Biochimica et Biophysica Acta, 662 (1981) 1–7
Elsevier/North-Holland Biomedical Press

BBA 69390

A STUDY WITH MODEL SUBSTRATES OF THE STRUCTURE OF THE SITES PHOSPHORYLATED BY RAT LIVER CASEIN KINASE TS

FLAVIO MEGGIO, ARIANNA DONELLA DEANA and LORENZO A. PINNA

Institute of Biological Chemistry, University of Padova, Via F. Marzolo 3, 35100 Padova (Italy)

(Received March 24th, 1981)

Key words: Casein kinase TS; Phosphorylated site structure; Model substrate; (Rat liver)

Two new sites phosphorylated by rat liver cyclic AMP-independent casein kinase TS have been identified in denatured pepsin and soybean antiprotease C-II, exhibiting the sequences: Cys-Ser-Ser(P)-Ile-Asp-Ser and His-Ser_3(P)-Asp-Asp-Glu, respectively. Their phosphorylation efficiency has been compared to that of previously identified sites and the effects of chemical modifications in the vicinity of the phosphorylatable residue have been studied. The results obtained support the following conclusions: 1. All sites affected by casein kinase TS conform to the sequence: Ser/Thr-X-Glu/Asp which is also believed to be required by the mammary gland casein kinase. Threonine appears to be less suitable for phosphorylation than serine. The presence of some additional residues on the C-terminal side also appears to be required. 2. X can be either an additional acidic residue or a neutral one, but not a basic residue. The contiguity of an acidic cluster to the C-terminal side of the target greatly improves the phosphorylation efficiency. 3. The residues N-terminal to the target one do not seem to be relevant for determining the site recognition by the protein kinase. 4. The predicted secondary structure constantly occurring at the phosphorylation sites is the β-turn: apparently the bend must include both the target residue and the acidic determinant at the $n + 2$ position.

Introduction

A very crucial problem in the field of protein phosphorylation concerns the factors determining the specificity of protein kinases toward their physiological targets, in terms of both substrates and site recognition. Such factors, for the cyclic AMP-dependent protein kinase have been successfully studied with the aid of model substrates like caseins [1], lysozyme [2], protamines [3] and synthetic peptides [4–6], making possible a detailed definition of the structural requirements of this enzyme.

A similar approach is especially suitable for the characterization of the several protein kinases whose natural substrates are still uncertain. In particular, studies concerning the site specificity of the two main rat liver casein kinases (belonging to the class of protein kinase *b*, according to the Weller classification (Ref. 7 p. 11)) have been previously undertaken with the aid of native and partially dephosphorylated casein fractions as model substrates [8–11]. The results obtained have clearly shown that the cytosol casein kinase TS constantly affects seryl and threonyl sites having two acidic residues adjacent to their C termini. These residues are to be considered also suitable for the physiological mammary gland casein kinase(s), which recognizes the amino acid triplet Ser-X-Glu/Asp [12–15], thus suggesting that casein kinases of the TS type might be phylogenetically, though not functionally related to the mammary gland enzyme(s).

The results of the present paper have been

Abbreviations: AE, aminoethylated; DMSO, dimethyl sulfoxide; TPCK, L-1-tosylamide-2-phenyl-ethyl-chloromethylketone.

0005-2744/81/0000–0000/$02.50

obtained by extending to other model substrates the studies concerning the site specificity of casein kinase TS and by comparing the suitability of sites differing for definite local features. Altogether they make possible a detailed definition of the structural factors determining the site recognition and influencing the efficiency of the protein kinase reaction.

Experimental

Casein kinase TS was isolated and purified from rat liver cytosol according to Meggio et al. [16], postponing however the Sepharose 6B gel filtration to the phospho-cellulose step. 50 μM phenylmethylsulfonyl fluoride was also present throughout the whole preparation.

