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A synthetic β-casein phosphopeptide and analogues as model substrates for casein kinase-1, a ubiquitous, phosphate directed protein kinase

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The phosphopeptide Ser(*P*)-Ser(*P*)-Ser-(*P*)-Glu-Glu-Ser[22]-Ile-Thr, reproducing the 17–24 segment of β-casein A[2] including the seryl residue (Ser-22) which is targeted by casein kinase-1 was synthesized and used as model substrate for this enzyme. Its phosphorylation efficiency is actually higher than that of intact β-casein (similar V_{max} and 14 μM vs 50 μM K_m). Conversely the fully dephosphorylated peptide SSSEESIT is not affected by CK-1 to any detectable extent and its glutamyl derivative EEEEEESIT displays a more than 50-fold higher K_m and a 5-fold lower V_{max} as compared to the parent phosphopeptide. The relevance of the individual phosphoseryl residues has been assessed by comparing the phosphorylation efficiencies of the phosphopeptides EESpEESIT, ESpEEESIT and SpEEEESIT: while the first is a substrate almost as good as the tris Ser (*P*)-peptide (K_m = 62 μM), and the third one is almost as poor as EEEEESIT (K_m = 1.55 mM), ESpEEESIT displays a intermediate efficiency (K_m = 277 μM). These data in conjunction with the finding that the phosphopentapeptide Ser(*P*)-Ser(*P*)-Ser-(*P*)-Ser-Ser(*P*), but neither Ser(*P*)-Ser(*P*)-Ser-Ser(*P*) nor Ser-Ser(*P*)-Ser(*P*)-Glu-Glu and Ser-Ala-Ala-Ser(*P*)-Ser(*P*), is readily phosphorylated by CK-1, support the concept that CK-1 is a phosphate directed protein kinase recognizing the Ser(*P*)-X-X-Ser-X and, less efficiently, the Ser(*P*)-X-X-X-Ser-X motifs.

Phosphopeptide; Protein kinase specificity; Casein kinase-1; β-Casein

1. INTRODUCTION

Casein kinase-1 and -2 (CK-1 and CK-2) are spontaneously active, Ca and cyclic nucleotide independent, ubiquitous Ser/Thr specific protein kinases termed after their preference for casein and phosvitin as in vitro substrates (reviewed in [1,2]). They can be differentiated from each other by a number of features which include substrate specificity. While the specificity determinants of CK-2 have been elucidated with the aid of model peptide substrates reproducing the phosphoacceptor sites for this enzyme in several protein targets [1], the study of the site specificity of CK-1 has been hampered by the paucity of information available about its physiological targets. In early studies which took advantage of artificial protein substrates we were able to identify the residues phosphorylated by CK-1 in different casein fractions [3,4], these residues being found to be located downstream from acidic clusters which invariably contained both glutamyl and O-phosphoseryl residues. Moreover the finding that prior dephosphorylation of such phosphoseryl residues, or their conversion into dehydroalanyl residues, prevented the subsequent phosphorylation by CK-1 [3] and that seryl residues located at the C-terminal end of sequences composed solely of glutamyl and aspartyl residues were unaffected by CK-1 [5], prompted us to propose that CK-1 might represent a 'secondary' protein kinase, which depends upon a 'primary' kinase creating its recognition site. However, a synthetic peptide corresponding to the phosphorylation site for CK-1 in β-casein in which the three phosphoseryl residues were replaced with aspartic acid was recently found to be a fairly good and highly specific substrate for CK-1 [6]. Here we show that the phosphorylation efficiency of similar peptides is dramatically increased if the carboxylic acid residues are replaced by phosphoseryl residues as they occur in β-casein sites. In particular it was found that an individual phosphoserine at position −3 and, to a lesser extent, at position −4 produced a striking reduction of K_m and rise of V_{max}.

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Abbreviations: CK-1, casein kinase-1; CK-2, casein kinase-2; Ser(*P*), phosphoserine; where one-letter symbols for amino acids are used; Sp denotes phosphoserine

2. MATERIALS AND METHODS

The Ser(*P*)-containing peptides were prepared by the use of Boc-Ser(PO_3Ph_2)-OH in the Boc/Bzl mode of solution-phase peptide synthesis followed by hydrogenation (platinum oxide) of the protected peptides in 50% TFA/AcOH [7–9]. The peptide, EEEEESIT-NHMe, was prepared by the Boc/Bzl mode of solution-phase peptide synthesis followed by hydrogenation (palladium) of the protected peptide in 50% TFA/AcOH. The peptide, SSSEESIT, was prepared by the

Fmoc/^{t}Bu mode of solid-phase synthesis on a ABI 431A Synthesizer followed by treatment of the peptide-resin with 5% H_2O/TFA. The structure of all six peptides was confirmed by FAB mass spectrometry.

