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# A single Gly114Arg mutation stabilizes the hexameric subunit assembly and changes the substrate specificity of halo-archaeal nucleoside diphosphate kinase

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**Abstract** **Nucleoside diphosphate kinase from extremely halophilic archaeon (HsNDK) requires above 2 M NaCl concentration for in vitro refolding. Here an attempt was made to isolate mutations that allow HsNDK to refold in low salt media. Such a screening resulted in isolation of an HsNDK mutant, Gly114Arg, which efficiently refolded in the presence of 1 M NaCl. This mutant, unlike the wild type enzyme, was expressed in *Escherichia coli* as an active form. The residue 114 is in close proximity to Glu155 of the neighboring subunit in the three dimensional hexameric structure of the HsNDK. It is thus possible that the attractive electrostatic interactions occur between Arg114 and Glu155 in the mutant HsNDK, stabilizing the hexameric subunit assembly.**


*Keywords:* Halophilic; Extremely halophilic archaea; Nucleoside diphosphate kinase; Mutation; Subunit assembly; Substrate specificity

## 1. Introduction

Salts at high concentration play a major role in the structure and stability of halophilic proteins [1–3]. We have shown before that nucleoside diphosphate kinase from *Halobacterium salinarum* (HsNDK), once folded, maintains the native and active structure at 0.2 M NaCl [4]. However, refolding of the heat denatured HsNDK requires high salt concentration, e.g. above 2 M [5]. Halophilic proteins are characterized by abundant acidic amino acids, especially on the protein surface [1–3,6–8]. High net charges at the protein surface, when not neutralized, generate unfavorable electrostatic free energy and destabilize the proteins [9]. Salts provide charge shielding, ion binding and salting-out effects in a salt-specific manner as well as according to the general preferential protein–salt interactions [10–15], all contributing to the enhanced packing and hence folding of the protein. To further understand the mechanism of salt effects on halophilic proteins, we initiated mutational analysis of the HsNDK. Here we used error-prone PCR random mutagenesis to generate mutant HsNDK and screened the mutant proteins for refolding in low salt media. Such mutation would afford folding of the HsNDK in the absence of concentrated salts. Screening resulted in isolation of novel mutant, which carries only a single mutation of Gly114 (Gly117 in *Dictyostelium discoideum* NDK) to arginine. This paper reports the first observation of the isolation of the mutant HsNDK and its characterization in terms of folding, oligomeric state and stability.

## 2. Materials and methods

### 2.1. Strains and medium

*Escherichia coli* JM109 was used for DNA manipulation. BL21 (DE3) and BL21 Star (DE3) were used for protein expression encoded on pET series vectors (Novagen). LB-ampicillin (100 μg/ml) was used. For preculture of the transformant harboring pET-derived vectors, LB-ampicillin containing 0.4% glucose was used.

### 2.2. Isolation, expression and screening of HsNDK mutants

Randomly mutagenized HsNDK genes were prepared by error-prone PCR method using forward primer 5′-CCCCATGGGCAGCAGCCATCATC-3′ (*Nco*I site was underlined), reverse primer 5′-CGGGCTTTGTTAGCAGCCGGATCCTCA-3′ (*Bam*HI site was underlined) and Gene Taq DNA polymerase (NIPPON GENE) in the reaction mixture (10 mM Tris–HCl buffer, pH 8.0, 1 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.3 mM $MnCl_2$ and 5% dimethyl sulfoxide). The PCR program was as follows: 30 cycles of 98 °C for 0.5 min., 50 °C for 0.5 min., and 72 °C for 1.5 min followed by incubation at 72 °C for 4 min. Amplified fragments were digested with *Nco*I/*Bam*HI and cloned to *Nco*I/*Bam*HI-digested pET15b.

Cell homogenates from 5 ml culture of transformants harboring HsNDK mutant plasmids were centrifuged and the supernatants applied to Ni-NTA column (Novagen) to purify His-HsNDK mutant proteins. The column was washed with 20 mM Na-phosphate buffer, pH 7.5, 2 mM $MgCl_2$, 1 M NaCl, and 15 mM imidazole. The bound proteins were then eluted with the same buffer containing 50 mM and 250 mM imidazole. The amounts of eluted proteins were estimated by Coomassie brilliant blue staining after SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

Screening of the desired mutants was carried out by subjecting the eluted proteins described above to heat denaturation at 90 °C for 5 min and refolding the heat denatured protein in the presence of 1.5 M NaCl. The refolded sample was incubated overnight at 4 °C and measured for enzyme activity. Enzymatic activity was assayed as described before [4], except for using a micro-titer plate (the volume of reaction mixture was 0.2 ml) and a plate reader (Benchmark Plus, Bio-Rad). The HsNDK mutant gene of interest thus isolated was

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re-cloned at *Nde*I/*Bam*HI site of pET3a to construct pG114R, analyzed by ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) for DNA sequence determination, and used for further characterization.

