SIMPLE AND RAPID IDENTIFICATION OF PHOSPHORYLATED PEPTIDES FROM BOVINE BRAIN MYELIN BASIC PROTEIN BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

S. SHOJI, J. OHNISHI, T. FUNAKOSHI and Y. KUBOTA*

Department of Biochemistry, Faculty of Pharmaceutical Sciences, Kumamoto University, Kumamoto 862 (Japan)

K. FUKUNAGA and E. MIYAMOTO

Department of Pharmacology, Kumamoto University Medical School, Kumamoto 860 (Japan)

and

H. UEKI

Department of Biochemistry, Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Fukuyama 729-02 (Japan)

(Received September 27th, 1984)

SUMMARY

The phosphorylation sites of the myelin basic protein from bovine brain were determined after phosphorylation with a cyclic 3′:5′-phosphate-dependent protein kinase from the same source. Three phosphorylated peptides were selectively and rapidly separated, before and after dephosphorylation, by reversed-phase high-performance liquid chromatography on a styrene 250 column under alkaline conditions. Partial sequencing of the peptides by automated Edman degradation revealed that the serine-115 residue located in the main encephalitogenic determinant of the protein was a phosphorylation site, in addition to the two phosphorylation sites established (threonine-34 and serine-55).
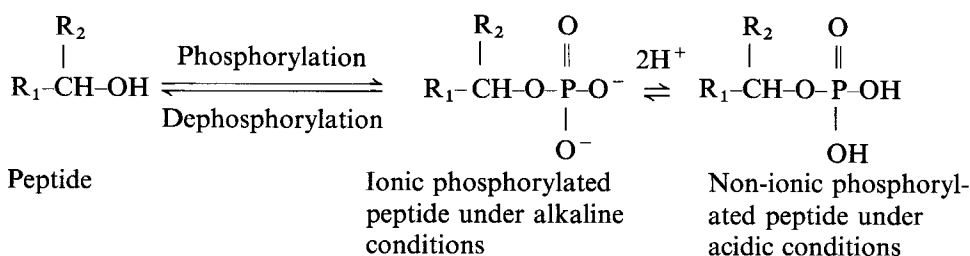
INTRODUCTION

The myelin basic protein isolated from central nervous tissue is a major structural protein in myelin and is known to be involved in experimental allergic encephalomyelitis, an autoimmune disease[1–3]. Some structural properties, the amino acid sequence and the main encephalitogenic determinant of the protein have been described by Eyler and co-workers[4–7]. Phosphorylation of the myelin basic protein by a cyclic 3′:5′-phosphate (cAMP)-dependent protein kinase and the resultant phosphorylated amino acids and peptides have also been reported[8–11].

Protein phosphorylation and dephosphorylation, which are primarily mediated by protein kinases and protein phosphatases, are well recognized to play a major general role in physiological functions[12–14]. Phosphorylated amino acids and peptides of protein phosphorylated by protein kinases were usually determined by paper chro-

matographic techniques using radioactive [γ-^{32}P]ATP[8] or by analysis of the paper electrophoretic mobility change (at pH 6.5) of a phosphorylated peptide before and after dephosphorylation[15]. The former technique requires special handing and apparatus for use of radioactive material. The latter is an excellent technique but is not suitable for amount of less than 10 nmole of amino acids and peptides.

Recently, many advantages in high-performance liquid chromatography (HPLC) have provided the most powerful means for both the analytical and preparative isolation of peptides and proteins[16,17]. HPLC is used not only for the speedy isolation of peptides and proteins but also for the selective separation of marked peptides such as S-methylmethionine-containing peptides in protein sequencing work[18]. Silica-based microporous particle packed columns are usually used under acidic conditions, *e.q.*, in 0.1% phosphoric acid or 0.1% trifluoroacetic acid (TFA)[19]. On the other hand, polystyrene-based microporous particle packed columns, which are currently available, can be used under both alkaline and acidic conditions. The selective separation of peptides containing phosphorylation sites by HPLC is more effective under alkaline than acidic conditions, as shown in the following scheme:

$$
\begin{array}{ccc}
R_2 & R_2 \quad O & R_2 \quad O \\
| \quad \text{Phosphorylation} & | \quad \| & 2H^+ \quad | \quad \| \\
R_1\text{--CH--OH} \;\rightleftharpoons\; & R_1\text{--CH--O--P--O}^- \;\rightleftharpoons\; & R_1\text{--CH--O--P--OH} \\
\quad \text{Dephosphorylation} & | & | \\
& O^- & OH
\end{array}
$$

