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Involvement of asparagine 118 in the nucleotide specificity of the catalytic subunit of protein kinase CK2

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Abstract Protein kinase CK2 is a heteromeric enzyme with catalytic (α) and regulatory (β) subunits which form an $\alpha_2\beta_2$ holoenzyme and utilizes both ATP and GTP as nucleotide substrate. Site-directed mutagenesis of CK2α subunit was used to study this capacity to use GTP. Deletion of asparagine 118 ($\alpha^{\Delta N118}$) or the mutant αN118E gives a 5–6-fold increase in apparent K_m for GTP with little effect on the affinity for ATP. Mutants αN118A and αD120N did not alter significantly the K_m for either nucleotide. CK2$\alpha^{\Delta N118}$ has an apparent K_i for inosine 5′ triphosphate 5-fold higher than wild-type and is very heat labile. These studies complement recent crystallographic data indicating a role for CK2α asparagine 118 in binding the guanine base.

Key words: Protein kinase CK2; Casein kinase 2; Nucleotide substrate specificity; Site-directed mutagenesis

1. Introduction

Protein kinase CK2 is a pleiotropic and ubiquitous enzyme that phosphorylates a large number of protein substrates. Abundant evidence indicates that this enzyme plays an important role in the regulation of gene expression and cell division (reviews [1–3]).

Protein kinase CK2 is normally found as a heterotetramer that contains catalytic (α and α′) and regulatory (β) subunits which are present in $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$ and $\alpha_2\beta_2$ combinations. The sequences of the catalytic subunits are highly conserved in a wide range of eukaryotic species and contain the typical 12 regions that are common to the large superfamily of protein kinase [4]. Considerable effort has been made in several laboratories to study the structure and function of both subunits [5–7]. Recently, important advances in the elucidation of this problem have been made through the solution of the crystal structure of the *Zea mays* CK2α subunit [8] and a truncated form of the human CK2β subunit [9].

One of the special characteristics of this protein kinase is the fact that it can efficiently use GTP, as well as ATP, as a phosphoryl donor. The crystal form of the *Z. mays* CK2α subunit contained one molecule of bound ATP. Unfortunately, the nucleotide triphosphate was partially disordered so that a definite picture of the binding of the purine base was not apparent [8]. This information would be highly relevant to answer the question as to the structural features that allow the CK2α subunit to use GTP as efficiently as ATP.

In an alternative approach, we reported [10]a a structural computer model of the CK2α structure based on the known structure of the cyclin-regulated protein kinase CDK2, the closest relative to CK2 in the protein kinase family. This approach allowed us to attempt to answer the question as to the lack of discrimination between the two purine nucleoside triphosphates [10]. In that work, it was concluded that a region involving Asn^{118} could be in close contact with the purine base, an idea that was strengthened by the fact that this asparagine is part of the sequence $N^{117}NTD^{120}$ that closely resembles the NKXD conserved sequence present in G proteins and other GTPases and that is known to participate in the recognition of the guanine base [11,12]. Deletion of Asn^{118} resulted in a significant increase in the apparent K_m for GTP and a smaller change in the apparent K_m for ATP [10].

In this report, a comparative study of this and other mutations that substitute for Asn^{118} is presented, demonstrating that this amino acid is involved in determining the specificity for the purine base of the phosphoryl donor. In addition, it is demonstrated that deletion of Asn^{118} greatly affects the stability of the enzyme.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP and GTP were obtained from NEN Life Science Products and dephospho-βcasein, protease inhibitors, nucleotides inosine 5′ triphosphate (ITP), XTP and GTP were obtained from Sigma. NTA-agarose was from Invitrogen. Nucleotide sequencing was performed using a 377 ABI automatic sequencer and oligonucleotides were synthesized by an ABI DNA synthesizer in the Oligopeptido Core Facility of the University of Chile.