### 2.3. Thermal stability and refolding assay of HsNDK proteins

The wild and mutant HsNDK proteins (0.1 mg/ml) were dialyzed against 50 mM Tris–HCl buffer, pH 8.0, 2 mM $MgCl_2$ containing 0.2 M or 3.8 M NaCl and heated at 30–90 °C for 5 min. After the heating, the samples were cooled and set on ice for 5 min and assayed for enzymatic activity as described previously.

The wild and mutant HsNDK proteins (0.37 mg/ml) were dialyzed against 50 mM Tris–HCl buffer, pH 7.5 and heated at 90 °C for 5 min for refolding experiments. Refolding was initiated by adding a 0.1 vol. of the heat-denatured sample into a 0.9 vol. of refolding buffer, 50 mM Tris–HCl buffer, pH 7.5, containing 0, 0.6, 1, 2 and 3 M NaCl. The sample was kept at 4 °C and the enzymatic activity assayed daily as described previously [5].

CD measurements were carried out on a Jasco J-715 spectropolarimeter equipped with a Peltier cell holder and a PTC-348WI temperature controller. A 0.1 cm cell and the protein concentration, 0.25 mg/ml, were used throughout the experiments. Thermal melting was carried out at a scan rate of 20 °C/h. The ellipticity at 216 nm was used to follow changes in the secondary structure.

### 2.4. Substrate specificity and kinetic parameters

Purified D-G114R ("D" stands for "digested", i.e., G114R expressed in *E. coli* with His-tag and digested with thrombin to remove His-tag) and D-HsNDK (HsNDK expressed in *E. coli* with His-tag and digested with thrombin to remove His-tag) were dialyzed against 50 mM Tris–HCl buffer, pH 8.0, 2 mM $MgCl_2$ and 0.2 M NaCl. Enzymatic activity was determined by luciferase assay method essentially according to Mizuki et al. [16] and Ishibashi et al. [17]. Briefly, 5 μl of enzyme was added to 95 μl of a solution containing 50 mM Tris–HCl buffer, pH 7.5, 10 mM nucleoside triphosphate (NTP), 10 mM ADP, 2.5 mM $MgCl_2$ and NaCl at the final concentration of 0.2 and 3.8 M and the mixture was incubated at 30 °C for 6 min. The reaction mixture was then diluted 200-fold with $H_2O$, and the diluted sample was assayed for the amount of ATP generated, using luciferase assay as follows: 5 μl of diluted sample was assayed in 50 mM Tris–HCl buffer, pH 8.0, 5 mM $MgCl_2$, 40% glycerol, 86 μM D-luciferin and 12 mM luciferase at 30 °C by Luminesencer-PSN AB-2200 (ATTO). The $K_m$ and $V_{max}$ were determined using GTP or UTP for a phosphate donor, and ADP as a phosphate acceptor.

### 2.5. Molecular mass determination by high-performance size-exclusion chromatography multi-angle laser light scattering (SEC-MALLS)

The chromatography system consisted of a Tohso model DP-8020 HPLC pump (Tohso, Japan), an on-line degassing system (model SD-8022, JASCO, Japan), a Rheodyne injection valve equipped with a 100-μl sample loop, and SEC columns (Tohso TSK-GEL 3000$SW_{XL}$, 7.8 mm × 30 cm and TSK $SW_{XL}$ guard column) housed in a column oven (JASCO, model CO-2060). SEC was carried out at 25 or 35 °C. Eluted proteins were monitored by a DAWN-EOS multi-angle laser light scattering detector and an Optilab rEX refractive index detector (Wyatt Technology, Santa Barbara, CA). Data collection and processing were performed using the Wyatt Technology ASTRA software version 4.9. The elution solvent (0.2 M NaCl, 2 mM $MgCl_2$, 50 mM Tris–HCl, pH 8.0) was pumped at 0.5 ml $min^{-1}$. Scattering data obtained at seven angles (60.0°, 69.3°, 79.7°, 90.0°, 100.3°, 110.7° and 121.2°) were used to construct the Debye plots by Zimm's method [18].