Peptide   Ionic phosphorylated   Non-ionic phosphoryl-
peptide under alkaline   ated peptide under
conditions   acidic conditions

The procedure proposed herein allows the rapid identification and selective separation of peptides containing the phosphorylation sites of the myelin basic protein from bovine brain by taking advantage of the residue-specific ionic change induced by the phosphorylation or dephosphorylation step. The protein is first phosphorylated with a cAMP-dependent protein kinase, then digested with trypsin and subjected to preliminary reversed-phase HPLC (polystyrene-based) under alkaline conditions. Phosphorylated peptides were distinguished and separated on the basis of the differences in the retention times of the tryptic products between the phosphorylated and the non-phosphorylated proteins. Treatment of phosphorylated peptides with alkaline phosphatase regenerated the respective free peptides with an increase, characteristic of each peptide, in the mobility in HPLC. Each phosphorylated peptide could thus be identified by measuring this increase.

In this study, the two phosphorylated sites (threonine-34 and serine-55) that had been described by Carnegie *et al.*[9] were selectively determined by the procedures described above. Further, phosphorylation of the serine-115 residue, which was located at the encephalitogenic determinant of the myelin basic protein, was found for the first time.

EXPERIMENTAL

Materials

The myelin basic protein and cAMP-dependent protein kinase (type II) were

isolated to homogeneity from bovine brain according to the methods of Deibler et al.[20] and Miyamoto et al.[21], respectively. Trypsin, purified by aminocaproyl-p-aminobenzamidine–Sepharose 4B chromatography[22], was a gift from Dr. Fujikawa (University of Washington, Seattle, WA, U.S.A.). Alkaline phosphatase (E. coli) was purchased from Worthington Biochemical (Freehold, NJ, U.S.A.). Aminopeptidase M was a product of Calbiochem-Behring (San Diego, CA, U.S.A). Sequential grade chemicals for sequenching and other solvents were obtained from Wako (Osaka, Japan).

Phosphorylation

[γ-³²P]ATP was prepared by the method of Post and Sen[23] using [³²P]orthophosphate obtained from Japan Radioisotope Assoc. (Tokyo, Japan).

The myelin basic protein was phosphorylated by the method of Miyamoto and Kakiuchi[24] with ATP or [γ-³²P]ATP, and radioactive phosphorylated amino acids were determined by thin-layer electrophoresis[25].

Tryptic digestion

The native and phosphorylated myelin basic proteins, 1.8 mg (100 nmol) of each, were suspended separately in 1 ml of 0.1 M ammonium hydrogen carbonate (pH 8.0) containing 10 mM calcium acetate. A solution of purified trypsin, 2.3 µg (1 nmol) in 10 µl of 0.1 N hydrochloric acid, was added to the protein suspension and the mixture was incubated at 37°C for 3 h with shaking and then lyophilized. The incubation with added calcium ions was used to overcome the resistance of an arginyl bond in the neighbouring acidic residues (or residues acidified by phosphorylation) to tryptic cleavage[26]; 10 mM calcium acetate overcame the resistance to tryptic attack of the peptide bond between arginine-32 and aspartic acid-33.

