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Facilitated folding and subunit assembly in *Escherichia coli* and in vitro of nucleoside diphosphate kinase from extremely halophilic archaeon conferred by amino-terminal extension containing hexa-His-tag

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Abstract **We have previously reported that nucleoside diphosphate kinase (HsNDK) from extremely halophilic archaeon *Halobacterium salinarum* was expressed in *Escherichia coli* as a soluble, but inactive form and required high salt concentrations for in vitro folding and activation. Here, we found that fusion of extra sequence containing hexa-His-tag at amino-terminus of HsNDK (His-HsNDK) facilitated folding and activation of HsNDK in *E. coli*. This is a first observation of active folding of halophilic enzyme from extremely halophilic archaeon in *E. coli*. The in vitro refolding rate of His-HsNDK after heat denaturation was greatly increased over the native HsNDK. Folded His-HsNDK isolated from *E. coli* formed a hexamer in both 0.2 M and 3.8 M NaCl at 30 °C, while the native HsNDK purified from *H. salinarum* dissociated to dimer in 0.2 M NaCl. The observed hexameric structure in 0.2 M NaCl indicates that amino-terminal extension also enhances dimer to hexamer assembly and stabilizes the structure in low salt. These results suggest that positive charges in fused amino-terminal extension are effective in suppressing the negative charge repulsion of halophilic enzyme and thus, facilitate folding and assembly of HsNDK.**

Keywords: Nucleoside diphosphate kinase; Halophilic enzyme; Extremely halophilic archaea; His-tag; Refolding; Subunit assembly

1. Introduction

Extremely halophilic archaea live in very salty environments such as the Dead Sea or Salt lakes and accumulate compatible concentrations of solute inside the cells [1–3]. Enzymes from extremely halophilic archaea are attractive in that they can function under such extreme conditions as high ionic strength where most enzymes from non-halophilic organisms cannot. In addition to the stability in high salt environments, several enzymes from halophilic archaea were reported to show stability against high temperature [4]. We have isolated nucleoside diphosphate kinase (HsNDK) from extreme halophile *Halobacterium salinarum* and shown that the enzyme is, as expected, active in high salt concentrations and stable against heat treatment when concentrated salts are present [5,6]. Most enzymes from extremely halophilic archaea rapidly lose their tertiary and quaternary structures, and hence activity, in low salt, e.g., below 1 M [1–3]. Although primary structure of HsNDK shows a typical characteristic of halophilic proteins, being highly acidic, it shows exceptional stability in low salt [5]. We have shown that HsNDK associates into a hexamer at 3.8 M NaCl and dissociates into a dimer at 0.2 M NaCl reversibly depending on the salt concentrations without converting into enzymatically inactive monomer [6]. We also studied the refolding of denatured-HsNDK to elucidate the salt effects on the structures of HsNDK, and indicated that salts enhance folding and enzymatic activity through both their electrostatic effects and their effects on hydrophobic interaction [7,8].

To further characterize this novel halophilic enzyme, we have attempted to generate a recombinant protein in *Escherchia coli*. Expression of the native protein, however, resulted in low expression of an inactive enzyme [5]. Here, we attempted to produce hexa-His-tag fusion of HsNDK. When an extra sequence containing His-tag was fused to the amino-terminus of HsNDK, unexpectedly the fusion resulted in a fully active enzyme with increased expression. Results of production and characterization of hexa-His-tag-HsNDK are reported in this paper.

2. Materials and methods

2.1. Bacterial strains and growth medium

Escherichia coli JM109 and BL21(DE3) were used for DNA manipulation and for expression of proteins encoded on pET series vectors (Novagen), respectively. 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. Construction of plasmids and expression of proteins

DNA manipulation was carried out using standard procedures [9]. Plasmid pETHsndk for expression of HsNDK in *E. coli* was described previously [5]. For the expression of amino-terminal His-tag-HsNDK (His-HsNDK), pETHisHsndk was constructed as follows. The cloned *ndk* gene was amplified by PCR using forward primer 5′-CCCATATGACCGATCACGACGAGCG-3′ which encodes an *Nde*I site (underlined) followed by the coding sequence starting at Met_1 to Glu_6, and reverse primer 5′-CCGGATCCTCAGTCGTGGTCTGCGAGGT-3′ which contains up to the termination codon of *ndk* gene followed by a *Bam*HI site (underlined). This amplified fragment was ligated to *Nde*I/*Bam*HI-digested pET15b to construct pETHisHsndk. This plasmid encodes a fusion protein containing HsNDK preceded by hexa-His-tag and a thrombin cleavage site (LVPRGS).

