RESEARCH ARTICLE

Crystal structures of NAC domains of human nascent polypeptide-associated complex (NAC) and its α NAC subunit

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ABSTRACT

Nascent polypeptide associated complex (NAC) and its two isolated subunits, α NAC and β NAC, play important roles in nascent peptide targeting. We determined a 1.9 Å resolution crystal structure of the interaction core of NAC heterodimer and a 2.4 Å resolution crystal structure of α NAC NAC domain homodimer. These structures provide detailed information of NAC heterodimerization and α NAC homodimerization. We found that the NAC domains of α NAC and β NAC share very similar folding despite of their relative low identity of amino acid sequences. Furthermore, different electric charge distributions of the two subunits at the NAC interface provide an explanation to the observation that the heterodimer of NAC complex is more stable than the single subunit homodimer. In addition, we successfully built a β NAC NAC domain homodimer model based on homologous modeling, suggesting that NAC domain dimerization is a general property of the NAC family. These 3D structures allow further studies on structure-function relationship of NAC.

KEYWORDS nascent polypeptide-associated complex, α NAC homodimer, β NAC, crystal structure

INTRODUCTION

Nascent polypeptide associated complex (NAC) heterodimer of α NAC (NACA) and β NAC (BTF3b) is characterized as the first cytosolic factor that binds nascent polypeptides emerging from the ribosome and prevents the peptides from incorrectly

binding with other cytosolic factors (Wiedmann et al., 1994). This complex is widely conserved from archaea to human. Some NAC mutants induce early embryonically lethal phenotypes in mice, fruit fly, and *Caenorhabditis elegans* (Deng and Behringer, 1995; Markesich et al., 2000). Experiments to determine NAC intracellular localization and distribution show that the vast majority of NAC is in the cytoplasm as a stable heterodimer, and no single subunit and homo-oligomer has been observed under physiologic conditions (Beatrix et al., 2000). However, drastic variations of relative concentration between the two NAC subunits are found in patients of Alzheimer's disease, Down syndrome, malignant brain tumors, AIDS, and ulcerative colitis (Zuo et al., 1997; Scheuring et al., 1998; Kroes et al., 2000; Kim et al., 2002), which indicates that imbalanced NAC subunits may result in pathological conditions. Sequence analysis predicts that the two NAC subunits share low yet recognizable amino acid sequence similarity in a region termed as the NAC domain. However, there is a UBA (ubiquitin-associated) domain, only in α NAC but not β NAC, C-terminal to the NAC domain, implicating different functions for the two subunits (Spreter et al., 2005). *Saccharomyces cerevisiae* genome encodes one α NAC homolog and two β NAC homologs (Shi et al., 1995; Reimann et al., 1999). In contrast, all sequenced archaeobacterial genomes have only one gene, named as aeNAC, homologous to human α NAC (Spreter et al., 2005). Therefore, NAC is likely to be an ancient protein complex with some latter-acquired functions.

At present, it is widely accepted that NAC plays an important role in cotranslational targeting of nascent polypeptides to endoplasmic reticulum (ER). Either of the two NAC subunits can bind directly with nascent polypeptides

emerging from the ribosome, albeit the binding of α NAC is rather weak. β NAC can directly bind ribosome protein near the exit site of nascent polypeptide using its N-terminal ribosome binding motif (Wegrzyn et al., 2006). In contrast, α NAC does not bind ribosome protein directly. Nevertheless, it can interact with ribosome by binding tightly with ribosome nucleic acid in a non-sequence-specific manner (Beatrix et al., 2000). Moreover, the NAC complex can start binding nascent polypeptide when the N terminus of the peptide is only 17-residues long from the peptidyl-transferase center, and NAC starts being released after the polypeptide becomes longer than 30 residues. Protease digestion assays confirm the NAC protection for nascent polypeptide (Wang et al., 1995). The binding and releasing of NAC for nascent polypeptide appear in quantal units instead of a residue-by-residue manner. This binding is independent of signal peptide sequences, and yet NAC cooperates with signal recognition particle (SRP) for correctly targeting and folding of nascent polypeptide in the cotranslational process (Lauring et al., 1995a, b; Powers and Walter, 1996; Moller et al., 1998b). However, yeast NAC homolog (termed as EGD complex) is not prerequisite for growth (Reimann et al., 1999), but is necessary for protein targeting to mitochondria (George et al., 1998; Fünfschilling and Rospert, 1999). In addition, in yeast both subunits including their NAC domains have been shown to be ubiquitinated in a lysine dependent manner (Panasenkov et al., 2006, 2009). Besides functioning in cytosol, NAC can tightly bind in nucleus with X-junction in DNA replication forks and homolog recombinant regions (Whitby and Dixon, 2001). Therefore, the heterodimer of NAC appears to be multifunctional.

