Journal of Chromatography, 253 (1982) 243–251
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,373

SELECTIVE MODIFICATION AND PURIFICATION OF PHOSPHOPEPTIDES FOR AUTOMATED SEQUENCE ANALYSIS

THOMAS R. SODERLING*
Howard Hughes Medical Institute, Department of Physiology, Vanderbilt University, Nashville, TN 37232 (U.S.A.)
and
KENNETH WALSH
Department of Biochemistry, University of Washington, Seattle, WA 98195 (U.S.A.)
(First received June 23rd, 1982; revised manuscript received September 9th, 1982)

SUMMARY

A method is described for the selective purification of ^{32}P-labeled peptides for sequence analysis. The procedure is based on the differential retention on reversed-phase high-performance liquid chromatography of the peptide containing phosphoserine *versus* dehydroalanine obtained by alkaline β-elimination of the phosphate. The method was tested using two synthetic phosphopeptides representing the phosphorylation site in pyruvate kinase and one of the sites in glycogen synthase.

INTRODUCTION

Protein phosphorylation is now firmly established as an important mechanism for regulation of cellular functions. Such diverse areas as metabolism, membrane transport, muscle contraction, protein synthesis, neuronal activity, cellular transformation by viruses, and others are involved[1,2]. Discoveries of phosphorylation-dependent cellular processes have been rapid in the past ten years and will probably continue for some time.

Frequently it is important to identify the amino acid sequence surrounding the residue which is phosphorylated. Three amino acids commonly phosphorylated are serine, tyrosine, and threonine. Studies of phosphorylation site sequences can yield information concerning the specificities of protein kinases such as the adenosine 3′:5′-monophosphate (cAMP)-dependent protein kinase which phosphorylates serines situated one or two positions C-terminal of two basic residues[3]. Such structural data are also useful to design synthetic peptide inhibitors of kinases which may be helpful for clinical as well as investigative purposes.

Determinations of phosphorylation site sequences have normally entailed purification of the ^{32}P-labeled peptide by conventional methods such as ion-exchange chromatography, gel filtration, and high-voltage electrophoresis. Such methods are tedious, unpredictable, and often result in low yields of pure peptide. A further major

0021-9673/82/0000–0000/$02.75

problem in the actual sequence determination is the apparent instability of the phosphate ester bond during the removal of the phosphoserine residue by the Edman degradation[4]. The phosphate group eliminates in the expected cycle of degradation and the serine residue is converted to the thiazolinone of dehydroalanine. The released inorganic ^{32}P does not extract well in the chlorobutane used to extract the thiazolinone derivates from the spinning cup sequenator. Typically only 1 to 5% of the radioactivity extracts from the cup at the correct position of the sequence with the remaining ^{32}P trailing off in subsequent cycles. This can cause problems in quantification, especially if there are multiple phosphorylation sites in close proximity.

EXPERIMENTAL

Materials

The synthetic Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) was obtained from Penninsula Lab or Boehringer Mannheim. N-Syntide (Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ser-Ser) was a gift from Dr. Bruce Kemp (University of Melbourne, Australia). cAMP-dependent protein kinase was purified as described[5]. Peptides were phosphorylated in 50 m*M* potassium phosphate buffer (pH 6.8) using 5 m*M* [γ-^{32}P]ATP, 2 m*M* peptide, 10 m*M* magnesium acetate and cAMP-dependent protein kinase (50,000 units/ml). [γ-^{32}P]ATP was synthesized as described[6]. Crystalline bovine serum albumin was from Sigma. Acetonitrile was obtained from Burdick and Jackson Labs., and trifluoroacetic acid from Pierce.