### 2.6. Others

Protein purity was examined by SDS–PAGE according to Laemmli [19]. Protein concentration was determined by Smith et al. [20].

## 3. Results

### 3.1. Refolding of HsNDK mutant proteins after heat-denaturation

Screening of the mutants, which enhance refolding of halophilic NDK in low salt environment, was carried out as described below. Since the crystal structure of HsNDK as well as a large volume of structure data exist for other NDKs [21–23], the observed mutation can be readily analyzed based on the three dimensional structure of the protein.

HsNDK from extremely halophilic archaeon *H. salinarum* exhibits a unique stability: i.e., it is active in the absence of salt as long as the enzyme is not exposed to elevated temperature. However, it requires high salt concentration, e.g., above 2 M NaCl, for refolding and reactivation from a heat-denatured form or from an inactive form, which occurs when expressed in *E. coli* cells [4,5]. To understand the mechanism of the salt-dependent refolding efficiency, we generated and screened mutant forms of HsNDK by error-prone PCR and high

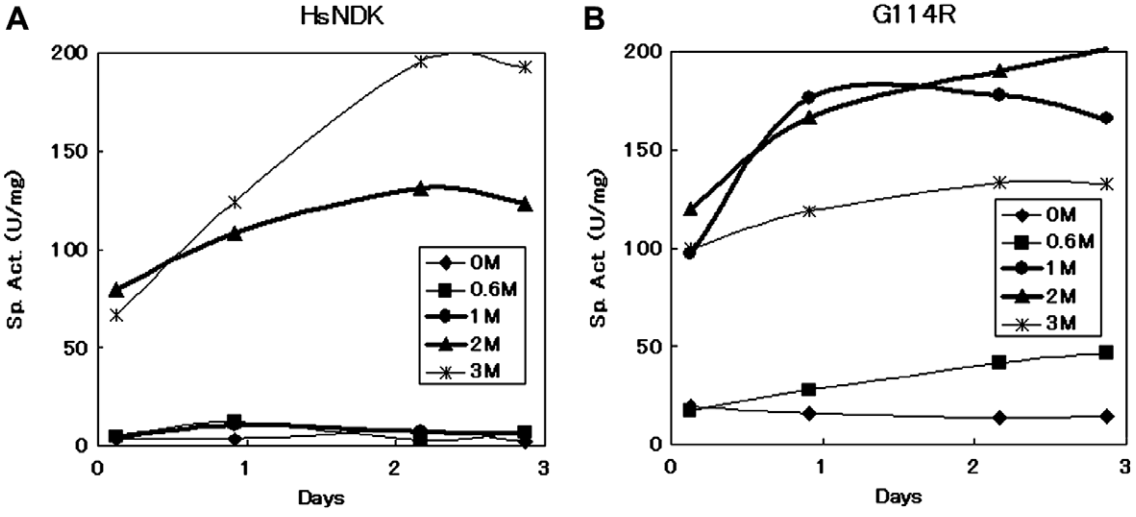


Fig. 1. Effects of salt concentrations on in vitro refolding of wild type and G114R mutant NDKs from heat-denaturation. Refolding experiments of wild type (A) and G114R mutant (B) NDKs were done as described in Section 2.

throughput refolding assay as described in Section 2. Such screening work resulted in isolation of a mutant HsNDK, which contained a single base mutation (G–A) at 340th base in *ndk* gene and hence resulted in amino acid change from Gly114 to Arg114 (G114R). As shown in Fig. 1B, this G114R mutant form of HsNDK refolded in vitro in the presence of 1.0 M NaCl and even at 0.6 M NaCl, although considerably more slowly. In contrast, the wild type HsNDK did not refold at such low NaCl concentrations (Fig. 1A). As expected, refolding of the wild type HsNDK increased when the salt concentration was increased to 2–3 M. Interestingly, the refolding efficiency of the G114R mutant was significantly reduced at 3 M NaCl (Fig. 1B), suggesting that high salt may interfere with the refolding of this mutant enzyme.

We have previously shown that the wild type HsNDK was expressed as an unfolded and inactive form in *E. coli* cells unless it was expressed with N-terminal His-tag [24]. Here, we cloned the G114R mutant gene without His-tag into a pET3a vector and expressed the protein in *E. coli*, resulting in its expression as a soluble, active form (specific activity with 391 U/mg protein). In addition, the expressed protein bound to ATP agarose column (Fig. 2), an indication of enzymatic activity; the wild type did not bind to ATP column (Fig. 2). It is worthy of note that the mobility of the G114R protein band on SDS–PAGE was faster than that of the wild type. A slow mobility on SDS–PAGE is one of the typical characteristics of halophilic proteins. These data indicated that G114R mutation resulted in reduced salt-dependency and halophilic characters.