In the absence of a hetero-partner, for example when overexpression or downregulation of a single subunit, the remaining dominant subunit may have functions independent of nascent peptide binding (Thiede et al., 2001; Spreter et al., 2005). For instance, isolated α NAC is a multiple functional protein taking part in transcription regulation (Moreau et al., 1998; St-Arnaud, 1998; St-Arnaud and Quelo, 1998; Yotov et al., 1998), cell proliferation, and differentiation (Al-Shanti et al., 2004; Lopez et al., 2005; Al-Shanti and Aldahoodi, 2006). Similarly, isolated β NAC (also called BTF3b) was initially identified as an alternative splicing isoform of the basic transcription factor (BTF3a). However, β NAC is transcriptionally inactive although it has the same ability as BTF3a to bind RNA polymerase II (Zheng et al., 1987, 1990). Structural basis of such distinct functions remains to be determined.

The crystal structure of α NAC shows that it forms a homodimer via an interaction between the NAC domains (Spreter et al., 2005). However, until now there has been no experimental data on how human NAC heterodimer is made from α NAC and β NAC and what the structural difference is between NAC and its subunit homodimer. In this study we used the crystallography method to address these questions. We determined the crystal structure of NAC hetero-dimeric

interaction core complex at 1.9 Å resolution and the crystal structure of α NAC NAC domain homodimer at 2.4 Å resolution. In addition, our structural analysis and model building suggested that β NAC subunits can form a homodimer similar to the NAC heterodimer. These structures provide detailed information of NAC heterodimerization and an explanation to why NAC is more stable than an α NAC homodimer.

RESULTS

Structure of human NAC complex

We coexpressed and copurified the full length recombinant proteins of human α NAC (GenBank: AAX14393.1, residues 1–215) and β NAC (GenBank: AAH08062.1, residues 1–162) from *E. coli*. Solution study showed that α NAC and β NAC recombinant proteins formed a stable heterodimer (Supplemental Fig. 1A). NAC crystals of good diffraction quality appeared six months after setting up the crystallization drops. The reservoir solution was 0.1 M sodium citrate (pH 5.6), 0.1 M NaCl, and 12% (w/v) polyethylene glycol (PEG) 4000. Regarding the prolonged crystallization time, we speculated that the protein sample was processed by some incidentally contaminated protease(s), which promoted the crystallization. Phases of the crystal structure were solved at 1.9 Å resolution using the single wavelength anomalous diffraction (SAD) data collected from a potassium iodine derivative. Subsequently, it was found that the crystal structure contained only the NAC domains from both α NAC (residues 79–132, designated as NAC $_{\alpha}$) and β NAC (residues 53–110, named as NAC $_{\beta}$) forming a heterodimer of dimensions of 30 × 34 × 45 Å (Fig. 1A). The solvent content of the crystal form was estimated to be 63% ($V_M = 3.3 \text{ Å}^3/\text{Da}$) with one such heterodimer per asymmetric unit. The crystal structure was refined to R factor of 0.196 (and R-free of 0.245) with excellent geometry (Table 1). Taken together, we obtained the crystal structure of a stable core of the human NAC heterodimer.

In this complex, NAC $_{\alpha}$ and NAC $_{\beta}$ subunits shared a similar V-shaped structure. The topology of the heterodimer is shown in Fig. 1B. NAC $_{\alpha}$ consisted of helix $\alpha 1$ and strands $\beta 1$ – $\beta 6$; and NAC $_{\beta}$ consisted of strands $\beta 1'$ – $\beta 6'$, helices $\alpha 1'$ and $\alpha 2'$ (structural elements of NAC $_{\beta}$ are denoted as primes hereafter). In NAC $_{\alpha}$, $\alpha 1$, $\beta 1$, $\beta 4$, and $\beta 5$ were arranged in one arm of the V-shaped structure, and $\beta 2$, $\beta 3$, and $\beta 6$ were arranged in the other arm. Similarly, in NAC $_{\beta}$, $\beta 1'$, $\beta 4'$, and $\beta 5'$ were assembled in one arm, and $\beta 2'$, $\beta 3'$, $\beta 6'$, $\alpha 1'$, and $\alpha 2'$ were in the other arm. The two V-shaped structures packed with each other in a head-to-head manner to generate a 12-stranded β -barrel-like heterodimer (NAC $_{\alpha}$ -NAC $_{\beta}$) with six strands in each of the two major β -sheets (Fig. 1A). Structure based sequence alignment (Fig. 1C) showed that there is neither insertion nor deletion between the NAC domains of α NAC and β NAC subunits. Although amino acid-sequence identity between the NAC domains of NAC $_{\alpha}$ and NAC $_{\beta}$ is low (10/48