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For the expression of HsNDK with carboxy-terminal His-tag, plasmid pETHsndkHis was constructed as follows. The *ndk* gene was amplified by PCR using the same forward primer as above and reverse primer 5′-CCCTCGAGGTCGTGGTCTGCGAGGTC-3′ which contains up to the carboxy-terminal Asp followed by an *Xho*I site (underlined). This amplified fragment was ligated to *Nde*I/*Xho*I-digested pET20b.

The plasmid was introduced into *E. coli* BL21(DE3) and the synthesis of protein was induced by the addition of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside.

2.3. Purification of His-HsNDK and native HsNDK, and assay of enzymatic activity

The standard method for the purification of His-HsNDK is as follows. *E. coli* cells expressing His-HsNDK were disrupted by sonication in E buffer (50 mM Tris–HCl buffer, pH 8.0 and 2 mM $MgCl_2$) containing 3 M NaCl, and supernatant after centrifugation at 14 000 rpm for 20 min was applied to an ATP-agarose (Sigma A2767) column. Bound protein was eluted with 3 mM ATP in E buffer containing 3 M NaCl. The presence of 3 M NaCl prevents binding of *E. coli* proteins to ATP-agarose. Native HsNDK was purified from *H. salinarum* using an ATP-agarose column in the presence of 0.2 M NaCl as described previously [5] and used for all experiments except Fig. 1. Enzyme activity was measured by coupling assay as described previously and one unit was defined as the activity which forms 1 μmol product/min [5].

2.4. Refolding assay in vitro in the presence of NaCl or trimethylamine N-oxide

Purified His-HsNDK and HsNDK were dialyzed against 50 mM Tris–HCl buffer, pH 7.5. Dialyzed sample was denatured either by heat-treatment at 90 °C for 5 min or by dialysis against 6 M urea/50 mM Tris–HCl buffer, pH 7.5, overnight. Heat-denatured sample (5 μl) was added to 45 μl of refolding solution (50 mM Tris–HCl buffer, pH 7.5 or pH8.3, containing 3.0 M NaCl or 4 M trimethylamine N-oxide (TMAO), respectively) and enzyme activity was measured. For experiments in Table 1, urea-denatured sample was dialyzed against 50 mM Tris–HCl buffer, pH 7.5, containing 0.2–3.0 M NaCl, and enzyme activity was measured. The protein amount in each refolding mixture was described in Table and Figure legends.

2.5. Thrombin digestion of His-HsNDK to remove His-tag

Purified His-HsNDK (1 mg) bound to Ni–NTA (Novagen) resin was digested with 100 U of bovine thrombin (Amersham Biotech) in 10 mM Na-phosphate buffer, pH 7.5, containing 2 mM $MgCl_2$ and 154 mM NaCl at 22 C overnight. Resulting His-tag-removed HsNDK was purified by ATP agarose column.

2.6. Circular dichroism spectroscopy

Circular dichroism (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 was used throughout the experiments. For wavelength scan, a scan rate of 10 nm/min was used at a time constant of 4 s and 10 scans were accumulated. The protein concentration was 0.2 mg/ml for all the CD measurements. The solvent spectrum was subtracted from the sample spectrum. The subtracted spectrum was then converted to the mean residue ellipticity using the mean residue weight (112), the path-length of the cell (0.1 cm) and the protein concentration (0.2 mg/ml). The CD spectra (expressed as the mean residue ellipticity) were analyzed by a secondary structure analysis program, CDNN version 2.1, Guid223 (10/9/98) [10]. Thermal melting was carried out at a scan rate of 20 °C/h. The ellipticity at 216 nm was used to follow conformational changes.

2.7. Cross-linking of His-HsNDK or HsNDK in the presence of 0.2 M NaCl

His-HsNDK or HsNDK (7.5 μg/50 μl) was dialyzed against 10 mM Na-phosphate buffer, pH 7.5, containing 2 mM $MgCl_2$ and 0.2 M NaCl. To these dialyzed samples, 5 μl of 50 mM 3,3′-dithio-bis(propionic acid *N*-hydrosuccinimide ester) (DSP) was added and incubated at 30 °C for 90 min. For cross-linking in Fig. 6B, His-HsNDK was preincubated at 30 or 40 °C for 10 min, and DSP was added to cross-link at 30 or 40 °C, respectively.