Casein kinase-1 was purified in excess of 2000-fold from rat liver as in [10]. Its specific activity was 19.4 U/mg, 1 U being defined as the amount of enzyme transferring 1 nmol P to casein per min. Reaction conditions for peptide phosphorylation assays were: 50 mM Tris-HCl pH 7.5, 12 mM $MgCl_2$, 100 mM NaCl, 20 μM [γ-^{32}P]ATP (sp. act. 1000–1500 cpm/pmol). The peptide concentration was variable, as indicated in figures and tables. The reaction was started by addition of CK-1 (0.01–0.02 units) and was performed at 37°C for 10 min (unless otherwise indicated). Reaction was terminated by addition of HCl and ^{32}P incorporation was evaluated by partial acid hydrolysis (6 N HCl, 4 h at 105°C) and quantitation of the radiolabeled phosphoamino acids resolved by high voltage paper electrophoresis as described in [11], procedure a. Phosphoserine invariably was the only radiolabeled amino acid detected.

3. RESULTS

A series of peptides derived from the phosphoacceptor site including β-casein Ser-22 have been assayed as substrates for CK-1. The kinetic constants are reported in Table I. For all peptides the only residue subject to phosphorylation was the seryl residue, the threonyl residue being completely unaffected (not shown). The lowest K_m (14 μM) and the highest V_{max} values are attained with the tris Ser(*P*)-peptide, SpSpSpEESIT, exactly reproducing the phosphoacceptor site, which is actually an even better substrate than its parent protein β-casein A_2. Substitution of the two phosphoserines at positions −4 and −5 with glutamic acid is quite well tolerated since the monophosphorylated peptide EESpEESIT displays just two-fold higher K_m and a slightly lower V_{max}. The phosphorylation efficiency of ESpEEESIT, with a $K_m \simeq 277\ \mu$M, is markedly reduced, but not as much as that of SpEEEESIT. The latter is a substrate almost as poor as the non-phosphorylated peptides EEEEESIT and DDDEESITRR, all these peptides having K_m values in the millimolar range. On the other hand, the peptides EEEEES and SSEESIT are not significantly phosphorylated by CK-1 even if their concentration is raised to 1 mM.

These results indicate that, relative to the target seryl residue, an individual phosphoseryl residue plays an especially crucial role when it is located at position −3, and is less efficient at position −4. That such a favourable effect of phosphoseryl residues is due to their phosphate moiety is indicated by the deleterious effect of prior enzymatic dephosphorylation of EESpEESIT (not shown) and by the failure of SSSEESIT (i.e. the dephosphorylated derivative of the best substrate) to undergo any detectable phosphorylation (Table I). On the other hand the striking superiority of a phosphate group over a carboxylic group as a phosphorylation determinant does not appear to be merely due to its higher negative charge at pH 7.5 since it is even more evident at pH 6 where the overall charge of EESpEESIT and EEEEESIT is expected to be almost the same (see Table I, values between brackets).

Table I

Kinetic constants of CK-1 for a series of β-casein derived peptides

Substrate	V_{max} (nmol/min/mg)	App. K_m (mM)	V_{max}/K_m
β-casein A^2	39.4	0.053	743.7
Ser(P)-Ser(P)-Ser(P)- Glu-Glu-Ser-Ile-Thr	51.2	0.014	3657.1
Ser(P)- Glu- Glu- Glu-Glu-Ser-Ile-Thr	23.0	1.550	14.8
Glu- Ser(P)- Glu- Glu-Glu-Ser-Ile-Thr	35.5	0.277	128.1
Glu- Glu- Ser(P)- Glu-Glu-Ser-Ile-Thr	38.3 (33.80)	0.062 (0.13)	617.7 (260.0)
Glu- Glu- Glu- Glu-Glu-Ser-Ile-Thr	9.7 (9.72)	1.173 (4.34)	8.2 (2.2)
Asp- Asp- Asp- Glu-Glu-Ser-Ile-Thr-Arg-Arg	25.0	1.000	25.0
Glu- Glu- Glu- Glu-Glu-Ser	N.D.	N.D.	-
Ser- Ser- Ser- Glu-Glu-Ser-Ile-Thr	N.D.	N.D.	-

K_m and V_{max} values were determined by double-reciprocal plots, constructed from initial rate measurements fitted to Michaelis-Menten equation. Average values from three or more experiments are shown. The standard error was ≤20%. Reaction conditions for peptide phosphorylation were as described in the experimental section except for the values between brackets which were determined at pH 6.0 (Tris-acetate buffer) instead of 7.5. The residue undergoing phosphorylation by CK-1 is underlined. Kinetic constants for the peptide Asp-Asp-Asp-Glu-Glu-Ser-Ile-Thr-Arg-Arg are drawn from [6]. N.D.: not determined due to the undetectable phosphorylation of the peptide up to 1 mM concentration.

