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The importance of Loop 7 for the activity of calcineurin

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Abstract Calcineurin (CN) is a heterodimer composed of a catalytic subunit (CNA) and a regulatory subunit (CNB). Loop 7 lies within the CNA catalytic domain. To investigate the role of Loop 7 in enzyme activity, we systematically examined all its residues by site-directed deletion mutation. Our results show that the Loop 7 residues are important for enzyme activity. Besides deleting residues V314, Y315 or N316, enzyme activity also increased dramatically when residues D313 or K318 were deleted. In contrast, almost all activity was lost when L312 or N317 were deleted. Ni^{2+} and Mn^{2+} were effective activators for all active mutants. However, whereas the wild-type enzyme was more efficiently activated by Ni^{2+} than by Mn^{2+} with ^{32}P-labeled R_{II} peptide as substrate, the reverse was true in all the mutants. We also found that the effect of Loop 7 on enzyme activity was substrate dependent, and involved interactions between Loop 7 residues and the unresolved part of the CN crystal structure near the auto-inhibitory domain and catalytic site.

Keywords: Catalytic subunit; Loop 7; Site-deletion mutant; Phosphatase activity; ^{32}P-labeled R_{II} peptide

1. Introduction

Calcineurin (CN, also known as protein phosphatase 2B, PP2B), a major serine/threonine protein phosphatase under the control of Ca^{2+}/calmodulin in the brain, is involved in many biological processes, including immune responses [1–5], the cAMP pathway [3,6], Na^+/K^+ ion transport in the nephron [7], cell cycle progression in lower eukaryotes [8], cardiac hypertrophy [9], and memory formation [10,11]. CN consists of a catalytic subunit (CNA) and a regulatory subunit (CNB). CN, unlike PP1 and PP2A [12–15], has three regulatory domains (about 170 extra residues) at its C-terminus: a CNB-binding domain (BBH), CaM-binding domain (CBD), and an autoinhibitory domain (AI) [18–21]. The X-ray crystal structure of CN revealed [16,17,22] that the catalytic center is formed by two β-sheets that create a β-sandwich with a loop (Loop 7) between β12 (in sheet 1) and β13 (in sheet 2). Loop 7 contains eight amino acids, from Y311 to K318. Previous studies have implicated this loop in CN activity and regulation [30].

Previously, we expressed rat CNA and its ΔV314 mutant in *Escherichia coli* [23]. We also constructed a truncated mutant of CNA (called CNAa) containing only the catalytic domain, and found that its activity (with pNPP as substrate) is much higher than that of wild-type CNA and is not regulated by CNB or CaM [24]. To systematically investigate the importance of each residue of Loop 7 for enzyme activity, in this paper we have created a series of Loop 7 deletion mutants for both CNA and CNAa, each of which contains a single amino acid deletion in Loop 7, and evaluated their activity using pNPP and ^{32}P-labeled R_{II} peptide as substrates. We have also examined the activation of wt-CNA and its mutants by the exogenous metal ions Mn^{2+} and Ni^{2+}. Our findings indicate that every residue in Loop 7 and consequently Loop 7 as a whole play a key role in CN activity and are particularly important for CN activation by CaM/CNB and exogenous metal ions. In contrast to previous studies [21,25], we have found Mn^{2+} to be a better activator for the CN mutants than Ni^{2+}. The activities of CNA and its mutants were generally higher than those of CNAa and its mutants with ^{32}P-labeled R_{II} peptide as substrate.

2. Materials and methods

2.1. Materials

The strains HMS174 (λDE3) and BL21 (λDE3), expression vector pET-21a (+), and the rat CNA-αcDNA clone were from our laboratory. pNPP was obtained from Sigma Chemical Corp. and R_{II} peptide was purchased from BioMoL Research Labs Inc. PO[OC(C_6H_5)=NCH=CC_6H_5] and POPOP{[OC(C_6H_5)=CHN=C]$_2C_6H_4$} were obtained from E. Merck. [γ-^{32}P]ATP was supplied by Beijing Furi Biology Engineering Corp. The catalytic subunit of cAMP-dependent protein kinase was purchased from Promega Chemical Corp., and all other reagents were of standard laboratory grade and the highest quality available from commercial suppliers.