Methods

Reversed-phase high-performance liquid chromatography (HPLC) was conducted using a Beckman Model 421 instrument with a Beckman Ultrasphere C_{18} column (25 cm $\times$ 4.6 mm, 5 μm particle size). Equilibration buffer was 0.1% trifluoroacetic acid in water, and gradients were established as indicated using acetonitrile in 0.1% aqueous trifluoroacetic acid. Absorbance was monitored at 206 nm, and the flow-rate was 1 ml/min. Peptides were hydrolyzed in 6 *M* hydrochloric acid at 110°C for 18 h, and amino acid analysis was performed on a Dionex analyzer. Sequence analysis was done on a Beckman Model 890 B Sequencer using the method described in ref. 7 in the absence of polybrene. In the presence of polybrene very little thiazolinone derivative of cysteic acid was extracted. β-elimination of phosphopeptides was in 0.1 *M* NaOH, 0.1 *M* $CaCl_2$ at 40°C for 2 h. Sulfite addition was in 0.1 *M* NaOH and 0.1 *M* Na_2SO_3 at 40°C for 2 h.

RESULTS AND DISCUSSION

The lability of the phosphate esters of serine and threonine in proteins to dilute alkali has been recognized for many years and constitutes a diagnostic test for the identification of such bonds (reviewed in ref. 8). More recently, alkaline β-elimination followed by nucleophilic addition to the unsaturated amino acid has been used to identify serine and threonine residues involved in phosphate and carbohydrate linkages[9–11].

Since the conversion of a phosphoserine to a dehydroalanine residue by β-elimination would be expected to have considerable influence on the hydrophobicity

of a small peptide, this procedure seemed well suited for selective purification of ^{32}P-labeled peptides using reversed-phase HPLC. Subsequent identification of the phosphorylation site is accomplished after [^{35}S]sulfite addition to the unsaturated dehydroalanine residue[10]. The resulting [^{35}S]cysteic acid is stable to conditions of the Edman degradation in contrast to the phosphoserine.

To establish the validity of this purification procedure, synthetic peptides of known sequence have been utilized. These peptides represent the phosphorylation site in pyruvate kinase (Kemptide)[3] and the amino-terminal phosphorylation site in glycogen synthase (N-syntide)[12,13]. Both are readily phosphorylated *in vitro* by the cAMP-dependent protein kinase. N-Syntide can also be phosphorylated by phosphorylase kinase in the absence but not the presence of ethylene glycol tetraacetate (data not shown).

Kemptide: Leu-Arg-Arg-Ala-Ser(^{32}P)-Leu-Gly
N-Syntide: Pro-Leu-Ser-Arg-Thr-Leu-Ser(^{32}P)-Val-Ser-Ser

Fig. 1 and Table I illustrate the ability of reversed-phase HPLC to separate the phosphoserine-peptides from the dehydroalanine-peptides which were generated by alkaline β-elimination of the phosphate group. In both cases the retention time of the dehydroalanine peptides were about double those of the phosphoserine-peptides. The β-elimination reaction was conducted in 0.1 *M* NaOH containing 0.1 *M* $CaCl_2$ since the divalent cation greatly increases the rate of phosphate release[14] as shown in Fig. 2.

