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# Synthetic peptide substrates for casein kinase 2. Assessment of minimum structural requirements for phosphorylation

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**Unlike the peptides SAEAAA and SEEAAA which are not substrates for casein kinase 2 (CK-2) their analogs SAAEAE and SAAEAA are still significantly phosphorylated. Their $K_m$ values, however, (13.3 and 18.9 mM, respectively) are almost two orders of magnitude higher than that of SEEEEE and their $V_{max}$ values are 3- and 14-fold lower than that of SAAEEE. The peptide ESEEEEE, but not ASEEEEE, is a slightly better substrate than SEEEEE, while both RSEEEEE and SEEEKE are very poor substrates compared to ASEEEEE and SEEEAE, respectively. SAAEAE is much more responsive to polylysine stimulation and polyphosphate inhibition than is SEEEEE. Taken together these data show that a single acidic residue at the third position from the C-terminal side of the phosphorylatable amino acid represents not only a necessary, but also a sufficient condition for site recognition by CK-2. Optimal phosphorylation efficiency, however, requires an extended C-terminal cluster of several acidic residues, and can be compromised by the presence of only a basic residue either inside the acidic cluster or adjacent to the N-terminal side of the phosphoacceptor amino acid. The structure of the phosphoacceptor site can greatly influence the efficacy of substrate-directed effectors of CK-2.**

## Introduction

Synthetic peptides proved useful for probing the site specificity of CK-2, an ubiquitous multifunctional protein kinase, also termed casein kinase TS or G, which is insensitive to cyclic nucleotides, calcium and any other known second messenger (reviewed in Refs. 1 and 2). In particular, it was shown that while the peptide SEEEEE, reminiscent of the phosphorylation site 5 of glycogen synthase and, to a lesser extent, its derivative SEEEAA were phosphorylated by CK-2, the peptide SEEAAA was no longer a substrate [3], suggesting that a cluster of three or more acidic residues downstream from the target amino acid is strictly required. A subsequent systematic study, however, taking advantage of a larger number of SEEEEE derivatives [4] disclosed the crucial relevance of the individual acidic residue occupying the third position on the C-terminal side of serine: actually, the hexapeptide SEEAEE is a very poor substrate compared to its four isomers differing

Abbreviations: CK-2, casein kinase 2; abbreviations used for amino acid derivatives and peptides are standard according to the IUPAC-IUB Commission on Biochemical Nomenclature, Biochemistry (1972) 11, 1726–1732. The amino acids used are of the L configuration.

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just for the position of the single alanine relative to the N-terminal serine.

This finding disclosed the possibility that the failure of SEEAAA to undergo phosphorylation might not be due to an insufficient number of glutamic acids, but rather to the lack of an acidic residue at position +3. In order to assess this point and to define the minimum structural requirements of CK-2, a new set of peptides has been prepared, including, among others, SAAEAE, SAAEAA and SAEAAA.

The strategy of their synthesis and their kinetic behaviour as substrates of CK-2 are described here.

## Materials and Methods

*Peptide synthesis.* Once we had chosen, as reference model substrate for CK-2, the peptide SEEEEE [3], our purpose was to build up a series of analogs systematically modified in the number and distribution of the residues along the sequence as well as the phosphorylation site. The fragment condensation strategy in solution, which allows the utilisation of several common intermediates for the preparation of the final peptides, was adopted. The standard procedures for peptide synthesis [5] were employed, generally using the classical combination benzyloxycarbonyl and *tert*-butyl groups for selective protection at the $\alpha$-amino and $\gamma$-carboxyl functions, respectively. The methyl or ethyl esters, from which the corresponding hydrazides are readily obtained, were used to mask the $\alpha$-carboxy function in the intermediate peptides. The Rudinger-modified azide procedure was therefore employed for fragment condensations, while mixed anhydrides or active esters (*p*-nitrophenyl or *N*-hydroxysuccinimido esters) were used for the preparation of the fragments. The protected final peptides were obtained by coupling the appropriate amino and carboxyl components. After convenient deprotections (catalytic hydrogenolysis in the presence of 10% palladized charcoal and/or exposure to 98% trifluoroacetic acid) the final peptides, when heterogenous, were purified by chromatographic procedures (ionic exchange and gel filtration), then converted into hydrochloride salts by lyophilisation from 5% HCl. The homogeneity of the final products was evaluated by thin-layer chromatography (TLC) on cellulose plates and reversed-phase high-pressure liquid chromatography (HPLC) on a VIOSFER C18 column, using *tert*-butyl amine as counter ion.