2.8. Other methods

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli [11]. The amount of protein was measured as described by Lowry et al. [12].

3. Results

3.1. Expression of HsNDK with hexa-His-tag in E. coli

HsNDK has been expressed in *E. coli* as a soluble, but inactive form [5]. The inactive form, which did not bind to ATP column (Fig. 1A, lane 1), was activated by treatment with 4.0 M NaCl, as shown by binding to the column (lane 2). In order

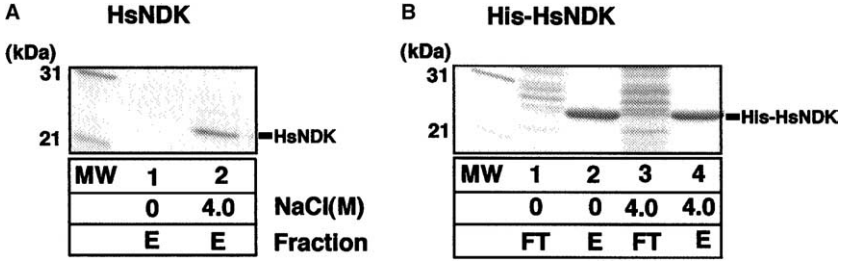

Fig. 1. ATP column chromatography of HsNDK and His-HsNDK expressed in *E. coli*. (A) Bound fractions of HsNDK expressed in *E. coli* to ATP column in the presence (lane 2) and absence (lane 1) of 4.0 M NaCl. (B) Flow through (FT, lanes 1 and 3) and bound (E, lanes 2 and 4) fractions of His-HsNDK to ATP column in the presence (lanes 3 and 4) and absence (lanes 1 and 2) of 4.0 M NaCl.

Table 1
Refolding of urea-denatured HsNDK and His-HsNDK in the presence of 0.2, 1.0 and 3.0 M NaCl for 24 h

Protein concentration in refolding mixture (μg/ml)	Specific activity (unit/mg)					
	HsNDK			His-HsNDK		
	NaCl (M)	0.2	1.0	3.0	0.2	1.0	3.0
170		13.5	14.5	316.8	12.3	90.8	330.6
340		11.0	19.8	408.0	10.9	54.6	544.4
340[a]		15.7[a]	33.0[a]	527.0[a]	11.9[a]	259.9[a]	437.0[a]
680		–	–	–	12.3	237.5	–
1100		–	–	–	10.8	232.7	–

[a] After 6 days refolding.

to facilitate expression and purification of HsNDK, here we have expressed HsNDK in *E. coli* as an amino-terminal fusion of an extra sequence containing hexa-His-tag, MGS-hexa-H-SSGLVPRGSH, provided by pET15b vector. His-HsNDK was expressed in large amount as a soluble form. (Fig. 2, lanes 5 and 6): the expression is ~3 mg/100 ml culture which is ~60-fold higher than that of HsNDK (Fig. 2, lanes 3 and 4). To our surprise, the His-HsNDK was found to bind to the ATP column before (Fig. 1B, lane 2) and after (lane 4) 4 M NaCl treatment, suggesting that His-HsNDK was expressed as an active form in *E. coli*. This is a first observation of active folding of halophilic enzyme from extremely halophilic archaeon in *E. coli*. The amounts of major chaperones, DnaK and GroE, in *E. coli* cells, when either HsNDK or His-HsNDK was expressed, were indistinguishable (data not shown). HsNDK with a carboxy-terminal His-tag was expressed in very low amount (not shown). Both *E. coli* -expressed His-HsNDK and refolded HsNDK showed a specific activity of 340 ± 10 unit/mg protein, identical to that of native HsNDK purified from *H. salinarum*.

