino acid. The reaction mixture contained 500 μl of 0.1 M-sodium pyrophosphate buffer, pH 8.4, 10 μl of 10 mg/ml horseradish peroxidase, 200 μl of 5 μM-scopoletin (7-hydroxy-6-methoxycoumarin), 50 μl of 0.1 mg/ml FAD, 50 μl of 2 mg/ml BSA and 100 μg of D-amino acid oxidase in a final volume of 1 ml. After 15 min at 37 °C, 4 ml of 0.1 M-sodium borate buffer, pH 10, was added and the relative decrease in fluorescence versus no-oxidase and no-substrate blanks was measured with a Perkin Elmer MPF44A spectrofluorimeter within 20 min. D-Alanine (10 nmol) was used as a positive control.

Other methods

A Varian 5000 LC or a narrow-bore system using components from Waters was used for h.p.l.c. procedures. The mobile phase was aq. 0.1% (v/v) trifluoroacetic acid and the modifier was acetonitrile [acetonitrile/water (4:1, v/v) for the narrow-bore system] containing 0.08% (v/v) trifluoroacetic acid. Amino acid analysis was by the Waters PicoTag system. An Applied Biosystems 470A or 477A sequencer with on-line 120A h.p.l.c. analysis was used for sequence analysis.

RESULTS

Catalysis of β-elimination of phosphoserine residues by group II metal ions

Prompted by the fact that the chemistry of phosphate in biological systems is intimately connected with the presence of a bivalent cation (Mg^{2+}), and that Ca^{2+} ions accelerated the rate of dephosphorylation of phosphoserine residues at high pH [5], other group II metal cations were assessed for their ability to catalyse this reaction.

Initially, the ^{32}P-labelled phosphopeptide Arg-Thr-Lys-Pro-[^{32}P]phosphoSer-Gly-Ser-Val-Tyr-Glu-Pro-Ile was incubated in alkaline solution with various group II cations at a constant concentration of OH^- ions (0.1 M), and the rate of release of acid-soluble [^{32}P]phosphate from the peptides was determined by a paper disc assay, which was performed essentially as described in ref. [10] except that P11 phosphocellulose paper (Whatman) was used and the filter discs were washed in 75 mM-H_3PO_4. Data when plotted semi-logarithmically were linear, indicating pseudo-first-order kinetics for the reaction (results not shown). The catalytic effect of the bivalent cations was apparent and was dependent on the position of the metal in the Periodic Table, i.e. the rates were ordered $Ba^{2+} > Sr^{2+} > Ca^{2+}$. Mg^{2+} and Be^{2+} were not examined because of their very limited solubilities in base.

To ensure that the cations were catalysing the β-elimination of phosphoserine to yield the desired $\alpha\beta$-desaturated derivative (dehydroalanine) and not merely catalysing the hydrolysis of the phosphoester, the presumed desaturated derivative was detected spectrally. The $\alpha\beta$-desaturated amino acids at above about pH 10 show a characteristic shoulder to the Soret band with $\lambda_{max.}$ 241 nm and ϵ 4200 $M^{-1} \cdot cm^{-1}$. This is due to tautomerization of the double bond from the side chain to the nitrogen atom in the main chain [11].

The 241 nm absorbance could be used to detect the formation of dehydroalanine spectrally on exposure of phosphoserine residues to dilute alkali (Fig. 1), thus establishing the catalysis of the β-elimination reaction under the conditions described. This technique was used to quantify accurately the rate enhancement afforded by the various group II metal ions by a simple continuous

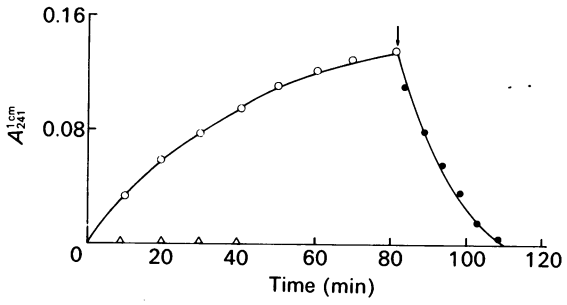

Fig. 1. Time course of modification of phosphoserine residues monitored spectrally