*Other experimental procedures.* Purification of rat liver CK-2, phosphorylation of peptides and determination of kinetic and inhibition constants have been either described or quoted in a previous paper [4]. In particular, the phosphorylation of peptides was constantly evaluated by procedure 'a', consisting of 6 M HCl hydrolysis of both [$\gamma$-$^{32}$P]ATP and $^{32}$P-labelled peptides and subsequent separation of [$^{32}$P]$P_i$ and [$^{32}$P]serine by high-voltage paper electrophoresis [3].

## Results

*Minimum requirements for catalytic phosphorylation by CK-2*

The recent finding that the hexapeptide SEEAEE is a very poor substrate of CK-2 as compared to its isomers carrying the single alanine at different positions [4] outlined the crucial role of the acidic residue lying in the third position on the C-terminal side of the phosphorylatable serine. Consequently, the previous observation that the peptide SEEEAA, but not SEEAAA, is a substrate for CK-2 [3] could be accounted for by the lack of the crucial acidic determinant at position +3 in the latter peptide, rather than merely by its lower number of acidic residues.

In order to assess the actual minimum number of acidic residues required, we first synthesised the hexapeptide SAAEAE, whose two glutamic acids are situated in those positions, namely +3 and +5, where the replacement of alanine for glutamic acid in the reference peptide SEEEEE proved more harmful [4]. This peptide as shown in Fig. 1, is still appreciably phosphorylated by CK-2, whereas its analog with the two acidic residues adjacent to the serine, is not.

Next, two monoglutamyl hexapeptides were synthesised and tested as substrates for CK-2, namely SAAEAA, with glutamic acid in the crucial +3 position, and, for comparison, SAEAAA where glutamic acid is instead, in the second position. As expected, the latter peptide was totally unaffected by the kinase; the former, however,

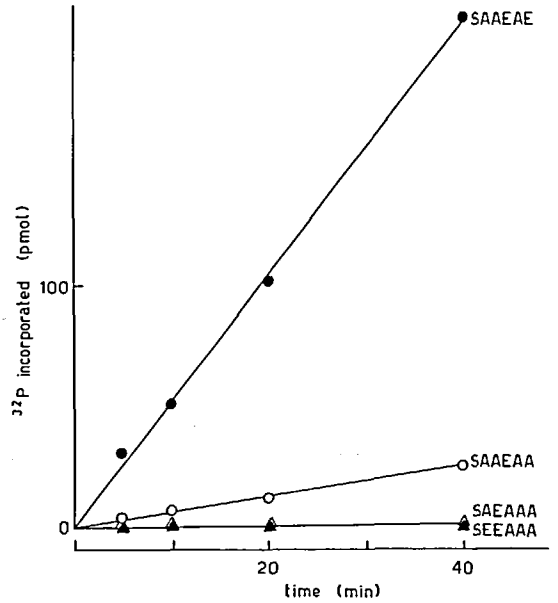


Fig. 1. Phosphorylation of hexapeptides including just one or two C-terminal acidic residues by CK-2. The peptide concentration was 2.5 mg/ml.

was still slowly but significantly phosphorylated (Fig. 1). This means that a single acidic residue at the third position downstream from the target amino acid represents not only a necessary requirement for efficient phosphorylation as previously shown [4], but also a sufficient condition for such a phosphorylation to occur.

Although the peptides SAAEAE and SAAEAA undergo detectable phosphorylation, their unfavourable kinetic constants, as compared to those of analogs bearing a larger number of acidic residues, corroborate the concept that a rather extended C-terminal cluster is required for optimising phosphorylation by CK-2: as shown in Table I, in fact, the $K_m$ values of SAAEAE and SAAEAA are almost two orders of magnitude higher than that of the reference hexapeptide with a cluster of five acidic residues, and their $V_{max}$ values are significantly lower than that of their analog with three acidic residues, SAAEEE. The poor suitability of the phosphorylatable peptides with just two and one acidic residues is even more evident if their kinetic parameters are compared with those of physiological targets of CK-2, such as glycogen synthase and troponin-T, whose $K_m$ values are 11 and 39 $\mu$M, respectively, and whose $V_{max}$ are of the same order as that of the pentaglutamyl peptide, SEEEEE [3].

