Volume 237, number 1,2, 225-228 FEB 06308 September 1988

Partially dephosphorylated phosphopeptide AcSer(P)-Ser(P)-Ser(P) is an excellent substrate for casein kinase-2

Flavio Meggio, John W. Perich*, Reginald B. Johns* and Lorenzo A. Pinna

Dipartimento di Chimica Biologica, Università di Padova, Padova, Italy and **Department of Organic Chemistry, University of Melbourne, Parkville, 3052 Victoria, Australia*

Received 29 July 1988

The synthetic phosphopeptide AcSer(P)-Ser(P)-Ser(P), reproducing a recurrent feature of casein and other phosphoproteins, once partially dephosphorylated by acid phosphatase, serves as an efficient substrate for casein kinase-2. Previous dephosphorylation beyond 30% hinders subsequent phosphorylation and the entirely dephosphorylated peptide is not a substrate at all. The kinetic constants of the partially dephosphorylated phosphopeptide are much more favourable than those of the synthetic peptides SEEEAA, SSEE and SEE, the latter one being totally inert. Optimal phosphorylation occurs at pH values that ensure complete ionization of the phosphoseryl side chains. These data provide incontrovertible demonstration that phosphoserine can replace carboxylic amino acids as specificity determinant for CK-2, being more effective than glutamic acid itself.

Casein kinase-2; Phosphopeptide; Phosphoserine; Specificity determinant; $[Ser\text{-}P]_n$ cluster

1. INTRODUCTION

Ubiquitous casein kinases are multifunctional enzymes independent of either cyclic nucleotides of Ca^{2+}, operationally termed after their preference for casein over histones as in vitro substrates. They can be grouped into two classes, namely type-1 (CK-1 or -S or -A), and type-2 (CK-2, or TS or G), according to their different structure, capability of using GTP besides ATP as phosphate donor, and response to various effectors, including heparin, polyamines and other polycations (reviews [1,2]).

The primary structures of several sites phosphorylated by CK-2 in its physiological targets have been elucidated: invariably they include several glutamic and/or aspartic acids close to the C-terminal side of the phosphorylatable serine (review [3]). Synthetic peptides reproducing such a recurrent feature proved good substrates for CK-2 and provided relevant information about the specificity determinants of this class of enzymes [3-6]: while multiple C-terminal carboxylic residues are required for high phosphorylation efficiency, the individual acidic residue at position +3 plays an especially crucial role, its absence causing a dramatic drop in phosphorylation rate [3].

The recent elucidation of the primary structure of phosvitin, which lacks seryl residues followed by either aspartic or glutamic acids but includes long stretches of consecutive phosphoserines [7] and the finding that this phosphoprotein becomes an excellent substrate for CK-2 upon slight dephosphorylation [8] strongly suggest that phosphorylated residues might replace carboxylic ones as specificity determinants of CK-2. The validity of such a concept is here incontrovertibly proven by showing that the synthetic phosphopeptide AcSer(P)-Ser(P)-Ser(P), reproducing a recurrent feature of most caseins, once partially dephosphorylated, becomes an excellent substrate for CK-2, displaying kinetic constants comparable to those of Ser-Glu-Glu-Glu-Glu-Ala and more favourable than those of Ser-Glu-Glu-Glu-Ala-Ala and Ser-Ser-Glu-Glu. This would also indicate that phosphoseryl residues are more powerful determinants than glutamic acids, being quite effective even if they do not reside in the +3 position.

Correspondence address: L.A. Pinna, Departimento di Chimica Biologica, Via Marzolo 3, I-35131 Padova, Italy

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50 © 1988 Federation of European Biochemical Societies

2. MATERIAL AND METHODS

Rat liver casein kinase-2 (TS) was purified to near homogeneity as in [9]. Its specific activity was about 10^6 U/mg, 1 unit being defined as the amount of enzyme transferring 1 pmol P from ATP to casein in 1 min under conditions detailed elsewhere [3]. Casein kinase-1 (S) was prepared as in [9]. GEF-CK, a casein kinase isolated from the Golgi-enriched fraction of guinea pig mammary gland [10], was kindly provided by A. Boulton (London). F_A kinase (glycogen synthase kinase-3) purified from rabbit skeletal muscle [11] was kindly provided by J.B. Vandenheede (Leuven).