### 3.2. Subunit structure of HsNDKs and their temperature dependency

We have previously reported that HsNDK forms dimer at 0.2 M NaCl and hexamer at 3.8 M NaCl by sedimentation equilibrium [25]. In this study, we used a high performance size exclusion chromatography-multi-angle laser light scattering (SEC-MALLS) in low salt buffer containing 0.2 M NaCl. Fig. 3 shows the SEC chromatogram of the wild type and mutant HsNDK in the standard buffer solution (50 mM Tris–HCl, pH 8.0, 2 mM $MgCl_2$ and 0.2 M NaCl) at two different temperatures. As shown in the lower panel (RI signal), the wild type HsNDK exhibits a major peak at 20 min and a minor peak at 13 min when SEC was done at 35 °C; note that the last peak at 27 min is due to salts. The molecular mass of the major peak was determined to be 36.6 kDa ± 3.3%, i.e., twice the monomer mass (18.16 kDa), indicating that it forms dimeric structure, consistent with the sedimentation analysis done at 0.2 M NaCl. The minor peak appears to be a mixture of aggregated proteins. On the contrary, SEC of the G114R mutant performed at 35 °C showed a single RI peak (except the salt peak), whose molecular mass was 108.3 kDa ± 0.1%, corresponding to the hexameric structure in the same solution (Fig. 3B). This data indicated that G114R mutation stabilized hexameric structure, against elevated temperature (35 °C), of HsNDK at low salt concentration.

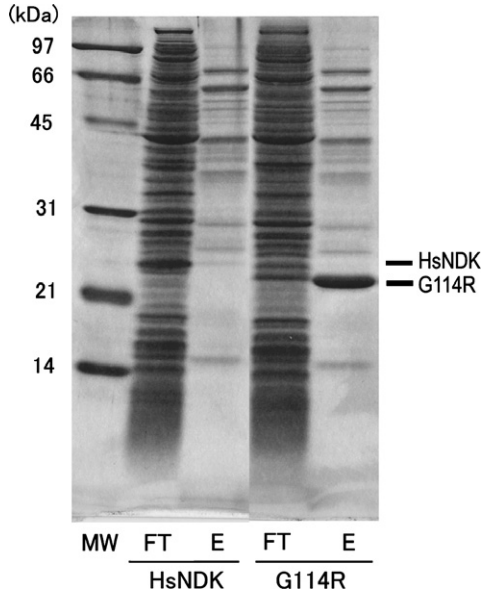


Fig. 2. Expression in *E. coli* and binding to ATP affinity column of wild type and G114R mutant NDKs. Wild type and G114R mutant NDKs were expressed in *E. coli* cells, and examined their binding to ATP affinity column. Proteins in flow through and eluted fractions from ATP column were analyzed by SDS–PAGE. MW, molecular weight marker; FT, flow through fraction; E, eluted fraction with ATP.

We further investigated the effect of temperature on subunit structure of wild type HsNDK. As shown in Fig. 3C, the wild type HsNDK showed a single peak (i.e., no aggregates) at 25 °C and was found to be 106.8 kDa ± 2.0%, corresponding to the hexameric structure. Thus, it is evident that the wild type HsNDK retains the native hexameric structure at 25 °C even in low salt medium of 0.2 M NaCl, but it dissociates to dimer at 35 °C. The hexameric HsNDK incubated at 25 °C exhibited enzymatic activity, but the dimeric structure, upon incubation at 35 °C, was inactive (see below). The dimeric molecular weight previously observed by sedimentation analysis is most likely due to accidental dissociation of the hexameric HsNDK, which may have occurred during sample storage and due to temperature fluctuation. Conversely, the G114R mutant maintains hexameric structure both at 25 and 35 °C (and probably even at higher temperature) with full enzymatic activity in low salt medium of 0.2 M NaCl (see below). For example, G114R mutant eluted as a hexamer with heat-treatment at 40 °C in the standard buffer solution, i.e., G114R mutation stabilized hexameric structure by ~15 °C.