Purified casein fractions were prepared according to Mercier et al. [17]. Low molecular weight soybean antiproteases were isolated according to Odani and Ikenaka [18]. Samples of antiproteases C-II, D-II and E-I were kindly provided by Dr. S. Odani. Pepsin was from Worthington: it was denatured by alkali exposure according to Tang and Hartley [19]. Reduction and carboxymethylation of antiproteases and pepsin were performed according to Odani et al. [20]. Aminoethylated pepsin (AE-pepsin) was prepared according to Chen et al. [21]. The synthetic peptides Arg-Arg-Ser-Thr-Glu-Ala and Ser-Thr-Glu-Ala were kindly provided by Prof. F. Marchiori.

Dephosphorylated α_{s1}-casein and pepsin were obtained by incubation with potato acid phosphatase under conditions previously described [10].

The enzymatic phosphorylation of proteins was performed by incubating them for 30 min at a final concentration of 2 mg/ml with casein kinase TS and [γ-^{32}P]ATP under conditions previously described [8]. For kinetic experiments the incubation time was 10 min.

CNBr, trypsin and chymotrypsin digestions were performed respectively as described [22,23,21]. Sometimes the pepsin fragment CB2 was submitted to further CNBr digestion at 37°C according to Chen et al. [21] for increasing the amount of cleavage at Met_{80}. Specific cleavage at tryptophan residues was obtained by DMSO/HBr treatment [24].

The CNBr-digested pepsin was submitted to gel chromatography through a Sephadex G-75 column (1.8 X 74 cm) equilibrated with 0.1 M NH_4HCO_3 by a procedure similar to that described by Chen et al. [21] whose nomenclature for pepsin fragments is used in the present paper.

The smaller fragments resulting from enzymatic and DMSO/HBr digestion were usually isolated by Sephadex G-50 gel chromatography in 15% formic acid and by high voltage paper electrophoresis at pH 1.9 (formic acid/acetic acid/H_2O, 55 : 156 : 2189) and 6.4 (pyridine/acetic acid/H_2O, 200 : 8 : 800). Ser-^{32}P and Thr-^{32}P were isolated as previously described [10].

The secondary structure at the phosphorylation sites was predicted according to the Chou and Fasman empirical model [25].

Results and Discussion

Phosphorylation site(s) in pepsin

All the phosphorylation sites of casein kinase TS previously identified either in casein fractions [8,9] or in soybean Bowmann-Birk trypsin inhibitor [26] are N-terminal to two or more acidic residues, though only a single acidic residue, in the position $n + 2$, is believed to be the determinant for the mammary gland casein kinase [13]. In order to check whether an additional acidic residue might represent a stringent requirement of rat liver casein kinase TS or not, pepsin was chosen as the next model substrate as it contains up to ten potential sites of phosphorylation exhibiting the sequence: Ser/Thr-X-Glu/Asp, where X however is constantly a residue other than an acidic one.

Unlike caseins which are rather unfolded molecules, pepsin is characterized by a compact tertiary structure stabilized through three disulfide bridges. Therefore, alkali-denatured pepsin was reduced and either aminoethylated or carboxymethylated before being submitted to phosphorylation by casein kinase TS. Its phosphorylation rate was found to be comparable to that of α_{s1}-casein, a fairly good substrate for casein kinase TS [8]. Ser-^{32}P accounts for more than 90% of the radioactivity incorporated in AE-pepsin (data not shown) thus indicating that none of the four threonine potentially suitable for casein kinase TS are appreciably affected by the enzyme. Such a finding fits with the previous suggestion [10] that threonine behaves less suitably as a target than serine.