Apparatus

The HPLC analysis was performed on a Waters liquid chromatograph (compact type) equipped with Model 660 and 6000 A solvent delivery systems, a Model U6K universal sample injector and Model 440 (280 nm) and a Model 441 (214 nm) UV detectors (Waters Assoc., Milford, MA, U.S.A.). The chromatographic separation was achieved under two different conditions: (1) on a TSK-gel styrene 250 column (polystyrene, particle size 10 µm, 600 × 4 mm I.D.; Toyo Soda, Tokyo, Japan), eluted with 0.01% ammonia solution containing 30 and 60% of methanol (alkaline conditions); (2) on a NOVA-PAK C_{18} cartilage column (particle size 5 µm, 100 × 8 mm I.D.) with a Waters RCM 100 radial compression model, eluted with 0.1% TFA containing 5 and 60% of acetonitrile (acidic conditions). The concentrations of methanol and acetonitrile were increased linearly from 30 to 60% and from 5 to 60%, respectively, over 30 min. The solvent flow-rate was set at 0.8 ml/min.

Amino acid analysis was conducted on a Waters ALC/GPC Model 420 amino acid analyser using o-phthalaldehyde[27]. Automated sample injections were performed with a Waters Model 710b intelligent sample processor.

Automated sequence analysis was performed on a JEOL Model JAS-47K sequence analyser (JEOL, Tokyo, Japan) according to the method of Edman and Begg[28] as modified by Brauer et al.[29] using polybrene[30], which gave consistent re-

petitive yields of 90–94%. The phenylthiohydantoin derivatives of amino acids were identified by reversed-phase HPLC[31].

RESULTS AND DISCUSSION

HPLC of tryptic digests from native and phosphorylated myelin basic protein

Fig. 1 shows the elution profiles of the tryptic digests of the native and phosphorylated proteins (referred to as N and P, respectively, for their fragments) obtained with the styrene 250 column under identical conditions. There are some differences in the profiles between the native and phosphorylated proteins. No fragment was detected corresponding to fragment N-2 or N-5 on the chromatogram of the tryptic digest of the phosphorylated protein (Fig. 1B), whereas the chromatogram of the digest of the native protein lacked fragments corresponding to P-2 and P-3 (Fig. 1A). Fragments P-2 and P-3 may be the phosphorylated forms of some peptides, as the phosphorylated forms of peptides migrate faster on the chromatogram than the corresponding dephosphorylated forms owing to their weak hydrophobicity. Frag-
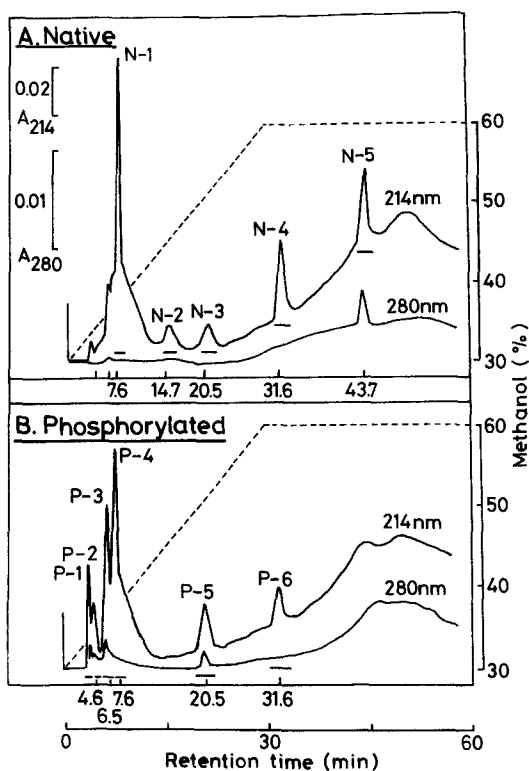

Fig. 1. HPLC of tryptic digests from native and phosphorylated myelin basic proteins. The native and phosphorylated myelin basic proteins (100 nmol each) were digested with trypsin (1 nmol) at 37°C for 3 h in 1 ml of 0.1 M ammonium hydrogen carbonate (pH 8.0) containing 10 mM calcium acetate. Aliquots (3 nmol) of the digests were chromatographed on a column of TSK-gel styrene 250 under the conditions described under Experimental. The fractions indicated by the horizontal bars were collected. The linear gradient is shown by the dashed line.