Phospho-Kemptide (35 μM) was β-eliminated in 0.1 M-$Ba(OH)_2$ (○). The resulting dehydroalanine residue was subjected to nucleophilic attack (by methylamine in this example) at the point indicated by the arrow (●). Unphosphorylated serine-containing and threonine-containing Kemptide were used as controls (△).

Table 1. Relative initial rates of β-elimination of phospho-Kemptide (100 μM, 1 nmol)

$k_{r(app.)}$ was calculated by using an ϵ value of 4200 $M^{-1} \cdot cm^{-1}$ for dehydroalanine [11].

Modification condition ([OH^-] = 100 mM)	$k_{r(app.)}$ (initial rate) (pmol/min)	Relative initial rate
NaOH	0.13	1
NaOH + Ca^{2+}*	2.38	18
$Sr(OH)_2$	6.67	51
$Ba(OH)_2$	15.42	118

*NaOH saturated with Ca^{2+}.

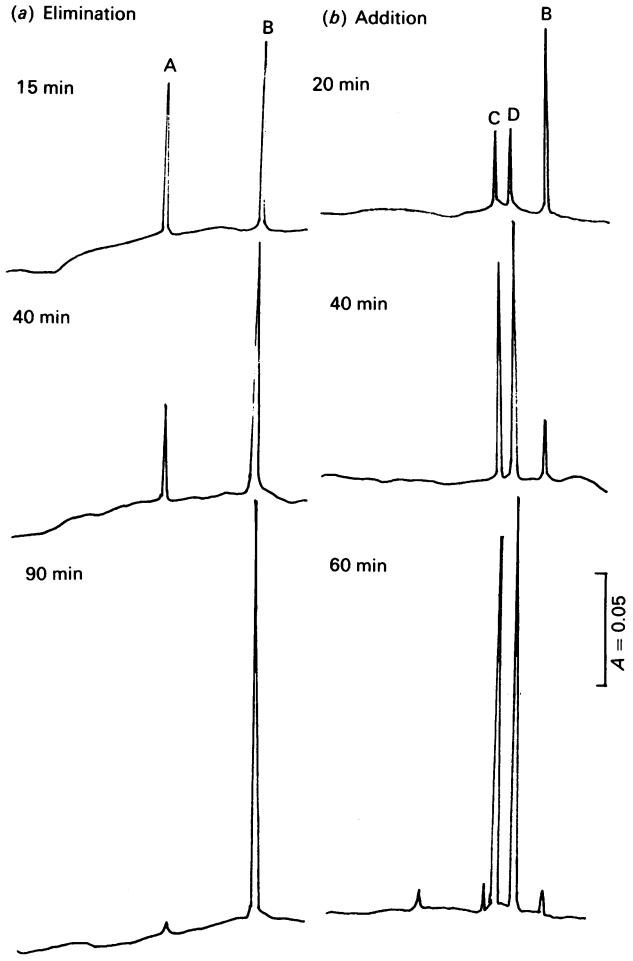

Fig. 2. Time course of modification of phosphoserine residues monitored by reverse-phase h.p.l.c.

Portions (20%) of the reaction mixture were removed and acidified at the indicated times and the reaction components were separated by reverse-phase h.p.l.c. The detector was set at 206 nm, and a linear gradient of 0–35% modifier in 20 min was used. Key: A, phosphopeptide; B, dehydroalanine-containing peptide; C and D, the two species of addition product.

independent method. The initial rate of formation of dehydroalanine was determined by monitoring the 241 nm absorbance of a solution of 0.1 mM phosphorylated Kemptide at a constant OH^- ion concentration with the various group II counterions (Table 1). This confirmed the dependence of the rate enhancement on the position of the metal in the Periodic Table, indicating that the electropositivity and/or ionic radius of the bivalent cation influences the reaction rate. It is likely that ion-pair formation between the cation and the phosphate group is involved in the mechanism. This effect would polarize the phosphate group and thus decrease the electronegativity of the ester oxygen atom. This would significantly increase the acidity of the $C_{(\alpha)}$ proton in the phosphoserine residue, resulting in the increased rate of elimination of the phosphate.