Figure 1. Structure of human NAC complex and TaNAC. (A) Ribbon diagram of the heterodimer NAC_α-NAC_β complex. NAC_α (magenta) consists of an N-terminal helix (labeled as α1) and six β-strands (β1–β6). NAC_β (yellow) consists of six β-strands (β1'–β6') and two short helices (α1' and α2') at the C-terminal. The pseudo dyad symmetry axis was marked as a black arrow in the left panel. (B) Topology of the NAC_α-NAC_β complex. α-Helices were drawn as cylinders, and β-strands were shown as arrows. Secondary structures are labeled and colored the same as panel A. (C) Sequence alignment of the two NAC subunits. Identical residues are highlighted with red color, and other conserved residues are highlighted with yellow color. Secondary structures of NAC_α and NAC_β were included on the top and at the bottom, respectively. Residues involved in heterodimerization were marked as following: #, residues with only main chain atoms being involved in dimerization; ★, residues with both main chain atoms and side chain atoms being involved; and ⊕, residues with only side chain atoms being involved. Note that in the NAC domain region, this alignment is consistent with a 3D structure-based sequence alignment. (D) Ribbon presentation of the TaNAC homodimer and homotetramer. On the left side two protomers (chain A, orange; and chain B, cyan) form a homodimer; and on the right side two homodimers (AB and CD; A and C colored in orange; B and D in cyan) form a tetramer. The tetramer was formed mainly through N-terminal 8 non-native residues (blue).

Table 1 Data collection, SAD phasing and refinement statistics

	TαNAC (KI derivative)	NAC complex (KI derivative)
data collection		
space group	P 2 ₁ 2 ₁ 2	P 6 ₅ 22
cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	54.6, 59.6, 69.1	59.8, 59.8, 156.8
α, β, γ (°)	90, 90, 90	90, 90, 120
wavelength	1.5418	1.0000
resolution (Å)	50–2.4 (2.49–2.40)	50–1.9 (1.97–1.90)
<i>R</i> _{sym} or <i>R</i> _{merge}	0.076 (0.282)	0.050 (0.533)
<i>I</i> /σ (<i>I</i>)	39.5 (9.1)	42.3 (3.98)
completeness (%)	99.8 (99.8)	98.8 (94.9)
redundancy	13.5 (12.3)	18.3 (13.4)
refinement		
resolution (Å)	29.9–2.4	29.4–1.9
No. reflections	9151	12,225
<i>R</i> _{work} / <i>R</i> _{free}	0.242/0.275	0.196/0.245
No. atoms		
protein	1830	868
solvent	43	48
<i>B</i> -factors		
protein	51.1	52.6
solvent	42.5	55.60
r.m.s deviations		
bond lengths (Å)	0.031	0.008
bond angles (°)	1.90	1.12
ramachandran plot ^a		
preferred region (%)	94.4	92.4
allowed region (%)	4.6	7.8
outlied region (%)	0.9	0.45

^a Calculated using the program Coot.

or 21%), they shared very similar 3D folding. The rmsd (root mean square deviation) of 44 Cα atom pairs was 1.05 Å (using a 3.0 Å cutoff, the same hereafter unless specified otherwise). There was a pseudo dyad symmetry in the heterodimer complex of NAC_α-NAC_β (Fig. 1A), and the corresponding rotation angle was 177° with a minor screw length (0.24 Å) between the two subunits.

The dimerization of NAC_α and NAC_β buried total of 2330 Å² solvent accessible surface from both subunits, corresponding to 36% of their total surface area. Interaction involved in the heterodimerization can be divided mainly into β-sheet formation contributed by main chain-main chain interaction and a hydrophobic core formed by side chain-side chain interaction. Residues of the first group were mainly distributed around the β2 and β5 strands (Fig. 1C), where one anti-parallel β-sheet was produced between β2 and β2',

and another one was generated by β5 and β5'. There were total 15 intermolecular hydrogen bonds between NAC_α and NAC_β. Nine of them were around the two β2 strands (Supplemental Table 1). It is worth to point out that there were a side chain-main chain hydrogen bond (Nε/Arg93–O/Gln109') and a side chain salt bridge (Nε/Arg97–Oε2/Glu61') in this group, which may be sensitive to mutations. In the main chain interface there was a bulge at Thr92 in the β2 strand of NAC_α (Supplemental Table 1). Because β2' was shorter at the N-terminal, this bulge was not conserved in βNAC. The rest six hydrogen bonds were around the two β5 strands (Supplemental Table 1). Hydrophobic side chains buried inside the β-barrel of the dimer included Val91, Val94, Ile96, Phe104, Ile106, Val111, Tyr120, Val122, Ala126, and Ile128 in molecule NAC_α and Ile56', Ile59', Val62', Met64', Ile72', Phe74', Val79', Leu83', Phe88', Ile90', and Ala94' in molecule

NAC $_{\beta}$ (Fig. 1C and Supplemental Fig. 2). Taken together, the two NAC domains have an extensive interaction which appears essential for the stability of each subunit.