3.2. In vitro refolding of denatured His-HsNDK in the presence of NaCl

We examined the refolding rate of denatured His-HsNDK and HsNDK in vitro. We have shown before that refolding of heat-denatured HsNDK takes days in 3.0 M NaCl [7]. As shown in Fig. 3, curve (a), His-HsNDK in the presence of 3.0 M NaCl almost completes refolding in 8 h, unlike HsNDK (curve (c)), indicating that amino-terminal extension sequence facilitates refolding of HsNDK. Removal of extension sequence by thrombin digestion resulted in decrease in refolding rate to the level of HsNDK (Fig. 3, curves (b) and (c)). In separate experiment, refolding was not facilitated in thrombin-digested His-HsNDK without removal of detached His-tag-portion from the refolding mixture (not shown), suggesting amino-terminal extension functions in *cis* position but not in *trans*. The final specific activity of both refolded His-HsNDK and HsNDK after 3 days incubation was the same, about 300 unit/mg protein, at 3.0 M NaCl (Fig. 3). Enhancement of refolding with fused His-tag was observed in the presence of 1.0 M NaCl, but not at 0.2 (Table 1) or 0.5 M (data not shown) NaCl. To prepare samples containing high concentration of protein, urea-denatured samples were used in Table 1. The refolding efficiency of His-HsNDK in salt increased with protein concentration in refolding mixture (Table 1). This result suggests that the rate-limiting step of activation in salt solution is oligomerization.

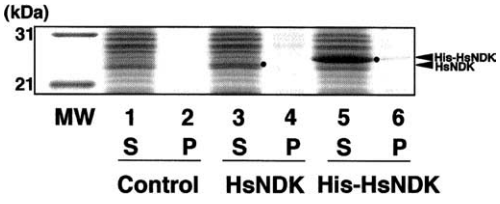

Fig. 2. Expression of HsNDK and His-HsNDK in *E. coli*. Crude homogenates of BL21(DE3, pET15b) (control, lanes 1 and 2), BL21(DE3, pETHsndk) (lanes 3 and 4), and BL21(DE3, pET-HisHsndk) (lanes 5 and 6) were analyzed by SDS–PAGE. S, soluble fraction; P, pellet fraction. Dots represent expressed HsNDK and His-HsNDK.

3.3. In vitro refolding of heat-denatured His-HsNDK in the presence of 4 M TMAO

We have shown that below pH 8.0, a non-ionic osmolyte, TMAO, can lead to a complete refolding of HsNDK at high concentrations, e.g., 4.0 M [7]. Above pH 8.0, however, 4 M TMAO is insufficient to induce refolding of HsNDK due to the pH effects on total negative charges, and hence an additional charge shielding, such as the addition of low concentration of NaCl, was required [7]. Here, we found that His-HsNDK was refolded at pH 8.3 in 4 M TMAO alone, i.e., no additional NaCl (Fig. 4), indicating that the amino-terminal extension sequence makes low concentration of NaCl unnecessary for refolding of His-HsNDK.

3.4. CD spectra and thermal melting of His-HsNDK

HsNDK purified from *H. salinarum* associates into a hexamer at 3.8 M NaCl and dissociates into dimer at 0.2 M NaCl reversibly depending on the salt concentrations [6]. The observed change of the subunit structure from hexamer to dimer was accompanied by a large decrease in α-helical content

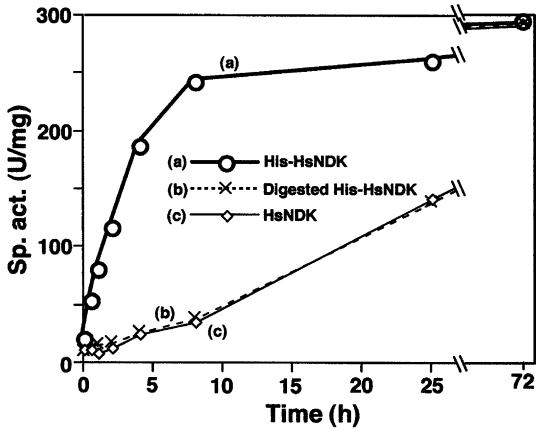

Fig. 3. Refolding of heat-denatured His-HsNDK and HsNDK in the presence of 3.0 M NaCl at pH 7.5. Protein amount in refolding mixture was 37 μg/ml. (a) His-HsNDK, (b) Thrombin-digested and purified HsNDK portion from His-HsNDK, (c) Native HsNDK purified from *H. salinarum*.

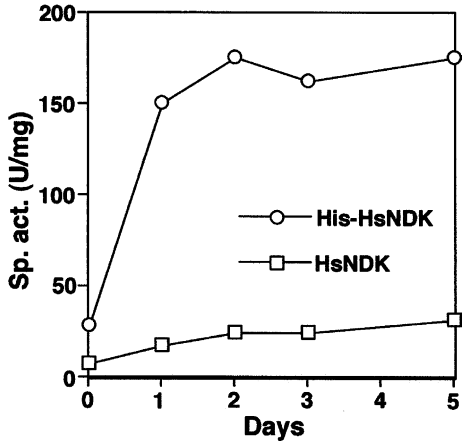

Fig. 4. Refolding of heat-denatured His-HsNDK and HsNDK in the presence of 4.0 M TMAO at pH 8.3. Protein amount in refolding mixture was 50 μg/ml.