In order to investigate whether acidic residues beyond the fifth position are still perceived as positive determinants capable of improving phosphorylation by CK-2, the hexapeptides SAEEEE and SAAEEE were extended on their C-terminal end with one and two additional glutamic acids, to give the heptapeptide SAEEEEE and the octapeptide SAAEEEEE, respectively. As shown in Table I, both are somewhat more efficiently phosphorylated than their analogs lacking the glutamic acids at positions +6 and +7, but they also exhibit $K_m$ values much higher than that of the reference hexapeptide SEEEEE. This would indicate that while acidic residues at positions +6 and +7 are still recognised as positive determinants, their favourable effect on binding is less pronounced than if they were adjacent to the C-terminal side of serine. In particular, the drop in affinity observed on replacing the first glutamic acid with alanine, can be only slightly counteracted by adding another glutamic acid after the sixth posi-

TABLE I

KINETIC CONSTANTS OF SYNTHETIC PEPTIDES USED AS MODEL SUBSTRATES FOR CK-2

Acidic residues are underlined, basic residues are typed in bold. Average values calculated from three or more experiments are reported. The standard error for all reported kinetic constants was less than 17%.

| Peptides | $V_{max}$ (nmol/min per mg) | $K_m$ (mM) |
|---|---|---|
| SEEEEE | 110.5 | 0.30 |
| SAAEEE | 114.0 | 4.85 |
| SAAEAE | 36.1 | 13.30 |
| SAAEAA | 7.2 | 18.90 |
| SAEAAA | undetectable | – |
| SEEAAA | undetectable | – |
| SAEEEEE | 83.1 | 1.33 |
| SAAEEEEE | 130.6 | 1.06 |
| SAEEEE | 106.9 | 3.40 |
| SEEEAE | 101.4 | 1.03 |
| SEEEVE | 95.8 | 0.80 |
| SEEEKE | 30.0 | 6.67 |
| ASEEEEE | 133.9 | 2.03 |
| ESEEEEE | 96.9 | 0.13 |
| RSEEEEE | 9.4 | 2.45 |

tion (compare SEEEEE with SAEEEE and SAEEEEE).

*Negative effect of lysine*

The above results, together with previous ones [4,6] would indicate that the minimum structural requirement for CK-2 consists of the sequence Ser(Thr)-X-X-Glu(Asp)-Xn, X representing either neutral residues, such as alanine, or additional acidic residues. In this latter case, the phosphorylation efficiency is greatly enhanced. On the other hand, basic residues appear to be hardly tolerated, since the replacement of the penultimate glutamic acid of the reference peptide SEEEEE with lysine gives rise to a derivative, SEEEKE, which is an extremely poor substrate for CK-2. While, in fact, neutral substitutions in that position increase the $K_m$ without affecting $V_{max}$, lysine also causes a dramatic decrease of $V_{max}$ (Table I).

*Influence of N-terminal residues*

Although amino acid residues upstream of the phosphorylatable one are obviously not among the minimum structural requirements of CK-2, if present, they can greatly influence the phosphorylation efficiency. This point has been systematically tested by adding either a neutral or acidic or basic residue to the N-terminal serine of phosphorylatable peptides (Table I). Surprisingly, addition of an alanine does not improve, but rather decreases the phosphorylation efficiency of SEEEEE, by raising the $K_m$ value several fold. No harmful effect is observed, however, if elongation is with a glutamic acid that actually improves phosphorylation, by significantly decreasing the $K_m$. Conversely, an arginine adjacent to the N-terminal side of serine is very deleterious giving rise to hardly phosphorylatable derivatives.

It may also be worth noting that the replacement of the N-terminal serine of SEEEEE with alanine gives rise to a hexapeptide (AEEEEE) that has lost not only phosphorylatability but also most of its binding capacity, as judged from its high $K_i$ value as compared with that of SEEEEE (8.19 mM and 0.25 mM, respectively, calculated using casein as phosphorylatable substrate).

*Response to effectors: influence of the structure of peptide substrate*

As previously shown [7], basic polypeptides such as protamines stimulate CK-2 activity by lowering the $K_m$ for the protein substrate. Such an effect, however, is markedly influenced by the structure of the peptide substrate used, being much more evident with the 'low affinity' SAAEAE peptide than with the 'high affinity' one, SEEEEE. In particular, while the $K_m$ value when using the former is lowered 10-fold by polylysine, that when using the latter is only reduced by a half (Fig. 2).