Reactivity of dehydroalanine

The characteristic u.v. absorbance of the dehydroalanine residue was used to monitor the overall reaction pathway of phosphoserine modification by using phospho-Kemptide. In these experiments the reaction was split into the two distinct phases: β-elimination in 0.1 M-$Ba(OH)_2$ and subsequent nucleophilic addition, by removing the $Ba(OH)_2$ as $BaCO_3$ with a stream of CO_2. Results of such an experiment are shown in Fig. 1. The chemistry of the nucleophilic Michael addition is such that any of a range of convenient nucleophiles may be used to modify the dehydroalanine formed during the elimination reaction. Analogous results were obtained with longer-chain aliphatic amine, ethylenediamine, hydroxylamine, aliphatic thiols and CN^-. Even the very slow addition of NH_3, an extremely poor nucleophile, could be quantified in this way. For the rapid addition of amines the pH of the reaction must be at least pH 12; addition of amines could not be detected below pH 11.5. The reaction rate increases approximately linearly with increasing pH above pH 11.5 (results not shown).

The spectrophotometric data could be readily correlated with analysis of the progress of the reaction by separating the reactants and products by reverse-phase h.p.l.c. Portions (20%) were withdrawn from the reaction mixture at appropriate times and acidified to pH 4 with aq. 0.1 M-acetic acid, and the components were resolved on an Altex Ultrasphere C_{18} column (Fig. 2). The chemical modifications described, when performed on a short phosphopeptide, result in significant changes in the overall hydrophobicity of the peptide that are easily detected on reverse-phase h.p.l.c. [8]. The formation and disappearance of the [dehydroalanine]Kemptide intermediate by β-elimination and subsequent addition was again readily detected. The h.p.l.c. traces demonstrate that peptide bond hydrolysis during the modification is minimal under the mildly alkaline conditions used. Amino acid analyses showed that the reactions occurred in almost quantitative yield. When the modification was done in a single reaction mixture, the dehydroalanine-containing peptide was readily detected both by reverse-phase h.p.l.c. analysis of the reaction mixture and by the 241 nm absorbance, indicating that the addition phase limits the rate of the overall reaction under the conditions used. Similar results were obtained with the alternative nucleophiles listed above. Identical results were obtained with other phosphorylated synthetic peptides.

Selectivity of the Ba^{2+}-catalysed reaction

Since the practical usefulness of a chemical modification of a peptide is dependent on its selectivity, other residues were examined for their reactivity under the protocol described. The β-elimination of serine or threonine could not be detected under the described conditions (Fig. 1). No destruction of arginine residues was observed in amino acid compositions of phosphopeptides exposed to the modification conditions. To explore the effects of the alkaline treatment on *O*-glycosidically linked carbohydrate groups, submaxillary-gland mucin (0.1 mg/ml) was denatured in 6 M-guanidinium chloride containing either 0.05 M-$Ba(OH)_2$ or 0.1 M-NaOH. The rate of formation of dehydroalanine residues was determined by the increase in the 241 nm absorbance. Although the β-elimination of the *O*-linked carbohydrate moieties to yield dehydroalanine could be quantified, the initial rate was less than 1% that of the initial rate of formation of dehydroalanine from phosphoserine. The rate of β-elimination of the carbohydrate groups was independent of the base counterion, i.e. the rates were identical when either NaOH or $Ba(OH)_2$ was used at the same OH^- ion concentration. This observation supports the role suggested for the bivalent cation in the catalysis of the phosphoserine β-elimination, since it would be expected that the bivalent cation could have no effect on increasing the rate of alkoxide elimination from the side chain.

