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β-Elimination of phosphate and subsequent addition of pyridoxamine as a method for identifying and sequencing peptides containing phosphoseryl residues

Teresa G. Hastings and Erwin M. Reimann

Department of Biochemistry, Medical College of Ohio, Toledo, OH 43699, USA

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Peptides containing phosphoseryl residues can be modified by removal of the phosphate groups via β-elimination followed by addition of pyridoxamine to the resulting dehydroalanyl residue. Peptides containing the modified residues can be detected at nanomole levels by monitoring absorbance at 328 nm or at picomole levels by monitoring fluorescence. Photolysis of the modified peptide converts the pyridoxamino adduct to a form which can be readily identified after Edman degradation.

Phosphoserine derivatization; Phosphopeptide; Sequence analysis; Pyridoxamine

1. INTRODUCTION

Because of the important role of protein phosphorylation in cellular regulation [1,2] it is often of interest to identify the phosphorylated residue. This task has proven difficult since phosphorylated residues are not readily identified in standard amino acid sequencing procedures [3]. The most satisfactory resolution to this problem to date has involved modification of the phosphoserine prior to sequence analysis. The procedure to modify the phosphoserine utilizes two steps in which the phosphoserine residue undergoes β-elimination under alkaline conditions to form a dehydroalanyl residue followed by addition of an appropriate nucleophile across the unsaturated bond to form a stable adduct. Some of the nucleophilic reagents that have been used in this procedure include methylamine [4,5], sodium sulfide [6], mercaptoethanol [5], and ethanethiol [5]. These nucleophiles react with the dehydroalanine intermediate forming β-methylamino-alanine, cysteic acid, *S*-ethanolcysteine and *S*-ethylcysteine residues, respectively.

In many cases, it is important to be able to easily identify phosphoserine-containing peptides, either to construct phosphopeptide maps or to facilitate their purification for sequence analysis. This is simple if the peptides are labelled with ^{32}P, but difficult if the peptides are not radioactive. Although peptide-bound phosphate can be determined colorimetrically, the method is tedious and requires nanomole quantities of peptides. It occurred to us that use of the β-elimination reaction followed by addition of a chromophore would enable one to identify and quantify the peptides of interest based on the absorbance or fluorescence properties of the chromophore. We report here on the successful use of pyridoxamine, one of the B_6-vitamers, as a reagent for identifying and sequencing phosphoserine-containing peptides.

2. MATERIALS AND METHODS

2.1. *Materials*

Kemptide (LRRASLG) and pyridoxamine dihydrochloride were obtained from Sigma. The cGMP-dependent protein kinase peptide substrate (cG-peptide) (RKRSRAE) was obtain-

Correspondence address: E.M. Reimann, Department of Biochemistry, Medical College of Ohio, Toledo, OH 43699, USA

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ed from Peninsula. The catalytic subunit of the cAMP-dependent protein kinase was prepared according to the procedure of Reimann and Beham [7]. 2,3-Diaminopropionic acid was purchased from Aldrich. The PTH-2,3-diaminopropionic acid derivative was prepared according to the procedure of Tarr [8].

2.2. *Peptide phosphorylation*

The synthetic peptide (300 μM) was incubated with 365 μM ATP, 18.2 mM magnesium acetate and 15–40 μg protein kinase (catalytic subunit)/ml for 60 min at 25°C.

2.3. *Preparation of the modified peptide*

β-Elimination of the phosphopeptide (260 μM) was carried out in the presence of 0.1 N NaOH and 0.1 M $SrCl_2$ [9] by incubating for 30 min at 40°C. The resulting dehydroalanyl residue was converted to a pyridoxaminoalanyl residue by adding 0.25 vols of 0.5 M pyridoxamine dihydrochloride, 0.75 vols of 1.0 M 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 11.0, 0.15 vols of 1 N NaOH and 0.35 vols of H_2O to the β-eliminated peptide (100 μM) solution and incubating for 180 min at 40°C. The reaction was stopped by acidification with HCl to approx. pH 4.

Formation of the β-eliminated peptides and final addition products was analyzed by shifts in the retention times of the modified peptides on reversed phase HPLC monitored at 206 nm. The peptides containing dehydroalanyl or pyridoxaminoalanyl residues also were monitored at 241 nm [10] and 328 nm, respectively. Fluorescence was monitored on an ABI Kratos Spectroflow 980 detector using a 295 nm excitation maximum and 370 nm cut off emission filter.