(Fig. 5B, [6]) and lowered thermal stability [6]. Here, we found that the helical content of His-HsNDK in 0.2 M NaCl is 17%, increasing to 22% in 3.8 M NaCl (Fig. 5A) and that the CD pattern of His-HsNDK at 0.2 M NaCl (Fig. 5A, curve (a)) was exactly identical to that of HsNDK at 3.8 M NaCl (Fig. 5B, curve (b)). This observation suggests that His-HsNDK may be a hexamer in 0.2 M NaCl. We examined the thermal melting of His-HsNDK at low- and high-salt conditions (Fig. 6). Melting curve in 0.2 M NaCl (curve (a)) shows a biphasic transition: the first transition occurs in 29–36 °C, while the second occurs in 42–52 °C. The second transition is almost the same as that of HsNDK in 0.2 M NaCl (43–53 °C, [6]), meaning that the first transition in this low salt condition is unique to His-HsNDK. It is likely that the first transition in low-salt was caused by the thermal melting of unstable His-HsNDK-hexamer formed in low salt at low temperature, as suggested by CD profile of His-HsNDK in low salts (Fig. 5A, curve (a)). Melting in 3.8 M NaCl (Fig. 6, curve (b)) occurs in 70–76 °C, which is slightly lower than that of HsNDK in 3.8 M NaCl (73–80 °C, [6]). CD of His-HsNDK showed a higher helical content than the HsNDK in 3.8 M NaCl. This may be related to the decreased stability of His-HsNDK in high salt condition, although the reason for the differences is not clear.

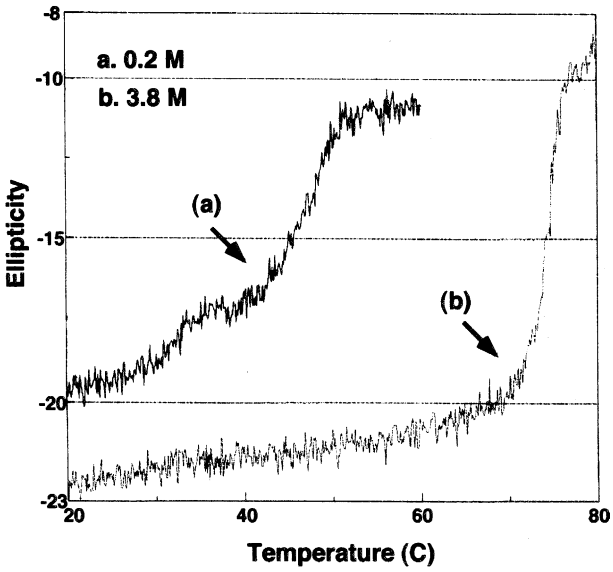

Fig. 6. Thermal melting of His-HsNDK in the presence of 0.2 M and 3.8 M NaCl. Thermal melting was measured in the presence of 0.2 M (a) and 3.8 M (b) NaCl.

3.5. Cross-linking of His-HsNDK subunit assembly

Cross-linking experiments were carried out to confirm the oligomeric structures of His-HsNDK inferred by CD: we found that a cross-linker, DSP, could function in the presence of 0.2 M NaCl (but not in 3.8 M NaCl). His-HsNDK and HsNDK were cross-linked with DSP in 0.2 M NaCl, and the resultant products were analyzed by SDS–PAGE. As shown in Fig. 7A, we detected a hexamer for His-HsNDK (lane 4) and a dimer for HsNDK (lane 3). We also detected a tetramer, a dimer as well as a monomer for His-HsNDK (lane 4) and only a monomer for HsNDK (lane 3). It is not possible to estimate the ratio of these oligomers from cross-linking experiments, which depend strongly on the cross-linking efficiency of two functional groups in the oligomers by the cross-linker used. We have reported that HsNDK forms homogeneous dimer in 0.2 M NaCl by means of an equilibrium centrifugation [6]. Some dimer molecules dissociated to monomer during chemical reactions of cross-linker in lane 3. Cross-linked oligomers were largely converted to the monomers on reducing SDS–PAGE (lanes 1 and 2) because of cleavage of disulfide bond in DSP. We then examined temperature effects on the subunit assembly of His-HsNDK in 0.2 M NaCl (Fig. 7B). At 30 °C, a hexamer of His-HsNDK was detected (lane 5), while a dimer was detected at 40 °C (lane 6). This result suggests that the first transition in CD thermal melting represents dissociation of hexamer to dimer and the second represents denaturation of dimer to monomer of His-HsNDK in 0.2 M NaCl (Fig. 6, curve (a)). The melting points of His-HsNDK dimer at 0.2 M NaCl and that of hexamer at 3.8 M NaCl were almost the same as those of HsNDK (Fig. 6, [6]).