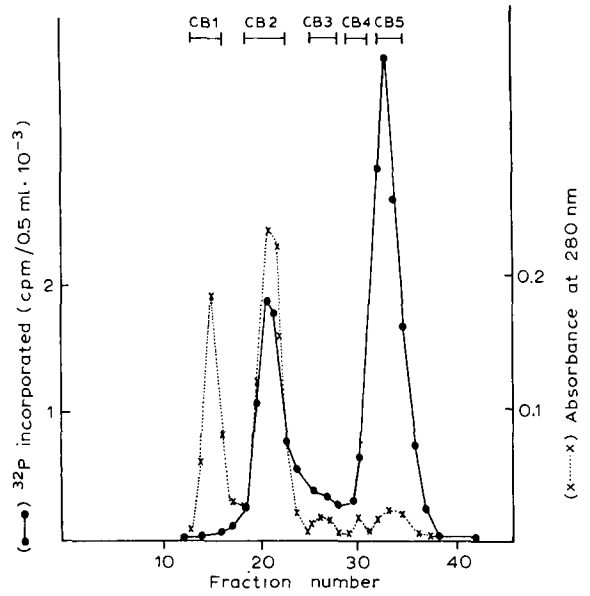

Fig. 1. Sephadex G-75 gel chromatography of CNBr-digested AE-pepsin phosphorylated by casein kinase TS. The same profile was obtained with pepsin previously dephosphorylated by acid phosphatase. Peptides are termed according to Chen et al. [21]. ●——●, ^{32}P incorporated and X ····· X, A_{280nm}.

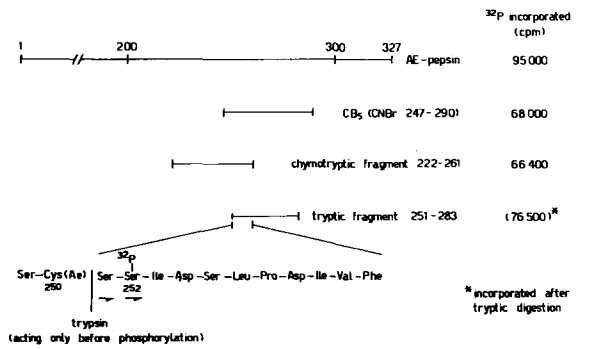

Fig. 2. Identification of the main phosphorylation site of casein kinase TS in AE-pepsin. The same sample of ^{32}P-labeled AE-pepsin was digested separately with CNBr and chymotrypsin. The CB5 fragment, obtained by CNBr digestion and gel filtration (see Fig. 1) was further purified [21]. The radioactive chymotryptic peptide was isolated in an essentially pure form by a single Sephadex G-50 gel filtration (in 15% formic acid) taking advantage of its uniquely large size compared to the remaining small chymotryptic products of pepsin. Its purity was confirmed by paper electrophoresis and aminoacid analysis, consistent with that of the 222-261 fragment of pepsin.

Upon CNBr digestion of ^{32}P-labeled AE-pepsin followed by gel filtration the main site(s) of phosphorylation was localized within the fragment 247-290 (CB5) (Fig. 1) accounting for 60–90% of the radioactivity incorporated also after further purification according to Chen et al. [21] (data not shown). Virtually the same profile was obtained with R-carboxymethylated pepsin and with samples of pepsin dephosphorylated with acid phosphatase before incubation with the casein kinase.

The procedure followed for the identification of the phosphorylated residue in CB5 is outlined in Fig. 2. The labeled site was firstly localized between Val_{247} and Phe_{261}, i.e., in the region of CB5 overlapped by the radioactive chymotryptic fragment 222-261. Since phosphorylation prevented trypsin from acting on aminoethylated Cys_{250} (suggesting the close proximity of this residue to the phosphorylated residue), the tryptic fragment 251-283 was obtained by 2 h digestion of unlabeled purified CB5 with trypsin (TPCK-treated, to minimize chymotryptic activity). The digested sample (boiled for 5 min to inactivate trypsin) was submitted to phosphorylation by casein kinase TS, freed from [γ-^{32}P]ATP by Sephadex G-25 gel filtration and resolved by Sephadex G-50 gel chromatography and high voltage paper electrophoresis: any phosphorylation of the two small N- and C-terminal fragments, Val-Ile-Ser-Cys-(AE) and Thr-Ser-Gly-Phe-Glu-Gly-Hse was ruled out. The largest predicted fragment 251-283, accounting for 80–90% of the ^{32}P incorporated, was submitted to two cycles of Edman degradation and released its radioactivity as $^{32}P_i$ during the second cycle. Since the phenylthiohydantoin derivative of phosphoserine spontaneously undergoes hydrolysis to inorganic phosphate [27] it can be concluded that the main target of casein kinase TS in AE-pepsin is Ser_{252}, included within the sequence:

-Cys(AE)-Ser-Ser(P)-Ile-Asp-Ser-

Attempts have also been made to localize the minor phosphorylation site(s) accounting for the radioactivity recovered in the large CNBr fragment 1-199 (CB2, see Fig. 1). The failure of previous dephosphorylation by acid phosphatase to enhance the ^{32}P incorporation into such a fragment ruled out the involvement of Ser_{68}, which is the only residue

phosphorylated in native pepsin [28] and which is also a good target for the mammary gland casein kinase [15]. Rather the phosphorylated residue(s) apparently lie in the 81–199 fragment obtainable by partial breakdown of Met_{80} by further CNBr digestion of CB2 at 37°C [21], since by such a procedure the radioactive fragment 81-199 isolated from labeled CB2 accounted for the whole radioactivity incorporated, after correction for its 25–30% yield (data not shown). Three potential sites for phosphorylation for casein kinase TS, Ser_{147}, Ser_{157} and Ser_{185}, are included in such a region. However the phosphorylation of Ser_{185} was ruled out by chemical cleavage of trytophan residues with DMSO/HBr reagent [24] giving rise to the unlabeled 182-190 fragment. It is likely therefore that Ser_{147} and/or Ser_{157} are the minor target(s) of casein kinase TS. Both these residues, as well as the main phosphorylation site Ser_{252}, are lying within predicted β-turns, which is also a common feature of most of the phosphorylated sites previously identified in casein fractions [9].

The available information about the suitability to casein kinase TS of the 10 potential sites of phosphorylation of pepsin are summarized in Table I. The phosphorylation of the four threonine residues and that of Ser_{68} and Ser_{185} (both located outside predicted β-turns) are definitely ruled out by our data. A minor phosphorylation of Ser_{147} and Ser_{157}, both included within predicted β-turns, though not directly demonstrated, is however consistent with our results. The main target is anyway Ser_{252} the suitability of which contrasts with the unfitness of Ser_{296} belonging to the unlabeled N-terminal CB1 fragment of pepsin (see Fig. 1). Though both these serine residues display the sequence Ser-X-Glu(Asp) and are included within predicted β-turns, it should be noted

TABLE I

SUITABILITY TO CASEIN KINASE TS OF THE PHOSPHORYLATION SITES OF PEPSIN EXHIBITING THE SEQUENCE: Ser/Thr-X-Glu/Asp

The target residues are numbered. Brackets include the predicted β-turns. Secondary structures were predicted to Chou and Fasman [25].

Sites	Predicted secondary structure	Suitable to casein kinase-TS
68 Ala-Thr-SerP-Gln-Glu	helical	Not phosphorylated
147 Leu-Val[Ser-Gln-Asp-Leu]	β-turn	Possibly phosphorylated [a]
157 Leu-Ser[Ser-Asn-Asp-Asp]	β-turn	Possibly phosphorylated [a]
185 Pro-Val-Ser-Val-Glu	coil	Not phosphorylated
252 Cys-Ser[Ser-Ile-Asp-Ser]	β-turn	Main phosphorylation site
296 [Thr-Ser-Ser-Gly]Glu	β-turn	Not phosphorylated
63 Asp-Ser-Thr-Phe-Gly	helical	Not phosphorylated
193 Gln-Ile-Thr-Leu-Asp	coil	Not phosphorylated
198 Ser-Ile[Thr-Met-Asp-Gly]	β-turn	Not phosphorylated
262 Val-Phe-Thr-Ile-Asp	β-sheet	Not phosphorylated

[a] The evidence for the phosphorylation of these sites is indirect, resting on the assumption that other residues not fulling the suitable primary structure cannot be affected by the kinase.

that Ser_{252} lies at the 1st position while Ser_{296} occupies the 3rd position within the tetrapeptide forming the β-bend, in such a way that the critical acidic determinant Glu_{298} is thus excluded.