### 3.3. Thermal stability of enzymatic activity and secondary structures of G114R mutant protein

The residual enzymatic activity of wild type and G114R mutant proteins after heat-treatment for 5 min at various temperatures is shown in Fig. 4A. At 0.2 M NaCl, the wild type HsNDK was inactivated by 35 °C heating, whereas the G114RHsNDK retained full activity up to 40 °C treatment. At 3.8 M NaCl, HsNDK was stable up to 70 °C, while G114RHsNDK was stable up to 80 °C. The observed activity loss is due to denaturation of the protein by heating, since no refolding should occur under the condition of enzyme activity measurements. In other words, these temperatures indicate the heat stability. Thus, it appears that the G114R mutation increased the stability of HsNDK by about 10 °C both in low and high salt conditions.

The thermal stability was examined by measuring the CD signal at 216 nm as a function of temperature. The melting of the G114R mutant showed a transition starting at 79 °C and ending at 85 °C for the high-salt conditions (Fig. 4B), consistent with the observed no loss of activity after heating

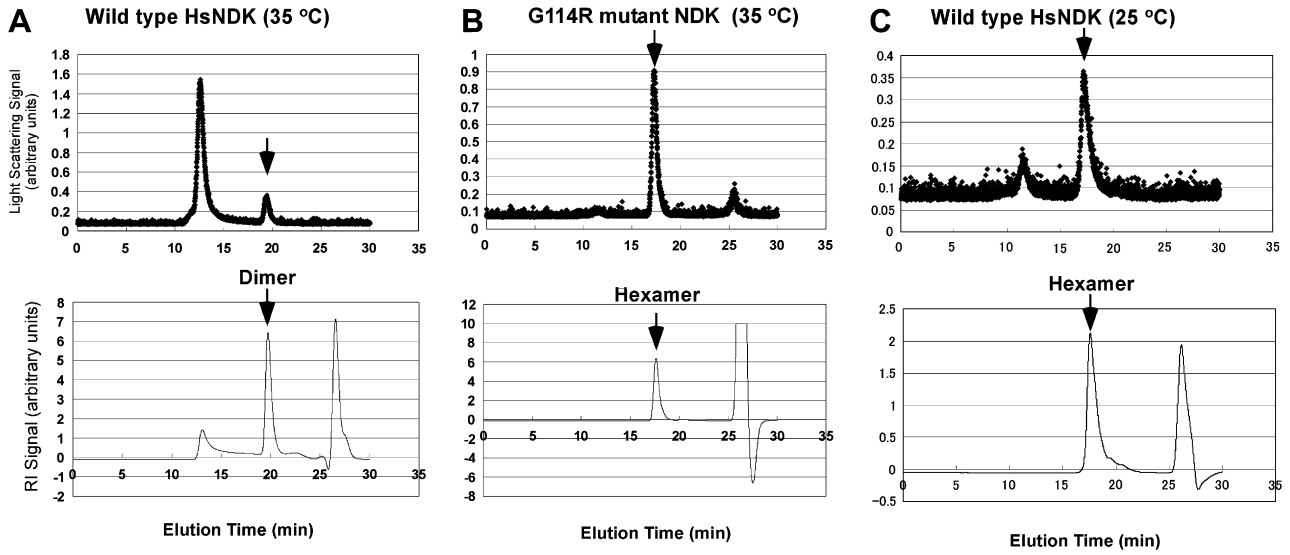


Fig. 3. Molecular mass determination of wild type and G114R mutant NDKs at 25 and 35 °C by SEC-MALLS. One hundred μl of protein sample (1.5–2.6 mg/ml) was used in each injection. The same sample was analyzed at least twice and average molecular mass was determined. Running buffer used was 50 mM Tris–HCl, pH 8.0, containing 2 mM $MgCl_2$ and 0.2 M NaCl. Upper panel, light scattering signal. Lower panel, refractive index signal.