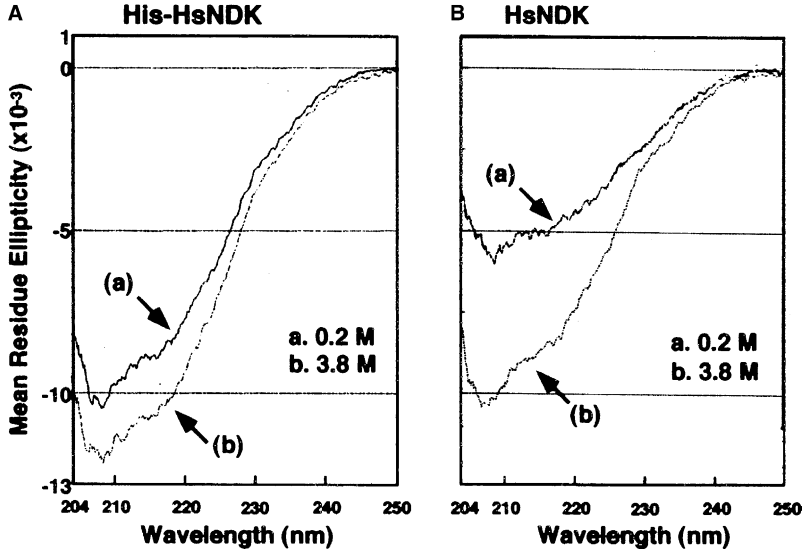

Fig. 5. CD spectra of His-HsNDK and HsNDK in the presence of 0.2 M and 3.8 M NaCl. CD spectra of His-HsNDK (A) and HsNDK (B) in the presence of 0.2 M (a) and 3.8 M (b) NaCl were measured.

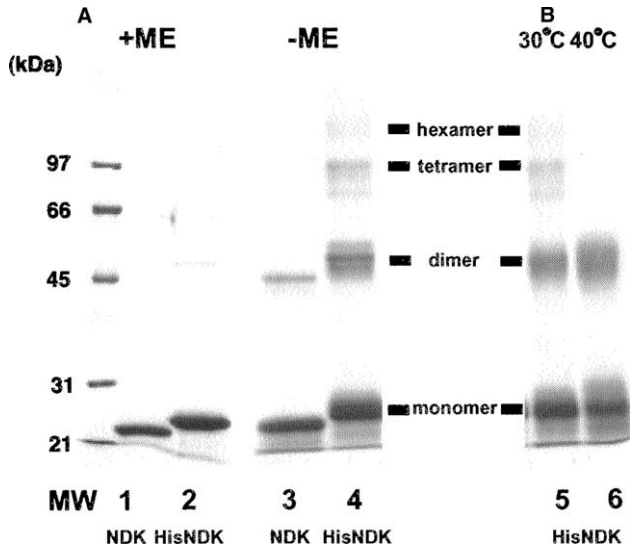

Fig. 7. Cross-linking of His-HsNDK and HsNDK in the presence of 0.2 M NaCl. (A) SDS–PAGE with (+ME) and without (−ME) addition of β-mercaptoethanol. Lanes 1 and 3, HsNDK; lanes 2 and 4, His-HsNDK. (B) Cross-linking was carried out at 30 °C (lane 5) and 40 °C (lane 6). SDS–PAGE was performed without addition of β-mercaptoethanol.