3. Methods

3.1. Construction of vectors

We generated a series of DNA fragments for both CNA and CNAa with single residue deletions in Loop 7 using the two-cycle PCR method. *Nde*I and *Hin*dIII restriction sites were introduced at the 5′- and 3′-ends of the primers, respectively. The mutant PCR products were sub-cloned into a pET-21a expression vector using the *Nde*I and *Hin*dIII restriction sites. DNA sequence was assayed by using dideoxynucleotide sequencing.

3.2. Expression and purification of the mutants

The mutants were expressed in *E. coli* $BL_{21}(DE_3)$. The cells were grown in Terrific Medium (1.2% tryptone, 2.4% yeast extract, 1% NaCl, 0.6% glycerol, and 50 μg/ml ampicillin) at 37

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°C to an optical density of 0.8 at 600 nm, and expression was then induced by adding 100 μM IPTG followed by growth for an additional 16 h at 25 °C. The cultures were centrifuged at 6000 × *g* for 20 min at 4 °C and the pellets stored at −20 °C.

CNA/CNAa and their mutants were purified as described previously [24,26] and their purity was assessed on 12% SDS gels.

3.3. Assay of phosphatase activity

The phosphatase activities of CNA/CNAa and their mutants were assayed using pNPP (the concentration in the assay buffer is 20 mM) and ^{32}P-labeled R_{II} peptide as substrates. pNPP activity was assayed as reported elsewhere [24,26]. Units (U) of pNPP activity are expressed as nanomoles of pNPP hydrolyzed per milligram enzyme per minute at 30 °C.

When assaying protein phosphatase activity, the enzyme solution (10 μl, with about 0.00015–0.00056 mg/ml enzyme) was mixed with 10 μl assay buffer (40 M ^{32}P-labeled R_{II} peptide, 100 mM Tris–HCl, 0.2 mg/ml BSA, 1 mM DTT and 1 mM Mn^{2+}, pH 7.4), both with and without 0.2 mM $CaCl_2$, 0.6 μM CaM and 0.2 μM CNB, at 30 °C for 10 min. The reaction was terminated by adding 0.18 ml of 83.3 mM H_3PO_4. Finally, the released ^{32}Pi was separated from R_{II} peptide and quantified by liquid scintillation spectrometry. Protein phosphatase activity was expressed as nanomoles of ^{32}Pi from R_{II} peptide released per milligram enzyme per minute.

When assaying the effect of metal ions, Mn^{2+}/Ni^{2+} were added to the assay buffer in the case of pNPP as substrate and to the enzyme solution when the substrate was ^{32}P-labeled R_{II} peptide (Mn^{2+} 1 mM; Ni^{2+} 5 mM).

Protein concentrations were determined by the Bradford method [27].

4. Results

4.1. Expression, purification and activities of CNA/CNAa and their Loop 7 single residue deletion mutants

By using a series of chromatographic steps, the aimed proteins were purified and the purified proteins were all electrophoretically pure (data not shown).

The activities of CNA/CNAa and their Loop 7 mutants are given in Fig. 1, together with the effects of CaM and CNB on enzyme activity.

From Figs. 1(a) and (b), it can be seen that deleting any one of the residues in Loop 7 has a pronounced effect on enzyme activity. When Y311, D313, V314, Y315, N316 or K318 was deleted, enzyme activity increased dramatically, especially with V314, Y315 or N316. In contrast, almost all activity was lost when L312 or N317 was deleted. The activity of V314 was the highest, showing 6.5-fold increase with respect to wt-CNA with pNPP and 14.7-fold with ^{32}P-labeled R_{II} peptide as substrates.

CaM and CNB induced activity in wt-CNA and the majority of the mutants when assayed with either substrate. Two exceptions to this rule were observed: with pNPP as substrate, ΔD313 was not activated by CaM and ΔV314 was not activated by CNB. The extent of activation by CNB of wt-CNA and its mutants with ^{32}P-labeled R_{II} peptide as substrate was in each case much greater than activation by CaM, but there was no obvious difference when pNPP was the substrate. In addition, CaM and CNB acted synergistically on wt-CNA and its mutants.

