Vol. 188, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1992 Pages 228-234

CASEIN KINASE-2 STRUCTURE-FUNCTION RELATIONSHIP: CREATION OF A SET OF MUTANTS OF THE ß SUBUNIT THAT VARIABLY SURROGATE THE WILDTYPE ß SUBUNIT FUNCTION

Brigitte Boldyreff[1], Flavio Meggio[2], Lorenzo A. Pinna[2] and Olaf-Georg Issinger[1]

[1]Institut für Humangenetik, Universität des Saarlandes, Homburg, Germany

[2]Dipartimento di Chimica Biologica Centro per lo Studio della Fisiologia Mitocondriale and C.N.R., Università di Padova, Padova, Italy

Received August 18, 1992

Nine mutants of human casein kinase-2 ß subunit have been created and assayed for their ability to assemble with the catalytic α subunit to give, at a 1:1 molar ratio, a fully competent CK-2 holoenzyme as judged by the following criteria: 1) the generation of an active heterotetrameric form of CK-2 exhibiting the expected sedimentation coefficient and 2) the enhancement of catalytic activity of CK-2 α. Extended deletions of 71 and 44 residues from the C-terminal end, but not a 7 residue deletion (including the cdc2 phosphorylation site) prevent both reconstitution of the holoenzyme and, consequently, stimulation of activity. This indicates that residue(s) located in the 171 - 209 sequence is essential for reconstitution. Also a four residue's N-terminal deletion (removing the autophosphorylation site) and single to quintuple substitutions of alanine for the acidic residues clustered in the 55 - 70 sequence give rise to mutants that still assemble with the α subunit to give a tetrameric holoenzyme. However, in the case of the mutants $A^{57,59}$, $A^{63,64}$, $A^{59-61,63,64}$ *in vitro* assembly with the CK-2 α subunit was not complete. There were also intermediate complexes, free α-subunit and ß-mutants found to sediment at various positions in the sucrose density gradient. In comparison to CK-2 $ß^+$, mutants $A^{57,59}$, A^{59-61} and $A^{59-61,63,64}$ show an increased stimulation of the catalytic activity supporting the view that these residues play a crucial role in determining the basal activity of reconstituted CK-2 holoenzyme. © 1992 Academic Press, Inc.

Casein kinase-2 is an ubiquitous Ser/Thr specific protein kinase implicated in a variety of cellular functions with special reference to gene expression and proliferation (reviewed in 1 - 3). An intriguing question about CK-2 is how this spontanously active protein kinase, independent of any known second messengers and not subjected to any tight control by either phosphorylation or dephosphorylation can nevertheless be regulated. Although CK-2

0006-291X/92 $4.00
Copyright © 1992 by Academic Press, Inc.
All rights of reproduction in any form reserved.

shares with protein kinase A a heterotetrameric structure including two catalytic (α and/or α') and two non-catalytic subunits (ß), it sharply differs because the tetrameric CK-2 holoenzyme represents its fully active, rather than inactive, form. CK-2 does not spontaneously dissociate under physiological conditions. By using the recombinant α and ß subunits expressed in *E.coli* it has been shown previously that the CK-2 subunits spontaneously self assemble to give a heterotetramer, indistinguishable from the native holoenzyme for all properties tested including its sedimentation coefficient (4). Association with the ß subunit confers to the catalytic subunit more ordered structure (5), reduced susceptibility to denaturing agents/conditions (6), enhanced sensitivity to polybasic effectors (7) and altered substrate specificity, as typically reflected by a decreased activity toward calmodulin as opposed to an increased activity toward most other substrates (4, 6). In order to scrutinize in detail the nature of the interaction between α and ß subunits and to localize domains and individual residues crucially required for conferring to the ß subunits its typical properties, so far nine mutants of the ß subunit have been created, expressed and purified with the aim of comparing their properties with those of the wildtype ß subunit.

