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ARTICLE *in* BIOCHEMISTRY · OCTOBER 1997

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Mutational Analysis of Residues Implicated in the Interaction between Protein Kinase CK2 and Peptide Substrates[†]

Stefania Sarno,[‡] Philippe Vaglio,[‡] Oriano Marin,[‡,§] Olaf-Georg Issinger,[||] Katia Ruffato,[‡,§] and Lorenzo A. Pinna*,[‡]

Dipartimento di Chimica Biologica, Universita' di Padova and Centro per lo Studio delle Biomembrane del Consiglio Nazionale delle Ricerche, 35121 Padova, Italy, CRIBI, Biotechnology Center, 35121 Padova, Italy, and Biokemisk Institut, Odense Universitet Campusvej 55, 5230 Denmark

Received March 13, 1997; Revised Manuscript Received June 27, 1997[⊗]

ABSTRACT: Sixteen derivatives of the optimal peptide substrate RRRA-DDSDDDDD in which aspartic acids were singly or multiply substituted by alanine have been assayed for their phosphorylation efficiency by either wild type protein kinase CK2 or CK2 α mutants defective in substrate recognition. With wild type CK2, the only detrimental single substitutions were those at positions +3 and +1. Each of these caused a 5-fold increase of K_m and a 2-fold decrease of the V_{max} values. If both aspartic acids at $n + 1$ and $n + 3$ were substituted however, the K_m rose 24-fold and the V_{max} decreased 16-fold. Multiple substitutions tend to have a more than additive effect even if they affect individually dispensable aspartic acids; thus, double, triple, and quintuple substitutions at positions $n - 2$ and -1, and $n + 2$, $+4$, and $+5$ had detrimental consequences comparable to those observed with substitutions at $n + 1$ and $n + 3$. These data indicate that additional acidic residues besides those at $n + 1$ and $n + 3$ are collectively required for efficient phosphorylation of CK2 substrates. They are also consistent with a flexible mode of binding of the substrate, where acidic residues may play interchangeable roles. Among twelve CK2 mutants in which basic residues suspected to be implicated in substrate recognition have been replaced by alanine, only K74−77A, K79R80K83A, R191,195K198A, and K198A showed substantially increased K_m values with the optimal substrate RRRA-DDSDDDDD, symptomatic of a reduced ability to bind it. However, if the suboptimal substrate RRRA-AASDDDDD was used, the single mutants K49A, K71A, K77A, R80A, and H160A also exhibited K_m values significantly higher than those of wild type CK2. Kinetic analysis with singly substituted derivatives of peptide RRRA-DDSDDDDD revealed that K49 is implicated in the recognition of the determinant at position $n + 2$, K77 cooperates with other residues nearby in the interaction with the determinants at $n + 3$ and $n + 4$, while K198 plays a prominent role in the recognition of the determinant at $n + 1$.

The reversible phosphorylation of seryl, threonyl, and tyrosyl residues of proteins represents a general mechanism by which nearly all cellular functions are regulated in eukaryotes. Protein kinases, responsible for such phosphorylation processes, make up a large superfamily of enzymes sharing catalytic domains with remarkable homologies (Hanks et al., 1988) and a similar bilobal architecture as judged from the protein kinases whose structures have been solved to date (Knighton et al., 1991a; De Bondt et al., 1993; Hu et al., 1994; Hubbard et al., 1994; Zhang et al., 1994; Xu et al., 1995; Goldberg et al., 1996). A striking property of protein kinases is their substrate specificity, enabling them to recognize just a few "right" targets amidst a multitude of "wrong" ones. Local structural features, notably the nature of the phosphoacceptor residue (allowing a preliminary subdivision into two subfamilies of Ser/Thr and Tyr specific kinases) and a limited number of key residues at definite positions nearby, contribute to such selectivity giving rise to consensus sequences that are specifically recognized by individual protein kinases (Pinna & Ruzzene, 1996).

On the basis of the nature of the specificity determinants, Ser/Thr kinases roughly fall into three categories: (i) basophilic, which are the majority of Ser/Thr protein kinases, (ii) proline-directed, and (iii) acidophilic, i.e. Asp/Glu- and sometimes phosphate-directed enzymes. Among these acidophilic kinases, one, termed protein kinase CK2[1] (formerly "casein kinase 2 or II"), is a ubiquitous and pleiotropic enzyme committed to the phosphorylation of a large number of proteins, many of which are implicated in signal transduction, gene expression, and cell proliferation [reviewed by Pinna (1990), Tuazon and Traugh (1991), and Allende and Allende (1995)]. The minimum consensus sequence of CK2 is S/T-X-X-D/E/Sp/Yp (Meggio et al., 1994), an acidic side chain at position $n + 3$ representing a sufficient condition for detectable phosphorylation (Marchiori et al., 1988); additional acidic residues however are required for efficient phosphorylation of peptide substrates (Meggio et al., 1984; Marin et al., 1986; Kunzel et al., 1987; Marchiori

[†] This work was supported by Grants to L.A.P. from the Italian Ministero dell'Universita' e della Ricerca Scientifica e Tecnologica, Consiglio Nazionale delle Ricerche (Target Project Applicazioni Cliniche della Ricerca Oncologica) Ministero della Sanita' (Project AIDS), and Associazione Italiana per la Ricerca sul Cancro and to O.-G.I. from the Danish Cancer Society (Grant 9610040).

* Address correspondence to this author at the following address: Dipartimento di Chimica Biologica, Viale G. Colombo 3, 35121 Padova, Italy. Fax: (39)-49-8073310. E-mail: pinna@civ.bio.unipd.it.

[‡] Universita' di Padova.

[§] CRIBI.

[||] Odense Universitet.

[⊗] Abstract published in *Advance ACS Abstracts,* September 1, 1997.

[1] Abbreviations: CK2, protein kinase CK2; PKA, cyclic AMP-dependent protein kinase; DEAE, diethylaminoethyl; CM, carboxymethyl; HPLC, high-performance liquid chromatography; FPLC, fast-performance liquid chromatography; CD, circular dichroism; erk, mitogen-activated protein kinase; cdk2, cyclin-dependent kinase 2; PKI, protein kinase inhibitor.