Despite of their folding similarity, the two subunits of NAC showed clear structural differences in a few regions. First, a short helix, $\alpha 1$, was located at the N terminus of NAC $_{\alpha}$, while there were two short helices, $\alpha 1'$ and $\alpha 2'$, at the C terminus of NAC $_{\beta}$. Based on their high sequence similarity at N-termini (Fig. 1C), we speculate that the reason why NAC $_{\beta}$ crystal structure missed an N-terminal short helix similar to that of NAC $_{\alpha}$ is a truncation of the peptide instead of high mobility of the N terminus. In contrast, the region C-terminal to NAC $_{\alpha}$ shares no sequence homology with NAC $_{\beta}$, and we do not expect similar secondary structures in the corresponding regions. Secondly, the lengths of $\beta 2$ and $\beta 2'$, of $\beta 3$ and $\beta 3'$, and of $\beta 4$ and $\beta 4'$ varied between the two subunits. In addition, positions of $\beta 1$ and $\beta 1'$ differed after overall superposition, and positions of the loops connecting $\beta 2$ and $\beta 3$ and connecting $\beta 4$ and $\beta 5$ strands also showed significant changes (Fig. 2A).

As shown in Fig. 1A, $\alpha 1'$ and $\alpha 2'$ of NAC $_{\beta}$ were away from the NAC core. Structure inspection showed that some hydrophobic residues including Phe65', Val71', Leu99', Met102', Leu103', Ile106', Leu107', and Leu110' of NAC $_{\beta}$ formed a small hydrophobic core between $\alpha 1'$, $\alpha 2'$, $\beta 2'$, and $\beta 3'$ (Supplemental Fig. 2A); additionally, O ϵ 1/Gln109' of $\alpha 2'$ formed a hydrogen bond with N δ 2/Asn63' of $\beta 2'$, and O/Gln109' of $\alpha 2'$ could form another hydrogen bond with N ϵ /Arg93 of NAC $_{\alpha}$ $\beta 2$ strand (Supplemental Fig. 2A). These intramolecular and intermolecular interactions likely contribute to the stability of helices $\alpha 1'$ and $\alpha 2'$ at the NAC $_{\beta}$ C-terminus in the crystal and presumably in solution too.

Structure of α NAC NAC domain homodimer

It has been reported that α NAC may perform certain function

in the absence of β NAC (Thiede et al., 2001; Spreter et al., 2005). To study the structure of human α NAC alone, we constructed a number of α NAC variants of different lengths, including the full length and the NAC domain alone (i.e., residues 80–133, named as TaNAC standing for truncated α NAC). In a solution study, size exclusion chromatography and analytical ultracentrifugation showed that both full length α NAC and TaNAC formed stable homodimers, separately (Supplemental Fig. 1B–E). While the recombinant proteins of full length and other constructs of α NAC did not give rise to crystals of high diffraction quality, TaNAC readily crystallized in 3–4 d in a hanging drop experiment using a reservoir solution of 0.2 M potassium citrate (pH 8.3) and 20% (w/v) PEG 3350. Similar to the above heterodimer crystal structure, the phases of the TaNAC crystal structure were determined at 2.4 Å resolution using SAD data from a potassium iodine derivative. Although the solvent content of the TaNAC crystal (41% with $V_M = 2.1 \text{ Å}^3/\text{Da}$) was lower than that of NAC $_{\alpha}$ -NAC $_{\beta}$ (63%), the resolution of diffraction data used in refinement was lower for the TaNAC crystal (2.4 vs. 1.9 Å). In fact, the TaNAC crystal indeed diffracted beyond 2 Å resolution. However, shapes of the diffraction spots were problematic, and the R-merge value would be intolerably high if higher resolution data were included. Therefore, we chose to lower the resolution limit to 2.4 Å during data processing.

The crystal structure consisted of four TaNAC protomers (chain A, residues 73–114 and 118–132; chain B, residues 74–133; chain C, residues 74–132; and chain D, residues 77–133) per asymmetric unit (Fig. 1D). Structures of the four TaNAC protomers were similar to each other, and any two of them had an rmsd ranged from 0.6 Å to 1.8 Å (for all common C α atoms, i.e., residues 77–114 and 118–132). Each TaNAC protomer (using molecule A as a representative in the following discussion, the same hereafter unless otherwise specified) consisted of identical secondary structures as NAC $_{\alpha}$ from the NAC $_{\alpha}$ -NAC $_{\beta}$ heterodimer (1.3 Å rmsd for 48 C α).