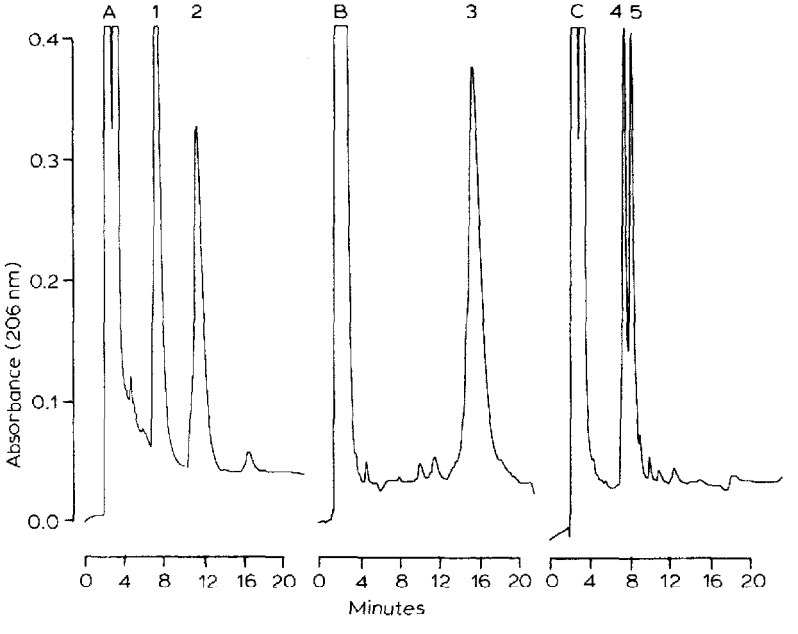

Fig. 1. HPLC of derivatives of Kemptide. A gradient of 15 % to 30 % acetonitrile in 0.1 % aqueous trifluoroacetic acid was run over 20 min. Peaks: A1 = phosphoserine-Kemptide; A2 = Kemptide; B3 = dehydroalanine-Kemptide; C4 and C5 = cysteic acid-Kemptide. The large peak(s) at 2–4 min are buffers and acetic acid.

TABLE I

RETENTION TIMES OF DERIVATIVES OF KEMPTIDE AND N-SYNTIDE

HPLC was as in *Methods* with an acetonitrile gradient of 15% to 30% in 20 min.

Peptide	*Retention time (min)*
Kemptide	11.5
Phosphoserine-Kemptide	7.6
Dehydroalanine-Kemptide	13.0
Cysteic acid-Kemptide	7.5
Syntide	11.0
Phosphoserine-Syntide	8.2
Dehydroalanine-Syntide	13.5
Cysteic acid-Syntide	9.4

Rather than incubating for 12–14 h to obtain complete β-elimination as is often necessary, conversion to the dehydroalanine-peptide was complete in 2–3 h in the presence of calcium.

Identification of the original site of phosphorylation in sequence analysis required that the dehydroalanine-residue be converted to a unique product since some phenylthiohydantoin-dehydroalanine is usually generated from phenylthiohydantoin-serine. Although there are reports[15] of reduction of dehydroalanine to alanine using $NaBH_4$, we were unable to reduce the dehydroalanine-peptide using $NaBH_4$. However, sulfite addition to form cysteic acid was rapid and quantitative yielding two peptides as illustrated in Fig. 1C. When these two peptides were resolved using an isocratic elution and subjected to amino acid analysis, both gave identical compositions of 2 Leu, 2 Arg, 1 Ala, 1 Gly, and 1 cysteic acid residues. We have tentatively concluded that these two peptides are diasteroisomers formed by the sulfite addition. It is interesting that the cysteic acid-peptide has a retention time very similar to that of the phosphoserine-peptide as would be expected.

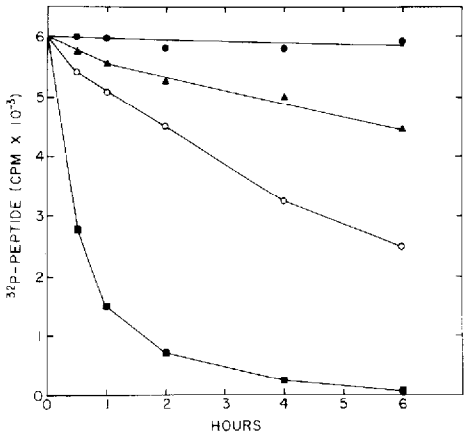

Fig. 2. Effect of Ca^{2+} on rate of β-elimination. [^{32}P]Kemptide was incubated at 30°C in water (●) or 0.25 *M* NaOH containing 0 (▲), 10 (○), or 100 (■) m*M* $CaCl_2$. Aliquots were spotted on phosphocellulose paper and washed in acetic acid to determine remaining [^{32}P]Kemptide[3].