S0006-2960(97)00577-1 CCC: $14.00

et al., 1988), and indeed, multiple acidic residues (quite often more than three) specify the natural phosphoacceptor sites affected by CK2 in its protein targets (Meggio et al., 1994). Although these acidic determinants may play a favorable role also on the N-terminal side of serine, notably at positions −1 and −2 (Kunzel et al., 1987; Meggio et al., 1994), they are maximally effective on the C-terminal side, at positions $n + 1$ to $n + 5$ or even farther away (Marchiori et al., 1988; Hubrey & Roach, 1990). These properties make CK2 a good model from which to gain insight into the molecular features that underlie the recognition of local specificity determinants by protein kinases. This information in fact would complement that drawn from PKA, the prototype of protein kinases, which recognizes basic, instead of acidic, determinants, located upstream, rather than downstream, from Ser/Thr. The PKA consensus sequence is R-R-X-S/T, and both mutational (Gibbs & Zoller, 1991a) and crystallographic studies (Knighton et al., 1991b) have shown that E127 and E170/E230 play a major role in interactions with the arginyl residues at positions $n - 3$ and $n - 2$, respectively. Interestingly, E170 is replaced in CK2 by a basic residue (H160) whose mutation to aspartic acid has been shown to impair the recognition of atypical peptide substrates, lacking the crucial determinant at $n + 3$, and whose phosphorylation is specified by an acidic side chain at $n - 2$ (Dobrowolska et al., 1994). These data supported the view that homologous residues of distinct kinases may play analogous roles in substrate recognition and prompted us to start a mutational analysis of CK2, focusing on conserved basic residues in it which are replaced by other residues in nonacidophilic protein kinases. This study led to the identification of clusters of three or four basic residues in the 74−77, 79−83, and 191−198 sequences whose simultaneous mutation to alanines was detrimental for the recognition of determinants at positions +3 and +4 (and/or +5) and +1, respectively (Sarno et al., 1996).

In this paper, we analyze the relative contribution of individual residues within these clusters and the potential implication of additional basic residues in the recognition of peptide substrates varying in the number and position of acidic determinants. The data are consistent with a flexible recognition module, where several specificity determinants may play interchangeable roles and the catalytic and phosphoacceptor sites adopt conformations that are the aptest for reciprocal interactions.

EXPERIMENTAL PROCEDURES

Materials. The synthetic peptides denoted in Table 1 with the numbers 1−20 were obtained in the solid phase with the Fmoc (FMOC, 9-fluorenylmethoxycarbonyl) strategy on 4-(hydroxymethyl)(phenoxymethyl)copolistirene/1% divinilbenzene-resin (0.96 mmol/g). The chain elongation was performed using a 5- or 10-fold excess of Fmoc-amino acid, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetrametyluronium hexafluorophosphate, and 1-hydroxybenzotriazole (1:1:1) in the presence of a 20-fold excess of *N*,*N*-diisopropylethylamine.

In particular, the syntheses of peptides 3, 4, 6, 9, 13, 16, 17, 19, and 20 were performed with a manual procedure in polypropylene columns as previously detailed (Sarno et al., 1995), while peptides 1, 2, 5, 7, 8, 10−12, 14, 15, and 18 were synthetized with an automatic peptide synthetizer (model 431-A from Applied Biosystems) as described by Lasa-Benito et al. (1996) and Marin et al. (1994). The acetylation of the N-terminal amine of peptide 12 (aimed at improving its solubility) was obtained with treatment of the peptidyl-resin with a 10% anhydride acetic in *N*-methylpyrrolidone solution for 10 min. The peptides were purified either by ion-exchange cromatography on Sepharose (DEAE or CM) Fast Flow resin (Pharmacia) or by semipreparative reverse-phase HPLC on a C18 column (Delta Pack, Waters, 15 μm, C18, 300 Å, 78 × 300 mm). The composition of peptides 1 and 2 was checked by amino acid analysis. All the purified peptides showed an HPLC purity of ≥95%.

Table 1: Effect of Substituting Aspartic Acids in the Reference Peptide Substrate RRRA-DDSDDDDD on Kinetic Constants for CK2-Catalyzed Phosphorylation[a]

		K_m (μM)	relative V_{max}	V_{max}/K_m
1	RRRA-DDSDDDDD	18	100.0	5.550
2	RRRA-**A**DSDDDDD	24	122.0	5.180
3	RRRA-D**A**SDDDDD	23	50.0	2.170
4	RRRA-DDS**A**DDDD	100	44.5	0.445
5	RRRA-DDSD**A**DDD	9	47.0	5.220
6	RRRA-DDSDD**A**DD	102	44.0	0.430
7	RRRA-DDSDDD**A**D	15	65.0	4.330
8	RRRA-DDSDDDD**A**	10	65.0	6.500
9	RRRA-DDSDDD**AA**	19	62.0	3.260
10	RRRA-DDSD**A**D**AA**	243	86.0	0.350
11	RRRA-DDS**A**D**A**DD	435	6.2	0.014
12	Ac**RRRA-DDSDAAAA**	2000	37.0	0.0185
13	RRRA-**AA**SDDDDD	66	52.5	0.795
14	RRRA-**AA**S**A**DDDD	147	2.2	0.015
15	RRRA-**AA**SDDDD**A**	87	37.0	0.425
16	RRRA-**AA**S**A**D**A**DD	870	3.8	0.004
17	RRRA-**AA**SD**A**D**AA**	135	7.6	0.056
18	RRRA-**EE**S**EEEEE**	17	45.0	2.650
19	RRRA-**EE**S**E**D**EE**	10	70.0	7.000
20	RRRA-**E**D**E**S**E**D**EE**	11	70.0	6.400

[a] Phosphorylation experiments were run with reconstituted CK2 wild type holoenzyme as described in Experimental Procedures. V_{max} values are expressed as the percent of the V_{max} value with the optimal peptide substrate, RRRA-DDSDDDDD (=1265 nmol min^{-1} mg^{-1}). All data are the mean of at least three separate determinations with a standard error of less than 15%. Substitution and addition are denoted by bold type. Ac denotes *N*-terminal acetylation (to improve solubility; see Methods).

Casein was from Sigma. [γ-^{32}P]ATP (2 mCi/mL) was from Amersham Corp. All the other reagents were of the hightest purity available.

Mutagenesis and Expression of CK2 Subunits. The new α mutants, K49A, K71A, K77A, K79A/A223T, R80A, K83A H160A, K198A, and H234,236A, were generated by oligonucleotide-directed mutagenesis as described in detail previously (Boldyreff et al., 1992). Expression of CK2 α and β subunits and of α mutants was performed as previosly described (Grankowski et al., 1991).

Reconstitution and Purification of CK2 Holoenzymes. The procedure followed was essentially the one described by Sarno et al. (1996); bacteria pellets of wild type α or mutated α were mixed with the same amount of bacteria pellet of the β subunit. The pellets were resuspended, sonicated, and centrifuged. P11 phosphocellulose and MonoQ (FPLC system) columns were used to get holoenzymes purified to >95% homogeneity as judged by Coomassie blue staining.

The specific activities of the mutated holoenzymes were as follows: K49A, 593 U mg^{-1}; K74−77A, 152 U mg^{-1}; K77A, 110 U mg^{-1}; K79R80K83A, 1832 U mg^{-1}; K79A/A223T, 137 U mg^{-1}; K83A, 3329 U mg^{-1}; H160A, 2500 U mg^{-1}; R191,195K198A, 59 U mg^{-1}; and K198A,144 U mg^{-1}

(1 unit being defined as the amount of enzyme transferring 1 nmol of P to the optimal peptide substrate RRRA-DDSDDDDD per minute under the experimental conditions detailed below). The recovery of R80A was too low to make reliable protein determination, and consequently, its specific activity could not be calculated. The specific activity of wild type CK2was 1245 U mg^{-1}. The specific activities of wild type CK2 and of mutants K74−77A and K79R80K83A are significantly higher than those previously reported (Sarno et al., 1996). The reasons for this discrepancy are not entirely clear. Seemingly, previous assays were run with an excess of enzyme protein, beyond the range of linear increase of activity.