2.4. *Reversed phase HPLC analysis*

Peptides were analyzed and isolated on C_{18} reversed phase columns (SynChropak RPP, 250 × 4.1 mm; Brownlee MPLC Analytical Cartridges, 30 × 4.6 mm and 100 × 4.6 mm). Following a 5 or 10 min isocratic wash with 0.1% trifluoroacetic acid (TFA), peptides were eluted with a gradient containing increasing concentrations of acetonitrile/0.08% TFA at a flow rate of 1 ml/min. Separation times varied depending on the column used.

2.5. *Irradiation of the peptides containing pyridoxaminoalanyl residues*

The peptide containing the pyridoxamino moiety was isolated on a reversed phase HPLC column, dried under vacuum, resuspended in 40 mM potassium phosphate, pH 7.0, containing 0.2 mM EDTA and exposed to a 30 W fluorescent light at 10 cm for 8–12 h. The peptide was reisolated by reversed phase chromatography for amino acid composition and amino acid sequence analysis.

2.6. *Amino acid composition*

The peptides were hydrolyzed in 6 N HCl at 110°C for 22 h. Amino acid composition analysis was obtained with a Beckman amino acid analyzer (model 6300) using ninhydrin chemistry.

2.7. *Amino acid sequence*

Amino acid sequence analysis was performed on an Applied Biosystems 470 A gas-phase sequencer. PTH-amino acid derivatives were analyzed on a Waters model 840 HPLC system and NOVA-PAK C_{18} reversed phase column, utilizing the Waters PTH-analysis program.

2.8. *Fast atom bombardment mass spectrometry*

Positive and negative ion fast atom bombardment mass spectra of modified Kemptide were obtained using a Kratos MS-50 mass spectrometer with a thioglycerol matrix, a post acceleration detector and calibration with CsI cluster ions. The accuracy was ± 0.5 Da.

3. RESULTS

The hexapeptide substrate for cAMP-dependent protein kinase, Kemptide (LRRASLG), was used as the model peptide for the development of the modification procedure. The various forms of the peptide, phosphokemptide, unmodified kemptide, and the dehydroalanyl form can be separated and

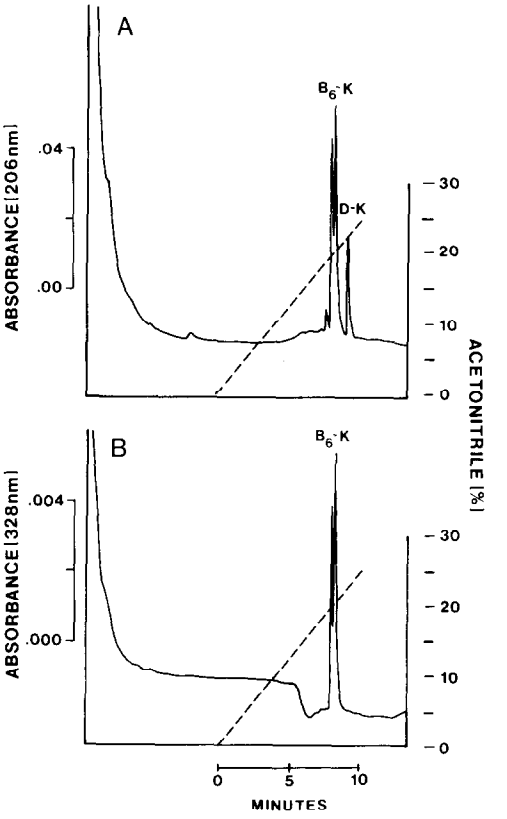

Fig.1. Analysis of kemptide containing pyridoxaminoalanine. 3 nmol of the dehydroalanine form of kemptide (D-K) were partially reacted with pyridoxamine to form the addition product (B_6-K) and the resulting mixture was separated on a reversed phase column and monitored at 206 nm (panel A) or 328 nm (panel B).

analyzed by C_{18} reversed phase HPLC. Using this analysis procedure, it was demonstrated that β-elimination of the phosphokemptide was complete within 30 min under the reaction conditions specified in section 2. The formation of the addition product (B_6-K) from the dehydroalanine form of the peptide (D-K) can be monitored at 206 nm (fig.1A) as well as the identifiable wavelength for the pyridoxamino adduct (328 nm) (fig.1B). The product appears as a doublet which presumably represents stereoisomers since analysis of each peak gave identical amino acid compositions. In some instances the two forms were not resolved.