Figure 2. Superposition of homologous structures of human and archaea NAC domains. (A) NAC domain protomers including NAC $_{\alpha}$ (magenta), NAC $_{\beta}$ (yellow), TaNAC (orange), and aeNAC (green). (B) NAC $_{\alpha}$ -NAC $_{\beta}$ heterodimer, TaNAC homodimer, and aeNAC homodimer.

The four TaNAC protomers formed two identical homodimers, denoted as AB and CD, with an rmsd of 1.1 Å for all 106 common Ca pairs. They were also similar to the NAC_α-NAC_β heterodimer (1.1 Å rmsd of 89 Ca pairs) (Fig. 2B). In the structure of TaNAC homodimer, stability of the TaNAC homodimer was contributed by total 14 intermolecular hydrogen bonds including a main chain-side chain one formed by O/Thr89/chain A and N^ζ/Lys98/chain B (Supplemental Table 1) and by the interaction of hydrophobic side chains of Val88, Val91, Val94, Ile96, Ile102, Phe104, Ile106, Pro109, Val111, Tyr120, Val122, Ala126, and Ile128 from both chains, which were buried inside the β-barrel (Supplemental Fig. 2B) just like the NAC_α-NAC_β heterodimer.

Furthermore, two such homodimers formed a tetramer in the crystal mainly through 8 non-native residues (i.e., ⁷³GPLGSPEF⁸⁰) which were left from the linker after proteolytically removing the N-terminal GST tag and differed from the native sequence in the corresponding region. Consistently, we observed a tetramer population of TaNAC in a solution study (Supplemental Fig. 1E). Similar to the NAC_α-NAC_β heterodimer, the two NAC domains of TaNAC homodimer were related by a pseudo dyad symmetry (178° rotation and 0.24 Å screw length in AB; and 179° rotation and 0.36 Å screw length in CD). Furthermore, the two homodimer were related by a 175° rotation angle and 0.12 Å screw length. The dimerization buried total of 1980 Å² and 2050 Å² solvent accessible surface in each of the two TaNAC homodimers, respectively. Formation of the tetramer further buried 2580 Å² solvent accessible surface. Comparing with corresponding native residues of αNAC (i.e., ⁷³EKKARKAM⁸⁰), we found that if we use native residues (⁷⁸KAM⁸⁰) to replace the corresponding non-native residues (⁷⁸PEF⁸⁰), there would be little influence on the backbone of TaNAC (e.g., their φ-φ angles). However, the rest residue substitution would disturb the local structure because of side chain collisions. Therefore, we concluded that the observed tetramer of TaNAC is more likely to be an artifact of crystal packing, and it probably has no physiologic significance.

Highly conserved NAC domain from Archaea to Human

To identify potential homologous structures of our NAC_α-NAC_β heterodimer, we compared our crystal structure with those in the Protein Data Bank (PDB) using the Dali search engine (Holm and Sander, 1993). The most similar folding found from this search was, as expected, the NAC domain homodimer from the archaea, *Methanothermobacter marburgensis* (Spreter et al., 2005), and no other significant hit emerged. Further analyses showed that this NAC domain of aeNAC is similar to our structures of NAC_α (1.0 Å rmsd for 42 Ca pairs), NAC_β (0.86 Å rmsd for 42 Ca pairs), and TaNAC (0.85 Å rmsd for 44 Ca pairs) at the protomer level (Fig. 2A) and to NAC_α-NAC_β (0.93 Å rmsd for 83 Ca pairs) and TaNAC-TaNAC (0.85 rmsd Å for 87 Ca pairs) at the dimer level (Fig. 2B).

DISCUSSION

Eukaryotic NAC is an important protein complex functioning as a stable heterodimer of αNAC and βNAC subunits in co-translation targeting (Lauring et al., 1995a, b; Powers and Walter, 1996; Moller et al., 1998b) and X-junction related pathways (Whitby and Dixon, 2001) among others. The crystal structure of homologous aeNAC from archaea, which has only one NAC-like gene, shows that aeNAC forms a homodimer through the NAC domains (Spreter et al., 2005). Deletion of the C-terminal UBA domain in human αNAC does not influence the NAC activity in binding nascent polypeptide ribosome complex (Spreter et al., 2005). Therefore, based on sequence homology it is anticipated that the dimerization of NAC was mainly mediated by the NAC domains of αNAC and βNAC subunits.

To study the three-dimensional structure of NAC, we coexpressed recombinant proteins of the two subunits of human NAC, αNAC and βNAC, in *E. coli*. The coexpression overcame problems of low yield associated with individually expressed proteins and eliminated the step of reconstitution of NAC from its recombinant components (Beatrix et al., 2000). Interestingly, although we set up the crystallization experiment with full length NAC heterodimer, the final crystal structure contained only the NAC domains after six-month crystallization. It suggests that other regions of the two subunits are more flexible and sensitive to degradation. The crystal structure of the heterodimer of the NAC domains of NAC_α and NAC_β indeed has a similar folding and dimer formation to the previously reported aeNAC dimer. It is clear that hydrogen bonding in two joint β-sheets and a hydrophobic core between the two sheets are the main driving force in dimerization. Therefore, an isolated NAC subunit of either NAC_α or NAC_β is unlikely to stay as stable, soluble monomers in solution because of unsaturated hydrogen bonding potential and extensively exposed hydrophobic surface.