Phosphorylation Assay. Phosphorylation of synthetic peptides and casein was performed with 10 min of incubation at 30 °C in 30 μL of a medium containing 50 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 100 mM NaCl, and 25 μM [γ-^{32}P]-ATP (specific activity, 500 cpm/pmol). The enzyme concentration (0.2−1.7 μg mL^{-1}) was always maintained within a linear range with activity. ^{32}P incorporation in synthetic peptides was evaluated with the phosphocellulose paper procedure (Glass et al., 1978); phosphorylation of casein was evaluated by trichloroacetic acid precipitation spotting 25 μL on Whatman 3MM chromatography paper and washing as described by Meggio and Pinna (1984). Kinetic constants were determined by double-reciprocal plots constructed from initial measurements fitted to the Michaelis−Menten equation.

CD Spectroscopy. CD spectra in the UV region (190−250 nm) have been obtained in 10 mM Tris-HCl buffer (pH 7.8) with a 1.5−2 μM concentration of either wild type CK2 or mutants, using a Jasco J-710 spectropolarimeter.

Molecular Modeling. Calculations were made on an Indy R4000 (Silicon Graphics) or an Indigo2 R8000 (Silicon Graphics) workstation. For CK2 α subunit modeling the crystal structures of PKA, erk2, and cdk2 were used (PDB codes latp, lerk and lhck, respectively). The sequences of these three kinases were aligned using the Quanta package (Molecular Simulation Inc.) with the protein homology tools. The proteins were first aligned according to their sequences; then, identical residues were matched, and the three structures were superimposed. Finally, a new alignment was made using the Cα distances as a weight for alignment, this procedure being applied twice. The second time, the sequence of human CK2α (residues 1−376) was aligned. Alignment was weighted by Momany secondary structure prediction and sequence homology. Then, the alignment of the CK2α sequence was adjusted by hand, using the result of a multiple alignment (clustalw) of the four sequences as a model. For the elaboration of the model of CK2α, the program Modeler (A. Sali) was used. Five structures were obtained from Modeler, and the one with the highest structural homology with respect the other kinases was used further. The model of CK2α was superimposed onto the structure of PKA plus the inhibitor peptide PKI (PDB code latp). The peptide was placed in CK2α, and the residues of the peptide were mutated to produce the sequence RRRA-DDSDDDDD (i.e. the parent peptide used in this study); then, the structure was minimized using the CHARMM program (MSI) until the energy reached a minimum.

RESULTS

(1) Mutational Analysis of Acidic Determinants within an Optimal Peptide Substrate. Previous studies have unambiguously shown that an individual acidic residue at position $n + 3$ plays an essential role as a minimum specificity requirement for phosphorylation by CK2 (Marin et al., 1986; Marchiori et al., 1988). While this feature represents a sufficient condition for phosphorylation, which becomes undetectable if this *unique* acidic residue is placed at different positions (e.g. +1, +2, +4, or +5) (Marchiori et al., 1988), additional acidic residues are required in order to optimize phosphorylation, as exemplified by comparing the K_m values of the "minimum substrate" SAAEAA (>18 mM), the "improved substrate" SEEEEE (0.27 mM) (Pinna, 1990), and the "optimal" substrates in which acidic residues are present at all positions from $n - 2$ to $n + 5$ (about 20 μM) (Marin et al., 1994). If however in the optimal peptide substrate RRRA-DDSDDDDD each of the seven aspartic acids is individually replaced by alanine, the only detrimental substitutions are those occurring at positions $n + 1$ and $n + 3$ (Table 1, peptides 4 and 6, respectively). Even these substitutions moreover are still compatible with appreciable phosphorylation efficiency. The modest effect of substitutions at $n - 2$, $n - 1$, $n + 2$, $n + 4$, and $n + 5$ does not mean, however, that acidic residues at these positions are unimportant, as exemplified by peptide 10 in which aspartic acids at positions $n + 2$, $n + 4$, and $n + 5$ are substituted, and which is a very poor substrate despite the aspartic acids at $n + 1$ an $n + 3$. Clearly, "subsidiary" acidic residues at positions +2, +4, and +5, although *individually* dispensable, are *collectively* required for efficient phosphorylation. Consequently, multiple substitutions tend to have a more than additive effect, consistent with the view that the suppression of an individual acidic residue is partially obviated by the presence of the other(s). This also applies to aspartyl residues at $n - 1$ and $n - 2$ whose simultaneous replacement by alanine gives rise to a substrate (peptide 13) worse than expected from the behavior of peptides 2 and 3 in which aspartyl residues at $n - 2$ and $n - 1$ were individually substituted.

It should also be noted that the lack of the two acidic determinants adjacent to the N-terminal side of serine [which is found in about 46% of CK2 sites (Meggio et al., 1994)] alters the effect of downstream substitutions. This is exemplified by the relatively modest effect on K_m of the $n + 1$ (compare peptides 13 and 14) and $n + 2/n + 4/n + 5$ substitutions (compare peptides 13 and 17) if residues at $n - 1$ and $n - 2$ are alanine as compared to the more drastic effect on K_m observed if the same substitutions are done in the parent peptide (compare peptides 4 and 10 with peptide 1). In contrast, the role of the acidic determinant at $n + 3$ is magnified by the lack of acidic residues upstream from serine (compare the K_m value of peptide 16 to that of peptide 14 and this latter to that of peptide 13).

Interestingly, although the "all-Asp" peptide RRRA-DDSDDDDD (peptide 1) is a better substrate than the "all-Glu" derivative RRRA-EESEEEEE (peptide 18), the peptide in which all aspartic acids but the one at +2 have been replaced by Glu (peptide 19) is actually better than both of these. This is in agreement with the optimal sequence predicted by an oriented peptide library (Songyang et al., 1994). In contrast to the library prediction, however, the addition of two extra acidic residues at positions −3 and −4 (ED instead of RA) does not improve the phosphorylation efficiency (compare peptides 19 and 20).

The data of Table 1 also disclose a tendency of substituted peptides to have decreased V_{max} values as compared to that

of the unsubstituted peptide substrate RRRA-DDSDDDDD. With some multiply substituted peptides, these decreases in V_{max} are striking (e.g. peptides 11, 14, 16, and 17). It seems possible that such low V_{max} values are, at least partially, due to the inhibitory potential of peptides in which D → A substitutions generate pseudosubstrate motifs, notably A-D-D-D, A-D-X-D, and A-X-X-D, which are not present in the parent peptide.