The effect of pH on the addition reaction is shown in fig.2. In the pH range 10.0–11.0 (fig.2A), using a sodium borate buffer instead of CAPS, the rate of the addition reaction increased steadily with increasing pH values. Beyond pH 11.0, where excess NaOH was added to raise the pH (fig.2B), the rate of the addition reaction decreased with increasing pH. This became especially apparent with longer reaction times, as a biphasic process was apparent around pH 12. A plot of the 50 min time points for all pH values tested demonstrated that over this period the reaction rate was maximal and relatively constant in the pH range 10.75–12.5 (fig.2C). To minimize adverse effects of high pH on the reaction rates a pH range of 10.75–11.0 appears optimal. The reaction rate was proportional to the concentration of pyridoxamine between 2 and 50 mM pyridoxamine.

Fig.3 correlates formation of the product with decreased amounts of the unreacted dehydroalanine form of the peptide. The data on fig.3 are plotted to demonstrate that the formation of product, monitored at both 206 nm and 328 nm, was closely paralleled by a decrease in the dehydroalanyl form monitored at 206 nm. At pH 10.75 and 50 mM pyridoxamine, the reaction was virtually complete in 180 min at 40°C. In the absence of pyridoxamine, the dehydroanalyl form of the kemptide was stable at pH 10.75 over the 5 h incubation period. To increase the buffering capacity, a CAPS buffer at pH 11.0 was used in subsequent studies with identical results.

Under the above conditions, modification of the cG-peptide resulted in the formation of two product peaks of approximately equal distribution. Only one peak showed absorbance at 328 nm, representing the pyridoxaminoalanyl form of the peptide. Amino acid compositional analysis showed that the other peak represented a lysinoalanyl form of the peptide where the ϵ-amino group of lysine added across the unsaturated bond of the dehydroalanyl residue [11]. Succinylation of the peptide prior to modification prevented the formation of this alternate form. Studies on another peptide indicated that formation of lysinoalanyl residues could be minimized if pyridoxamine was present during β-elimination.

The sensitivity of the analysis procedure, monitoring absorbance at 328 nm, is limited to approx. 0.2 nmol of the modified peptide. Since

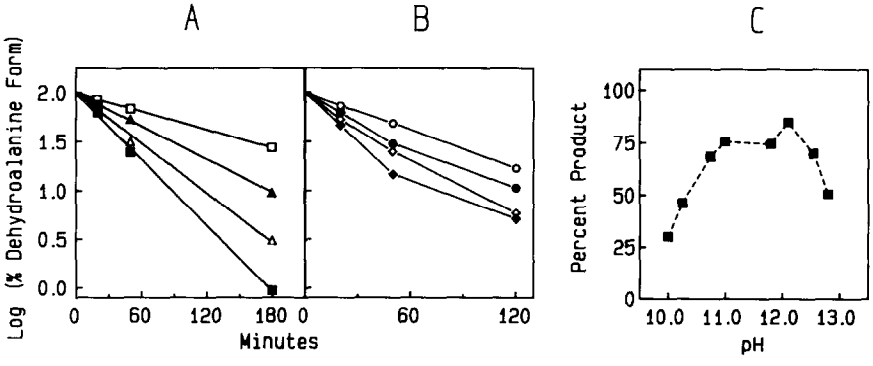

Fig.2. The effect of pH on the rate of the addition reaction. The dehydroalanine form of kemptide was modified as in section 2 except that borate buffer or NaOH was added to the indicated pH. 3 nmol aliquots of the peptide were removed from the reaction mixture at the indicated times and were analyzed by reversed phase HPLC. Results from A and B are presented as percent of the dehydroalanine form remaining. (A) A 0.15 M sodium borate buffer system was used for pH 10.0 (□), 10.25 (▲), 10.75 (△), and 11.0 (■). (B) Excess NaOH was used in the higher pH range, pH 11.8 (◇), 12.1 (◆), 12.5 (●), and 12.8 (○). (C) Amount of product formed in 50 min as a function of pH.