2.2. Mutants of protein kinase CK2α subunit

The deletion mutant CK2$\alpha^{\Delta N118}$ was generated by overlapping primer extension PCR [13] using clones of CK2α in vector pT7-7 [14,15]. The initial amplification reactions to generate the two overlapping fragments used oligonucleotide pairs (5′-TTCGAACATGTCAACACAGATTTTAA-3′) and the T7 primer (5′-AATACGACTCACTATAG-3′), and for the second fragment (5′-TTAAAAATCTGTGTTGACATGTTCGAA-3′) with the reverse T7 primer (5′-ATTGGTAACTGTCAGACCAAG-3′).

In the final amplification reaction, the T7 and reverse T7 primers were used with the mixture of the products of the first two PCR reactions. The products were digested with *Nde*I and *Sal*I and subcloned into pT7-7H6 which contains a six histidine tag to facilitate the purification [15].

Mutants N118A, N118E and D120N were prepared as given for ΔN118 using the same T7 primers and the appropriate oligonucleotides for the initial overlap extension PCR reactions were as follows:

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mutant N118A: 5′-AACATGTCAACGCTACAGATT-3′ and 5′-AATCTGTTAGCGTTGACATGTT-3′, mutant N118E: 5′-AACATGTCAACGAAACAGATT-3′ and 5′-AATCTGTTTCGTTGACATGTT-3′, mutant D120N: 5′-CAACAATACAAATTTTAAGCAGT-3′ and 5′-ACTGCTTAAAATTTGTATTGTTG-3′.

To generate the double mutation ΔN118,D120N, mutant ΔN118 was used as template with the following primers: 5′-GTCAACACAAATTTTAAGCAG-3′ and 5′-CTGCTTAAAATTTGTGTTGAC-3′ with a similar procedure as given above. In all cases, the complete nucleotide sequence of each recombinant clone was confirmed by sequencing using a 377 ABI automatic sequencer.

2.3. Preparation of mutant CK2α recombinant proteins

The wild-type and mutant CK2α were cloned in vector pT7-7H6 and expressed in *Escherichia coli* strain BL21-DE3 after induction at 37° with 1 mM IPTG as previously described [15]. The bacterial pellet was lysed in 1/10 of the volume of the culture in a buffer containing 50 mM sodium phosphate buffer pH 8.0, 300 mM NaCl, 10 mM imidazole, 20 mM β-mercaptoethanol and 0.5% Triton. In addition, the buffer contained a mixture of protease inhibitors including 4 μg/ml of pepstatin, leupeptin and antipain, 5 μg/ml of aprotinin and 1 mM PMSF. Immediately after lysis, recombinant CK2β subunit, prepared as described previously [15], was added (8 nmol per l of culture) to stabilize the activity of the mutant CK2α preparations. After centrifugation of the extract for 30 min at 6000×*g*, the His-tagged proteins were purified from the soluble fraction using a Ni^{+}-NTA-agarose column as indicated by the manufacturer. The eluted fractions were analyzed for their catalytic activity and the presence of proteins of the expected molecular mass by sodium dodecyl sulfate gel electrophoresis.

2.4. Protein kinase CK2 assay

The standard assay mixture contained in a final volume of 30 μl: 50 mM HEPES, pH 7.7, 100 mM NaCl, 10 mM $MgCl_2$ 0.5 mM dithiothreitol, 10 mg/ml dephospho-βcasein and 50 μM of either [γ-^{32}P]ATP (1300 cmp/pmol) or [γ-^{32}P]GTP (2000 counts per min (cpm)/pmol). 5 pmol of CK2α wild-type or mutant α subunits was used per assay and, in the case of the wild-type enzyme, an equimolar amount of recombinant CK2β [15] was added to the preparation to give maximal stimulation of the basal activity of CK2α (usually 6–8-fold stimulation). The maximal stimulation by CK2β was confirmed by titration of the catalytic activity to assure the presence of the heterotetrameric $\alpha_2\beta_2$ form. In the case of the recombinant CK2α mutants, the CK2β subunit was added during the preparation of the expressed protein, as described above. Reactions were carried out for 10 min at 30°C and terminated by the absorption of an aliquot to Whatman P81 paper, followed by washing three times in 75 mM phosphoric acid. Results are expressed as pmol of [^{32}P]phosphate incorporated into protein substrate per min.