(2) Mutational Analysis of Basic Residues Implicated in Substrate Recognition. Previous mutational studies (Sarno et al., 1995, 1996) based on the simultaneous replacement of multiple basic residues with alanine led to the identification of three basic stretches of the CK2 α subunit which are implicated in substrate recognition. In particular, the quadruple mutation of four consecutive lysyl residues, K74−K77, did not prevent either association with the β subunit or the thermostability of the reconstituted enzyme, while it caused a dramatic decrease in phosphorylation efficiency, which can be attributed mainly to a large increase in K_m. This result was especially notable considering that the double mutation of either K74 and K75 or K75 and K76 was previously shown to have an only marginal effect on substrate phosphorylation (Hu & Rubin, 1990; Gatica et al., 1994). It was possible therefore that the mutation of the fourth lysine, K77, could have been critical for determining the detrimental effect of the quadruple mutation. To check this possibility, K77 was replaced by alanine and this mutant was compared to the quadruple one, K74−77A. Since the data in the literature concerning the 74/75 and the 75/76 double mutations were obtained using casein as the phosphorylatable substrate (Hu & Rubin, 1990; Gatica et al., 1994), casein was used in our experiments as well. As shown in Figure 1, in contrast to double mutants K74,75E and K75,76E, the single mutant K77A is severely defective in casein phosphorylation, with an affinity 12-fold lower than that of the wild type, a decrease comparable to that of the quadruple mutant K74−77A.

However, if the same comparison is made using the optimal peptide RRRA-DDSDDDDD as substrate (Table 2) the increase in K_m promoted by the single K77 → A mutation (about 2-fold) is negligible as compared to that promoted by the quadruple mutation of the whole K74−77 stretch (about 40-fold). It should be noted that the sites affected by CK2 in casein (Pinna et al., 1990) are much less acidic than the polyaspartyl stretch surrounding serine in the optimal peptide, and in particular, they lack acidic residues adjacent to the N-terminal side of Ser/Thr. Consequently, the two mutants, K77A and K74−77A have also been compared using a doubly substituted derivative of the optimal peptide in which aspartyl residues at positions −2 and −1 were replaced by alanine (peptide 13 of Table 1). As shown in Table 2, with this peptide substrate, K77A exhibits a 9-fold higher K_m value than wild type CK2, denoting a remarkable drop in affinity, though not as dramatic as that observed with the quadruple mutant K74−77A.

These results corroborate the concept that acidic specificity determinants may play interchangeable roles so that the presence of acidic residues upstream may partially obviate the lack of other interactions between the kinase and acidic determinants located downstream from serine. Consequently, the assay with peptide RRRA-AASDDDDD may prove to be more sensitive than that with the optimal peptide RRRA-DDSDDDDD in order to highlight the effects of mutations

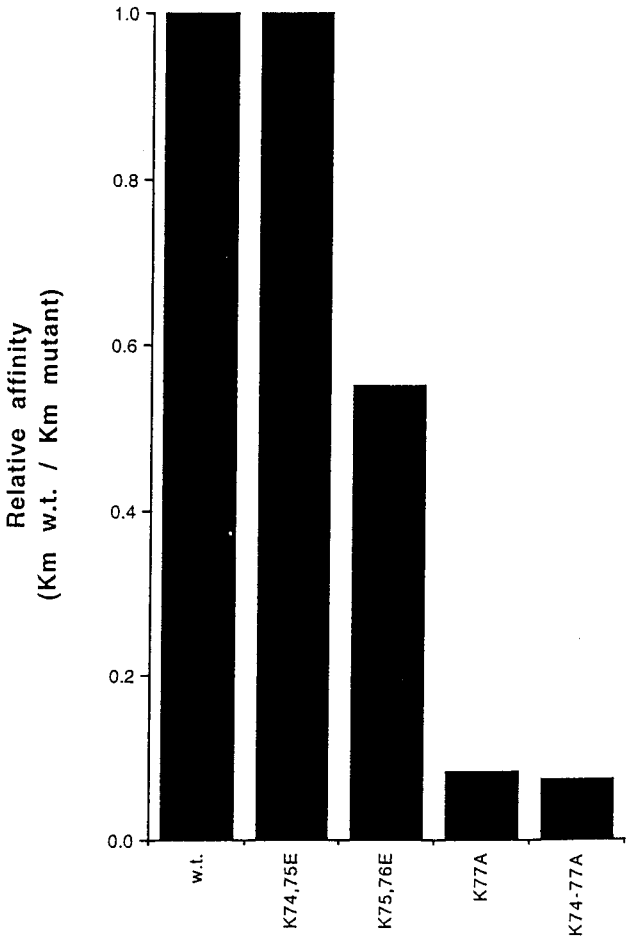

FIGURE 1: Effect of altering the 74−77 basic cluster on the relative affinity of protein kinase CK2 for casein. Affinity is expressed as the ratio between K_m with wild type CK2 and K_m with CK2 mutants. The data for mutants K74,75E and K75,76E have been drawn from Hu and Rubin (1990) and Gatica et al. (1994), respectively.

on the recognition of acidic residues on the C-terminal side of serine.

Consistent with this prediction, a number of mutations of basic residues in CK2 cause significant decreases in affinity toward the RRRA-AASDDDDD peptide whereas they are almost ineffective if the substrate is RRRA-DDSDDDDD (see Table 2). These include, besides K77, also K49, K71, a homologue to PKA K81, supposed to lie in the $n + 3$ pocket (Songyang et al., 1996), R80, and H160. On the other hand, the mutations of K79, K83, and H234 plus H236, these latter included in the "small insert", whose implication in substrate recognition was suggested by Tiganis et al. (1995), have little effect on the K_m values with either the optimal or the suboptimal peptide substrates. Only one single mutant, K198A, is severely defective with both peptide substrates.

A more detailed analysis of individual acidic determinants recognized by mutated residues was done using a series of peptide substrates in which the aspartic acids at positions −2 to +5 in the parent peptide RRRA-DDSDDDDD were replaced by alanine (peptides 2−8 of Table 1). As pointed out elsewhere (Sarno et al., 1996), by determining the kinetic constants for these peptides, it is possible to gain information about which are the positions where specificity determinants are primarily recognized by the residues mutated in the kinase. The simultaneous substitution of specificity determinants and of the residues committed to their recognition is in fact less detrimental than substitutions that suppress determinants recognized by residues which are conserved

Table 2: Kinetic Constants of CK2 Holoenzyme α-Mutants with an Optimal and a Suboptimal Peptide Substrate[a]

	RRRA-DDSDDDDD			RRRA-**AA**SDDDDD		
	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})	V_{max}/K_m	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})	V_{max}/K_m
wild type α	18.0	1265.0	70.30	69.0	1015.0	14.71
K49A	19.6	1462.0	74.60	400.0	1649.5	4.12
K71A	19.0	1185.0	62.37	338.0	840.0	2.48
K74−77A	769.0	1170.5	1.52	1818.0	64.3	0.03
K77A	30.0	1650.0	55.00	645.0	389.0	0.60
K79R80K83A	100.0	2611.0	26.11	1818.0	294.0	0.16
K79A/A223T	11.5	153.0	13.30	115.0	102.0	0.89
R80A	43.0	nd	nd	714.0	nd	nd
K83A	12.0	3747.0	312.25	238.0	3103.5	13.04
H160A	7.0	2566.5	366.64	345.0	3556.0	10.30
R191,195K198A	420.0	563.0	0.13	3333.0	58.0	0.02
K198A	232.5	495.5	2.13	952.0	100.0	0.10
H234,236A	16.0	1518.0	94.87	82.5	996.0	12.07

[a] Phosphorylation experiments and calculation of kinetic constants were performed as described in Experimental Procedures. nd = not determined because it was impossible to measure the protein concentration of mutant R80A (see Experimental Procedures).