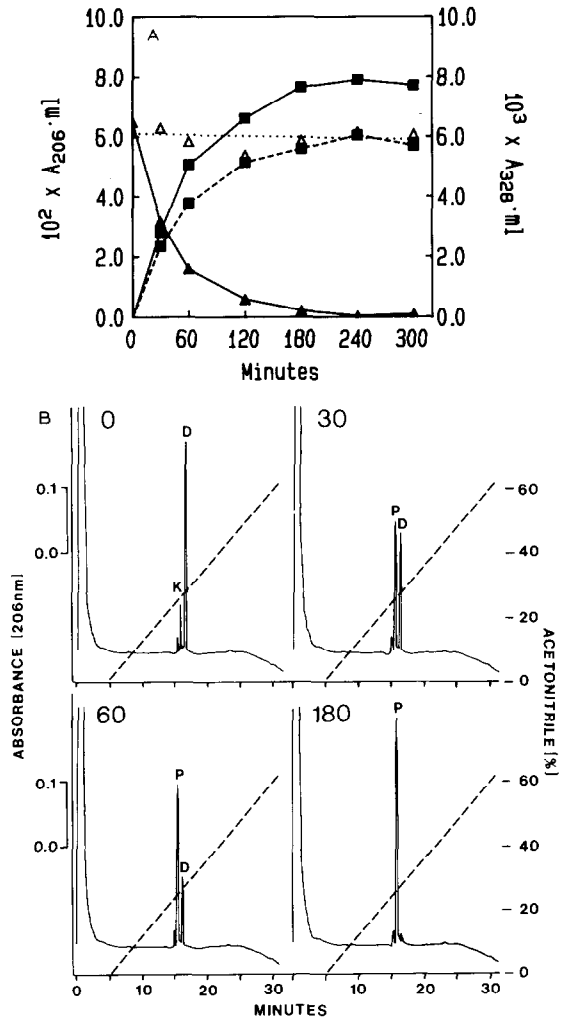

Fig.3. (A) Time course of the addition reaction. Dehydroalanine form of kemptide (0.1 mM), 0.15 M sodium borate pH 10.75, and 50 mM pyridoxamine were incubated at 40°C for the indicated times. Peptides containing dehydroalanyl (▲—▲) and pyridoxaminoalanyl (■—■) residues were monitored at 206 nm on reversed phase HPLC as shown in panel B. Pyridoxaminoalanyl form was also monitored at 328 nm (■---■). The stability of the dehydroalanine intermediate at this pH was measured in the absence of pyridoxamine (△ ··· △). (Absorbance values are corrected to a volume of 1 ml for each of the peaks.) (B) HPLC chromatograms of some of the time course data (0, 30, 60, 180 min) represented in panel A. Unmodified form (K), dehydroalanyl form (D), and pyridoxaminoalanyl form (P) of kemptide were monitored at 206 nm.

pyridoxamine and its derivatives are known to be fluorescent [12], increasing the detection sensitivity by monitoring fluorescence of the addition product was examined. Fluorescence monitoring gave approx. 100-fold greater sensitivity over absorbance measurements at 328 nm as shown by the detection of 11 pmol of the modified kemptide (fig.4).

Automated sequence analysis of the peptide modified with pyridoxamine failed to yield an identifiable product for the modified residue, indicating that the PTH derivative is not stable, is not extracted in the standard Edman degradation method, or is not detected by the PTH analysis procedure. In order to circumvent this problem we exposed kemptide containing the pyridoxamino moiety to fluorescent light, since photolysis of proteins containing reduced, pyridoxylated lysyl residues regenerates free lysyl residues [13]. Light exposure of modified kemptide resulted in a time-dependent decrease in the amount of peptide absorbing at 328 nm and an increase in the amount of a slightly more hydrophilic form of the peptide which absorbed only at 206 nm. Photolysis of the peptide was complete in 8 h with a 30 W fluorescent light. When the light-exposed modified kemptide was subjected to standard Edman degradation, the PTH-derivative of the modified phosphoserine appeared in cycle 5 as a strong signal eluting just prior to DPTU (fig.5). The modified residue is resolved from the other amino

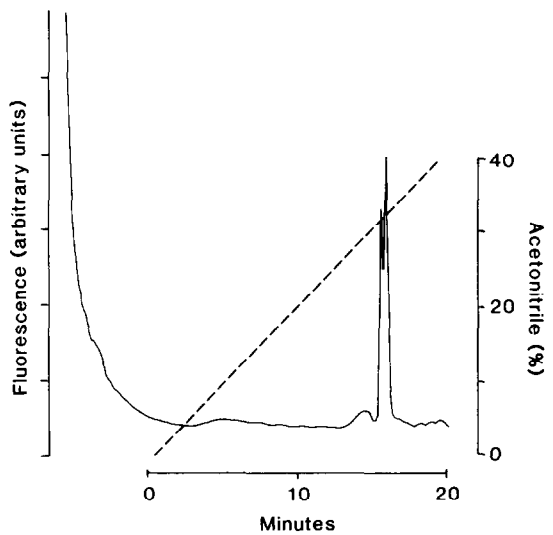

Fig.4. Fluorescence monitoring of the pyridoxamine peptide. An aliquot of a reaction mixture containing 11 pmol of the pyridoxaminoalanine form of kemptide was analyzed by reversed phase HPLC and monitored for fluorescence.