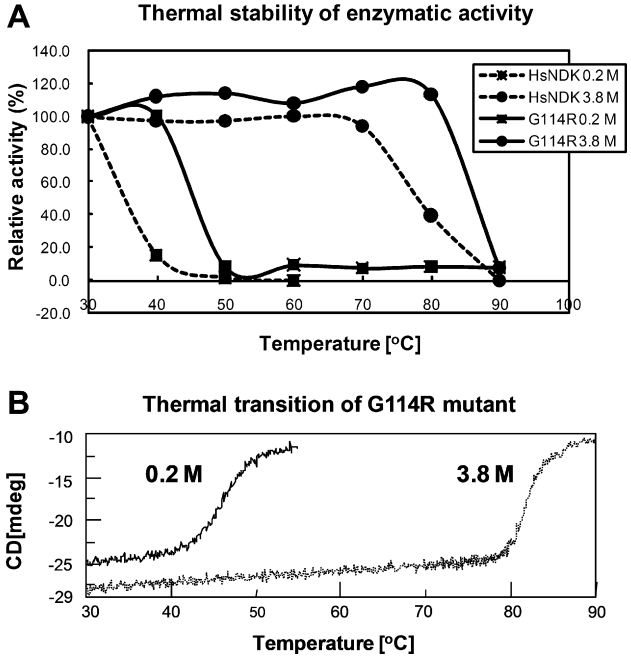


Fig. 4. Thermal stability of enzymatic activity and thermal melting of secondary structure. Residual enzymatic activity of wild type and G114R NDKs after heat-treatment for 5 min (A) and thermal transition of G114R NDK monitored by CD (B) were measured in the presence of 0.2 and 3.8 M NaCl.

at 80 °C. We have shown in the previous paper that the wild type HsNDK showed a transition starting at 73 °C and ending at 80 °C for the high salt-conditions, also consistent with the observed loss of enzyme activity after heating at 80 °C. The structure was therefore stabilized by ~6 °C by the mutation based on CD melting at 3.8 M NaCl. In the presence of 0.2 M NaCl, G114R mutant showed a transition starting at 41 °C and ending at 51 °C (Fig. 4B), similarly to the observed activity loss between 40 and 50 °C (Fig. 4A). However, in 0.2 M NaCl, the melting profile of the mutant is similar to the wild type HsNDK [25], which showed the onset temperature at 40 °C and end temperature at 53 °C. Thus, under the low-salt conditions, the wild and mutant HsNDKs showed the almost same denaturation profile.

### 3.4. Substrate specificity and kinetic parameters of G114R mutant protein

Since the amino acid residue at position 114 faces to substrate binding pocket [21], we examined the possibility that G114R mutation might affect substrate specificity. Fig. 5A shows that the wild type HsNDK exhibited highest activity for GTP as a phosphate donor, but lower activity for pyrimidine nucleoside triphosphates, UTP, CTP and TTP: UTP, CTP and TTP showed 24%, 21% and 24% activity relative to the activity toward GTP. In contrast, Fig. 5B shows that the G114R mutant preferred pyrimidine nucleoside triphosphate to GTP as a phosphate donor: UTP, CTP and TTP showed 319%, 446% and 404% activity relative to GTP. The kinetic parameters were shown in Table 1. Both wild and mutant HsNDK showed similar $K_m$ values to each substrate at both low and high salt concentrations. $V_{max}$ value of wild type HsNDK for GTP as a phosphate donor or ADP as a phosphate acceptor were 2–4 times higher than that of G114R mutant; however, $V_{max}$ for UTP was about the half that of G114R mutant regardless of salt concentrations. Therefore, higher activity of wild type HsNDK with GTP than with UTP depends on higher $V_{max}$.

## 4. Discussion

A majority of proteins from extreme halophiles require high salt concentrations, at least above 1 M, for the stability of the

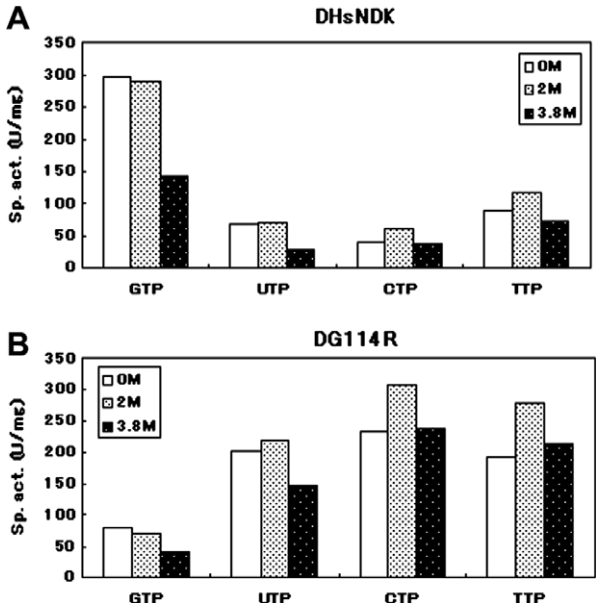


Fig. 5. Substrate specificity of wild type and G114R NDKs for a phosphate donor. Enzymatic activity was measured as described in Section 2 using GTP, UTP, CTP and TTP as a phosphate donor and ADP as a phosphate acceptor. The amount of ATP generated was determined using luciferase assay. (A) DHsNDK (wild type HsNDK expressed in *E. coli* with N-terminal His-tag and digested with thrombin to remove His-tag) was assayed at 0, 2 and 3.8 M NaCl. (B) DG114R mutant NDK (expressed with His-tag and removed it as above) was assayed at 0, 2 and 3.8 M NaCl. One unit was defined as the activity which forms 1 μmol product/min.