Table 3: Kinetic Analysis of CK2 Mutants with Peptide Substrates Bearing Individual Substitutions of Specific Determinants[a]

		CK2 α-mutants									
peptide substrate		wild type α	K49A	K74−77A	K77A	K79R80K83A	K79A/A223T	K83A	H160A	R191,195K198A	K198A
RRRA-DDSDDDDD	K_m	18.0	19.6	769.0	30.0	100.0	11.5	12.0	7.0	417.0	232.5
	V_{max}	1265.0	1462.0	1170.5	1650.0	2611.0	153.0	3747.0	2566.5	563.0	495.5
RRRA-**A**DSDDDDD	K_m	24.0	9.5	2222.0	200.0	417.0	58.0	17.0	13.0	645.0	227.0
	V_{max}	1540.0	570.0	805.0	3017.0	3389.0	184.0	3583.0	2460.0	307.0	219.0
RRRA-D**A**SDDDDD	K_m	23.0	29.5	1667.0	333.0	833.0	83.0	45.0	36.0	896.5	278.0
	V_{max}	632.0	506.0	126.0	1178.0	901.0	123.0	2530.0	1720.0	111.5	62.0
RRRA-DDS**A**DDDD	K_m	100.0	122.0	4000.0	488.0	667.0	167.0	100.0	48.0	105.0	91.0
	V_{max}	563.0	314.0	84.0	519.0	324.5	72.0	1656.0	924.0	676.0	390.0
RRRA-DDSD**A**DDD	K_m	9.0	2.7	526.0	80.0	67.0	13.5	17.0	13.0	714.0	178.5
	V_{max}	591.0	535.0	1560.5	2929.0	2186.0	128.0	4056.0	2387.0	578.0	291.0
RRRA-DDSDD**A**DD	K_m	102.0	143.0	952.0	200.0	200.0	200.0	86.0	119.0	571.0	1333.0
	V_{max}	556.0	399.0	875.0	1438.0	3281.0	67.0	1433.0	1266.0	13.4	35.0
RRRA-DDSDDD**A**D	K_m	15.0	10.5	385.0	51.0	100.0	9.5	16.0	7.0	833.0	294.0
	V_{max}	819.0	718.0	2201.5	3362.0	3013.0	127.0	3166.0	2493.0	447.0	536.0
RRRA-DDSDDDD**A**	K_m	10.0	12.0	500.0	55.5	83.0	8.0	13.0	7.2	263.0	185.0
	V_{max}	822.0	697.0	1535.5	3410.0	2410.0	124.0	3367.0	2539.5	245.0	329.0

[a] The alanyl residues replacing aspartic acid are bold. All data are the mean of at least three separate determinations with a standard error of less than 14%. Additional details are given in Experimental Procedures. K_m is expressed in micromolar V_{max} is expressed in nanomoles per minute per milligram.

in the mutants, as first shown by "Ala scanning mutagenesis" of PKA (Gibbs & Zoller, 1991b). Thus, relative phosphorylation efficiencies higher than those calculated for the wild type kinase are symptomatic of substitutions affecting determinants recognized by residues mutated in the kinase.

For comparison, we have reconsidered in our current kinetic analysis also three multiple mutants (K74−77A, K79R80K83A, and R191,195K198A) whose kinetic constants had been calculated in a previous study (Sarno et al., 1996). These have now been assayed using the newly synthesized peptide substrates singly substituted at $n + 4$ and $n + 5$, instead of the peptide bearing a double substitution at $n + 4$ and $n + 5$, used before. A synopsis of the K_m and V_{max} values of all the mutants with all the peptide substrate derivatives is shown in Table 3. The data obtained with mutants K74−77A, K79R80K83A, and R191,195K198A are in good agreement with those previously calculated (Sarno et al., 1996), except for the V_{max} values of K74−77A and K79R80K83A, which are higher, probably due to previous underestimation of the specific activity of recombinant CK2 holoenzymes (see Methods).

The data of Table 3 have been used to construct the histograms of Figure 2, where the relative phosphorylation efficiencies of substituted peptides were normalized to the relative efficiency of the same peptide with wild type CK2. As discussed elsewhere (Sarno et al., 1996), this mode of representation highlights position(s) where the acidic determinants recognized by the mutated residue(s) are located. The results of this analysis, besides confirming previous data obtained with multiply substituted mutants (Sarno et al., 1996), also disclose the role of three individual lysyl residues, K49, K77, and K198, in the recognition of acidic determinants located at $n + 2$, $n + 3$ (and $n + 4$), and $n + 1$, respectively. The patterns induced by individual mutations of other basic residues whose substitution alters the kinetic parameters with the peptide RRRA-AASDDDDD (e.g. K79, K83, and H160) do not display significantly distinctive features denoting clear-cut interactions with given specificity determinants. Thus, the molecolar basis for altered substrate recognition by these mutants (Table 2) remains unclear.

To check whether point mutations that affect substrate binding promote structural alterations of CK2, CD spectroscopy of mutants K198A and K77A, defective in the recognition of the important specificity determinants at $n + 1$ and $n + 3$ (plus $n + 4$), respectively (see Figure 2), was carried out in the far-UV region (190−250 nm). This technique is a sensitive probe of secondary structure conformation (Woody, 1995) and was successfully used for monitoring conformational changes wild type CK2 underwent upon assembly of its subunits (Issinger et al., 1992;