Initial velocity measurements for determination of the apparent K_m were performed under the standard conditions except for the varied nucleotide substrate concentrations (ATP or GTP, 10–500 μM) and varying the radiolabel between 400 and 10 000 cmp/pmol. Controls were performed in the absence of enzyme for each level of radioactivity. Apparent K_m values were determined from Lineweaver–Burk double reciprocal analysis and using the program Microcal Origin 2.8. K_i values were obtained from the slopes observed at the different inhibitor concentrations. All reactions were performed in duplicate and the data represent results obtained from 3–5 individual experiments.

Table 1
Apparent K_m values for substrates ATP and GTP of reconstituted holoenzyme protein kinase CK2 and holoenzymes formed from several CK2α mutants

CK2α subunit	K_m ATP (μM)	K_m GTP (μM)
α^{WT}	19 ± 3	30 ± 5
$\alpha^{\Delta N118}$	49 ± 2	149 ± 3
α^{N118A}	16 ± 3	39 ± 1
α^{N118E}	20 ± 4	199 ± 4
α^{D120N}	22 ± 1	54 ± 0.3
$\alpha^{\Delta N118,D120N}$	41 ± 3	158 ± 7

The formation of the mutant holoenzymes and assay conditions are given in Section 2.

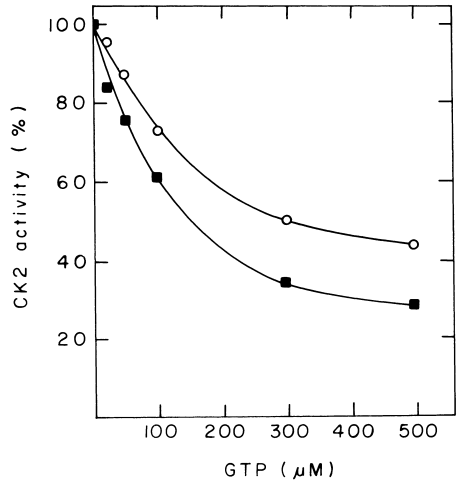

Fig. 1. Inhibition by the alternate nucleotide substrate GTP of recombinant wild-type protein kinase $CK2\alpha_2\beta_2$ holoenzyme and holoenzyme formed from $CK2\alpha^{\Delta N118}$ and CK2β. Using the standard assay described in Section 2 and 50 μM [γ-^{32}P]ATP as labeled nucleotide substrate, activities of wild-type (■) and ΔN118 mutant holoenzyme (○) were measured in the presence of varying levels of unlabeled GTP. The 100% values of incorporation of [^{32}P]phosphate into substrate casein were 48 pmol per min for wild-type enzyme and 17 pmol per min for the mutant.

3. Results

Mutants of recombinant CK2α subunit were prepared as indicated in Section 2. Due to the extreme thermal lability of the mutant in which the asparagine 118 has been deleted, the regulatory β subunit of CK2, which in a great measure stabilizes the enzyme, was added immediately after lysis to all the bacterial extracts that expressed the α subunit mutants. After purification of the recombinant reconstituted holoenzymes through Ni^{+}-NTA-agarose that retains the histidine-tagged proteins, their apparent K_m values for ATP and GTP were determined.