Structure based sequence alignment shows that there are ten pairs of identical residues between the observed structures of the two subunits (Fig. 1C). Among them, Gly90, Pro109, Gly124 in NAC_α and corresponding Gly58', Pro77', and Gly92' in NAC_β were located at the bottom of V-shaped structure, connecting each of the three pairs of β-strands, β1-β2, β3-β4, and β5-β6 from the two arms. The special main chain properties of these conserved glycine and proline residues likely contribute to the correct folding of the subunits. This notion is further supported by an observation that these residues are also conserved in αNAC homologs from different species (Fig. 3). Some of the remaining conserved residues may contribute to formation of the hydrophobic core of the NAC domain dimer.

Isolated αNAC subunits play several important roles distinct from those of the NAC complex. For example, αNAC can function as a transcription coactivator (Moreau et al., 1998; St-Arnaud, 1998; St-Arnaud and Quelo, 1998; Yotov et al., 1998); it is a positive regulator in human

erythroid-cell differentiation (Lopez et al., 2005) and a negative regulator in the proliferation, differentiation, and cytotoxic activation of CD8 (+) T cells (Al-Shanti et al., 2004; Al-Shanti and Aldahoodi, 2006). Furthermore, α NAC can interact with several disease related proteins (Goatley et al., 2002; Mossabeb et al., 2002; Li et al., 2005; Mittermann et al., 2008). What does isolated α NAC look like? And how does it function in the absence of β NAC? To address these questions, we expressed recombinant proteins of a full length α NAC and its NAC domain (TaNAC). Solution studies on both α NAC and TaNAC showed that they all exist in solution as stable homodimers, suggesting that the homodimer of full length α NAC shares the same interface with the TaNAC homodimer. More importantly our crystal structure of the

TaNAC homodimer shows a very similar folding and dimerization to the NAC_α - NAC_β heterodimer as well as the previously reported aeNAC homodimer (Fig. 2).

Our solution study showed that full length β NAC can form a homodimer (Supplemental Fig. 4). It is interesting to ask whether β NAC also forms the homodimer via its NAC domain. In this context, we made a model of a homodimer of β NAC NAC domain (T β NAC, Supplemental Fig. 3) using the SWISS-MODEL online server (Guex and Peitsch, 1997; Schwede et al., 2003; Arnold et al., 2006) and the NAC_α - NAC_β heterodimer as the template structure. We assessed packing quality of this T β NAC model using the atomic empirical mean force potential implemented in the program ANOLEA (Melo and Feytmans, 1998) and using the program

Figure 3. Multiple sequence alignment of NAC homologs from human to archaea. NAC_α - NAC_β secondary structure elements are also labeled. (A) Alignment of α NAC homologs: hNACA, human α NAC, GenBank ID AAX14393.1; mouse_NACA, mouse, AAH99375.1; D.m_NACA, fruit fly, AAM68653.1; S.c_Egd2, yeast, AAA92080.1; and aeNAC, archaea, NP_275320. (B) Alignment of β NAC homologs: h_BTF3b, human β NAC, AAH08062.1; mouse_BTF3, mouse, AAH64010.1; D.m_BTF3, fruit fly, AAF06076.1; S.c_BTT1, yeast, CAA55370; and S.c_egd1p, yeast, CAA55371.1.

PROCHECK (Laskowski et al., 2005). The overall stereochemical quality was good, and energy environment for majority residues were favorable (data not shown). It suggests that β NAC can form a homodimer in a manner similar to the NAC heterodimer as well as the α NAC homodimer.

With our current constructs, the recombinant proteins of full length α NAC (23.3 kDa) and β NAC (17.7 kDa) and different species of their combinations could be distinguished with a number of techniques based on molecular weight. During the purification process, we observed a stable heterodimer of α NAC and β NAC with a 1:1 stoichiometry of the two subunits as reported previously (Beatrix et al., 2000), which migrated as a single macromolecule, but no other combinations of the two species appeared (Supplemental Fig. 1A). Meanwhile, individually purified α NAC, β NAC or T α NAC formed stable homodimers in a number of tested solutions shown in size chromatography and analytical ultracentrifugation (Supplemental Fig. 1B–E). These observations raise a question: Why does the heterodimer become the dominant species when α NAC and β NAC co-exist? In other words, why is the NAC heterodimer complex more stable than the α NAC or β NAC homodimer?