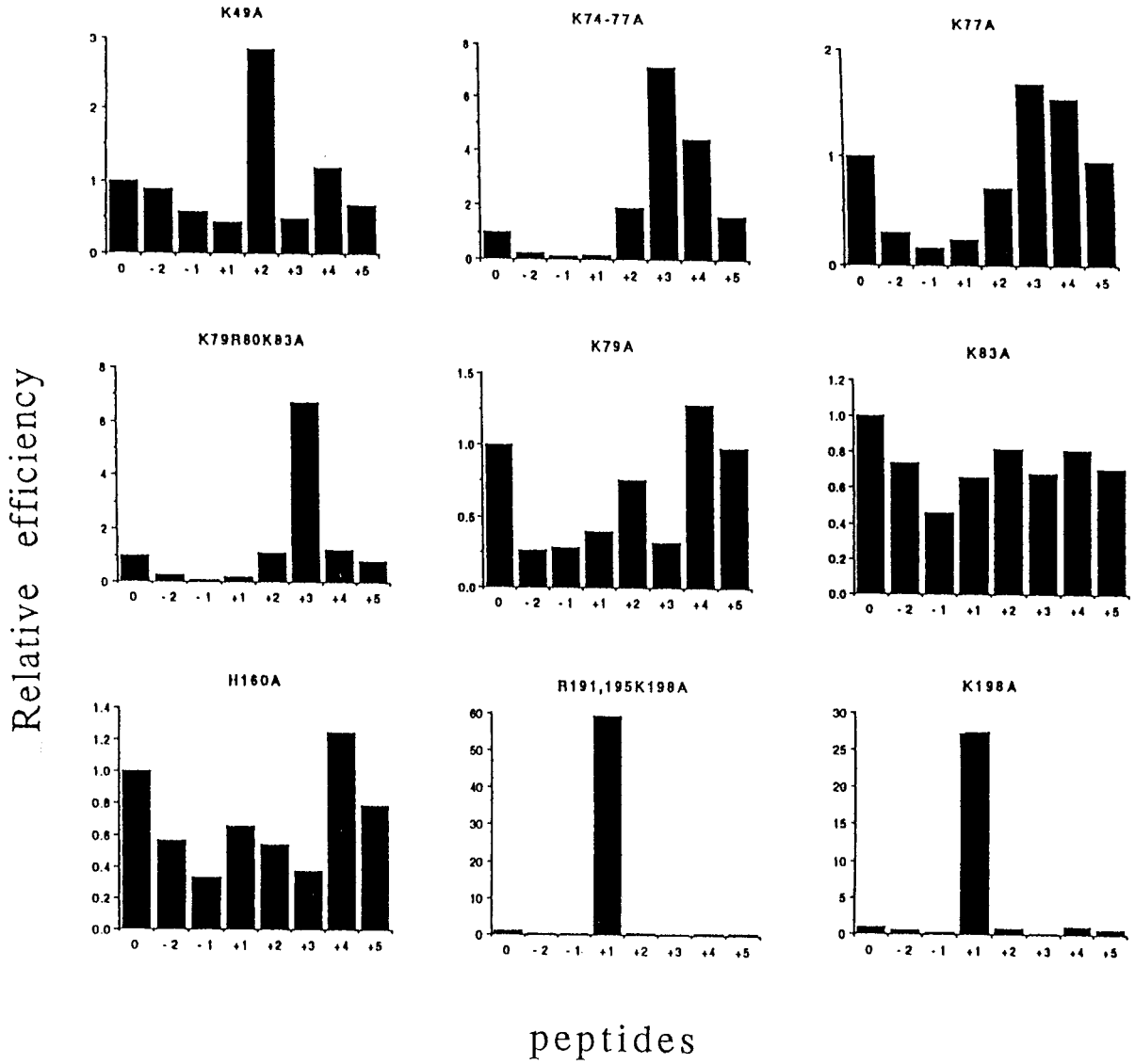

FIGURE 2: Relative phosphorylation efficiencies of synthetic peptides by wild type and mutated forms of protein kinase CK2. The histograms have been constructed with efficiencies (V_{max}/K_m) drawn from Table 3. Relative phosphorylation efficiencies, calculated by dividing the phosphorylation efficiency of each peptide by that of the reference peptide, have been normalized to the relative phosphorylation efficiencies calculated with wild type CK2 (=1). The numbers on the abscissa denote either the parent peptide, RRRA-DDSDDDDD, bearing no substitutions (0) or the derived peptides with an individual D → A substitution at positions between −2 and +5, as indicated.

Jacobi & Traugh, 1995). The CD spectra of both mutants K77A and K198A are almost identical in both shape and ellipticity values at 222 nm to that of wild type CK2, which, in turn, can be superimposed to the ones previously reported by Issinger et al. (1992) and Jacobi and Traugh (1995) (data not shown). This would indicate that, within the limit of experimental error, the secondary structure content is not changed upon Lys to Ala mutation. This finding corroborates the concept that higher K_m values of protein and peptide substrates for these mutants are directly due to impaired substrate recognition rather than being a consequence of alterations in the overall enzyme conformation.

DISCUSSION

The experiments described in the first part of this paper support the view that the mode of binding of phosphoacceptor substrates to the CK2 catalytic site is not rigidly predetermined but may vary according to the number and position of the acidic residues acting as specificity determinants. Indeed, whenever these are numerous, as in the case of the great majority of CK2 phosphoacceptor sites (Meggio et al., 1994), they can play, to a variable extent, interchangeable roles. Even the specificity determinant at $n + 3$, which is crucial whenever other acidic residues are lacking (Marchiori et al., 1988), is not absolutely indispensable if embedded in a series of six additional acidic residues, its individual substitution causing only a 5-fold increase in K_m, still compatible with the appreciable phosphorylation efficiency. Incidentally, this accounts for the observation that the crucial determinant at $n + 3$ is lacking in about 15% of naturally occurring CK2 sites (Meggio et al., 1994) where additional acidic residues are present at different positions. Even more remarkable is the observation that those acidic determinants that appear to be individually dispensable because their single substitutions are nearly ineffective are nevertheless collectively important since their multiple substitution is detrimental. This applies, for example, to the three determinants at positions +2, +4, and +5 and to the two at upstream positions −2 and −1, whose substitutions have more than additive effects on phosphorylation efficiency. It should be noted that acidic determinants at n − 2 and −1 are lacking in a large proportion of CK2 natural

substrates, a circumstance that appears to alter significantly the relative importance of acidic determinants downstream from serine (see Table 2). The concept that the relevance of individual residues in determining site specificity is variable and that, consequently, determinants that are normally dispensable may become crucial under special conditions, and vice versa, also applies to other protein kinases, as discussed elsewhere (Pinna & Ruzzene, 1996).

In the second part of this work, we have analyzed the contribution of individual basic residues of the CK2 catalytic subunit to the recognition of acidic determinants present in the phosphoacceptor substrates. Our results, in conjunction with those of previous studies (Sarno et al., 1996; Dobrowolska et al., 1995), suggest the implication of the following residues.

K49. This residue, included in the "glycine rich loop", is not basic in most protein kinases other than CK2. Its homologue in PKA is Ser53 whose backbone amide is implicated in the binding of ATP (Bossemeyer et al., 1993), while in cdc2, this residue is a threonine (Thr14) whose phosphorylation contributes to down-regulation. In CK2, substitution of K49 with alanine is detrimental to the phosphorylation of peptide RRRA-AASDDDDD (Table 2) and the relative efficiency analysis (Figure 2) clearly outlines the implication of K49 in the recognition of the acidic determinant at $n + 2$.

Cluster K74−K77. Previous studies had shown that the quadruple mutation of these residues, unique to CK2, has a dramatic effect on substrate recognition by altering the binding of the crucial determinant at $n + 3$ and of the more dispensable one(s) located at $n + 4$ and/or $n + 5$ (Sarno et al., 1996). Our present data add two new pieces of information. First, the determinant which is recognized in addition to $n + 3$ is that at $n + 4$ rather than that at $n + 5$; second, the last residue of this stretch, K77, plays a prominent role in the recognition of substrates lacking acidic residues on the N-terminal side of serine, like casein and the peptide RRRA-AASDDDDD.