As had been shown previously [10], the deletion of Asn^{118} ($CK2\alpha^{\Delta N118}$) causes an important increase in the apparent K_m for GTP but a considerably lower increment in the K_m value for ATP (Table 1). The mutation of Asn^{118} to alanine ($CK2\alpha^{N118A}$) does not affect the affinity for either nucleotide. Similarly, the mutation of Asp^{120} to asparagine ($CK2\alpha^{D120N}$) does not affect significantly the K_m values. On the other hand, the mutation of the Asn^{118} to glutamic acid ($CD2\alpha^{N118E}$) causes a 6-fold increment in the K_m for GTP without affecting at all the apparent K_m for ATP. The double mutant with deleted Asn^{118} and mutated Asp^{120} to Asn ($CK2\alpha^{\Delta N118,D120N}$) has properties very similar to the single deletion of Asn^{118}.

The different affinities of the $CK2\alpha^{\Delta N118}$ for the nucleoside triphosphates were also studied through competition experiments using purine nucleoside triphosphates. Fig. 1 shows the inhibition of the CK2 activity by the presence of non-radioactive GTP, when [γ-^{32}P]ATP is used as substrate. It is clearly observed that GTP is a weaker inhibitor when the $CK2\alpha^{\Delta N118}$ is used as compared to the wild-type, confirming the lower affinity of this mutant for GTP.

Kinetic studies on the inhibition by ITP of both the wild-type and the $CK2\alpha^{\Delta N118}$ mutant using GTP as a substrate (Fig. 2) demonstrated that ITP, as expected, is a competitive inhibitor. These experiments allowed us to calculate the ap-

parent K_i for ITP which for the wild-type is 75 μM, while for the CK2α$^{\Delta N118}$, it is 360 μM. Similar experiments using XTP as inhibitor showed that CK2α$^{\Delta N118}$ activity is likewise less affected than wild-type enzyme by this purine analogue (not shown).

Our previous observation as to the extreme lability of CK2α$^{\Delta N118}$ led us to study the effect of exposing the holoenzymes of these mutants to preincubation at 45°C. Fig. 3 shows that, while the CK2α^{N118A}, CK2α^{N118E} mutants behave very much as the wild-type enzyme, the CK2α$^{\Delta N118}$ is much more labile, reaching complete inactivation after 10 min at this temperature.

4. Discussion

The results presented here extend our previous observations that indicated that Asn^{118} is involved in determining the unusual nucleotide specificity of protein kinase CK2 [10]. The function of this amino acid residue was examined considering that a computational model of CK2α constructed on the basis of the tridimensional structure indicated that Asn^{118} might be in close proximity to the nucleotide base. Additional interest was suggested by the fact that this residue is part of a sequence (N^{117}NTD^{120}) which is analogous to the NKXD sequence that has been shown to play a critical role in the specificity for GTP of G proteins and other known GTPases [11,12]. The hypothesis that this sequence plays a similar role in CK2 as it does in G proteins has been discarded by the experiments presented above. In G proteins and in GTPases, such as protein synthesis elongation factors and transducin, the aspartic acid that is part of that sequence plays a key role in stabilizing the guanine moiety through hydrogen bonding with the N1 nitrogen and the amino group in ring position 2. The change of aspartic acid to asparagine drastically modifies the nucleotide specificity of these proteins [11]. On the other hand, in the present case, mutation of the equivalent Asp^{120} to Asn in CK2α did not affect its affinity for GTP or ATP and the double mutant CK2α$^{\Delta N118,D120N}$ has specificity properties similar to that of the CK2$^{\Delta N118}$ single mutant.