A significant difference between the heterodimer and homodimer comes from their interface charge distribution as shown in Fig. 4. The pI values of NAC $_{\alpha}$ and NAC $_{\beta}$ are 9.8 and 5.5, respectively (estimated using the program DNASTAR (Madison, WI)). These two subunits carry opposite electric charges under neutral pH. Thus, on top of other favorable intermolecular interactions the electrostatic interaction contributes favorably to the overall binding energy between NAC $_{\alpha}$ -NAC $_{\beta}$. In contrast, in a homodimer both T α NAC subunits, for example, carry positive charges, and the electrostatic term of the binding energy between the two subunits in a T α NAC homodimer is unfavorable, although it may not cancel completely other favorable terms in the total binding energy. In particular, on the interface of NAC $_{\alpha}$ -NAC $_{\beta}$ heterodimer, Arg93, Arg97, and Asp118 of NAC $_{\alpha}$ are close to the Ile110' carboxyl oxygen, Glu60' (and Glu61') side chain, and His93' side chain of NAC $_{\beta}$, respectively, thus providing favorable binding energy. In contrast, on the interface of T α NAC homodimer, Arg93 and Arg97 of subunit A are close to Arg97 and Arg93 from subunit B, thus contributing negatively to the dimerization. Therefore, electrostatic interaction is likely to play a key role in stability difference between the T α NAC homodimer and NAC $_{\alpha}$ -NAC $_{\beta}$ heterodimer.

To investigate the generality of our crystal structures, we aligned amino acid sequences of homologs of α NAC and β NAC from archaea (*Methanothermobacter marburgensis*), yeast (*Saccharomyces cerevisiae*), fruit fly (*Drosophila melanogaster*), mouse (*Mus musculus*), and humans (*Homo sapiens*). As reported before (Spreter et al., 2005), α NAC homologs share two conserved domains. One is the NAC domain which is observed in our crystal structures, and the

other is a UBA (ubiquitin-associated) domain which would be C-terminal to the NAC domain but was lost during crystallization (Fig. 3A). Similarly, β NAC homologs share two conserved regions. One is the NAC domain, and the other is N-terminal to the NAC domain (Fig. 3B). The latter conserved region is potentially responsible for conserved functions such as ribosome binding (Wegrzyn et al., 2006). It is quite probable that an ancestor gene coding a NAC-like domain was duplicated into two copies. Subsequently, one evolved to α NAC homologs, and the other to β NAC homologs. They all preserved the NAC domain presumably to maintain the dimerization ability and other NAC associated functions while allowing new functions to be developed by additional structural features.

In summary, we determined two crystal structures of NAC $_{\alpha}$ -NAC $_{\beta}$ and T α NAC at 1.9 Å and 2.4 Å resolutions, respectively. Different NAC domains show very similar folding at both protomer and dimer levels despite of the low sequence identity between α NAC and β NAC subunits. Analyses on the charge distributions suggest that electrostatic interaction between subunits contribute to the stability difference between the NAC heterodimer and α NAC homodimer. However, may there be any other function in the NAC domain of NAC subunits in addition to dimerization? Are the positively charged surface residues of NAC $_{\alpha}$ related to nucleic acid binding of α NAC? Such questions warrant further investigation.

MATERIALS AND METHODS

Expression and purification of the human NAC complex and the NAC domain of α NAC

The coding sequences of full length human α NAC and β NAC were amplified by PCR from an in-house human cDNA library and inserted between the *Eco*RI and *Sal*I restriction sites and between the *Nde*I and *Xho*I restriction sites of the pETDuet-1 coexpression vector (Novagen), respectively. The recombinant His $_6$ - α NAC and β NAC were coexpressed in *E. coli* strain BL21 (DE3). The cell culture was induced at 0.6 OD $_{600}$ with 0.5 mM Isopropyl-beta-D-thiogalactopyranoside (IPTG). The cells were disrupted with a French Press instrument (Sim-Aminco) at 15,000 psi in a lysis buffer of 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 5% (v/v) glycerol. The lysate was centrifuged at 30,000 *g* for 30 min. The supernatant after centrifugation was loaded onto a Ni affinity column (GE Healthcare), followed by washing with 20 column volume of the lysis buffer. His $_6$ - α NAC and β NAC were co-eluted with 200 mM imidazole in the lysis buffer. Recombinant NAC was further purified using sequential HiTrap Heparin HP, ion-exchange Mono Q, and gel-filtration Superdex 200 10/300 GL (GE Healthcare) chromatography. The purified NAC from Superdex 200 was concentrated to 33 mg/mL (BCA Protein Assay Kit, GeneStar Biosolutions Co., Ltd.) in a storage buffer of 20 mM Tris-HCl (pH 8.0) and 350 mM NaCl.