As shown in Figs. 1(c) and (d), the activity of the CNAa ΔK318 mutant was almost zero, in sharp contrast to the

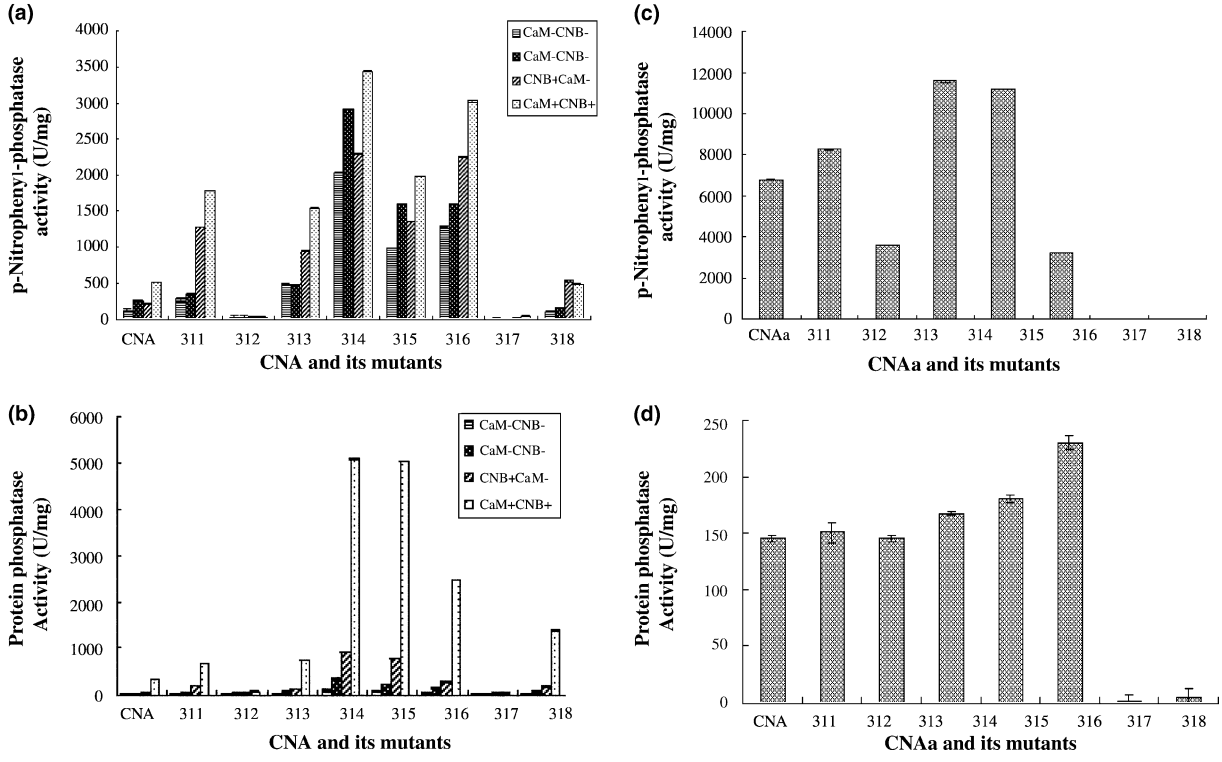

Fig. 1. Activities of CNA/CNAa and their mutants: (a) activities of CNA and its mutants with pNPP as substrate; (b) activities of CNA and its mutants with ^{32}P-labeled R_{II} peptide as substrate; (c) activities of CNAa and its mutants with pNPP as substrate; and (d) activities of CNAa and its mutants with ^{32}P-labeled R_{II} peptide as substrate. Note: All data are the averages of three testing data.

equivalent CNA mutant that had an activity higher than the wt-CNA. In the case of the CNAa ΔL312 mutant, the situation was reversed. Apart from these two mutants, there were no major differences between the activities of CNAa and its mutants when ^{32}P-labeled R_{II} peptide was used as the substrate. With pNPP as substrate, however, dramatic differences in CNAa activity were observed.