Phosphorylation efficiency at different sites

In order to check the relevance of the N-terminal residues for the site recognition by casein kinase TS, the rates of phosphorylation of Ser_{252} in carboxymethylated, aminoethylated and aminoethylated-trypsin-digested pepsin were compared since all these modifications affect Cys_{250}, near to the N terminus of Ser_{252}. As shown in Fig. 3 the phosphorylation rates are not significantly different indicating that the replacement of a basic group for an acidic one or even the detachment of all the N-terminal residues but one, do not critically influence the protein kinase reaction. The preservation of the disulfide bridge between Cys_{250} and Cys_{283} however almost completely prevents the phosphorylation of Ser_{252}, even in alkali-denatured pepsin, probably due to some local steric hindrance.

Since pepsin Ser_{252} is the only identified residue phosphorylated by casein kinase TS which is characterized by just one acidic residue at the $n + 2$ position, rather than by two or three C-terminal acidic residues, it was interesting to compare its phosphorylation efficiency with those of other potential

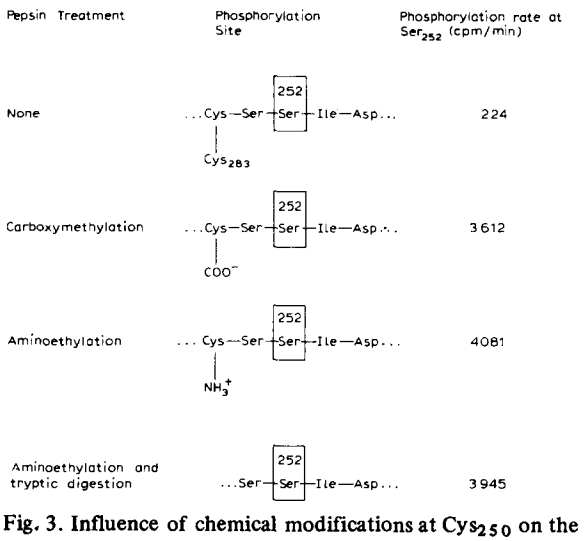

Fig. 3. Influence of chemical modifications at Cys_{250} on the phosphorylation rate of pepsin Ser_{252}.

TABLE II

KINETIC CONSTANTS FOR SOME SUBSTRATES OF CASEIN KINASE TS

Phosphorylatable residues are numbered. Acidic residues are underlined. One unit is defined as 1 pmol ^{32}P transferred to protein in 1 min under our conditions. K_m and V were estimated by the double-reciprocal plot method, assuming that the molecular weights of the proteins were 24 000 (α_{s1}- and β-casein) and 35 000 (pepsin).

Substrate	Phosphorylation site	K_m app. (μM)	V (units)
AE-pepsin	252 Ser-Ile-Asp-Ser	253	160
50% dephospho-α_{s1}-casein	68 Ser-Glu-Glu-Ile	57	246
β-casein	41 Thr-Glu-Asp-Glu	60	225
α_{s1}-casein	49 Thr-Glu-Asp-Gln	57	36
Soybean antiprotease E-1	72 Ser-Arg-Asp-Asp	–	0
Synthetic peptide	1 Ser-Thr-Glu-Ala	–	0
Synthetic peptide	3 Arg-Arg-Ser-Thr-Glu-Ala	–	0

sites of phosphorylation previously identified. As shown in Table II pepsin Ser_{252} is not as good as substrate as most of the residues N-terminal to two or three acidic residues. Actually the relevance of C-terminal, negatively-charged groups is stressed by the high phosphorylation of Thr_{41} of β-casein, occurring 10-times faster than that of Thr_{49} of α_{s1}-casein which differs only for a glutamine replaced for a glutamic acid at $n + 3$ position, and by the failure to phosphorylate the soybean antiprotease E1 whose potential site of phosphorylation exhibits a positively charged Arg between Ser and Asp. It should also be noted that the synthetic peptides Arg-Arg-Ser-Thr-Glu-Ala and Ser-Thr-Glu-Ala, at concentrations up to 1000 μM, are not affected by casein kinase TS.