MATERIAL AND METHODS

Oligonucleotide directed mutagenesis- For creating mutants with amino acid exchanges, the 0.58 kb EcoRI/HindIII fragment of expression plasmid pBB4 (4) containing the coding region of the human CK-2 ß cDNA from nucleotide 58 till the end was excised and cloned into vector M13. *E.coli* strain CJ236 was used to prepare single-stranded, uracil-containing template DNA. Oligonucleotides were constructed to mutate acidic amino acids (Glu, Asp) to alanine according to the alanine-scanning mutagenesis technique (8). The heteroduplex region of each oligonucleotide was flanked by 10 - 12 complementary nucleotides. Oligonucleotide directed mutagenesis was carried out using the "Bio-Rad Muta-Gene" *in vitro* mutagenesis kit according to the manufacturer's instructions. Mutants were selected in *E.coli* strain MV1190, and they were identified by dideoxy-chain termination DNA sequencing (9). The mutated fragments were then excised from M13 and inserted into the EcoRI/HindIII site of pBB4. For creating mutants with N- or C-terminal deletions the expression plasmid pBB4 containing the coding region of the human CK-2 ß cDNA was used as a template for polymerase chain reactions. Mutagenic primers consisted of a 20 bp complementary stretch starting at the first codon not to be deleted. In case of the N-terminal deletions the primer contained additional nucleotides representing a NdeI cutting site and an ATG start codon. In the case of the C-terminal deletions the primer contained additional nucleotides representing a stop codon and a HindIII site. The second primers used in the PCR were complementary over 20 bp to the 5' or 3' end of the cDNA and contained additional nucleotides representing either a NdeI site in case of the 5' end or a HindIII site in case of the 3' end. PCR was

carried out with Vent polymerase (New England Biolabs) under the following conditions: 1 min 93°C, 1 min 52°C, 1 min 72°C, 16 cycles. The PCR product was cut with NdeI/HindIII and cloned into vector pT7-7.

Expression and purification of mutated CK-2 ß subunits- Expression and purification was done as described earlier (4,6); but instead of the phosphocellulose chromatography step, HPLC chromatography on MA7Q ion exchange column (Biorad) was carried out. Elution of the ß-subunits was with a salt gradient from 0.3 - 1.5 M NaCl in 25 mM Tris/HCl pH 8.5. The preparations contained > 95 % pure ß subunits as judged by Coomassie Blue staining.

Sucrose density gradient analysis- A linear gradient (3.5 ml) of sucrose 5-25% w/w was prepared in 25 mM Tris/HCl pH 8.5 containing 300 mM NaCl. Protein samples containing 30 µg recombinant human CK-2 (rhCk-2) α subunit, prepared as described earlier (4) and equimolar amounts of wildtype or mutated rhCK-2 ß subunit were loaded on top of the gradient and centrifuged at 48,000 rpm in a Kontron TST 60 rotor for 14 h at 4°C. 20 fractions were collected and monitored by Coomassie staining, immunoblotting and enzyme activity as described earlier (4).

Protein kinase assay- CK-2 activity was assayed as described earlier (4) except that we used 25 mM Tris/HCl pH 8.5 instead of 20 mM MES/NaOH pH 6.9. Incubation was for 5 min at 37°C.

RESULTS

The mutants of the ß subunit of human CK-2 which were made for this study are schematically outlined in Fig. 1. The mutations made can be grouped into 3 categories: 1) a deletion from the N-terminal end removing just 4 residues (including the autophosphorylation site); 2) deletions from the C-terminal end removing either 7 residues (including the site phosphorylated by cdc2) or larger segments of 44 and 65 residues, respectively; 3) single or multiple Glu/Asp → Ala mutations within the acidic stretch 55-70 including 8 acidic amino acids out of 16. These acidic residues are identical or conservatively replaced in the ß subunit from all known species, while none of them is conserved in the product of the stellate gene, a functionally unrelated protein exhibiting otherwise a