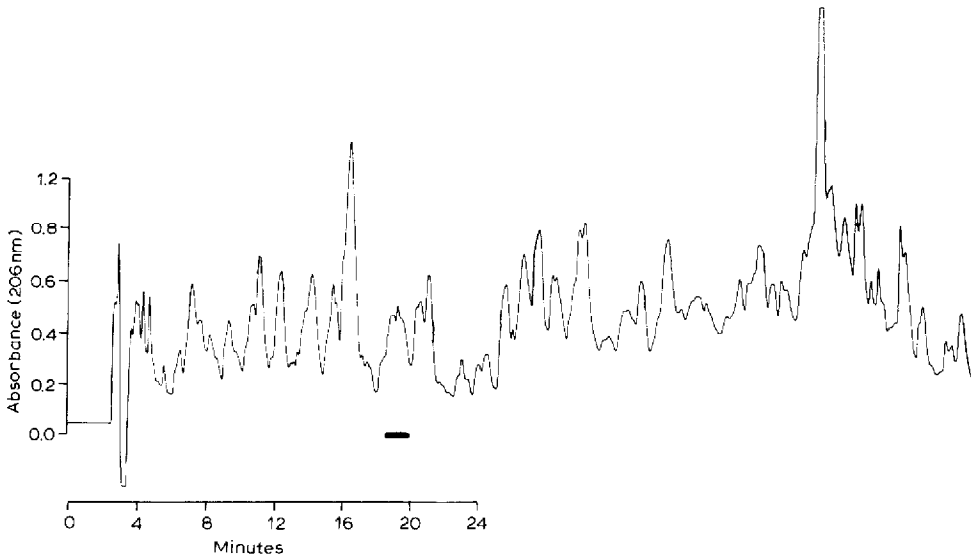

Fig. 3. HPLC of tryptic digest of bovine serum albumin containing [^{32}P]Kemptide. A mixture of 35 nmol each of [^{32}P]Kemptide and tryptic digest of bovine serum albumin was made. Aliquots (11.7 nmol) were subjected to HPLC using an acetonitrite gradient as follows: 5–15 % in 15 min, 15–25 % in 20 min, 25–50 % in 20 min. The peaks at 19–20 min (above the bar) contained the radioactivity and were pooled.

Recoveries of the various peptide forms from reversed-phase HPLC were between 50 and 80 %, based on integration of peak areas, recoveries of radioactivity, and amino acid analyses.

To test this method for selective purification of a phosphoserine-peptide from a complex mixture, 35 nmol of [^{32}P]Kemptide were mixed with 35 nmol of a tryptic

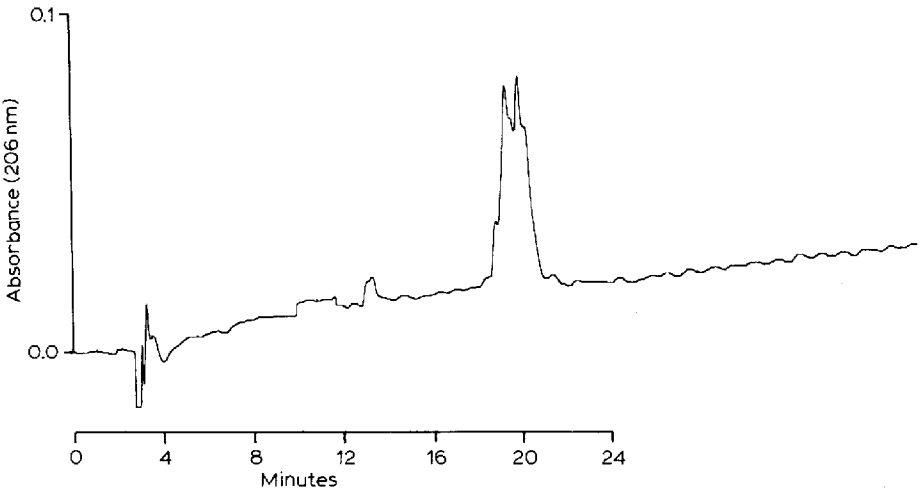

Fig. 4. HPLC of peptides before β-elimination. An aliquot (5 %) of the peptides pooled in Fig. 3 were run on the same gradient as in Fig. 3.

