Mapping the residues of protein kinase CK2 α subunit responsible for responsiveness to polyanionic inhibitors

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Abstract The quadruple mutation of the whole basic cluster, $K^{74}KKK^{77}$ conserved in the catalytic subunits of protein kinase CK2 and implicated in substrate recognition, not only abolishes inhibition by heparin but even induces with some peptide substrates an up to 5-fold stimulation by heparin in the 0.5–5 μg/ml concentration range. Two other mutants defective in substrate recognition, R191,195K198A and K79R80K83A, display either a 100-fold reduction or no alteration at all in heparin inhibition, respectively. In contrast sensitivity to heparin inhibition is increased 30-fold by a single mutation affecting Arg-228 while it is not altered by a triple mutation in the small insert of subdomain XI (mutant R278K279R280A). The effect of the same mutations on inhibition by pseudosubstrate EEEEEYEEEEEEE is different, the mutant displaying the most reduced sensitivity being R191,195K198A, followed by K74–77A and K79R80K83A; the other mutants are almost indistinguishable from CK2 wild type. Substantial reduction of inhibition by poly(Glu,Tyr)4:1 is only observable with mutant R191,195K198A, whereas R228A is significantly more sensitive to inhibition. These data show that the mode of inhibition of CK2 by polyanionic compounds occurs through substantially different mechanisms involving residues that are variably concerned with substrate recognition.

Key words: Protein kinase CK2; Mutants of CK2; Heparin; Poly(Glu,Tyr)4:1; CK2 pseudosubstrate; Inhibitors of CK2

1. Introduction

Protein kinase CK2 (formerly termed casein kinase-2 or -II) is a ubiquitous, Ser/Thr specific protein kinase responsible for the phosphorylation of a myriad of protein substrates and supposed to play a central albeit still enigmatic role in cell regulation (reviewed in [1–4]). Its activity is invariably elevated in proliferating and transformed tissues [3] and recent experiments with transgenic mice showed that CK2 catalytic subunit may act like an oncogene [5]. CK2 is normally composed of two catalytic (α and /or α') and two non catalytic β-subunits tightly associated to give a fully active stable heterotetrameric holoenzyme. Although CK2 is spontaneously active toward most of its substrates and it is not subjected to any strict control mediated by either known second messengers or phosphorylation, it is generally accepted that its activity can be subtly modulated by its non catalytic β-subunit. Reconstitution experiments with mutants and synthetic fragments of the β-subunit have disclosed a dual function of this protein which on one side protects the catalytic subunits against denaturing agents and increases its basal activity [6], on the other side exhibits an intrinsic down-regulation, becoming especially evident if calmodulin is the substrate [7]. Down-regulation by the β-subunit is mediated by acidic residues clustered in its N-terminal moiety whose mutation to alanines consequently gives rise to hyperactivating β-subunits [8,9]. This would imply the presence of complementary basic residues in the catalytic subunits. A crucial role of basic residues in CK2 is highlighted by other properties as well, notably its unusual site specificity, determined by rows of acidic residues mostly located down-stream from the target aminoacid [1] and its extreme sensitivity to polyanionic inhibitors, such as heparin [10] and the random polymers poly(Glu,Tyr)4:1 [11]. In order to identify the structural elements responsible for these peculiar properties of CK2 we have started to mutate the conserved basic residues of human α-subunit that are divergent from the homologous residues in the majority of Ser/Thr protein kinases, which are basophilic instead of acidophilic like CK2. In a previous report we have analysed the catalytic activity of six such mutants and found that three of them were defective in substrate recognition [12]. Here these mutants, purified to near homogeneity, are exploited to localize the residues responsible for the binding of different classes of anionic inhibitors.

2. Materials and methods

Expression of human CK2 α and β subunits and of mutated CK2 α subunits, was performed as previously described [12–14]. Soluble extracts of bacteria expressing CK2 α subunits, either wild type or mutants were combined with the extracts containing the β subunit and the holoenzymes, resulting from equimolar combination of α and β subunits, were purified by phosphocellulose chromatography followed by Mono Q FPLC, by a procedure that will be detailed elsewhere. By such a procedure all five mutants were purified to near homogeneity as heterotetrameric enzymes as judged from both PAGE/SDS coomassie patterns showing two prominent bands of α and β subunit in about the same ratio as CK2 wild type, and sucrose gradient ultracentrifugation, revealing peaks of activity with the same sedimentation coefficient as CK2 wild type. The specific activities were: CK2 wild type 300 U/mg, K74–77A 27 U/mg, K79R80K83A 93 U/mg, R191,195K198A 40 U/mg, R228A 450 U/mg, R278K279R280A 370 U/mg, one unit being equivalent to one nmol P incorporated into the peptide substrate, RRRADDSDDDDD, per min, under our experimental conditions. Synthetic peptides were prepared as described or referenced in [12]. The assay of CK2 activity was performed as in [15], by 10 min incubation at 37° C