All these data support the view that a cluster of negatively-charged groups adjacent to the C terminus of the target residue represents the optimal condition for casein kinase TS activity, whereas the mammary

gland casein kinase(s), also recognizing the same Ser/Thr-X-Glu/Asp triplet, apparently does not display such a preferential requirement [13]. Interestingly however extremely acidic sequences have been found on the C-terminal side of the serine residues which are phosphorylated through a cyclic AMP-independent mechanism in nucleolar non-histone proteins of Novikoff hepatoma [29]. It is quite conceivable therefore that a protein kinase of the casein kinase TS class may be responsible for such phosphorylations.

Phosphorylation site in soybean antiprotease C-II

The finding that the low molecular weight soybean antiprotease C-II behaves as an oustandingly suitable model substrate of casein kinase TS [30] prompted us to the identification of the residue(s) responsible for such a fast phosphorylation, in order to obtain additional information on the structural features improving the phosporylation efficiency. ^{32}P-labeled, reduced and carboxymethylated C-II was thus digested with trypsin and chymotrypsin and the digestion products fractionated essentially according Odani and Ikenaka [31]. The whole radioactivity incorporated was accounted for by the peptide T1A, corresponding to the N-terminal residues and still including six serine residues [31]. Its N-terminal serine however was not phosphorylated since it could be removed by Edman degradation without any appreciable loss of radioactivity. On the other hand the phosphorylation of Ser_{10-11} can be excluded since the homologous serine residues of BBI, included within an identical sequence of 10 consecutive residues [31], is not affected by casein kinase TS [26]. Hence the phosphorylation site must be located within the cluster Ser_{4-6}, and most likely corresponds to Ser_5 and/or Ser_6, since Ser_4 lacks the typical acidic determinant at the $n + 2$ position. The primary structure at the phosphorylation site of C-II shown in Table III, corroborates the conclusion that a cluster of acidic residues C-terminal to the target residue enhances the casein kinase reaction.

Moreover, the negligible phosphorylation efficiency observed with inhibitor D-II (Table III) is noticeable considering its striking homologies with C-II [32] including a very similar phosphorylation site in its N-terminal region. Such a site however, also shown in Table III, is characterized by a tyrosine inserted between the serine and a block of four acidic residues. As shown by the phosphorylation of pepsin Ser_{252} a neutral residue in such a position should not prevent the protein kinase reaction. In the case of D-II however the tyrosine residue causes a shift toward the left of the predicted β-turn, which still includes the serine residues but leaves out the acidic residues, while in C-II two acidic residues are still lying in the bend (Table III). These data are consistent with those obtained with pepsin and altogether they support the hypothesis that the inclusion of both the target residue(s) and the acidic determinant(s) within the same β-turn might indeed represent a structural requirement of this type of protein kinase.

Acknowledgments

The skilful technical assistance of Mr. Giuseppe Tasinato and the helpful secretarial aid of Ms. Maurizia Cuccia are gratefully acknowledged.

TABLE III

PHOSPHORYLATION SITE IN SOYBEAN ANTIPROTEASE C-II

The phosphorylation rates are relative to dephospho-α_{s1}-casein (100%) at substrate concentration of 2 mg/ml, and were determined by 10 min incubation. The potential sites of phosphorylation are numbered and the C-terminal acidic residues are underlined. The predicted β-turns including the phosphorylation sites are included within brackets.