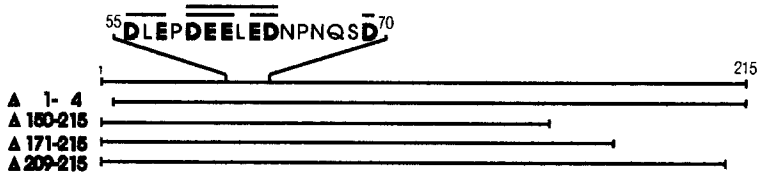

Fig.1. Schematic representation of the ß subunit showing the deleted sequences and the acidic stretch 55-70. The residues mutated to alanine are in bold face. *Overlined* charged residues were mutated together.

remarkable homology with the ß subunit (see 1, 10). We have previously shown that a synthetic peptide including such an acidic stretch could either mimick or counteract some of the properties of the ß subunit (6).

Each of the mutants created was tested for its ability to combine with the α subunit and give rise to an active holoenzyme, as judged by sucrose density gradient analysis. The results are summarized in Table 1. From the position in the sucrose density gradient we can distinguish four types of mutants: (i) a normal reconstitution comparable to that occuring with the wildtype ß subunit is observed with the two deletion mutants Δ1-4 and Δ209-215, respectivly as well with the Glu/Asp → Ala mutants, A^{59-61} and A^{70}. (ii) mutants $A^{55,57}$ and $A^{59-61,63,64}$ give rise only partially to the heterotetramer and in addition to an intermediate form sedimenting between the free subunits and the tetrameric form. (iii) in the case of the mutant $A^{63,64}$ three activity peaks are found distributed over the gradient. One was found at the position where the holoenzyme was located, one at the position of free α and ß subunits and one at an intermediate position. The presence of the mutated ß at all three positions was verified by Coomassie staining and immunoblotting. (iv) no reconstitution whatsoever was detected with the large deletion mutants Δ150-215 and Δ171-215 (Table 1).

The failure of the deleted mutants Δ150-215 and Δ171-215 to assemble with the catalytic subunit is also reflected by their lack to surrogate the wildtype ß subunit as enhancer of the catalytic activity (Table 2). As also shown in Table 2, however, the other

Table 1 . Sucrose density gradient analysis of mixtures from rhCK-2 α (rα) and mutated forms of rhCK-2 ß. As references a 1:1 molar mixture from rhCK-2 α and wildtype ß, pure α and ß subunits were run in parallel.

	$\alpha_2\beta_2$	Intermediate	Free α & ß
Δ1-4	+	-	-
Δ209-215	+	-	-
A^{59-61}	+	-	-
A^{70}	+	-	-
$A^{55,57}$	+	+	-
$A^{59-61,63,64}$	+	+	-
$A^{63,64}$	+	+	+
Δ150-215	-	-	+
Δ171-215	-	-	+

Table 2. Increase of rCK-2 α (rα) catalytic activity upon addition of equimolar amounts of wildtype and mutant forms of rhCK-2 ß with calmodulin peptide (RKMKDTDSEEEIR; 100 μM final concentration) as a substrate.

CK-2 subunits	Stimulation (fold)	
	no NaCl	+ 0.1 M NaCl
rα	1	1
rα + ß⁺	8.5	6.8
rα + Δ1-4	5.0	7.2
rα + Δ209-215	9.1	7.5
rα + Δ150-215	1.8	1.1
rα + Δ171-215	1.2	1.0
rα + A^{70}	9.9	6.5
rα + $A^{63,64}$	7.5	5.0
rα + $A^{55,57}$	27.9	15.7
rα + A^{59-61}	16.7	15.4
rα + $A^{59-61,63,64}$	15.0	4.5

mutants that are still capable to reconstitute CK-2 holoenzyme, promote quite variable stimulations of the catalytic activity depending on their structure. Stimulation compared to wildtype ß was observed with mutants: Δ1-4, Δ209-215, $A^{63,64}$ and A^{70}. On the other hand the substituted mutants $A^{55,57}$, A^{59-61} and $A^{59-61,63,64}$ are more effective than the wildtype ß subunit in enhancing the kinase enhancing the kinase activity. Whereas the former two mutants showed this hyperactivation irrespective of the salt conditions, the latter showed only higher activity in the absence of salt (Table 2).