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in the presence of 100 μM peptide substrate (unless differently indicated). The inhibitory pseudosubstrate peptide EEEEEYEEEEEEE was prepared by Fmoc solid-phase chemistry using an automatic peptide synthesizer (Model 431-A Applied Biosystem).

Heparin (average M_r=14,000) and the random polymer poly(Glu/Tyr)4:1 (average M_r=49,100) were from Sigma. All the other chemicals were of the highest quality commercially available.

3. Results and discussion

Fig. 1 shows the effect of increasing concentrations of heparin on the activity of CK2 wild type and five mutants in which basic residues have been variably replaced by alanines. All the assays were performed using the best known peptide substrate for CK2 [16] RRRADDSDDDDD, in which all the positions between −2 and +5, where acidic residues are known to act as positive determinants [1], are occupied by aspartic acid. While the inhibition of two mutants, K79R80K83A and R278K279R280A, is indistinguishable from that of CK2 wild type (IC_{50} values around 0.1 μg/ml) the other mutants display deeply altered heparin sensitivity. Mutant R228A is more readily inhibited than CK2 w.t.; in contrast mutant R191,195K198A is less susceptible and mutant K74–77A is totally refractory to inhibition. The behaviour of K74–77A is especially remarkable since it not only is insensitive to inhibition (up to 120 μg/ml heparin) but even is significantly stimulated by heparin concentrations around 1 μg/ml.

The relevance of the substrate in determining such a paradoxical stimulation by heparin of CK2 mutant K74–77A is highlighted by the data of Fig. 2 showing that the extent of stimulation is enhanced to up to 5-fold if the optimal peptide substrate is replaced by its derivative in which a single aspartyl residue at position −2 (rather unimportant for substrate recognition) is replaced by alanine. In contrast stimulation is nearly undetectable with other two peptide substrates. With one of these, RRKDLHDDEEDEEMSETADGE, lacking the crucial acidic determinant at position +3, but still having the one at position +1, no stimulation occurs while a slight inhibition is observable reaching about 40% with 120 μg/ml heparin.

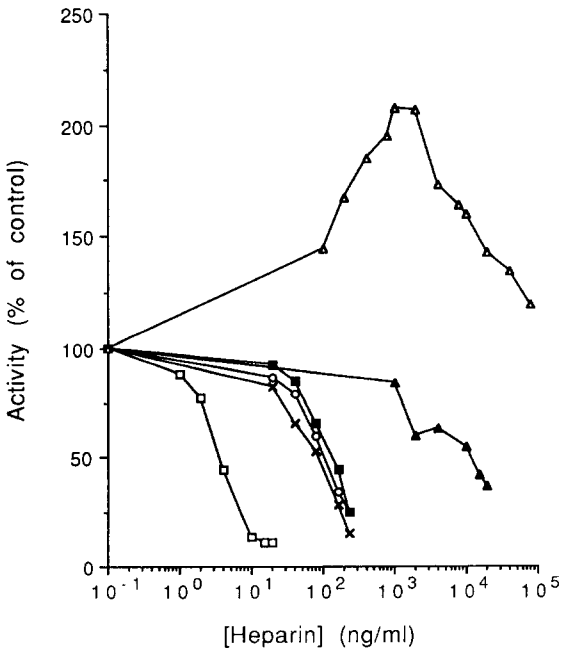

Fig. 1. Effect of increasing heparin concentration on the catalytic activities of wild type and variably mutated CK2. CK2 activity was assayed on the peptide substrate RRRADDSDDDDD [100 μM]. The same amount (0.1 μg) of either CK2 wild type (○) or mutants K74–77A (△), K79R80K83A (×) R191,195K198A (▲) R228A (□) and R278K279R280A (■) was used in all experiments.