Another effect of alkaline treatment of peptides or proteins is the β-elimination of free thiol groups to yield dehydroalanine residues. As expected, this reaction was very rapid at the pH used to β-eliminate phosphoserine. In the presence of 0.1 M-NaOH the rate of β-elimination of 1 nmol of cysteine residues at 100 μM

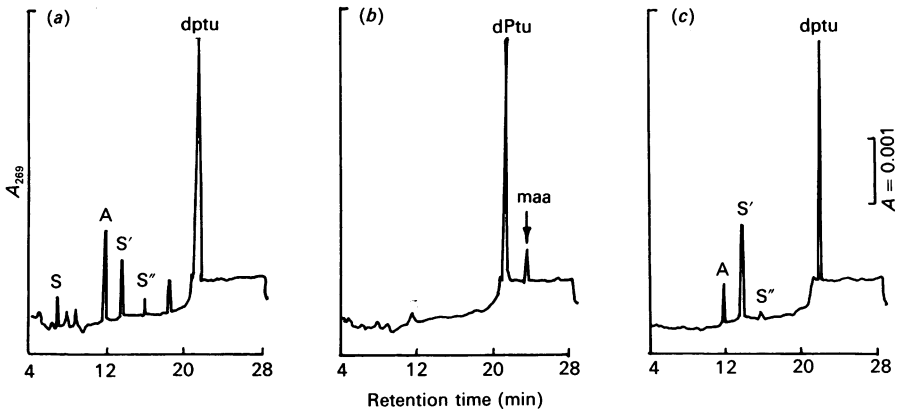

Fig. 3. Sequence analysis of peptides

(*a*) Cycle 4 (phosphoserine) of sequence of tryptic phosphopeptide Ile-Gln-Ala-Ser-Phe-Arg derived from 'neuromodulin' stoichiometrically phosphorylated with protein kinase C (approx. 150 pmol loaded). Abbreviation: dptu, diphenylthiourea. (*b*) Cycle 4 (β-methylaminoalanine) from tryptic phosphopeptide derived from 'neuromodulin' modified as described (approx. 50 pmol loaded). Abbreviation: maa, β-methylaminoalanine phenylthiohydantoin derivative. (*c*) Cycle 5 (dehydroalanine) of phospho-Kemptide after prior quantitative elimination of the phosphate (approx. 150 pmol loaded). In the chromatograms A labels the carry-over from the previous sequencer cycle (alanine in all three runs).

concentration (as GSH) was 3.2 pmol/min (i.e. approx. 25-fold faster than phosphoserine residues). The use of $Ba(OH)_2$ with the same concentration of OH^- ions accelerated the initial rate by 26-fold compared with the rate obtained with NaOH. This observation supports the concept that the catalytic effect of the bivalent cation is due to ion-pair formation with the ionized form of the eliminated species. In practice, the β-elimination of free thiol groups is unimportant, as they are converted into stable alkyl derivatives before structural analysis. Carboxymethylcysteine (as carboxymethyl-GSH), as expected, was stable to the conditions described for the modification of phosphoserine. The behaviour of cysteine under these conditions was not investigated, as the reactions of the disulphide bridge at high pH are multiple and rather complex [12]. Thus disulphide bonds must be reduced and converted into alkyl derivatives before the described procedure.

The chemistry of the aromatic phosphoester in phosphotyrosine is inherently different from that of the phosphoesters of the primary and secondary aliphatic alcohols in phosphoserine and phosphothreonine. This residue is thus acid-labile and base-stable. Phosphotyrosine was examined for its stability towards hydrolysis under the modification conditions by estimating the shift in the aromatic absorbance from 270 nm to 293 nm due to the formation of the tyrosinate anion on base hydrolysis. Under the conditions described, as expected, no hydrolysis of phosphotyrosine was observed.