digest of bovine serum albumin. This mixture was subjected to HPLC, and aliquots of fractions were counted for radioactivity. The radioactivity was found to elute in the peptide peaks between 19 and 20 min (Fig. 3). These fractions were pooled and lyophilized. When an aliquot (5%) was reapplied to HPLC, the profile shown in Fig. 4 was obtained. The remaining 95% was dissolved in 0.1 ml of 0.1 *M* NaOH containing 0.1 *M* $CaCl_2$. After incubation at 40°C for 2 h, the sample was again applied to HPLC using a different gradient (Fig. 5). The peaks eluting at 12–14 min represented contaminating peptides which contained less than 5% of the applied radioactivity. The flow through fractions contained 95% of the applied radioactivity. The peak at 21.5 min, representing the dehydroalanine-peptide, was lyophilized, dissolved in 0.1 ml of 0.1 *M* NaOH plus 0.1 *M* Na_2SO_3, and incubated for 2 h at 37°C. This mixture was applied to HPLC and the two peaks eluting at 13 min (Fig. 6) were pooled separately, lyophilized, and subjected to amino acid analysis. The molar ratios, based on 2 leucines, were:

Peak I: Leu (2.0), Ala (1.11), Arg (1.70), cysteic acid (1.04), Ser (0.93), Gly (2.35)

Peak II: Leu (2.0), Ala (0.96), Arg (1.80), cysteic acid (1.02), Ser (0.40), Gly (2.22)

The high values for serine and glycine (expected 0 and 1, respectively) reflect background contamination from the HPLC system. The recovery of cysteic acid-peptide was only about 5 nmol or 14% of the original ^{32}P-labeled peptide. Assuming equal recovery of the peptides at each stage of HPLC, this would calculate to about

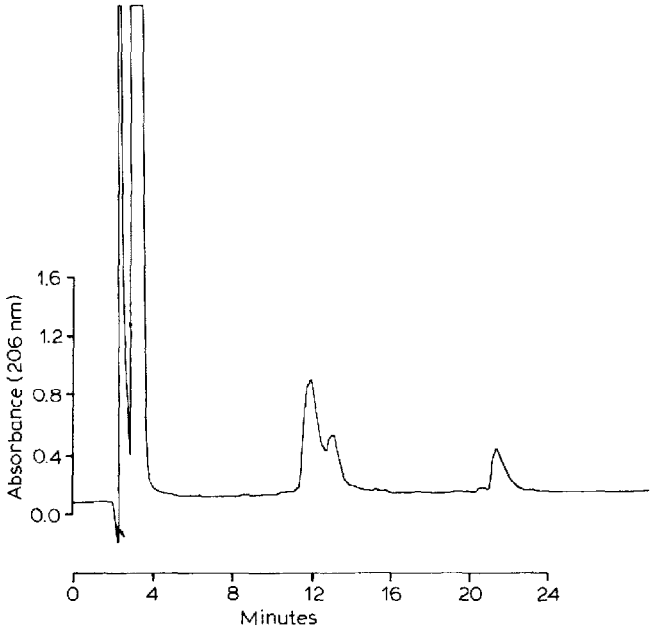

Fig. 5. HPLC of peptides after β-elimination. The peaks pooled in Fig. 3 were subjected to β-elimination (see *Methods*) and chromatographed with a 10–30% acetonitrile gradient over 30 min. The peak at 21.5 min was pooled.