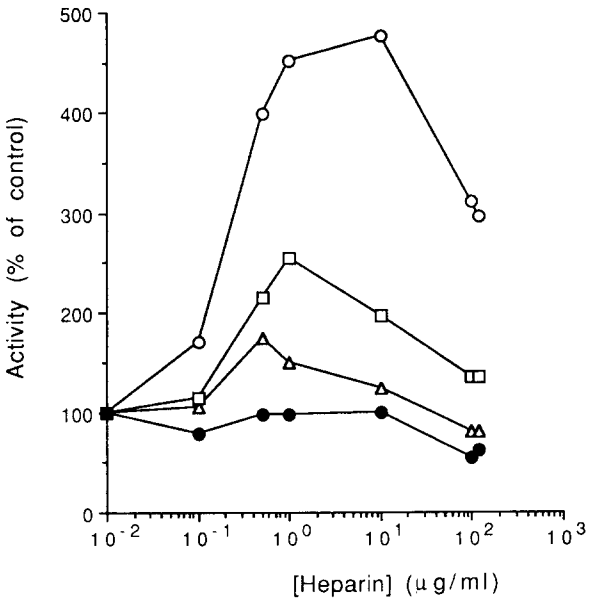

Fig. 2. Stimulation of the catalytic activity of CK2 mutant K74–77A by heparin is dependent on the structure of the peptide substrate. Experimental conditions are the same as in Fig. 1 except for the replacement of peptide RRRADDSDDDDD with RRRAADSDDDDD (○), RRRADDSDADDD (△), RRRADDSDDADD (□) and RRKDLHDDEEDEEMSTADGE (●).

The notion that the 74–80 basic stretch unique to CK2 is implicated in heparin inhibition was already provided by double mutations of K74/K75 [16] and K75/K76 [17] giving rise to enzymes defective in this property. Interestingly however these mutations caused a 70- and 13-fold increase of IC_{50} values, respectively, but they did not suppress heparin inhibition as it is observed with the quadruple mutated K74–77A mutant used here. This may suggest that all the four, 74, 75, 76, and 77 basic residues are implicated in heparin binding and the mutation of all of them is necessary to minimize heparin inhibition and to disclose the paradoxical stimulation by heparin, which is evident with some peptide substrates (see Fig. 2). In striking contrast the basic residues just down-stream from K77, namely K79, R80 and R83, very important for substrate recognition [12] are not concerned at all with heparin inhibition, since their triple mutation does not alter the IC_{50} value (see Table 1).

It should be concluded therefore that the highly conserved 74–80 basic stretch unique to CK2 and having the hallmark of a nuclear localisation signal (NLS) [5], is composed by two functionally distinct entities: an N-terminal moiety mostly concerned with heparin inhibition, as well as with down-regulation by β-subunit (unpublished data), and a carboxyl terminal part implicated in the recognition of the crucial specificity determinant at position +3 [13] but irrelevant to heparin.

Though the 74–77 quartet appears to be the main site of high affinity heparin binding, additional basic residues cooperate with it, as indicated by the two orders of magnitude increase

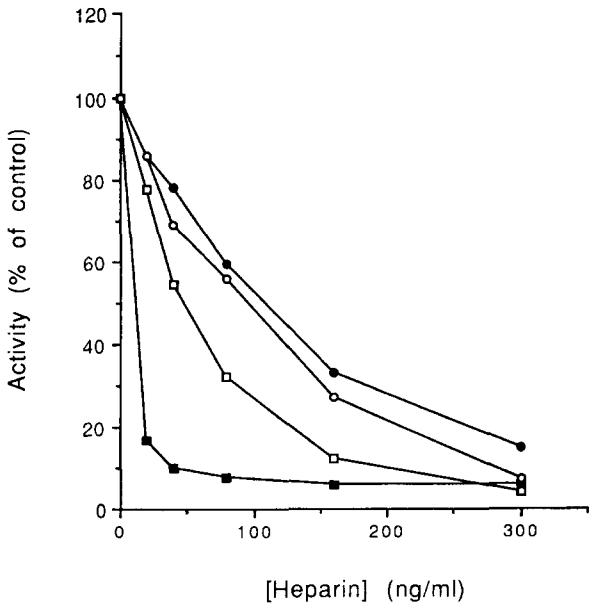

Fig. 3. Opposite effect of holoenzyme reconstitution on the susceptibility to heparin inhibition of CK2 α wild type and its mutant R228A. The catalytic activity of CK2 α wild type (circles), either alone (○) or combined with equimolar amounts of β subunit (●) and of CK2 α mutant R228A (squares), either alone (□) or combined with equimolar amounts of β subunit (■) was assayed with the peptide substrate RRRADDSDDDDD (100 μM) as in Fig.1.

of IC_{50} observed with mutant R191,195K198, in which the basic stretch 74–77 is preserved. The effect of this mutation is actually comparable to that induced by the mutation of just K74 and K75 inside the 74–77 stretch [17], although a precise comparison is hampered by the different substrate (casein) used in that study.