β-Elimination of phosphothreonine

The threonine analogue of Kemptide (Leu-Arg-Arg-Ala-Thr-Leu-Gly) was phosphorylated by using cyclic AMP-dependent protein kinase and subjected to the Ba^{2+}-catalysed β-elimination reaction as described for the phosphoserine-containing peptide. As expected, elimination of phosphate from phosphothreonine was much (approx. 20-fold) slower than for phosphoserine. All attempts to form analogous nucleophilic adducts with the resulting α-aminocrotonic acid residue were unsuccessful. This is readily explained by the effect of the methyl group on the $C_{(\beta)}$ atom; thus this residue will react much less rapidly with nucleophiles than the corresponding structure derived from phosphoserine.

Modification of phosphoserine racemizes the $C_{(\alpha)}$ atom

When the reaction products of the modification were analysed by reverse-phase h.p.l.c., two peptide species were consistently observed as reaction products in approximately 1:1 yield (Fig. 2). This phenomenon was seen with all phosphopeptides and nucleophiles examined and was independent of any manipulation of the reaction conditions. Both species behaved identically on sequence analysis and their amino acid compositions were identical. These observations suggest a mechanistic artifact. During the nucleophilic addition, the re-protonation of the $C_{(\alpha)}$ atom will not be stereospecific and will thus result in racemization of the modified residue, and hence 50% of the product peptide will contain a D-amino acid residue at the site of modification. In a peptide this will result in the formation of a diastereoisomeric pair that, for a small peptide, can be readily separated by reverse-phase h.p.l.c.

To test this hypothesis, 10 nmol portions of each of the product peptide species were digested with Pronase. Enzymic hydrolysis was chosen to eliminate artifactual scrambling of the stereochemical configuration of the $C_{(\alpha)}$ atoms, which will occur to some extent on chemical hydrolysis. Samples of each digest were incubated with D-amino acid oxidase and the relative extent of oxidation of the digests was assayed fluorimetrically. This showed that the peptide eluted first contained a D-amino acid residue, whereas the second did not. Thus for phospho-Kemptide modified with methylamine the first peptide species of the doublet eluted from the reverse phase column is the *allo* peptide containing a stereochemically inverted amino acid, whereas the second species is the *threo* peptide of the diastereoisomeric pair. The racemization of the modified residue provides independent confirmation that the reaction mechanism is an elimination/addition.

Behaviour of phosphopeptides and their derivatives on sequence analysis

Phospho-Kemptide and its modified forms, the dehydroalanine- and β-methylaminoalanine-containing peptides, were sequenced. To demonstrate the sensitivity of the method, an experimental sample, the tryptic phosphopeptide containing the protein kinase C phosphorylation site (Ile-Gln-Ala-[^{32}P]phospho-Ser-Phe-Arg) [13] of the neurospecific calmodulin-binding protein 'neuromodulin' (GAP-43, B-50, F-1, P-57) was also

sequenced before and after the modification (Figs. 3*a* and 3*b*). Approx. 80 pmol was committed to the modification, and the peptide was desalted (approx. 65% yield) on a 100 mm × 2.1 mm Aquapore RP-300 C_8 reverse-phase h.p.l.c. column. As expected, the unmodified phosphoserine residues showed the characteristic increased yield of the dithiothreitol adduct (S′) presumed to be formed by β-elimination of the phosphate in the sequencer chemistry (Fig. 3*a*; identical results obtained for the Kemptide derivative) compared with what is normally seen for an unphosphorylated serine. That enhanced β-elimination of the phosphoserine is the cause of this phenomenon was confirmed by sequence analysis of the dehydroalanine derivative of Kemptide, where there was near-quantitative recovery of the S′ species (Fig. 3*c*).