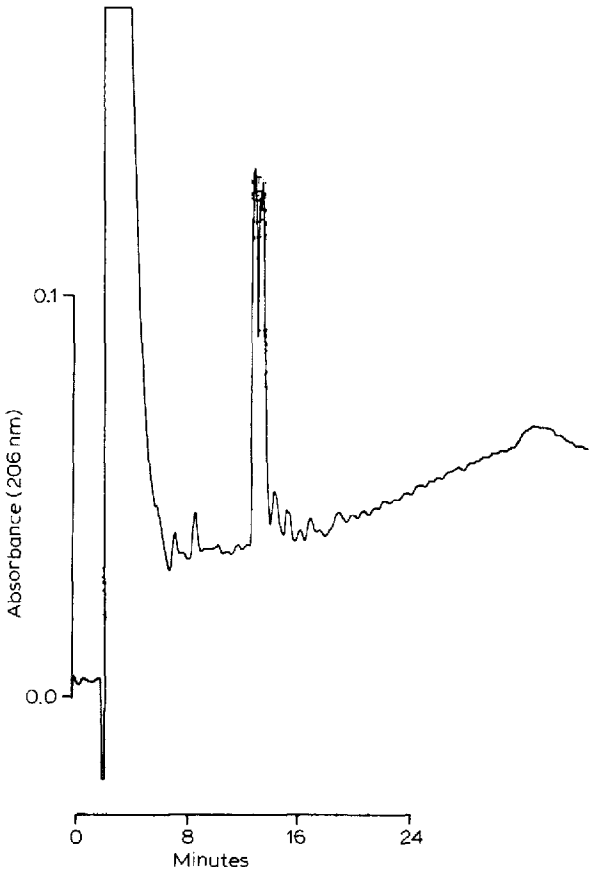

Fig. 6. HPLC of peptides after sulfite addition. The peak pooled in Fig. 5 was reacted with sulfite (see *Methods*), and this reaction mixture was subjected to HPLC with a 10–30% acetonitrile gradient over 30 min.

50% recovery at each cycle. These rather low recoveries may be due to binding of peptide to glass surfaces. It should be pointed out that for purposes of selective purification of the peptide the last HPLC step could be omitted since it only serves to remove excess sulfite and other ions. In this case the yield would be about 25% of the starting material.

In terms of sequence analysis, one remaining problem is extraction of the polar thiazolinone derivative of cysteic acid from the liquid sequenator cup. Using performic acid-oxidized β-chain of insulin, which contains a cysteic acid 7 residues from the amino terminus, only 15–18% of the cysteic acid was recovered on sequence analysis (data not shown). While this is not very good, if one uses [^{35}S]sulfite one can easily identify sites which originally were phosphoserine residues. More quantitative results could be obtained by stopping the sequenator at the cycle where a preliminary run indicated release of radioactivity. An aliquot could be removed from the sequenator and run on high-voltage electrophoresis to quantitate the amount of ^{35}S which migrates as phenylthiohydantoin-cysteic acid. Alternatively, the peptides could be sequenced on a solid phase sequenator with a more polar extraction buffer.

Previous studies[9–11] as well as those in this paper demonstrate that alkaline β-elimination coupled with nucleophilic addition is an excellent procedure for identification of phosphoserine modifications in proteins. However, certain precautions must be kept in mind to avoid potential problems. For example, cysteine is known to readily undergo β-elimination in alkali[16]. This problem can be avoided by performic acid oxidation of the protein or peptide mixture prior to the β-elimination of the phosphoserine. Another potential problem is the addition of an adjacent lysine residue to the dehydroalanine to form lysinoalanine[16,17]. However, if one uses tryptic hydrolysis of the phosphoprotein, lysine N-terminal to the phosphoserine would be cleaved from the phosphopeptide thereby decreasing this side reaction. Hydrolysis of the guanido group of arginine to form ornithine can occur with prolonged exposure to alkali[18]. This did not appear to be a problem with the relatively short treatments which we used since no significant loss of arginine was observed. Another potential problem, if one wanted to effect the β-elimination–sulfite addition on the intact protein, is peptide bond hydrolysis in alkali. Studies have shown that Gly–Gly and Gly–Ser linkages are somewhat labile in alkali[19] as is the dehydroalanine bond itself (see p. 232 of ref. 20). With our model peptides the dehydroalanine peptide derivative was stable in 0.1 *M* NaOH for up to 6–8 h with no indication of peptide hydrolysis. It has been reported that β-elimination in a dimethylsulphoxide–ethanol mixture greatly reduces peptide bond hydrolysis[11]. Lastly, any O-glycosidic bonds would also β-eliminate in alkali. Most of the above problems only become serious if the exposure to alkali is for periods of 6–24 h. Since β-elimination of phosphate from small peptides requires 4 h or less in the presence of calcium, these potential problems are greatly minimized. Thus, when certain precautions as outlined above are taken, this method should allow for selective purification and sequence analysis of phosphorylation sites involving serine residues. The method should also be applicable for phosphothreonine residues, but it may not be as quantitative and is untested at this time.