Cluster 79−83. Unlike the preceding 74−77 basic quartet, the three basic residues within this segment are not unique to CK2, since K83 is conserved in many kinases and, if a manual alignment is followed (Sarno et al., 1996), K79 is homologous to PKA K83. These residues are also functionally distinct from the 74−77 quartet since their mutation to alanine does not affect inhibition by heparin which is conversely compromised by mutations within the 74−77 quartet (Hu & Rubin, 1990; Gatica et al., 1994; Vaglio et al., 1996). The triple mutation of the basic residues within this sequence correlates with the reduced capability to recognize the determinant at $n + 3$ (Sarno et al., 1996; see also Figure 2). Our present data show that the relevance of this triplet in substrate recognition increases if the peptide substrate lacks the acidic determinants at $n - 1$ and $n - 2$ (see Table 2). The somewhat paradoxical increase of V_{max} with peptide RRRA-DDSDDDDD appears to be accounted for by the mutation of K83, since its individual substitution (K83A) promotes alone a remarkable increase of V_{max} with all peptide substrates (see Tables 2 and 3).

H160. CK2 H160 is homologous to PKA E170, a residue shown from both mutational studies (Gibbs & Zoller, 1991a) and crystallographic evidence (Knighton et al., 1991b) to bind the crucial arginine at position $n - 2$. Consequently, a H160D mutant was found to be significantly defective in the recognition of short peptide substrates lacking the canonical S-X-X-E/D consensus sequence, while bearing an acidic residue at $n - 2$ (Dobrowolska et al., 1994). Our present data with a similar mutant, H160A, rule out any significant role of H160 in the recognition of either the reference peptide with seven aspartic acids or any of its monosubstituted derivatives (see Table 3), reinforcing the view that an aspartic acid at $n - 2$ plays a marginal role in the recognition of the very acidic peptides used for the kinetic analysis of Table 3.

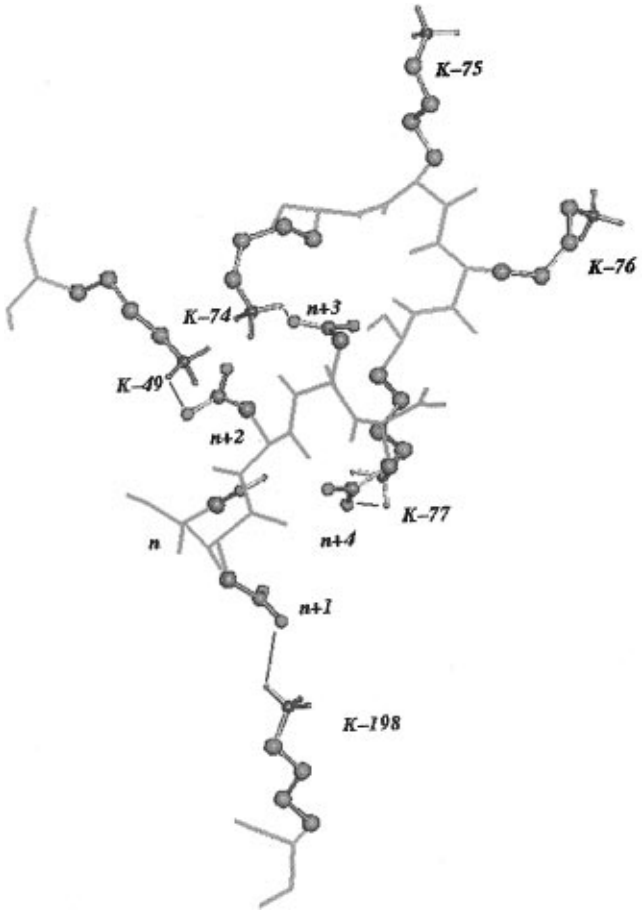

FIGURE 3: Recognition of acidic determinants of the peptide substrate (green) by basic redidues of CK2 (pink). Drawn from CK2 modeling based on the crystal structures of PKA, Erk2, and cdk2 (for details, see Methods). The interactions between the peptide substrate aspartyl residues at positions $n + 1$ to $n + 4$ and lysyl residues of CK2 are shown. n denotes the position of the phosphorylatable N-terminal serine. Distances between charged side chain atoms are as follows: K198−D($n + 1$), 2.3 Å; K49−D($n + 2$), 2.9 Å; K74−D($n + 3$), 1.9 Å; and K77−D($n + 4$), 2.5 Å. Distances between D($n + 1$) and R191 and R195 (not shown) are 3.2 and 6.0 Å, respectively. The crucial determinant, D($n + 3$), apart from being in close contact with K74, projects into a positively charged cavity surrounded by the quartet K74−K77.

R191−K198 Loop. This sequence includes three basic residues, R191, R195, and K198, which are homologous to hydrophobic residues, just downstream from the PKA activation loop, shown to make contacts with a hydrophobic residue present at position $n + 1$ in the inhibitor peptide cocrystallized with PKA (Knighton et al., 1991b). While to our knowledge no mutational study of these residues in PKA has been done, the triple substitution of the homologous basic residues of CK2 with alanine gave rise to a mutant defective in substrate recognition for its inability to interact with the acidic determinant at position $n + 1$ (Sarno et al., 1996). Molecular modeling suggests that the third residue of this triplet makes the major contact with the determinant

at position $n + 1$ while the other two residues (R191 and R195 in CK2) are not so important (Songyang et al, 1996; see also Figure 3 and its legend). Mutational analysis unequivocally corroborates this conclusion, supporting the notion that the nature of the residue homologue to PKA L205 (=CK2 K198) is diagnostic of the specificity determinant at position $n + 1$. [discussed by Pinna and Ruzzene (1996)].

A molecular modeling of the interactions between a peptide substrate bearing acidic determinants at positions $n + 1$, $n + 2$, $n + 3$, and $n + 4$ and basic residues of CK2α suggested by our study to be implicated in substrate recognition is shown in Figure 3. The model, constructed assuming a similar overall architecture of CK2 and PKA, appears to be quite consistent with the results of our mutational analysis. In particular, the interactions of aspartic acids at position $n + 1$, $n + 2$, and $n + 3$ or $n + 4$ with K198, K49, and the K74−77 cluster, respectively, as suggested by the kinetic analysis (Table 3 and Figure 2), are also supported by the molecular simulation. This latter would favor major contacts between K74 and D($n + 3$) and between K77 and D($n + 4$). Our mutational analysis, in conjunction with previous data (Hu & Rubin, 1990; Gatica et al., 1994), are more consistent with an interchangeable role of the residues in the K74−77 quartet, where K77 appears to be important for both $n + 3$ and $n + 4$ recognition (see Figure 2), while K74 appears to be dispensable (Hu & Rubin, 1990). This discrepancy possibly reflects the fact that, as discussed above, the peptide can bind in a flexible way, whereas the picture provided by the model is necessarily a rigid one. Further refinement of this model will be possible after the crystal structure of the CK2 cataytic subunit is solved.