Table II

Phosphorylation rate of phosphopeptides by CK-1

Peptide	relative phosphorylation rate
AcSer(P)-Ser-Ser(P)	n.d.
AcSer(P)-Ser(P)-Ser-Ser(P)	11
AcSer(P)-Ser(P)-Ser(P)-Ser-Ser(P)	101
AcSer-Ser(P)-Ser(P)	n.d.
AcSer(P)-Ser(P)-Ser	n.d.
Ser-Ser(P)-Ser(P)-Glu-Glu	n.d.
Ser-Ser-Ser-Ser(P)-Ser(P)	n.d.

Peptide concentration was 1 mM. The phosphorylation rate is expressed relative to that of the reference phosphopeptide SpSpSpEESIT (= 100%). n.d. = not detectable (i.e. phosphorylation rate < 1%). Underlining denotes the phosphorylatable residues.

The crucial requirement of CK-1 for a phosphoseryl residue at position −3 is corroborated by the behaviour of another series of short peptides composed by stretches of variably phosphorylated serines. As shown in Table II the phosphorylation rate of SpSpSpSSp is 10-fold higher than that of SpSpSSp, while SSSSpSp, SSpSpEE, SpSSp, SSpSp and SSSp are not affected at all.

4. DISCUSSION

The data presented here clearly demonstrate that the efficient phosphorylation of casein fractions by CK-1 is critically dependent on the constitutively phosphorylated serines located on the N-terminal side of those residues which are targeted by this enzyme. Not all the phosphorylated side chains however are equally important for site recognition by CK-1 as shown by variably replacing them with glutamic acid. While EESpEESIT is still almost as good as the parent tris Ser(*P*)-peptide, ESpEEESIT is less efficiently phosphorylated and SpEEEESIT is almost as poor a substrate as EEEEESIT. These data in conjunction with the finding that SpSpSpSSp (but neither SpSpSSp nor a series of peptides with phosphoseryl residues on the C-terminal side of serine) is a good substrate for CK-1 lead to the deduction that position −3 and, to a lesser extent, position −4 are the crucial residues sites, while phosphoseryl residues at position −1, −2 and −5 and on the carboxyl terminal side of serine are nearly ineffective. Seemingly however one or more residues on the C-terminal side of serine are also required considering the failure of EEEEES, but not of EEEEESIT, to undergo phosphorylation by CK-1 at Ser-5. The above conclusions are in agreement with the recent finding that the PK-A dependent phosphorylation of Ser-7 in the glycogen synthase 1–14 peptide PLSRTLSVASLPGL-amide is a prerequisite for subsequent efficient phosphorylation of Ser-10 by CK-1 [12]. In that study it was also shown that by varying the spacing between the phosphorylated Ser-7 and the acceptor serine the peptide with three residue spacing was virtually not phosphorylated. The data presented here, conversely, support the view that a 3-residue spacing is still compatible with fairly efficient phosphorylation by CK-1. This finding incidentally accounts quite well for the phosphorylation of α_{s2} casein Ser-135 [3,5] at a site with a Ser(*P*)-X-X-X-Ser rather than a Ser(*P*)-X-X-Ser structure. The apparent discrepancy between our data and those reported in [12] is probably due to technical hindrances encountered in the latter study where the amount of phosphorylated peptide available was conditioned by the preliminary enzymatic reaction with PK-A. As a consequence the phosphorylation rates were determined at very low substrate concentrations (10 μM) and the kinetic constants of peptide substrates whose K_m value exceeded 25 μM escaped accurate evaluation [12]. This drawback highlights the practical advantage of a methodology which uses synthetic phosphopeptides in which the various phosphorylated residues are placed at any given position with 100% certainty.

While the superiority of phosphoseryl residues over carboxylic amino acids as specificity determinants for CK-1 is incontrovertible, the question remains as to whether efficient phosphoacceptor sites for CK-1 could be also created by other structural features capable of surrogating the phosphorylated residue. Actually multiple carboxylic residues encompassing the crucial −3 and −4 positions do act as positive determinants, as

outlined by the appreciable phosphorylation of the peptides DDDEESITRR [6] and EEEEESIT as opposed to SSSEESIT, which is not a substrate (Table I). Such an effect of N-terminal carboxylic groups, however, is much weaker than that exerted by Ser(*P*). The optimizing effect of the phosphorylated chains moreover cannot be merely attributed to their doubly negative charge (at pH 7.5) since it is fully maintained at pH 6 where the phosphoseryl residue bears a reduced negative charge similar to that of Glu and Asp. This behaviour, among others, differentiates CK-1 from CK-2, which is very responsive to changes of pH which reduce the negative charge of its acidic determinants [13,14].

In conclusion, CK-1 is clearly a 'phosphate-directed' protein kinase but apparently not so 'acidophylic' as CK-2, which conversely displays no special requirement for phosphoseryl residues, whose potency as specificity determinants for CK-2 is comparable to that of carboxylic acid residues [15].

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