The activities of mutants Δ313, ΔV314, ΔY315, ΔN316, and ΔK318 were all much higher in CNA than in CNAa (Fig. 1). The activity ratios of CNA/CNAa differed with different mutants, such as wt-CNA/wt-CNAa 2.4, ΔD312 5.1, ΔV314 30.5, ΔY315 28.1 and ΔN316 10.8.

4.2. Effects of Mn^{2+}/Ni^{2+} on CNA/CNAa and their single site Loop 7 deletion mutants

As shown previously, the activities of CN and CNA both require the presence of exogenous metal ions, such as Mn^{2+} or Ni^{2+}. Of these two ions, Ni^{2+} has been reported to be the more effective one [21,25]. Fig. 2(a) confirms this observation for the wt-CNA, but it also shows that the relative effectiveness of Mn^{2+} and Ni^{2+} is reversed for all single residue Loop 7 deletion mutants of CNA when using ^{32}P-labeled R_{II} peptide as substrate, and especially so in mutants ΔY311, D313, ΔV314, ΔY315, and ΔK318. With pNPP as the substrate, Ni^{2+} was more effective than Mn^{2+} with CNA and all its mutants, except for ΔY311 and ΔV314 (data not shown). With CNAa and its mutants, activation by Mn^{2+} was likewise more effective than by Ni^{2+} with ^{32}P-labeled R_{II} peptide as substrate (Fig. 2(b)).

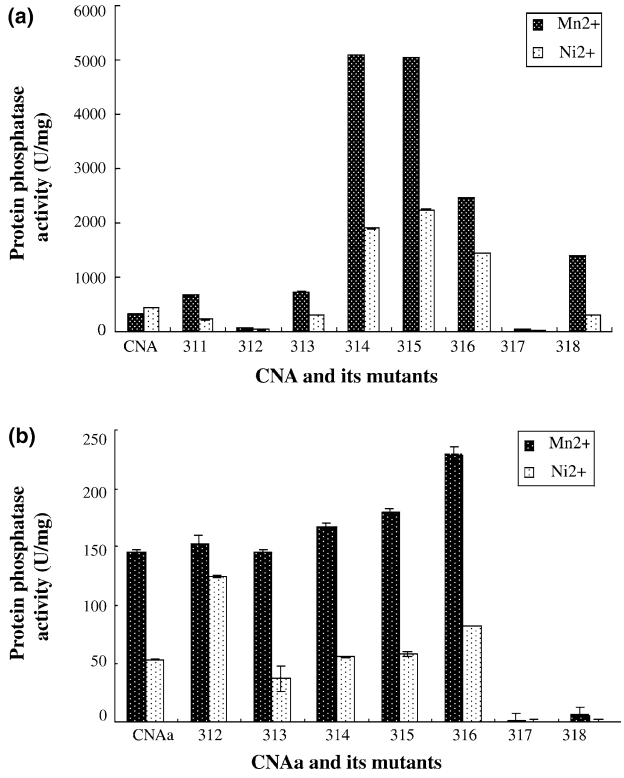

Fig. 2. Activation of phosphatase activities by 1 mM Mn^{2+} or 5 mM Ni^{2+}: (a) activity of CNA with ^{32}P-labeled R_{II} peptide as substrate; (b) activity of CNAa with^{32}P-labeled R_{II} peptide as substrate. Note: All data are the averages of three testing data.

5. Discussion

In protein phosphatase-1 (PP1), the loop equivalent to Loop 7 in CNA is very important for regulation of its activity. Its structure and conformation determine the effect on its binding to toxins and endogenous proteins. Moreover, it has been shown that this loop in the PP1 catalytic subunit mediates the binding inhibition of enzyme to I-1 and NIPP-1 [28,29]. In our previous reports [30,31], we pointed out that CNA Loop 7 might be an important structural element in the conformation of the catalytic domain and in regulating the enzyme. In this report, we have shown that each residue of Loop 7 is important for enzyme activity and further identified the key role status of Loop 7 in the enzyme activity, though the effects of deleting various Loop 7 amino acids differ. Furthermore, the effects of the exogenous metal ions Mn^{2+}/Ni^{2+} on enzyme activity are also affected by the changes in Loop 7.