native conformation. Halophilic proteins are characterized by high content of acidic amino acids and relatively low proportion of hydrophobic amino acids [2,26]. Fukuchi et al. demonstrated by the genome-wide computational analysis that the content of apolar amino acid residues (Ile, Leu, Met, Phe, Trp and Val) at the protein surface was 15.1% of total residues for extreme halophiles and 18.1% for non-halophiles, while that in the interior was 41.5% for extreme halophiles and 45.6% for non-halophiles, respectively [26]. High salt concentrations provide charge shielding of large number of negative charges present on the protein surface, binding of ions, and salting-out effects. The last salt effect compensates for the lack of hydrophobic amino acid residues necessary for packing of the folded structure of halophilic proteins. It is interesting to study how high salt concentrations affect the stability of oligomeric proteins from extreme halophiles. Here we have investigated the role of particular amino acid residues in the assembly of oligomeric HsNDK. We used random mutagenesis and refolding assay to screen mutants of HsNDK, assuming that higher refolding efficiency of the mutant, if obtained, may be due to stabilization of the hexameric structure. Such screening in fact resulted in isolation of novel mutant, which afforded an efficient refolding of the mutant HsNDK in low salt medium.

The observed mutant has a single base change, resulting in mutation of Gly114 to arginine: the mutation G114R is located in the C-terminal part of the Kpn loop [22]. The hexameric NDK structure consists of a trimeric ring structure of the dimeric unit [22]. Analysis of the three dimensional structure of the HsNDK [21] showed that this amino acid at position 114 is in the close proximity to Glu155 of the other subunit in the trimeric ring of HsNDK (Fig. 6); the distance between the alpha-carbons of Gly114 and Glu155 is 5.60 angstrom according to the MOE software analysis, as shown in Fig. 6. Such a charged amino acid may be destabilizing to the protein–protein interaction, unless electrically neutralized, in particular in the non-polar environment of low dielectric constant. Arginine, instead of glycine, at this position can satisfy this condition. The favorable electrostatic interaction in the mutant may be responsible for the observed efficient refolding in low salt medium after heat denaturation at 90 °C (Fig. 1). The observed enhanced stability of the mutant oligomeric structure suggests an important role of the electrostatic interactions between Arg114 and Glu155, since these residues reside in different subunits of the trimeric ring structure. Thus, the mutation stabilizes the trimeric structure, but not the basic NDK dimer.

The mutation also enhanced resistance to heat treatment. Both heat inactivation and CD melting experiments showed that the mutant protein is significantly more stable at 3.8 M NaCl (Fig. 4). The thermal unfolding of the mutant at 3.8 M NaCl occurs around 80 °C, consistent with the observed activity loss. Conversely, the wild type begins to lose activity around 70 °C, which is also consistent with the observed melting.

The results in 0.2 M NaCl are somewhat puzzling. For the mutant, the activity loss begins around 40 °C, at which this protein begins to melt (Fig. 4). The wild type protein completely lost the activity at 40 °C, indicating that the mutant is more stable in 0.2 M NaCl as well. A puzzling data is the CD melting of the wild type; i.e., it begins to melt at ~40 °C [25], similarly to the mutant. We have shown before that HsNDK is a dimer in 0.2 M NaCl by sedimentation equilibrium analysis [25]. However, we showed here that the HsNDK is a hexamer at 25 °C and dimer at 35 °C. It is thus most likely that the activity loss during heating reflects a transition from the active hexamer to the inactive dimer, while the

Table 1
Kinetic parameters of wild and G114R mutant NDKs

| | 0 M NaCl | | | 3.8 M NaCl | | |
|---|---|---|---|---|---|---|
| | GTP (donor) | UTP (donor) | ADP (acceptor) | GTP (donor) | UTP (donor) | ADP (acceptor) |
| $K_m$ (mM) | | | | | | |
| HsNDK | 3.0 | 6.1 | 0.6 | 3.8 | 8.1 | – |
| G114R | 4.4 | 4.8 | 0.7 | 5.5 | 6.0 | – |
| $V_{max} \times 10^{-3}$ (μmolls) | | | | | | |
| HsNDK | 24.0 | 5.2 | 23.0 | 7.6 | 4.6 | – |
| G114R | 6.9 | 12.0 | 7.2 | 3.8 | 11.0 | – |

Experiments were done as described in Section 2 using luciferase assay method [16,17].