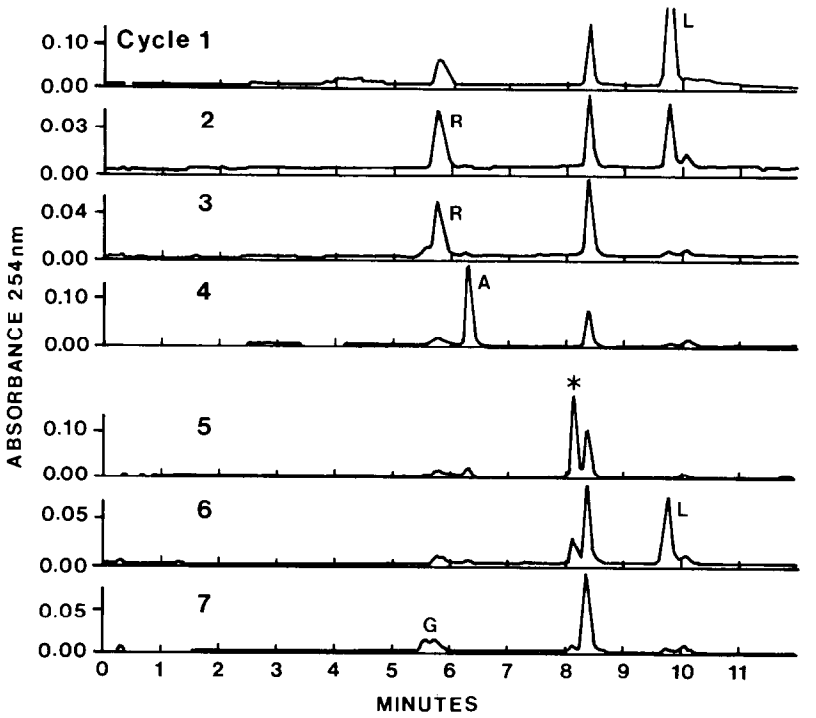

Fig.5. Sequence analysis of the pyridoxaminoalanyl form of kemptide after light exposure. 11 nmol of the modified peptide were subjected to Edman degradation and PTH-amino acid analysis. The peak in cycle 5 (*) represents the PTH-derivative of the modified phosphoserine.

acids and elutes between valine and tryptophan PTH-derivatives (fig.6). Similar analysis of the modified cG-peptide gave the same PTH derivative at the appropriate cycle.

The structure of the photolysis product has not been established with certainty but a tentative identification can be made. Based on the findings of Ritchey et al. [13] the anticipated product is aminoalanine. Fast atom bombardment analysis of kemptide after modification and photolysis indicated a mass of 770 as expected if an aminoalanine residue were generated. Furthermore the PTH derivative of the modified serine eluted in the same position as the PTH derivative formed from PITC and β-aminoalanine. However, acid hydrolysis of three different phosphopeptides that had been subjected to β-elimination, addition of

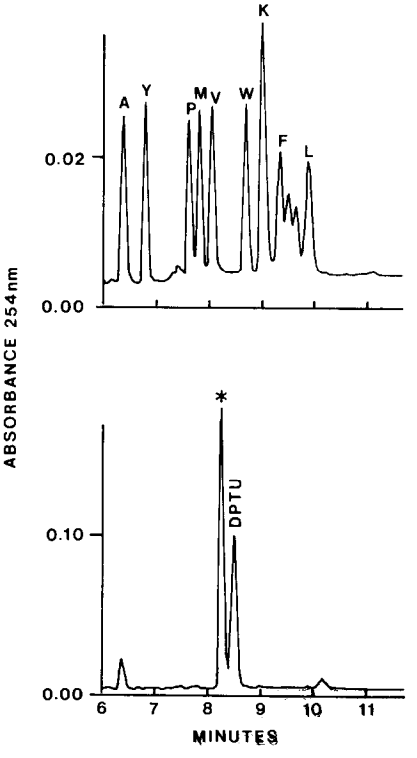

Fig.6. Separation of the PTH-derivatives of the modified and standard amino acyl residues. The PTH-derivative (lower panel) of the light-exposed pyridoxaminoalanyl residue (cycle 5, fig.5) was resolved from the standard PTH-amino acids (upper panel).

pyridoxamine and photolysis failed to yield an amino acid corresponding to β-aminoalanine whereas β-aminoalanine subjected to the conditions of acid hydrolysis yielded a ninhydrin-positive peak that eluted just after histidine when analyzed as described in section 2. One possible explanation for these results is that the product is α-aminoalanine rather than β-aminoalanine and that α-aminoalanine is not stable under the conditions of acid hydrolysis.