Presumably the product of the Edman degradation of the β-methylaminoalanine residue is the phenyl(thiocarbamoyl)ated residue/phenylthiohydantoin derivative resulting from the coupling of phenyl isothiocyanate to the side-chain secondary amine, placing the derivative in the latter part of the 120A analyser chromatogram. The inferred phenyl(thiocarbamoyl)ated residue/phenylthiohydantoin derivative migrates very close to the phenylalanine phenylthiohydantoin derivative in the standard analyser program (Fig. 3*b*; identical results obtained for the Kemptide derivative). It can be readily resolved from phenylthiohydantoin derivatives of other amino acids by using the off-line system described by Ericsson *et al.* [14], in which it runs in an unequivocal position midway between the tryptophan phenylthiohydantoin derivative and the phenyl(thiocarbamoyl)-lysine/lysine phenylthiohydantoin derivative. If only the 120A system is available, the same peptide can be run in modified and unmodified forms (if there is sufficient materials, as in this example), or the 120A analyser program could eventually be modified to resolve these phenylthiohydantoin derivatives. Alternatively, the utility of other nucleophiles yielding stable derivatives could be more fully explored.

On Waters PicoTag amino acid analysis the presumed bis[phenyl(thiocarbamoyl)] derivative of β-methylaminoalanine ran as expected, just before bis[phenyl(thiocarbamoyl)]-lysine but significantly later than a spurious but persistent artifactual peak (probably diphenylthiourea) seen in the chromatograms. The modified residue was readily identified with the standard analyser program.

DISCUSSION

The exploitation of the catalysis of the β-elimination reaction by alkaline-earth-metal hydroxides results in very rapid and essentially quantitative formation of the reactive $\alpha\beta$-desaturated dehydroalanine residue. Peptide bond hydrolysis is negligible under the relatively mild and brief alkaline treatment required to complete the β-elimination reaction in the presence of the bivalent cation. Thus, by contrast with other described protocols [6–8], dissolution of the peptides in mixtures of aprotic solvents (to minimize peptide bond hydrolysis by lowering the proticity of the solvent) is unnecessary. Shorter reaction times and lower temperatures can be used, the reaction being usually complete in about 90 min at 30 °C. The sensitivity of the protocol is determined by the desalting h.p.l.c. step. With the use of a narrow-bore column, sufficient modified peptide for sequence analysis could be obtained from less than 100 pmol of available phosphopeptide.

The exploitation of catalysis of the β-elimination reaction by group II cations contributes to the selectivity of the modification. Provided that disulphide groups and free thiol groups are reduced (if necessary) and alkylated, the β-elimination reaction is specific for phosphoserine and phosphothreonine, although the latter reacts much more slowly. Serine and threonine are unaffected by these conditions. Glycosyl groups *O*-linked to serine react much too slowly under the described conditions to interfere with the detection of a phosphoserine residue.

The reaction chemistry results in the following inherent limitations. Firstly, for small peptides, the resolution by reverse-phase h.p.l.c. of the diastereoisomeric pair of peptides formed during the racemization phase may result in h.p.l.c. profiles that might be initially difficult to interpret, particularly if multiple sites are phosphorylated. Secondly, *N*-terminal and *C*-terminal phosphoserine residues cannot be detected in this manner. *N*-Terminal dehydroalanine is unstable and slowly spontaneously deaminates to yield an *N*-pyruvoyl group, thus blocking the peptide to the Edman degradation. *C*-terminal dehydroalanine is not a good Michael substrate, presumably because the free carboxylate group lacks the electron-withdrawing effect of the carbonyl group present in the amide bond; glycyl-dehydroalanine is entirely refractory to nucleophilic attack (results not shown). Thirdly, the α-aminocrotonic acid residue formed by the slow β-elimination of phosphothreonine is too weak a Michael substrate to react readily by the nucleophilic addition mechanism. This inherent lack of reactivity has precluded extension of this methodology to facilitate the identification of phosphothreonine residues.

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