The phosphotripeptide AcSer(P)-Ser(P)-Ser(P)-NHMe (Z) was prepared from Boc-Ser(PO_3Ph_2)-Ser(PO_3Ph_2)-Ser(PO_3Ph_2)-NHMe (X) using the Boc mode of solution-phase peptide synthesis following the strategy in [12]. Ac-Ser(PO_3Ph_2)-Ser(PO_3Ph_2)-Ser(PO_3Ph_2)-NHMe (Y) was deprotected by hydrogenolysis over platinum oxide in 50% TFA/AcOH [12,13]

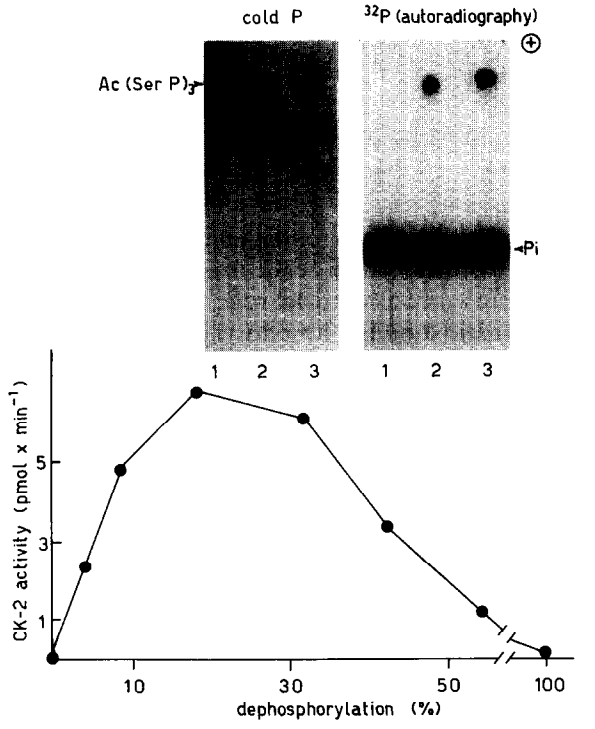


Fig.1. Effect of previous dephosphorylation on CK-2-catalyzed phosphorylation of the synthetic peptide AcSer(P)-Ser(P)-Ser(P). The preparation of variably dephosphorylated phosphopeptide and conditions for enzymatic phosphorylation with [^{32}P]ATP in the presence of CK-2 are detailed in section 2. (Inset) pH 1.5 high-voltage electrophoretic runs of fully phosphorylated (lane 1), 9% dephosphorylated (lane 2) and 18% dephosphorylated (lane 3) phosphopeptide, after incubation with [^{32}P]ATP and CK-2. Phosphopeptides were demonstrated with the method of Hanes and Isherwood for phosphorylated compounds [16] (left) and ^{32}P incorporated was detected by autoradiography (right).

and the purity of the tripeptide was determined to be >99% as judged by ^{13}C-NMR spectroscopy and fast atom bombardment mass spectrometry [14]. The synthesis of Ser-Ser-Glu-Glu and Ser-Glu-Glu was accomplished using the Boc mode of solution-phase peptide synthesis, as will be detailed elsewhere.

Partial dephosphorylation of the phosphopeptide was performed by incubating 100-μg aliquots thereof in 25 μl of 40 mM Na acetate buffer (pH 5.2) with 5 μg potato acid phosphatase (Boehringer) at 37°C for variable time periods. The reaction was blocked by 5 min boiling and the extent of dephosphorylation was evaluated by determining the amount of P_i released [15].

Enzymatic phosphorylation of peptide substrates was routinely assayed by 15 min incubation at 37°C in a medium containing 50 mM Tris-HCl buffer (pH 7.5), 12 mM $MgCl_2$, 100 mM NaCl, 10 μM [γ-^{32}P]ATP (spec. act. 650 cpm/pmol) and 20–40 units of CK-2 or other protein kinases. The peptide concentration, unless otherwise indicated, was 0.5 mg/ml. The reaction was stopped by adding HCl (6 N final concentration). The samples were heated for 4 h at 110°C in order to convert the phosphopeptides into free phosphoserine which was separated from $^{32}P_i$ by pH 1.9 high-voltage paper electrophoresis, according to procedure a of [4].