On the other hand, the effect of polyphosphates which are competitive inhibitors of CK-2 (unpublished data) is much less evident with SEEEEE than with SAAEAE (Fig. 3).

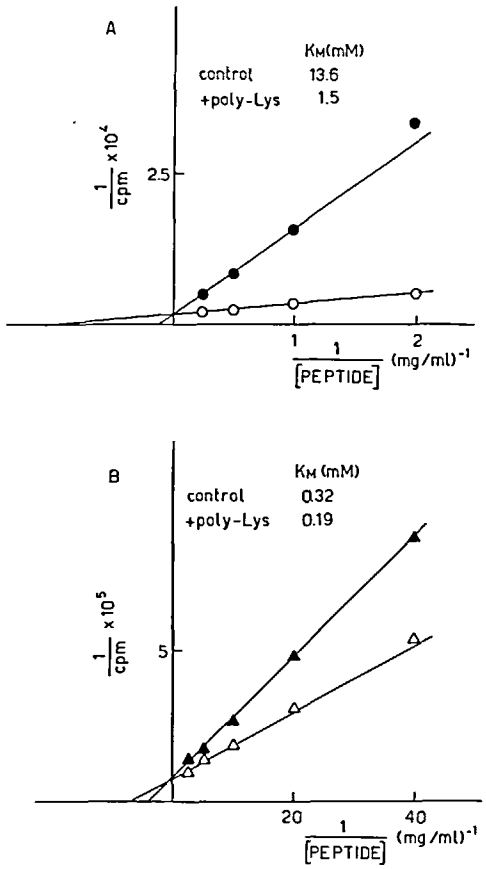


Fig. 2. Double-reciprocal plots for stimulation of CK-2 activity by polylysine using the peptides SAAEAE (A) or SEEEEE (B) as phosphorylatable substrate. Filled symbols, control; open symbols, plus 0.1 mg/ml polylysine.

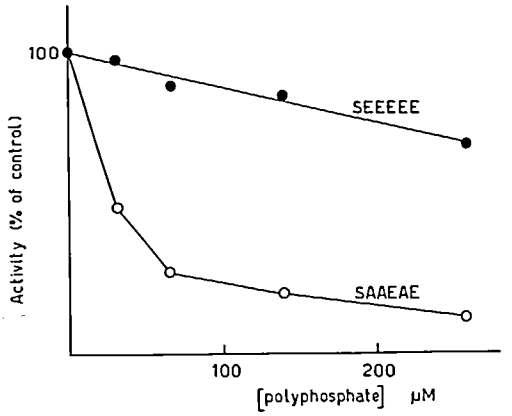


Fig. 3. Inhibition by polyphosphate of CK-2-catalysed phosphorylation of peptides SEEEEE (●) and SAAEAE (○). The sodium phosphate polymer was type 15 (average chain length 15±3 phosphates) from Sigma. The concentration of the peptide substrate was 1 mM.

*Failure of synthetic peptides to undergo phosphorylation by casein kinase 1*

The peptide SEEEEE and several other synthetic peptides described here have been also assayed as substrates for type-1 casein kinase (CK-1), whose site specificity, definitely distinct from that of CK-2, is not yet understood [8]. None of them, however, could be significantly phosphorylated (not shown). In particular, the failure to phosphorylate the peptide EEEEES and ESEEEEE would indicate that N-terminal acidic residues, which are supposed to represent a positive determinant for site recognition by CK-1 [9] are not a sufficient condition for the phosphorylation of a peptide substrate by this enzyme.

## Discussion

In two previous reports [3,4], 17 peptides reproducing with suitable modifications the phosphorylation sites for CK-2 in physiological substrates, have been employed to disclose the basic structural determinants of CK-2, leading to the conclusion that this enzyme can recognise serine and, less readily, threonine residues, that are located on the N-terminal side of acidic clusters, the acidic residue lying in the third position downstream from the phosphorylatable amino acid playing an especially crucial role.