DISCUSSION

The data presented in this paper represent a first step towards the elucidation of the structure-function relationship of the non-catalytic ß subunit of CK-2, which is normally associated with the catalytic subunits to give fully active heterotetramers $\alpha_2\beta_2$. The 9 mutated forms of ß subunit described here allow to draw the following main conclusions:

According to the degree of reconstitution we have tentatively assigned four groups of mutants: 1) Removal of the

autophosphorylation site (mutant Δ1-4) or the cdc2 phosphorylation site(Δ209-215) apparently have no influence on the reconstitution and activity of the holoenzyme. The same is true for the single exchange mutant Asp^{70} to Ala. Mutant A^{59-61} which also reconstitutes to a normal heterotetramer indicates that these residues are not crucial for self-assembly. However, this mutant shows a hyperactivation of the α subunit when compared to the wildtype ß. Since this hyperactivation is also found with mutants $A^{55,57}$ and $A^{59-61,63,64}$ we conclude that especially the acidic residues 55-61 normally keep the basal activity of CK-2 at a lower level than it would be if they were replaced uncharged residues.

4. The most crucial substitution influencing the self-assembly seems to involve the acidic residues at positions 55, 57 and 63, 64. This is exemplified by the behaviour of mutants $A^{55,57}$, $A^{63,64}$ and $A^{59-61,63,64}$ which all show beside the normal assembly to a heterotetramer also intermediate complexes (eventually α/ß dimers). In the case of the $A^{63,64}$ mutant we also find free subunits indicating that the exchange of residues 63 and 64 strongly interferes with the reconstitution and that in the case of the quintuple mutant the presence of $Ala^{63,64}$ may be responsible for the uncomplete formation of heterotetramers which is not observed with mutant A^{59-61}.

4) Larger deletions (Δ150-215 and Δ171-215) fully prevent both the reconstitution of the holoenzyme and the stimulation of activity. This suggests that residue(s) located in the 171-209 sequence play a crucial role in the reconstitution of CK-2 heterotetramer, suggesting that more than one domain of the ß subunit is involved in the formation of the holoenzyme. It should be noted that these deletions deeply affect the overall charge of the ß subunit whose basic residues are concentrated in the C-terminal moiety.

Taken everything into consideration, the mutants of the ß subunit used for this study will prove useful for inspecting many other properties of CK-2, such as (auto)-phosphorylation, response to polyamines and to polybasic peptides.

ACKNOWLEDGMENTS

This work was supported by grants to LAP from CNR (grant 91.00302.CT04, Target Projects on Biotechnology and Bioinstrumentation and ACRO) and from AIRC and to FM from MURST and CNR (Grant 92.015056.CT04). BB was supported by SFB246/B3 to OGI and DFG Bo 1151/1-1.

REFERENCES

1. Pinna, L. A. (1990) Biochim. Biophys. Acta 1054, 267-284.
2. Tuazon, P. T., and Traugh, J. A. (1991) Adv. Second Messenger Phosphoprotein Res. 23, 123-164.
3. Schneider, H.R., and Issinger, O.-G. (1989) BiotechForum 6, 82-88.
4. Grankowski, N., Boldyreff, B., and Issinger, O.-G. (1991) Eur. J. Biochem. 198, 25-30.
5. Issinger, O.-G., Brockel, C., Boldyreff, B., and Pelton, J. T. (1992) Biochemistry, 31, 6098-6103.
6. Meggio, F., Boldyreff, B., Marin, O., Pinna, L. A., and Issinger O.-G. (1992) Eur. J. Biochem. 204, 293-297.
7. Meggio, F., Boldyreff, B., Marin, O., Marchiori, F., Perich, J. W., Issinger, O.-G., and Pinna L. A. (1992) Eur. J. Biochem. 205, 939-945.
8. Cunningham, B. C., and Wells, J. A. (1989) Science 244, 1081-1085.
9. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
10. Livak, K. J. (1990) Genetics 124, 303-316.