In any case our data unambiguously show that the triple mutation in the 191–198 loop is much less effective on heparin inhibition than the quadruple mutation of the 74–77 cluster, which not only abolishes but even subverts the heparin effect under comparable conditions (see Fig. 1). Interestingly the opposite is true in the case of CK2 inhibition by poly(Glu,Tyr)4:1 and by the pseudosubstrate peptide EEEEEYEEEEEEE, the efficacy of both being reduced more with mutant R191,195K198A than with mutant K74–77A (Table 1). It should be noted that inhibition by EEEEEYEEEEEEE is also significantly reduced by the triple mutation in the 79–83 sequence (Table 1), which is ineffective on heparin and only slightly effective on poly(Glu,Tyr)4:1 inhibition. The somewhat different modes of action of the two polyglutamyl inhibitors is also outlined by the observation that sensitivity to EEEEEYEEEEEEE is compromised much more severely than that to the random co-polymer (compare the alterations in IC_{50} values in Table 1). In particular the generally modest changes in IC_{50} values of poly(Glu,Tyr)4:1, as compared to EEEEEYEEEEEEE and heparin, may suggest that additional basic residues not mutated in this study are majorly implicated in the mechanism of poly(Glu,Tyr)4:1 inhibition. In contrast the binding of EEEEEYEEEEEEE seems to be almost entirely dependent on the basic residues clustered in the 191–198 and 74–83 sequences, whose variable substitutions with alanine causes rises of IC_{50} value ranging between 10- and 590-fold, suggesting that the simultaneous mutation of all these basic residues might fully suppress the inhibition by EEEEEYEEEEEEE. This would be not unexpected considering that the same basic residues account for the recognition of the main substrate specificity determinants, notably those at positions +3 and +1 [12], which are presumably responsible also for the binding of the pseudosubstrate.

The different effects of mutations on heparin and pseudosubstrate inhibition may reflect the observation that while the latter is a purely competitive inhibitor with respect to the phosphoacceptor substrate, the actual mode of heparin inhibition remains a matter of debate, as discussed in [1], where a number of arguments supporting the possibility that heparin might interact with site(s) partially distinct from the catalytic one are cited. Consistent with this interpretation would be also the finding that two CK2 mutants partially altered in the 74–77 basic quartet are not seriously defective in substrate recognition, despite their drastically reduced sensitivity to heparin [18,19].

An intriguing outcome of this study is the increased sensitivity to heparin and to poly(Glu,Tyr)4:1 inhibition of mutant R228A which does not exhibit any marked alteration in substrate recognition [12]. How might the suppression of a positively charged side chain increase the affinity for polyanionic inhibitors is hard to explain, unless assuming an indirect effect. It seems conceivable that R228 contributes to attain a molecular conformation that makes the binding sites less accessible to polyanionic inhibitors. Interestingly the paradoxical effect of mutating R228 into alanine is partially dependent on the β-subunit, being less pronounced with the isolated α-subunit (Fig. 3). In other words the β subunit that normally slightly protects CK2 α against heparin inhibition [7], dramatically increases the sensitivity to heparin of this CK2 α mutant. This would mean that the conformational changes induced by the mutation of R228 and leading to an increased sensitivity to heparin inhibition, are amplified by the β subunit. It is conceivable therefore

Table 1
IC_{50} values for the inhibition of CK2 wild type and the mutants by heparin, the pseudo-substrate EEEEEYEEEEEEE and poly(Glu,Tyr)4:1

	IC_{50} values		
	Heparin (μg/ml)	EEEEEYEEEEEEE (μM)	Poly(GLu,Tyr)4:1 (nM)
Wild type	0.1040	2.0	3.00
K74-77A	≫120.0000	600.0	23.00
K79R80K83A	0.0860	20.0	4.80
R191,195K198A	11.4000	1180.0	180.00
R228A	0.0036	1.0	0.42
R278K279R280A	0.1300	3.5	3.00

All the experiments were performed under comparable condition, with the same concentration of the peptide substrate RRRADDSDDDDD (100 μM) and of the enzymes (3.3 μg/ml). The values shown are the mean of at least 3 experiments with a standard error never exceeding 15%.

that R228 is implicated in some kind of functional interaction with the β subunit.

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