The DNA sequence encoding for the NAC domain of α NAC (i.e., T α NAC, residues 80–133 based on the best refinement result at the time) was subcloned into the pGEX-6P-1 vector (Amersham

Figure 4. Different binding interfaces of NAC α -NAC β heterodimer and TaNAC homodimer. In the surface presentation, blue, red, and white colors represent positively charged region, negatively charged region, and neutral zone, respectively. (A) Surface presentation of electrostatic potential of the binding surface between NAC α and NAC β . On the left side and in the middle, the corresponding binding partner is shown in a ribbon presentation. The right side panel shows the dimer surface model. (B) Surface representation of TaNAC protomer. The left panel has a similar orientation to that of NAC α in panel A. The right panel shows a semi-transparent surface model of the homodimer superimposed with its ribbon presentation.

Biosciences) between *EcoRI* and *SalI* restriction sites. Recombinant fusion protein with an N-terminal glutathione S-transferase (GST) was expressed in *E. coli* strain BL21 (DE3) and extracted using glutathione sepharose affinity column (Amersham Biosciences) in the same lysis buffer as above. TaNAC was proteolytically released from GST with PreScission Protease (Pharmacia Biotech) and was further purified by sequential chromatography including HiTrap Heparin HP and Superdex 75 10/300 GL (GE Healthcare). The purified TaNAC sample was concentrated to 8.3 mg/mL, assayed as above.

Crystallization and structure determination

Crystals of the NAC complex were grown at 16°C using the hanging drop vapor diffusion method. The protein solution was mixed in a 1 μ L + 1 μ L format with reservoir solution (500 μ L). Crystal screening was carried out using Hampton Research crystal screening kits. Crystals of good diffraction quality appeared in 0.1 M NaCl, 0.1 M sodium citrate tribasic dehydrate (pH 5.6), and 12% (w/v) polyethylene glycol

(PEG) 4000 (i.e., No. 17 of MembFacTM, named as mother liquid A hereafter) after long time incubation (about 6 months). They were cryoprotected by being quickly dipped into a cryoprotectant solution (i.e., mother liquid A supplemented with 12.5% (v/v) glycerol). Iodide derivatives were prepared by soaking native crystals in the mother liquid A plus 0.75 M KI for 2 min and then 1 M KI for 2 min.

TaNAC crystals grew more quickly than the NAC complex, despite of following the same crystallization protocol. It took only 3–4 d for high diffraction-quality crystals to grow up in mother liquid B (0.2 M potassium citrate tribasic monohydrate (pH 8.3) and 20% (w/v) PEG 3350). Heavy atom derivative crystals were prepared similarly to the above, except that TaNAC crystals were soaked only 1 min in mother liquid B plus 0.4 M KI.

Crystals were screened for good diffraction from native and heavy atom derivatives with an in-house FR-E VariMax X-ray source (Rigaku). High resolution data sets were collected at the beam line 17A of KEK Photon Factory, Japan. All data sets were processed using the program HKL2000 (Otwinowski and Minor, 1997). Phases of the crystal structures of both NAC complex and α NAC NAC domain

were determined using SAD method and the Phenix software suite (Adams et al., 2010) (including Phenix.autosol and Phenix.autobuild) and the CCP4 program package (Potterton et al., 2003). Manual model building was carried out using the Coot program (Emsley and Cowtan, 2004), and refinement alternated between cycles using the Phenix software suite and Refmac5 (Murshudov et al., 1997). Data collection, phasing, and refinement statistics are summarized in Table 1. Secondary structures were defined using the DSSP program (Kabsch and Sander, 1983). Structural analyses including superposition of three dimensional structures, calculation of rotation angles, screw lengths, and rmsd, and analyzing dimerization interface were carried out using the program EdPDB (Zhang and Matthews, 1995). Structural ribbon diagrams and molecular surface diagrams were prepared with the program PyMol (Delano, 2002). Sequence alignment was performed using the program ClustaX2.0 (Larkin et al., 2007), and the alignment figures were generated using the online ESPript server (Gouet et al., 1999).

COORDINATE DEPOSITION

Coordinates of the refined models of NAC α -NAC β complex and TaNAC and their experimental structural factors have been deposited to the RCSB Protein Data Bank (<http://www.rcsb.org/pdb/>). The accession ID is 3MCB and 3MCE, respectively.

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ABBREVIATIONS

α NAC, alpha subunit of NAC; β NAC, beta subunit of NAC; ER, endoplasmic reticulum; GST, glutathione S-transferase; IPTG, Isopropyl-beta-D-thiogalactopyranoside; NAC, nascent polypeptide-associated complex; PEG, polyethylene glycol; rmsd, root mean square deviation; SAD, single wavelength anomalous diffraction; SRP, signal recognition particle; TaNAC, truncated α NAC; UBA domain, ubiquitin-associated domain

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