We are currently working to apply this methodology to the selective purification of phosphopeptides generated by tryptic digestion of ^{32}P-labeled glycogen synthase. This enzyme contains 5 to 6 phosphorylation sites per subunit[21]. It is hoped the method will be of general use to investigators purifying phosphopeptides or studying *in vivo* phosphorylation.

ACKNOWLEDGEMENT

This work was supported by Grants AM 17808 and GM 15731 from the National Institutes of Health.

REFERENCES

1 E. G. Krebs and J. A. Beavo, *Annu. Rev. Biochem.*, 48 (1979) 923.
2 O. M. Rosen and E. G. Krebs (Editors), *Protein Phosphorylation, Cold Spring Harbor Conference on Cell Proliferation*, Vol. 8, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1981.
3 B. E. Kemp, D. J. Graves, E. Benjamin and E. G. Krebs, *J. Biol. Chem.*, 252 (1977) 4888.
4 C. G. Proud, D. B. Rylatt, S. J. Yeaman and P. Cohen, *FEBS Lett.*, 80 (1977) 435.
5 T. R. Soderling, M. F. Jett, N. J. Hutson and B. S. Khatra, *J. Biol. Chem.*, 252 (1977) 7517.
6 T. Walseth and R. Johnson, *Biochim. Biophys. Acta*, 562 (1979) 11.
7 A. W. Brauer, M. N. Margolies and E. Haber, *Biochemistry*, 14 (1975) 3029.

8 G. Taborsky, *Advan. Protein Chem.*, 28 (1974) 1.
9 D. L. Simpson, J. Hranisavljevic and E. A. Davidson, *Biochemistry*, 11 (1972) 1849.
10 R. C. Clark and J. Dijkstra, *Int. J. Biochem.*, 11 (1980) 577.
11 P. M. Steinert, M. L. Wantz and W. W. Idler, *Biochemistry*, 21 (1982) 177.
12 T. R. Sonderling, V. S. Sheorain and L. H. Ericsson, *FEBS Lett.*, 106 (1979) 181.
13 N. Embi, P. J. Parker and P. Cohen, *Eur. J. Biochem.*, 115 (1981) 405.
14 T. A. Sundararajan, K. S. V. Kumar and P. S. Sarma, *Biochim. Biophys. Acta*, 28 (1958) 148.
15 F. Downs, A. Herp, J. Moschera and W. Pigman, *Biochim. Biophys. Acta*, 328 (1973) 182.
16 Z. Bohak, *J. Biol. Chem.*, 239 (1964) 2878.
17 J. R. Whitaker and R. E. Feeney, *Advan. Exp. Biol.*, 86B (1977) 155.
18 K. Ziegler, I. Melchert and C. Lurken, *Nature (London)*, 214 (1967) 404.
19 C. J. Jarboe, B. W. Noll and L. F. Hass, *Biochem. Biophys. Res. Commun.*, 43 (1971) 1029.
20 B. Witkop, *Advan. Prot. Chem.*, 16 (1961) 221.
21 T. R. Soderling and B. S. Khatra, in W. Y. Cheung (Editor), *Calcium and Cell Function*, Academic Press, New York, Vol. 3, 1982, in press.