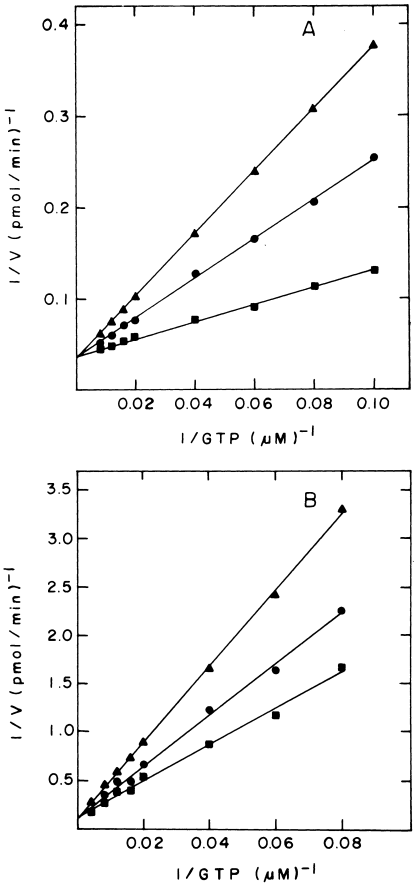

Fig. 2. Comparison of the inhibition of recombinant holoenzyme $\alpha_2\beta_2$ protein kinase CK2$\alpha_2\beta_2$and holoenzyme formed from CK2α$^{\Delta N118}$ and CK2β by the substrate analog ITP. Double reciprocal plots of 1/velocity vs. 1/GTP concentration. Substrate [γ-^{32}P]-GTP, between 10 and 125 μM, was used as variable substrate in the otherwise standard conditions given in Section 2. A: Wild-type holoenzyme assayed without additions (■) or with 100 (●) or 200 (▲) μM ITP. B: Holoenzyme formed from CK2α$^{\Delta N118}$ and assayed without additions (■) or with 150 (●) or 300 (▲) μM ITP.

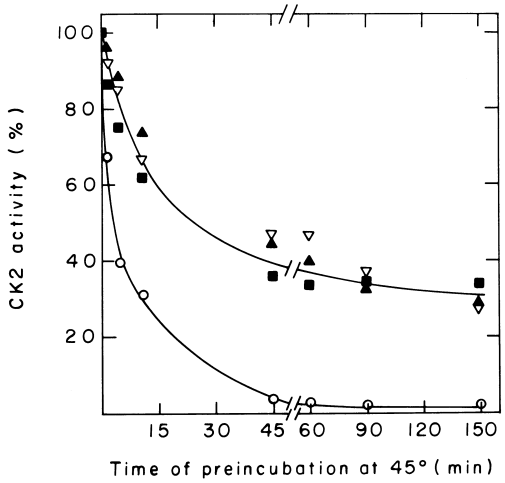

Fig. 3. Heat inactivation of recombinant wild-type holoenzyme protein kinase CK2$\alpha_2\beta_2$ and holoenzyme prepared using mutant CK2α subunits. Wild-type holoenzyme CK2$\alpha_2\beta_2$ (■) or mutant α$^{\Delta N118}$ (○), αN118A (▲) or αN118E (▽) were preincubated for different time periods at 45°C and assayed using 500 μM substrate GTP (133 cpm/pmol) and otherwise standard conditions as given in Section 2.

The observation that the CK2α^{N118A} behaves as the wild-type suggests that the Asn side chain may not be playing a major role for GTP usage, whereas the observation that the CK2α^{N118E} behaves as the deletion mutant that eliminates Asn^{118} in selectively lowering the affinity for GTP indicates that an acidic side chain in that position can disturb the function that the backbone of Asn^{118} may play in GTP recognition.

Not surprisingly, the lower affinity of CK2α$^{\Delta N118}$ for GTP extends to other purine nucleotides, in particular ITP. The Asn^{118} deletion raises the apparent K_i for ITP from 75 μM to 360 μM.

It is interesting that the CK2α$^{\Delta N118}$ and CK2α^{N118E} which have similar properties with regard to nucleotide specificity, but they differ markedly in their response to heat treatment. Thus the deletion of Asn^{118} causes a dramatic labilization of the enzyme while the substitution of asparagine for glutamic acid gives a protein with heat stability similar to the wild-type. In the computational model, Asn^{118} appears as an 'insertion segment' that changes the local structure of the nucleotide pocket when compared to other protein kinases [10]. This apparent distortion would seem to be important in maintaining the stability of the native enzyme.