Radiolabeled AcSer(P)-Ser(P)-Ser(P) could be also separated as such from [γ-^{32}P]ATP and $^{32}P_i$ by pH 1.5 high-voltage paper electrophoresis (see inset to fig.1). The acetylated phosphopeptides that do not react with ninhydrin, were localized on the paper by spraying with the reagent of Hanes and Isherwood for phosphorylated compounds [16]. Radioactive spots were detected by autoradiography, excised and quantitated by liquid scintillation

3. RESULTS

Samples of either fully phosphorylated or variably dephosphorylated tripeptide AcSer(P)-Ser(P)-Ser(P) were subjected to enzymatic phosphorylation by CK-2 in the presence of [γ-^{32}P]ATP. As shown in fig.1, while the entirely phosphorylated peptide is not affected by CK-2, a remarkable radiolabeling of partially dephosphorylated samples is observed. Previous dephosphorylation beyond 30% however impairs subsequent enzymatic phosphorylation, and the fully dephosphorylated tripeptide is not a substrate at all.

Similar experiments were performed with other protein kinases supposed to require acidic groups as specificity determinants, namely, casein kinase-1, GEF-CK, a physiological casein kinase isolated from the Golgi-enriched fraction of mammary gland [10] and glycogen synthase kinase-3 (F_A kinase) which has been recently shown to affect peptide substrates which had been previously phosphorylated by CK-2 [17]. Detectable amounts of radiolabeled AcSer(P)-Ser(P)-Ser(P) could be

demonstrated only with GEF-CK (not shown). The rate of such phosphorylation however was almost negligible in comparison to that observed with CK-2 if the activities of the two enzymes were normalized using the common peptide substrate SEEEEE as a reference.

The inset to fig.1 shows that the product of enzymatic phosphorylation has the same electrophoretic mobility as AcSer(P)-Ser(P)-Ser(P). This finding, together with the observation that previous dephosphorylation beyond 30% gives rise to product(s) that are less suitable for enzymatic phosphorylation, supports the view that the substrate of CK-2 is the doubly phosphorylated, rather than singly phosphorylated, tripeptide. Assuming, moreover, that dephosphorylation of AcSer(P)-Ser(P)-Ser(P) occurs randomly, three doubly phosphorylated isomers should be expected, namely AcSer-Ser(P)-Ser(P), AcSer(P)-Ser-Ser(P) and AcSer(P)-Ser(P)-Ser. Most likely the first one will be the preferred target considering that the acidic determinants of CK-2 must be located downstream of serine [3]. This would mean that the K_m value calculated for 30% dephosphorylated phosphopeptide (table 1) is at least 3-fold overestimated, due to the presence of the two 'inert' isomers, without taking into account the presence of fully phosphorylated and entirely dephosphorylated derivatives which would not be suitable for enzymatic phosphorylation.

Even considering the experimental kinetic constants without corrections, anyway, the phosphorylated tripeptide turns out to be a much better substrate than tetra- and hexapeptides with two

Table 1

Kinetic constants of acidic peptide substrates for CK-2

Peptide	V_{max} (pmol · min^{-1})	K_m (mM)
30% dephosphorylated AcSer(P)-Ser(P)-Ser(P)	57	2.9
Ser-Glu-Glu	n.d.	–
Ser-Ser-Ser	n.d.	–
Ser-Ser-Glu-Glu	12	23.1
Ser-Glu-Glu-Glu-Ala-Ala	22	5.9
Ser-Glu-Glu-Glu-Glu-Ala	28	4.5

K_m and V_{max} were determined from double-reciprocal plots constructed from initial rate measurements fittet to the Michaelis-Menten equation. n.d., no detectable phosphorylation with 4 mM peptide substrate. Data relative to peptides Ser-Glu-Glu-Glu-Ala-Ala and Ser-Glu-Glu-Glu-Glu-Ala are drawn from [3]