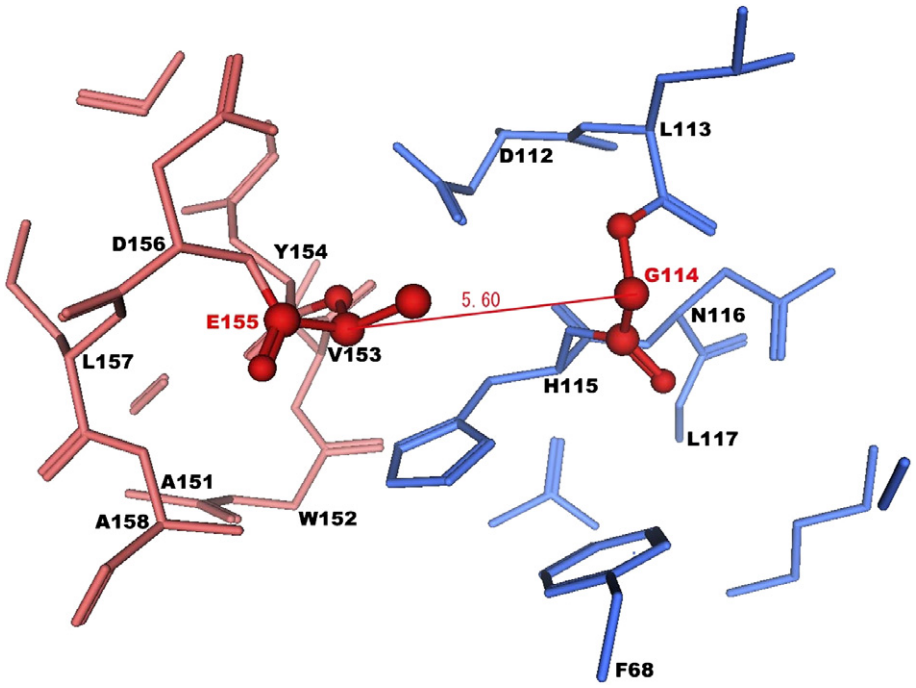


Fig. 6. Three dimensional configuration around Gly114 and Glu155 in HsNDK hexameric structure. This model was illustrated by MOE (Canada Chemical Computing Group Inc.) software according to the crystal structure of HsNDK (PDB 2AZ1, the longest complete chain comprises residues 4-158) [21]. Dark pink color represents amino acid residues in one subunit and blue represents the other subunit in hexameric structure. Amino acid residues located within 7 Å region from alpha-carbon of Glu155 and Gly114 were shown. The distance of alpha-carbons between Glu155 and Gly114 was 5.60 Å. Glu155 and Gly114 were highlighted with red color. In the G114R mutant NDK, Gly114 was replaced by arginine.

CD melting reflects a transition from the inactive dimer to the unfolded structure. This is consistent with the unstable trimeric structure of the wild-type enzyme. The observed identical temperature for heat inactivation and thermal melting for the mutant indicates a simultaneous loss of the hexameric structure and secondary structure at an identical temperature. Such altered stability of the hexamer has been observed for a lethal *kpn* mutation in *Drosophila* enzyme [27]. Proline at position 97 is involved in subunit contact and is highly conserved [22,28]. Mutation of this proline to serine has little effect on the enzyme activity, but is lethal with prune mutation [27–29], due to destabilization of the hexameric structure by the mutation [27,28]. It is interesting to note that refolding efficiency appears to decrease at 3 M NaCl relative to the lower concentration only for the mutant (Fig. 1). Since the favorable electrostatic interaction between the Arg114 and Glu155 is considered to be responsible for the stable subunit interaction, the observed results for the mutant suggests that NaCl at such high concentration may disrupt the favorable electrostatic interactions between Arg114 and Glu155.

Gly114 appeared to be not optimal for the stability of the hexameric structure in low salt environment. It is not clear whether other factors, which enhance the stability of the wild-type enzyme or make the choice of the residue at position 114 trivial in the enzyme, are involved in the non-halophilic organisms.

The mutation also caused changes in substrate specificity. It increased the affinity for pyrimidine and decreased the affinity for purine. This may be due to bulky arginine residue in place of glycine at near the substrate binding pocket, which made an entry of larger purine more difficult. However, such speculation is based on the assumption that the mutation does not grossly alter the structure of the NDK. Both exact structural and functional consequences of the mutation require determination of the crystal structure of the mutant enzyme.

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