Here, ten additional peptides have been assayed as substrates and/or inhibitors of CK-2, providing more detailed information about the actual minimum structural requirements of this enzyme and some features of the phosphorylation site influencing, either positively or negatively, the catalytic efficiency. The most relevant new data obtained can be summarised as follows:

(1) Although peptides with extended acidic clusters on the C terminal side of serine are by far preferred, a single acidic residue at the third position is sufficient for phosphorylation to occur, provided negative determinants are absent. Actually, while SEEAAA and SAEAAA are not substrates at all, the peptides SAAEAA and, to an even greater extent, SAAEAE, are still significantly phosphorylated by CK-2. Their $K_m$ values, however, are two orders of magnitude higher and their $V_{max}$ values are much lower than those of SEEEEE. These data are in good agreement with the recent finding that CK-2 can phosphorylate fibrinogen [10] and eIF-2 ($\beta$ subunit) [11] at sites including just two acidic residues, one of which is at position +3; but they also account for the very favourable kinetic parameters of nucleolar protein substrates of CK-2, in which the phosphorylated residues are followed by long acidic stretches [12]: it should be noted on this matter that the sequence downstream from the phosphorylatable Ser-209 of nucleolin is exactly reproduced in our peptide, SEEEEE.

(2) The phosphorylation of 'poor' peptide substrates, fulfilling just the minimum requirements, is much more responsive to polylysine stimulation than that of optimal substrates with extended acidic clusters. Thus, the $K_m$ for SAAEAE is lowered from 13 to 1.5 mM, wheraes that for SEEEEE is just reduced to half. Likewise, inhibition by polyphosphates is more readily evident with SAAEAE than with SEEEEE. These observations provide a structural basis for the interpretation of the observed differential effects of stimulators and inhibitors of CK-2 on the phosphorylation of individual substrate proteins (e.g., Refs. 13 and 14). Conceivably, the structure and amino acid composition of the phosphoacceptor sites at least partially account for such 'substrate-directed' regulations playing an especially critical role in the case of multifunctional protein kinases, such as CK-2.

(3) The peptide SEEEEE is a much better sub-

strate than SAAEEEEE, which, in turn, is better than SAAEEE: this means that the substrate-binding site of CK-2 recognises positive acidic determinants until the sixth and seventh positions downstream from the phosphorylatable residue, albeit, in such positions, they are much less effective than when in the first and second position.
(4) While the interruption of the acidic cluster by a neutral residue at positions other than +3 is well tolerated, a basic residue is deleterious: the peptide SEEEKE, in fact, is a very poor substrate compared to both SEEEAE and SEEEVE. The harmful effect of lysine could be reinforced through internal neutralisation of the adjacent carboxylic group at position +3, which is essential for efficient phosphorylation [4]. In agreement with this observation, basic residues are always lacking on the C-terminal side of phosphorylation sites for CK-2 (see Ref. 4).
(5) N-terminal amino acid residues are not required for ensuring optimal phosphorylation of serines by CK-2: actually, the upstream elongation of SEEEEE with alanine results in a remarkable increase of $K_m$, and an arginine residue in that position is even more harmful, virtually preventing phosphorylation. Conversely, however, N-terminal elongation of SEEEEE with an additional glutamic acid, rather than with alalnine and arginine, does not decrease, but actually increase the phosphorylation efficiency. This clearly indicates that the N-terminal residue(s), though not representing a minimum requirement, can, nevertheless, greatly influence the efficiency of serine phosphorylation by CK-2 with their side chains.

Clearly, the specificity determinants of CK-2 sharply differentiate this enzyme from 'basic-residue-requiring' serine/threonine-specific protein kinases including, among others, cAMP and cGMP-dependent protein kinases, Ca/calmodulin dependent protein kinases and protein kinase C, all of which recognise basic, rather than acidic residues, either on the N- or C-terminal sides of the target amino acid (reviewed in Ref. 18). Moreover, the site specificity of CK-2 apparently is also different from those of other 'acidic-residue-requiring' protein kinases, such as mammary gland casein kinases, requiring an acidic residue at position +2 rather than +3 [15,16] and casein kinase 1 whose structural requirements are still obscure, but which proved definitely unable to phosphorylate the typical peptide substrates of CK-2. More generally, our data provide experimental evidence that the suitability of an individual phosphoacceptor site for a protein kinase ultimately depends not only on its fulfilment of the minimum structural requirements, which in the case of CK-2 appear to be the sequence Ser(Thr)-X-X-Glu(Asp)-X, but also on other features among which are the following: (i) Additional positive determinants allowing optimum efficiency (such as multiple acidic residues on both sides of the target amino acid, in the case of CK-2. (ii) The inclusion of negative determinants (such as basic residues, in the case of CK-2) preventing the phosphorylation of otherwise suitable sites. (iii) The presence of substrate-directed effectors whose influence on the phosphorylation efficiency is shown here to be also largely dependent on local structural features.

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