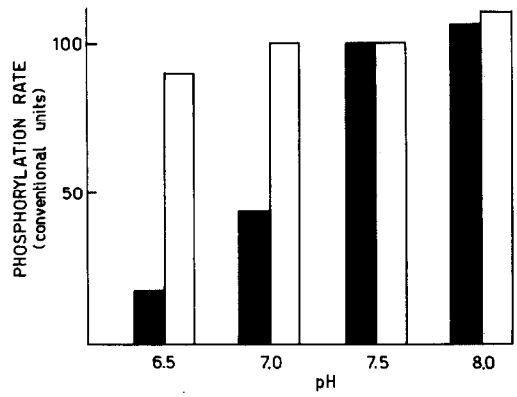


Fig.2. pH specifically affects the enzymatic phosphorylation of AcSer(P)-Ser(P)-Ser(P). A sample of 30% dephosphorylated phosphopeptide was subjected to phosphorylation by CK-2 in the presence of [^{32}P]ATP at the indicated pH values under conditions described in section 2 except that the buffer was Tris-acetate instead of Tris-HCl. For comparison the phosphorylation of the peptide Ser-Glu-Glu-Glu-Glu-Glu was studied under identical conditions. Phosphorylation rate is expressed as percent of that observed at pH 7.5 (= 100%). Filled bars: AcSer(P)-Ser(P)-Ser(P). Open bars: Ser-Glu-Glu-Glu-Glu-Glu.

and three glutamic acids on the C-terminal side of serine, and at least as good as a hexapeptide bearing four glutamyl residues (table 1). This could be due to the presence of two almost entire negative charges, at pH 7.5, on the side chain of phosphoserine, instead of just one as in the case of glutamic acid. This point of view is strongly supported by the pH dependence experiments summarized in fig.2: while in fact the phosphorylation rate of glutamyl peptides is nearly unaffected by varying the pH of the reaction medium between 6.5 and 8.0, a dramatic rise of activity is observed with the 30% dephosphorylated derivative of AcSer(P)-Ser(P)-Ser(P) if the pH is increased from 6.5 to 8.0, i.e. in a range encompassing the second pK of the phosphoserine side chain. Additional factors, however, might be involved in determining the special efficacy of phosphoserine: e.g. the length of its side chain is closer to that of aspartic acid, which has been shown to be a better determinant than glutamic acid for CK-2-catalyzed phosphorylation [5].

4. DISCUSSION

The synthetic phosphopeptide AcSer(P)-Ser(P)-Ser(P) whose enzymatic phosphorylation is described in this report, reproduces a typical se-

quence which is recurrent in many casein fractions [18], as well as in other phosphoproteins, including basic ones, like protamines [19]. To our knowledge it represents the shortest and simplest model substrate whose phosphorylation by a Ser/Thr-specific protein kinase has been described so far. In particular its rapid phosphorylation by CK-2 following partial dephosphorylation, as opposed to its incapability of acting as a substrate once fully dephosphorylated, further corroborates the concept that acidic residues are strictly required for target recognition by this enzyme [3–6], providing, at the same time, an unambiguous demonstration that phosphoserine can replace carboxylic residues as specificity determinant. A comparative analysis of the kinetic constants makes it clear that phosphoserine is indeed a much more powerful specificity determinant than glutamic acid; this is due, at least in part, to the larger negative charge of phosphoserine side chain, at pH values beyond 7, as indicated by the pH dependence histograms of fig.2. Phosphoserine, moreover, can act as an effective positive determinant even if it does not occupy position +3, which is crucial whenever the determinant is glutamic acid [3,6]: just two phosphoserines in fact represent a sufficient condition for the efficient phosphorylation of a seryl residue adjacent to them. Conversely, the corresponding tripeptide with two glutamic acids, Ser-Glu-Glu, is not a substrate at all (table 1).

The finding that a partially phosphorylated cluster of three consecutive seryl residues entirely fulfils the structural requirements for being recognized and phosphorylated by CK-2 opens new perspectives on the biological commitments of this class of enzymes. For example, mammary gland GEF-CK, whose consensus sequence is the triplet Ser-X-Glu [20], proved unable to carry out alone the complete phosphorylation of the peptide Leu-Ser-Ser-Ser-Glu-Glu which is fully phosphorylated in β-casein (unpublished); our data suggest that a reasonable candidate for such a task could be CK-2. Likewise, the Ser-Ser-Ser cluster present in most fish protamines can be only partially phosphorylated by cAMP-dependent protein kinase [21], whereas all three residues were found to be phosphorylated in vivo [19]. Here again one could hypothesize the involvement of CK-2 as secondary phosphorylating agent, though in this case the arginyl residues flanking the seryl cluster might be expected to hinder phosphorylation by an 'acidic determinant' requiring enzyme, like CK-2. It will be interesting in this matter to investigate how the CK-2-dependent phosphorylation of the peptides including the $(Ser\text{-}P)_n$ cluster could be influenced by addition of different amino acids on either side.

Acknowledgements: This work was partially supported by grants from Italian Ministero della Pubblica Istruzione and Consiglio Nazionale delle Ricerche (grant no. 87.00197.04).

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