4. DISCUSSION

The procedure for chemically modifying phosphoserine residues differs from previously reported β-elimination-addition methods in the use of a chromophore. Pyridoxamine was selected as the chromophore so that the modification could be utilized for both peptide mapping and amino acid sequencing of phosphoserines contained in peptides and proteins. Photolysis of the pyridoxamino moiety renders the peptide suitable for amino acid sequence analysis and also renders the peptide slightly more hydrophilic, which generally should allow one to separate it from any contaminating peptides via reversed phase chromatography. This would be a particularly useful final purification step when the modified peptides are isolated from whole protein digests.

In all peptides and proteins examined thus far, 60 min was sufficient for complete β-elimination of all phosphoserine residues. However, in some instances the modification reaction may not go to completion because the rate of β-elimination for a given residue differs based on its surrounding amino acid environment [14]. Although the β-elimination reaction is relatively specific for phosphoserine, phosphothreonine residues have been shown to undergo β-elimination, but at a reduced rate when compared to phosphoserines [14]. Likewise, *O*-glycosylated residues will also undergo β-elimination and, in the case of *O*-glycoserine, will form the same dehydroalanine derivative observed with phosphoserine [10]. Cysteine residues may also β-eliminate under alkaline conditions, forming the dehydroalanine intermediate [10]. The sulfhydryl group of an intact cysteine or the ε-amino group of a neighboring lysine may act as an internal nucleophile and add across the unsaturated dehydroalanine bond, forming a lanthionine [10] or lysinoalanine residue [11], respectively. Therefore, modification of cysteine and/or lysine residues prior to β-elimination may be required to avoid these complications.

The reduced reactivity of some phosphoserines and the lack of absolute specificity are not serious limitations for many of the potential applications of this method. This modification procedure has the potential for peptide mapping of phosphoproteins as well as sequence analysis of the resulting phosphopeptides. It does not require the use of radioisotopes or prior knowledge of the primary structure of the protein to detect phosphoserine containing peptides and, utilizing the fluorescent characteristics of the chromophore, can be used to detect pmol amounts of the modified peptides.

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REFERENCES

[1] Krebs, E.G. (1986) in: The Enzymes (Boyer, P.D. and Krebs, E.G. eds) vol.17, pp.3–20, Academic Press, Orlando, FL.
[2] Cohen, P. (1985) Eur. J. Biochem. 151, 439–448.
[3] Proud, C.G., Rylatt, D.B., Yeaman, S.J. and Cohen, P. (1977) FEBS Lett. 80, 435–442.
[4] Annan, W.D., Manson, W. and Nimmo, J.A. (1982) Anal. Biochem. 121, 62–68.
[5] Meyer, H.E., Hoffman-Posorske, E., Korte, H. and Heilmeyer, L.M.G. jr (1986) FEBS Lett. 204, 61–66.
[6] Soderling, T.R. and Walsh, K. (1982) J. Chromatogr. 253, 243–251.
[7] Reimann, E.M. and Beham, R.A. (1983) Methods Enzymol. 99, 51–55.
[8] Tarr, G.E. (1986) in: Methods of Protein Microcharacterization (Shively, J.E. ed.) pp.183–185, Humana Press, Clifton, NJ.
[9] Sundararajan, T.A., Kumar, K.S.V.S. and Sarmon, P.S. (1958) Biochim. Biophys. Acta 28, 148–158.
[10] Whitaker, J.R. and Feeney, R.E. (1977) Adv. Exp. Biol. 86B, 155–175.
[11] Bohak, Z. (1964) J. Biol. Chem. 239, 2878–2887.
[12] Forrey, A.W., Olsgaard, R.B., Nolan, C. and Fischer, E.H. (1971) Biochimie 53, 269–281.
[13] Ritchey, J.M., Gibbons, I. and Schachman, H.K. (1977) Biochemistry 16, 4584–4590.
[14] Kemp, B.E. (1980) FEBS Lett. 110, 308–312.