	Aminoacid sequence at the phosphorylation sites	Relative phosphorylation rate
	5 6	
Antiprotease C-II	Ser-Asp-His-Ser[Ser-Ser-Asp-Asp]Glu-Ser-Ser	155
	6	
Antiprotease D-II	Ser-Asp-Glu[Ser-Ser-Ser-Tyr]Asp-Asp-Asp-Glu	3.4
	67 68	
50% dephospho-α_{s1}-casein	SerP-Ile[Ser-Ser-Ser-Glu]Glu-Ile	100

References

1 Kemp, B.E., Bylund, D.B., Huang, T. and Krebs, E.G. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3448–3452
2 Bylund, D.B. and Krebs, E.G. (1975) J. Biol. Chem. 250, 6355–6361
3 Shenolikar, S. and Cohen, P. (1978) FEBS Lett. 86, 92–98
4 Zetterqvist, O., Ragnarsson, V., Humble, E., Berglund, L. and Engstrom, K. (1976) Biochem. Biophys. Res. Commun. 70, 696–703
5 Kemp, B.E., Graves, D.J., Benjamini, E. and Krebs, E.G. (1977) J. Biol. Chem. 252, 4888–4894
6 Feramisco, J.R., Glass, D.B. and Krebs, E.G. (1980) J. Biol. Chem. 255, 4240–4245
7 Weller, M. (1979) Protein Phosphorylation, Pion Ltd., London
8 Meggio, F., Donella Deana, A. and Pinna, L.A. (1978) FEBS Lett. 91, 216–221
9 Pinna, L.A., Donella Deana, A. and Meggio, F. (1979) Biochem. Biophys. Res. Commun. 87, 114–120
10 Donella Deana, A., Meggio, F. and Pinna, L.A. (1978) Biochem. J. 179, 693–696
11 Meggio, F., Donella Deana, A. and Pinna, L.A. (1979) FEBS Lett. 106, 76–80
12 Mercier, J.C., Grosclaude, F. and Ribadeau-Dumas, B. (1972) Milchwissenschaft 27, 402–408
13 Mercier, J.C. (1981) Biochimie 63, 1–17
14 MacKinlay, A.G., West, D.W. and Manson, W. (1977) Eur. J. Biochem. 76, 233–243
15 Bingham, E.W. and Groves, M.L. (1979) J. Biol. Chem. 254, 4510–4515
16 Meggio, F., Donella Deana, A., Pinna, L.A. and Moret, V. (1977) FEBS Lett. 75, 192–196
17 Mercier, J.C., Maubois, J.L., Poznanski, S. and Ribadeau-Dumas, B. (1968) Bull. Soc. Chim. Biol. 50, 521–530
18 Odani, S. and Ikenaka, T. (1977) J. Biochem. (Tokyo) 82, 1513–1522
19 Tang, J. and Hartley, B.S. (1970) Biochem. J. 118, 611–623
20 Odani, S., Koide, T. and Ikenaka, T. (1972) J. Biochem. (Tokyo) 71, 831–838
21 Chen, K.C.S., Tao, N. and Tang, J. (1975) J. Biol. Chem. 250, 5068–5075
22 Brignon, G., Ribadeau-Dumas, B. and Mercier, J.C. (1976) FEBS Lett. 71, 111–116
23 Grosclaude, F., Mercier, J.C. and Ribadeau-Dumas, B. (1970) Eur. J. Biochem. 14, 98–107
24 Savige, W.E. and Fontana, A. (1977) Methods Enzymol. 47, 459–469
25 Chou, P.Y. and Fasman, G.D. (1978) Annu. Rev. Biochem. 47, 251–276
26 Meggio, F., Donella Deana, A. and Pinna, L.A. (1979) J. Biochem. (Tokyo) 86, 261–264
27 Proud, C.G., Rylatt, D.B., Yeaman, S.J. and Ph. Cohen, (1977) FEBS Lett. 80, 435–442
28 Tang, J., Sepulveda, P., Marciniszyn, J., Chen, K.C.S., Huang, W.Y., Tao, N., Liu, D. and Lanier, J.P. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 3437–3439
29 Mamrack, M.D., Olson, O.J. and Bush, H. (1979) Biochemistry 18, 3381–3386
30 Meggio, F., Donella Deana, A. and Pinna, L.A. (1981) Anal. Biochem. 111, 158–162
31 Odani, S. and Ikenaka, T. (1977) J. Biochem. (Tokyo) 82, 1523–1531
32 Odani, S. and Ikenaka, T. (1978) J. Biochem. (Tokyo) 83, 737–745