ACKNOWLEDGMENT

We thank Dr. V. De Filippis (CRIBI, Padova) for performing CD analyses of wild type CK2 and mutants and Dr. L. Cesaro for help in drawing the model of CK2−peptide substrate interaction.

REFERENCES

Allende, J. E., & Allende, C. C. (1995) *FASEB J. 9*, 313−323.
Boldyreff, B., Meggio, F., Pinna, L. A., & Issinger, O.-G. (1992) *Biochem. Biophys. Res. Commun. 188*, 228−234.
Bossemeyer, D., Engh, R. A., Kinzel, V., Ponstingl, H., & Huber, R. (1993) *EMBO J. 12*, 849−859.
Cox, S., Radzio-Andzelm, E., & Taylor, S. S (1994) *Curr. Biol. 4*, 893−901.
De Bondt, H. L., Rosenblatt, J., Jancarik, J., Jones, H. D., Morgan, D. O., & Kim, S.-H. (1993) *Nature 363*, 595−602.
Dobrowolska, G., Meggio, F., Marin, O., Lozeman, F. J., Li, D., Pinna, L. A., & Krebs, E. G. (1994) *FEBS Lett. 355*, 237−241.
Gatica, M., Jedlicki, A., Allende, C. C., & Allende, J. E. (1994) *FEBS Lett. 339*, 93−96.
Gibbs, C. S., & Zoller, M. J. (1991a) *Biochemistry 30*, 5329−5334.
Gibbs, C. S., & Zoller, M. J. (1991b) *J. Biol. Chem. 266*, 8923−8931.
Glass, D. B., Masaracchia, R. A., Feramisco, J. R., & Kemp, B. E. (1978) *Anal. Biochem. 87*, 566−575.
Goldberg, J., Nairn, A. C., & Kuriyan, J. (1996) *Cell 84*, 875−887.
Grankowski, N., Boldyreff, B., & Issinger, O.-G. (1991) *Eur. J. Biochem. 198*, 25−30.
Hanks, S. K., Quinn, A. M., & Hunter, T. (1988) *Science 241*, 42−51.
Hu, E., & Rubin, C. S. (1990) *J. Biol. Chem. 265*, 20609−20615.
Hu, S. H., Parker, M. W., Lei, J. Y., Wilce, M. C., Benian, G. M., & Kemp, B. E. (1994) *Nature 369*, 581−584.
Hubbard, S. R., Wei, L., Ellis, L., & Hendrickson, W. A. (1994) *Nature 372*, 746−754.
Hubrey, T. W., & Roach, P. J. (1990) *Biochem. Biophys. Res. Commun. 172*, 190−196.
Issinger, O.-G., Brockel, C., Boldyreff, B., & Pelton, J. T. (1992) *Biochemistry 31*, 6098−6103.
Jakobi, R., & Traugh, J. A. (1995) *Eur. J. Biochem. 230*, 1111−1117.
Knighton, D. R., Zheng, J. H., Ten Eyck, L. F., Ashford, V. A., Xuong, N. H., Taylor, S. S., & Sowadski, J. M. (1991a) *Science 253*, 407−414.
Knighton, D. R., Zheng, J. H., Ten Eyck, L. F., Xuong, N. H., Taylor, S. S., & Sowadski, J. M. (1991b) *Science 253*, 414−420.
Kunzel, E. A., Mulligan, J. A., Sommercorn, J., & Krebs, E. G. (1987) *J. Biol. Chem. 262*, 9136−9140.
Lasa-Benito, M., Marin, O., Meggio, F., & Pinna, L. A. (1996) *FEBS Lett. 382*, 149−152.
Marchiori, F., Meggio, F., Marin, O., Borin, G., Calderan, A., Ruzza, P., & Pinna, L. A. (1988) *Biochim. Biophys. Acta 971*, 332−338.
Marin, O., Meggio, F., Marchiori, F., Borin, G., & Pinna, L. A. (1986) *Eur. J. Biochem. 160*, 239−244.
Marin, O., Meggio, F., & Pinna, L. a. (1994) *Biochem. Biophys. Res. Commun. 198*, 898−905.
Meggio, F., & Pinna, L. A. (1984) *Eur. J. Biochem. 145*, 593−599.
Meggio, F., Marchiori, F., Borin, G., Chessa, G., & Pinna, L. A. (1984) *J. Biol. Chem. 259*, 14576−14579.
Meggio, F., Perich, J. W., Johns, R. B., & Pinna, L. A (1988) *FEBS Lett. 237*, 225−228.
Meggio, F., Marin, O., & Pinna, L. A. (1994) *Cell. Mol. Biol. Res. 40*, 401−409.
Perich, J. W., Meggio, F., Reynolds., E. C., Marin, O., & Pinna, L. A. (1992) *Biochemistry 31*, 5893−5897.
Pinna, L. A. (1990) *Biochim. Biophys. Acta 1054*, 267−284.
Pinna, L. A., & Ruzzene, M. (1996) *Biochim. Biophys. Acta 1314*, 191−225.
Pinna, L. A., Meggio, F., & Marchiori, F. (1990) in *Peptides and Protein Phosphorylation* (Kemp, B. E., Ed.) pp 145−169, CRC Press, Boca Raton, FL.
Sarno, S., Boldyreff, B., Marin, O., Guerra, B., Meggio, F., Issinger, O.-G., & Pinna, L. A. (1995) *Biochem. Biophys. Res. Commun. 206*, 171−179.
Sarno, S., Vaglio, P., Meggio, F., Issinger, O.-G., & Pinna, L. A. (1996) *J. Biol. Chem. 271*, 10595−10601.
Songyang, Z., Blechner, S., Hoagland, N., Hoekstra, M. F., Piwnica-Worms, H., & Cantley, L. C. (1994) *Curr. Biol. 4*, 973−982.
Songyang, Z., Lu, K. P., Kwon, Y. T., Tsai, L.-H., Filhol, O., Cochet, C., Brickey, D. A., Soderling, T. R., Bartleson, C., Graves, D. J., De Maggio, A. J., Hoekstra, M. F., Blenis, J., Hunter, T., & Cantley, L. C. (1996) *Mol. Cell. Biol. 16*, 6486−6493.
Tiganis, T., House, C. M., Tech, T., & Kemp, B. E. (1995) *Eur. J. Biochem. 223*, 647−653.
Tuazon, P. T., & Traugh, J. A. (1991) *Adv. Second Messenger Phosphoprotein Res. 23*, 123−164.
Vaglio, P., Sarno, S., Marin, O., Meggio, F., Boldyreff, B., Issinger, O.-G., & Pinna, L. A. (1996) *FEBS Lett. 380*, 25−28.
Woody, R. W. (1995) *Methods Enzymol. 246*, 34−71.
Xu, R. M., Carmel, G., Sweet, R. M., Kuret, J., & Cheng, X. (1995) *EMBO J. 14*, 1015−1023.
Zhang, F., Strand, A., Robbins, D., Cobb, M. H., & Goldsmith, E. J. (1994) *Nature 367*, 704−711.

BI9705772