When this manuscript was in preparation, a publication by Niefind et al. appeared [16]. This work is crucial to our understanding of the nucleotide specificity of CK2α since it solved the crystal structure of the α subunit bound to non-hydrolyzable analogues of ATP and GTP in the presence of Mg^{+2}. This work demonstrates that CK2 uses both purine bases by allowing a displacement of the position of the guanine base relative to the adenine position along the backbone of the enzyme and that water molecules play key roles in the fit of the guanine base.

Relevant to the work presented here, the results of Niefind et al. [16] demonstrate that Asn^{118} is hydrogen-bonded through its backbone nitrogen to the backbone carbonyl of valine 16, a link that is especially important for binding of the guanine base and which is not present in other kinases. In addition, it is found that the side chain of Asn^{118} is linked to the ribose-2′O of GTP through a water molecule. Our results on the involvement of Asn^{118} in the determination of the nucleotide specificity and the efficiency of GTP usage by holoenzyme CK2 complement and are fully in accord with the crystallographic data.

There has been some debate as to the physiological meaning of this almost unique capacity of CK2 to use efficiently GTP as a phosphoryl donor. Although this matter has not been resolved, the availability of mutants such as $CK2\alpha^{\Delta N118}$ and $CK2\alpha^{N118E}$ which are almost normal for ATP usage but have a much lower affinity for GTP could be very useful in trying to address this question.

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References

[1] Allende, J.E. and Allende, C.C. (1995) FASEB J. 9, 313–323.
[2] Pinna, L.A. and Meggio, F. (1997) in: Progress in Cell Cycle Research 3 (Meijer, L., Guidet, S. and Philippe, M., Eds.), pp. 77–97, Plenum Press, New York.
[3] Guerra, B. and Issinger, O.-G. (1999) Electrophoresis 20, 391–408.
[4] Hanks, S.K. and Quinn, A.M. (1991) Methods Enzymol. 200, 38–62.
[5] Sarno, S., Vaglio, P., Meggio, F., Issinger, O.-G. and Pinna, L.A. (1996) J. Biol. Chem. 271, 10595–10601.
[6] Sarno, S., Vaglio, P., Cesaro, L., Marin, O. and Pinna, L.A. (1999) Mol. Cell Biochem. 191, 13–19.
[7] Hinrichs, M.V., Gatica, M., Allende, C.C. and Allende, J.E. (1995) FEBS Lett. 368, 211–214.
[8] Niefind, K., Guerra, B., Pinna, L.A., Issinger, O.-G. and Schomburg, D. (1998) EMBO J. 17, 2451–2462.
[9] Chantalat, L., Leroy, D., Filhol, O., Nueda, A., Benitez, M.J., Chambaz, E.M., Cochet, C. and Dideberg, O. (1999) EMBO J. 18, 2930–2940.
[10] Srinivasan, N., Antonelli, M., Jacob, G., Korn, I., Romero, F., Jedlicki, A., Dhanaraj, V., Sayed, M.F.-R., Blundell, T.L., Allende, C.C. and Allende, J.E. (1999) Protein Eng. 12, 119–127.
[11] Hwang and Miller, D.L. (1987) J. Biol. Chem. 262, 13081–13085.
[12] Noel, J.P., Hamm, H.E. and Sigler, P.B. (1993) Nature 366, 654–663.
[13] Ho, S., Hunt, H., Horton, R., Pullen, J. and Pease, L. (1989) Gene 77, 51–59.
[14] Hinrichs, M.V., Jedlicki, A., Téllez, R., Pongor, S., Gatica, M., Allende, C.C. and Allende, J.E. (1993) Biochemistry 32, 7310–7316.
[15] Cosmelli, D., Antonelli, M., Allende, C.C. and Allende, J.E. (1997) FEBS Lett. 410, 391–396.
[16] Niefind, K., Pütter, M., Guerra, B., Issinger, O.-G. and Schomburg, D. (1999) Nat. Struct. Biol. 6, 1100–1103.