In the CN crystal structure [16,17], residues V314, Y315 and N316 are situated close to the AI domain of CNA, part of which, unfortunately, is crystallographically unresolved. Loop 7 is in direct contact with the AI domain and also CBD. Chief among various contacts is a strong hydrogen bond formed between Y315 with D477 of the AI domain (~2.7 Å). In addition, there are van der Waals contacts between V314 and A473 (~3.9 Å), and aromatic–aromatic interactions between Y315 and F470 (~2.9 Å). Because the crystal structure of CBD is incomplete (~30% missing), there are probably more contacts involving residues of Loop 7 (especially from D313 to N316) and CBD.

When D313 is deleted, the hydrogen bond between D313 and H339 is lost. V314 would take the "equivalent" position of D313. By doing so, the position of the key residue Y315 would be changed, weakening or even completely losing the hydrogen bond to the AI residue D477, the most critical interaction. The same would happen in the case of V314 deletion. Of course, deletion of Y315 will abolish the hydrogen bond. L312 is a little farther away from Y315 and the effect on Y315 may be less profound than other residues mentioned. Furthermore, if D313 takes the "equivalent" position of L312, there is a possibility that D313 can move closer to the AI helix and form an interaction. This would make it harder for AI to disassociate from the catalytic domain, resulting in inhibition. Deletion of N316 or N317 should, in theory, generate the same mutant. It is therefore not clear why one is active and another is not. From our other experiments, such as the characters of fluorescence spectroscopy and circular dichroism spectroscopy, we find that N317, N316 and CNA are almost the same in their characters of circular dichroism spectroscopy, but different in the characters of fluorescence spectroscopy (data not shown). It indicates that losing the activity of mutant ΔN317 may be resulted from the changes of three-dimensional structure.

Sequential deletion of single amino acid of Loop 7 results in activation by CaM and/or CNB to varying extent; the same is true for the activity ratio of CNA/CNAa. These results further reinforce the notion that the activations of enzyme by CaM and/or CNB are related to Loop 7. It is known that the activation of CN/CNA by CaM and/or CNB is mediated by the interaction between AI domain and catalytic domain [32,33]. Therefore, Loop 7 plays a key role in the activation of enzyme by CaM and/or CNB.

Deleting individual residues of Loop 7 and the resulting enzyme activity change are mediated not only by the AI domain, but also by the CBD and BBH domains, as well as the catalytic site. Either direct or indirect interactions among CaM binding domain, CNB binding domain and Loop 7 would be necessary for such effects. Based on the structural analysis, we are able to explain the activity of most, though not all, mutants. Those mutations that disturb or abolish a critical hydrogen bonding interaction between Y315 of Loop 7 and the auto-inhibition domain would facilitate its dissociation and result in enhanced activity.

CN is a metalloenzyme that is regulated by various metal ions, including both the intrinsic metal ions at the active site (Fe^{3+} and Zn^{2+}) and exogenous metals [21,25]. Both $Mn^{2+}$and Ni^{2+} are very important for structural stability and activity with either the non-protein substrate pNPP, or with phosphoprotein substrates. Generally, Ni^{2+} is more effective than Mn^{2+} [26]. A novel finding here is that Mn^{2+} is more effective than Ni^{2+} in activating the CNA deletion mutants of Loop 7, and results with CNAa and its deletion mutants are similar. These results indicate that the Loop 7 residues affect activation by exogenous metal ions and have different effects with different metal ions. The relative efficacy of Mn^{2+}/Ni^{2+} differs in various CNA/CNAa deletion mutants and, therefore, each residue has a different effect on the interaction between enzyme and Mn^{2+}/Ni^{2+}. In addition, the extents of activation by Mn^{2+}/Ni^{2+} on CNA and its mutants are higher than that on CNAa and its mutants, suggesting that the regulatory fragment of CNA, in the presence of CaM and CNB, facilitates the activation of Mn^{2+}/Ni^{2+}. It indicated that the recognition and binding of enzyme molecule to substrates (would be detailed in another paper) is also important to the enzyme activities and sometimes it is a key factor; it is also for the sake of the difference of the activity between CNA and CNAa with R_{II} as the substrate.

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