4. Discussion

In this paper, three significant observations have been made. First, His-HsNDK is folded and active when expressed in *E. coli* under the conditions, in which the native protein, i.e., without an amino-terminal His-tag, is soluble, but inactive. Second, refolding of His-HsNDK after denaturation is much more efficient than is the HsNDK. Third, the His-HsNDK is a hexamer in 0.2 and 3.8 M NaCl, while HsNDK is a hexamer only in 3.8 M NaCl. The observed sensitivity of HsNDK to salt concentration was ascribed to two factors unique to halophilic proteins, insufficient hydrophobic amino acids and excess negative charges [1–3,13,14]. Stabilization of the hexamer and hence enzyme activity of the HsNDK and enhanced refolding by NaCl at high concentration are ascribed at least in part to charge shielding effects of the salt. The observed activity and hexameric structure of His-HsNDK in low salt may be ascribed to neutralization of negative charges by an amino-terminal fused extension sequence containing 6 histidine residues. This extra sequence contains 6 histidine and one arginine residues for providing positive charges. At the pH of the experiments, however, histidine ($pK_R = 6.04$) is theoretically only partially protonated. This means that the effects of charge neutralization by the 6 histidine residues can occur if they have unusually high pK values. Alternatively, even with low protonation, a cluster of 6 histidine residues could interact with a critical region of HsNDK which is responsible for the formation of a correct higher-order structure. Another possibility is that an arginine ($pK_R = 12.48$) at thrombin-cleavage site might function solely or cooperatively with hexa-His-tag to neutralize negative charges of HsNDK. Upon thrombin cleavage, both hexa-His-tag and arginine are removed from the HsNDK sequence, resulting in the loss of the effects of the amino-terminal extension. It is still possible that factors other than charge neutralization are involved in the hexa-his-tag effects on His-HsNDK.

Although the active form of His-HsNDK is formed in *E. coli*, unfolded His-HsNDK cannot be refolded in low salt media. This suggests that during in vivo folding in *E. coli*, certain factors, not present in in vitro folding, assist folding of His-HsNDK. We have shown before that charge shielding by salts is not the only factor in facilitating in vitro refolding. For example, $MgCl_2$ induces refolding at low salt concentration, which is offset by a salting-in effects of this salt at 2 M or above [8]. Unfavorable interactions of a non-ionic TMAO with HsNDK can facilitate refolding, in which case no charge shielding is involved [7]. The observed in vivo refolding may be ascribed to molecular crowding in the cytoplasm of *E. coli* [15,16]. Molecular crowding effect is identical to the effect conferred by TMAO, since solutes causing crowding effect interact with the proteins unfavorably, just as in the case for TMAO. Another possible in vivo factor assisting folding of His-HsNDK is the presence of molecular chaperones. Chaperones and foldases might facilitate folding of HsNDK discriminating one with amino-terminal extension sequence from the one without.

Halophilic enzymes such as HsNDK are more active in concentrated salt solutions. Concentrated salt solutions are not compatible with most enzymes from non-halophilic organisms including *E. coli*. This may allow the use of crude preparations of halophilic enzymes expressed in *E. coli* in the presence of concentrated salts, which should inactivate *E. coli* enzymes. In addition to optimal activity, halophilic enzymes are resistant to thermal melting at high salt concentrations [4–6]. Both salt and heat resistance should allow a simple preparation of recombinant halophilic enzymes expressed in non-halophilic organisms such as *E. coli*.

One problem of expressing HsNDK in *E. coli* was the incomplete folding and hence low activity due to low salt environment of the cytoplasm. Although limited to HsNDK, the addition of amino-terminal tag was shown sufficient to neutralize the excess acidic charges and lead to the production of active protein. In addition, His-tag should allow a simple purification of recombinant enzymes. Thus, His-tag can be a versatile technology for expressing and purifying recombinant halophilic enzymes in an active form using non-halophilic organisms as a host. These characteristics of halophilic enzymes, along with hexa-His-tag fusion, make them attractive for industrial applications.

As a final remark, many studies have been carried out to elucidate the mechanism of salt requirements for halophilic proteins; e.g., structure analysis in concentrated salt solutions [17–19], analysis of protein refolding [7], and mutational analysis [20]. These studies gave us a clue as to the uniqueness of halophilic enzymes in interacting with salts, but no clue as to the way to reduce salt dependence while keeping halophilic natures. This study is consistent with the notion that acidic characteristic confers halophilic properties to halophilic proteins and demonstrates that a simple addition of hexa-His-tag is sufficient for in vivo folding of HsNDK without losing halophilic character. Fusion of a short sequence to the mature proteins may be one approach to be considered for production of folded structure, in the current demand of recombinant proteins in genomic and proteomic era.

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