ments N-1 and P-4 appeared with the same retention time (7.6 min), as also did fragments N-4 and P-6 (31.6 min). The fragments chromatographed with the same retention time may contain either identical peptides or different peptides also chromatographed with the same retention time (20.5 min), whereas they differed from each other in their absorption at 280 nm. No fragment was detected corresponding to fragment N-5 (280 nm positive) on the chromatogram of the tryptic digest of the phosphorylated protein. Fragment P-5, the phosphorylated form of fragment N-5, probably fell at the position of fragment N-3.

The separation of the tryptic products on the NOVA-PAK C_{18} column under acidic conditions was excellent, and the fragments could each be identified as the segments composed of peptides in the protein by amino acid analysis. No difference was, found, however, in the chromatogram of the fragments between the native and phosphorylated proteins under acidic conditions and phosphorylated and non-phosphorylated peptides could not be distinguished by their chromatographic mobilities in 0.1% TFA (data not shown). The phosphorylaated and non-phosphorylated forms of the peptides could be distinguished under alkaline conditions as described above. On the basis of these results, fragments P-2, P-3 and P-5 were conducted to be the phosphorylated peptides.

HPLC of tryptic products before and after dephosphorylation

Dephosphorylation of the products was carried out by the method of Shoji et al.[15] using alkaline phosphatase (E. coli, 37°C for 60 min in 0.1 M ammonium hydrogen carbonate, pH 8.0). As Fig. 2 shows, dephosphorylation caused increases in the retention times of P-2, P-3 and P-5. Fragment P-2 (4.5 min) appeared at a retention time of 14.7 min, which was the same as that of fragments N-2, after treatment with alkaline phosphatase. After dephosphorylation, fragments P-3 (6.5 min) and P-5 (20.5 min, the 280 nm positive fragment) moved to the same positions as those of N-1 (7.6 min) and N-5 (43.7 min), respectively. Neither changes in the mobility of
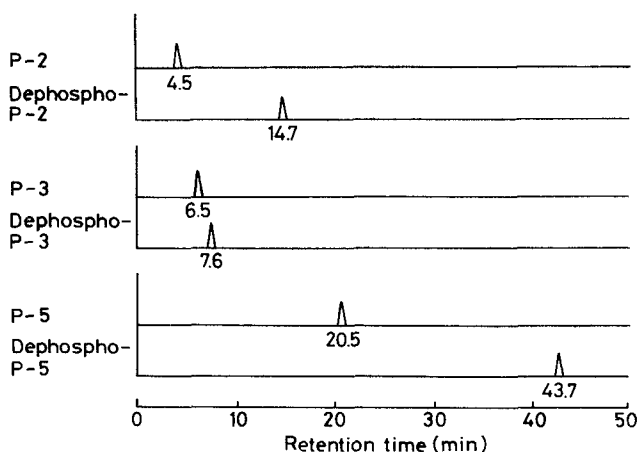

Fig. 2. Retention times of phosphorylated peptides before and after dephosphorylation. The phosphorylated fragment (3 nmol) was treated with alkaline phosphatase (0.6 nmol, 37°C, 60 min) in 50 µl of 0.1 M ammonium hydrogen carbonate (pH 8.0) and then chromatographed under the conditions described in Fig. 1.

TABLE I

AMINO ACID COMPOSITION OF PHOSPHORYLATED PEPTIDES

The samples (3 nmol) were hydrolysed in 6 N HCl at 110°C for 24 h under vacuum, and the hydrolysates were analysed with an amino acid analyser using o-phthalaldehyde[27] as described under Experimental.

Amino acid residues	Residues per molecule*		
	P-2, 53–57	P-3, 33–42	P-5, 114–130
Asx, D and N		1.99(2)	
Thr, T		0.97(1)	
Ser, S	0.81(1)	0.80(1)	0.80(1)
Glx, E and Q			2.01(2)
Pro, P			1.19(1)
Gly, G	2.00(2)	2.01(2)	6.00(6)
Ala, A			1.00(1)
Met, M			
Ile, I		1.00(1)	
Leu, L		2.07(2)	
Tyr, Y			1.08(1)
Phe, F			1.90(2)
His, H			
Lys, K	0.80(1)		0.76(1)
Arg, R	0.80(1)	1.05(1)	1.10(1)
Trp, W**			Trace(1)
No. of residues	5	10	17
Yield (%)	28	30	36

  * Number of residues determined by amino acid analysis. Numbers in parentheses are those in the sequence[6].
  ** Determined by the method of Delange[32].

P-1, P-4 or P-6 nor peptide bond cleavage of any fragments were detected on treatment with alkaline phosphatase.

These differences in the retention times before and after dephosphorylation apparently resulted from elimination of the phosphoryl group from the phosphorylated peptides by alkaline phosphatase attack. The phosphorylated peptides could be purified selectivity from the complex digests by taking advantage of the changes in hydrophobicity and chromatographic mobility caused by the elimination of the phosphoryl group.

Amino acid composition and partial amino acid sequence of phosphorylated peptides

The phosphorylated fragments were selectively purified after dephosphorylation by HPLC under the same conditions as described above. Portions of purified fragments were hydrolysed in 6 N HCl at 110°C for 24 h under vacuum and submitted to amino acid analysis. Other portions were hydrolysed with aminopeptidase M[32] in order to determine tryptophan. The remainder of the fragments (5 nmol) were subjected to amino acid sequence analysis. The amino acid compositions of the three phosphorylated peptides are given in Table I. The compositions agree with those of the peptides composed of residues 53–57 33–42, and 114–130. These fragments were identified as peptides P-2, P-3 and P-5 from their partial sequences: peptide P-2 sur-

```
            10                    20                        30
Ac  A  S  A  Q  K  R  P  S  Q  R  S  K  Y  L  A  S  A  S  T  M  D  H  A  R  H  G  F  L  P  R


        *           40                    50          *           60
    H  R  D  T  G  I  L  D  S  L  G  R  F  F  G  S  D  R  G  A  P  K  R  G  S  G  K  D  G  H
        ────────────────────                               ────────────────
            P-3                                                P-2
            70                    80                        90
    H  A  A  R  T  T  H  Y  G  S  L  P  Q  K  A  Q  G  H  R  P  Q  D  E  N  P  V  V  H  F  F


            100                   110          *           120
    K  N  I  V  T  P  R  T  P  P  P  S  Q  G  K  G  R  G  L  S  L  S  R  F  S  W  G  A  E  G
                                                            ─────────────────
                                                                  P-5
            130                   140                       150
    Q  K  P  G  F  G  Y  G  G  R  A  S  D  Y  K  S  A  H  K  G  L  K  G  H  D  A  Q  G  T  L
    ──────────────────────────────


            160                   170
    S  K  I  F  K  L  G  G  R  D  S  R  S  G  S  P  M  A  R  R
```
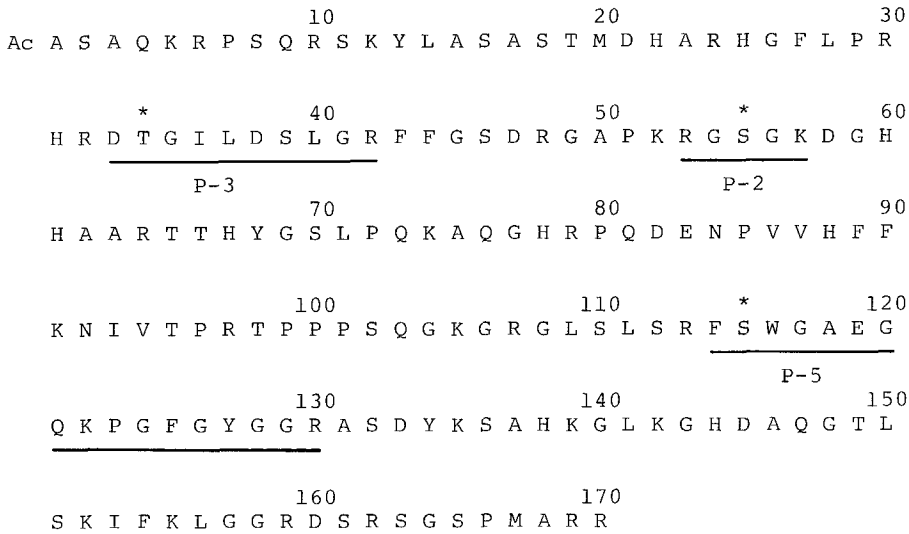
Fig. 3. Amino acid sequence of a myelin basic protein from bovine brain[6]. Ac = acetyl. The one-letter code is shown in Table I. The phosphorylated segments generated by tryptic attack were determined by this procedure and are shown as horizontal bars. Threonine-34, serine-55 and serine-115 residues marked with asterisks are phosphorylation sites.

rounding phosphorylated serine-55, Arg–Gly–Ser*–Gly–Lys; peptide P-3 surrounding phosphorylated threonine-34, Asp–Thr*–Gly–Ile–Leu–Asp–Ser–Leu–Gly–Arg; peptide P-5 surrounding phosphorylated serine-115, Phe–Ser*–Trp–Gly–Ala–Glu–Gly–Gln–Lys–Pro–Gly–Phe–Gly–Try–Gly–Gly–Arg. These sequences are found in the complete sequence of the myelin basic protein Fig. 3)[6]. The asteriks and the arrows represent the phosphorylation sites and the results of the sequencing run, respectively. In this study, the incorporation of [^{32}P]phosphates was determined to be 2.96 mol per enzyme and phosphorylated serine and phosphorylated threonine were also identified by thin-layer electrophoresis[25]. The serine-55 and -115 residues of peptide P-2 and P-5 should have been phosphorylated, because only one serine residue is included in each fragment, as shown in the complete sequence (Fig. 3). On the other hand, fragment P-3 has two potential sites of phosphorylation (at threonine-34 and serine-39); of these, the threonine residue was confirmed to be a phosphorylation site by thin-layer electrophoretic analysis[25] of its acid hydrolysate.

Carnegie et al.[9] reported that threonine-34, serine-55 and serine-110 in the myelin basic protein were the sites of in vitro phosphorylation with the cAMP-dependent protein kinase from bovine brain. In this study, we identified serine-115 as well as threonine-34 and serine-55 as the phosphorylation sites, but could not detect phosphorylation at serine-110. Turner et al.[11] could not identify serine-110 as the phosphorylation site of the protein and suggested the phosphorylation of serine-115 instead of serine-110.

Martenson et al.[33] recently reported that in a myelin basic protein from rabbit brain, the serine-113 residue that corresponds to serine-115 of the bovine protein was also phosphorylated in vivo by a cAMP-dependent protein kinase from rabbit brain.

In this study, we isolated selectively the phosphorylated peptides by the simple

and rapid chromatographic method and identified threonine-34, serine-55 and serine-115 as phosphorylation sites. The serine-115 residue, located in the encephalitogenic segment of the protein, is a newly found site of *in vitro* phosphorylation by the cAMP-dependent protein kinase from bovine brain. Phosphorylation and dephosphorylation at this residue, in addition to the threonine-34 and serine-55 residues, may be related to the physiological function of the myelin basic protein.

REFERENCES

1 E. H. Eylar, J. Salk, G. C. Bereridge and R. L. V. Brown, *Arch. Biochem. Biophys.*, 132 (1969) 34.
2 E. H. Eylar, J. Caccam, J. J. Jackson, F. C. Westall and A. B. Robinson, *Science*, 168 (1970) 1220.
3 E. H. Eylar, F. C. Westall and S. Brostoff, *J. Biol. Chem.*, 246 (1971) 3418.
4 E. H. Eylar and M. Thompson, *Arch. Biochem. Biophys.*, 129 (1969) 468.
5 Y. Oshiro and E. H. Eylar, *Arch. Biochem. Biophys.*, 138 (1970) 606.
6 E. H. Eylar, *Proc. Nat. Acad. Sci. U.S.*, 67 (1970) 1425.
7 F. C. Westall, A. B. Robinson, J. Caccam, J. Jackson and E. H. Eylar, *Nature (London)*, 229 (1971) 22.
8 P. R. Carnegie, B. E. Kemp, P. R. Dunkley and A. W. Murray, *Biochem. J.*, 135 (1973) 569.
9 P. R. Carnegie, P. R. Dunkley, B. E. Kemp and A. W. Murray, *Nature (London)*, 249 (1974) 147.
10 E. Miyamoto, S. Kakiuchi and Y. Kakimoto, *Nature (London)*, 249 (1974) 150.
11 R. S. Turner, R. L. Raynor, C.-H. Jen Chou, B. E. Kemp, R. F. Kibler and J. F. Kuo, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 42 (1983) 900.
12 E. G. Krebs and J. A. Beavo, *Annu. Rev. Biochem.*, 48 (1979) 923.
13 C. S. Rubin and O. M. Rosen, *Annu. Rev. Biochem.*, 44 (1975) 831.
14 P. Cohen, *Nature (London)*, 296 (1982) 613.
15 S. Shoji, K. Titani, J. G. Demaille and E. H. Fisher, *J. Biol. Chem.*, 254 (1979) 6211.
16 K. A. Walsh, L. H. Ericsson, D. C. Parmelee and K. Titani, *Annu. Rev. Biochem.*, 50 (1981) 261.
17 K. Titani, T. Sasagawa, K. Resing and K. A. Walsh, *Anal. Biochem.*, 123 (1982) 408.
18 T. Sasagawa, K. Titani and K. A. Walsh, *Anal. Biochem.*, 128 (1982) 371.
19 W. C. Mahoney and M. A. Hermodson, *J. Biol. Chem.*, 255 (1980) 11199.
20 G. E. Deibler, R. E. Martenson and M. W. Kies, *Prep. Biochem.*, 2 (1972) 139.
21 E. Miyamoto, G. L. Petzold, J. F. Kuo and P. Greengard, *J. Biol. Chem.*, 248 (1973) 179.
22 K. D. Jany, W. Keil, H. Meyer and H. H. Kiltz, *Biochim. Biophys. Acta*, 453 (1976) 62.
23 R. L. Post and A. K. Sen, *Methods Enzymol.*, 10 (1967) 773.
24 E. Miyamoto and S. Kakiuchi, *J. Biol. Chem.*, 249 (1974) 2769.
25 R. A. Nemenoff, Y. C. Kwok, G. I. Shulman, P. J. Blackshear, R. Osathanondh and J. Avruch, *J. Biol. Chem.*, 259 (1984) 5058.
26 S. Shoji, L. H. Ericsson, K. A. Walsh, E. H. Fischer and K. Titani, *Biochemistry*, 22 (1983) 3702.
27 P. Bohlen and M. Mellet, *Anal. Biochem.*, 94 (1979) 313.
28 P. Edman and G. Begg, *Eur. J. Biochem.*, 1 (1967) 80.
29 A. W. Brauer, M. N. Margolies and E. Haber, *Biochemistry*, 14 (1975) 3029.
30 G. E. Tarr, J. F. Beecher, M. Bell and D. J. Mckean, *Anal. Biochem.*, 84 (1978) 622.
31 J. Hermann, K. Titani, L. H. Ericsson, R. D. Wade, H. Neurath and K. A. Walsh, *Biochemistry*, 17 (1978) 5672.
32 R. J. Delange, *J. Biol. Chem.*, 245 (1970) 907.
33 R. E. Martenson, M. J. Law and G. E. Deibler, *J. Biol. Chem.*, 258 (1983) 930.