International Journal of Biological Macromolecules 49 (2011) 778–783

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Interaction of hexa-His tag with acidic amino acids results in facilitated refolding of halophilic nucleoside diphosphate kinase

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ARTICLE INFO

Article history:
Received 18 March 2011
Received in revised form 15 July 2011
Accepted 19 July 2011
Available online 5 August 2011

Keywords:
Nucleoside diphosphate kinase
Archaea
Halophile
Refolding
His-tag

ABSTRACT

We have previously reported that amino-terminal extension sequence containing hexa-His facilitated refolding and assembly of hexameric nucleoside diphosphate kinase from extremely halophilic archaeon *Halobacterium salinarum* (NDK). In this study, we made various mutations in both the tag sequence and within NDK molecule. SerNDK, in which hexa-His was replaced with hexa-Ser, showed no facilitated folding. In addition, HisD58GD63G, in which both Asp58 and Asp63 in NDK were replaced with Gly, also showed no refolding enhancement. These results suggest that hexa-His in His-tag interact cooperatively with either Asp58 or Asp63 or both. Furthermore, G114D mutant, which formed a dimer in low salt solution, was strongly stabilized by His-tag to form a stable hexamer.

1. Introduction

Halobacterium salinarum belongs to extremely halophilic archaea, which can grow under concentrated solutions of NaCl, even above 2.5 M NaCl. Their cytoplasmic enzymes require high salt concentrations for stability, its activity and folding [1–4]. We have isolated a hexameric enzyme, nucleoside diphosphate kinase (NDK), from *H. salinarum*. To our surprise, its structure and activity were stable in low salt solutions, while most enzymes from extremely halophilic bacteria would be rapidly inactivated under similar conditions [4,5]. However, once denatured and disassembled, refolding of the NDK was slow even in the presence of salt. When NDK was expressed as His-tag fusion (HisNDK), surprisingly, it was expressed as an active form in *E. coli* under the condition, in which recombinant NDK was inactive and requires high concentration of salts for refolding *in vitro* [4]. Refolding of HisNDK in 3 M NaCl after heat-denaturation was much more efficient than the NDK [6]. In this paper, we attempt to understand how the His-tag facilitates refolding of recombinant NDK by introducing mutations into the His-tag sequence and within the NDK molecule.

2. Materials and methods

2.1. Strains and medium

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

2.2. Site-directed mutagenesis, expression and purification of NDK mutants

Site-directed mutagenesis was performed as follows: For the construction of pSNDK and pSGNDK, the DNA fragment encoding NDK was amplified by PCR using primer sets shown in Table 1, and then ligated to NcoI/BamHI-digested pET15b to construct pSNDK and pSGNDK, respectively. For others (Table 1), Quick Change Site-Directed Mutagenesis Kit (Stratagene) was used according to the manufacturer's instruction with the primer sets described (Table 1). *E. coli* BL21 Star (DE3) harboring each plasmid was grown in LB-ampicillin containing 0.4% glucose at 37 °C overnight and the 1% culture was added to LB-ampicillin. After OD600 reached 0.8 at 37 °C, synthesis of mutants was induced for 2 h by the addition of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were disrupted in ice-cold E buffer (50 mM Tris–HCl (pH8.0)/2 mM $MgCl_2$) containing 4 M NaCl by sonication (SMT UH-150 sonifer with a

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doi:10.1016/j.ijbiomac.2011.07.014

Table 1
Template and primer sets for the construction of plasmid.

Protein (plasmid)	Template	Primer	Primer sequence
HisGNDK (pHGNDK)	pETHisHsndk	RGS (43mer)	ACAGCAGCGGCCTGGTGCCGGGCGGCAGCCATATGACCGATCA
		RGAS (43mer)	TGATCGGTCATATGGCTGCCGCCCGGCACCAGGCCGCTGCTGT
SerNDK (pSNDK)	pETHisHsndk	6H6S (40mer)	CCCCATGGGCAGCAGCTCATCATCATCATCATCAAGCAGC
		NDKMR (27mer)	CGGGCTTTGTTAGCAGCAGCAGCCGGATCCTCA
SerGNDK (pSGNDK)	pHGNDK	6H6S (40mer)	CCCCATGGGCAGCAGCTCATCATCATCATCATCAAGCAGC
		NDKMR (27mer)	CGGGCTTTGTTAGCAGCAGCAGCCGGATCCTCA
HisD112S (pHD112S)	pETHisHsndk	D112SF (34mer)	CGGCGACTACGGCAACAGCCTCGGGCACAACCTC
		D112SR (34mer)	GAGGTTGTGCCCGAGGCTGTTGCCGTAGTCGCCG
HisD58G (pHD58G)	pETHisHsndk	D58GF (34mer)	GCATTACGCCGAGCACGAGGGCAAGCCGTTCTTC
		D58GR (34mer)	GAAGAACGGCTTGCCCTCGTGCTCGGCGTAATGC
HisD63G (pHD63G)	pETHisHsndk	D63GF (37mer)	CAAGCCGTTCTTCGGCGGCCTGGTGTCGTTCATCACG
		D63GR (37mer)	CGTGATGAACGACACCAGGCCGCCGAAGAACGGCTTG
HisD58GD63G (pHD58GD63G)	pHD58G	D63GF (37mer)	CAAGCCGTTCTTCGGCGGCCTGGTGTCGTTCATCACG
		D63GR (37mer)	CGTGATGAACGACACCAGGCCGCCGAAGAACGGCTTG
HisG114D (pHG114D)	pETHisHsndk	G114DF (45mer)	GGCGACTACGGCAACGACCTCGATCACAACCTCATCCACGGCAGC
		G114DR (45mer)	GCTGCCGTGGATGAGGTTGTGATCGAGGTCGTTGCCGTAGTCGCC

5 mm tip) for 3 min with a 40% pulse, and soluble and pellet fractions were obtained by centrifugation at 12,000 × *g* for 15 min. The soluble fraction was applied to an ATP-agarose (Sigma A2767) column. Bound protein was eluted with 3 mM ATP in E buffer containing 4 M NaCl. The presence of 4 M NaCl prevents binding of *E. coli* proteins to ATP-agarose. As for purification of HisG114D, HisTrap HP column (0.7 cm × 2.5 cm, GE Healthcare) chromatography was performed on an Akta prime chromatography system (Amersham, USA). The bound proteins were eluted with a linear gradient of imidazole from 15 to 250 mM in 20 mM Na–phosphate buffer (pH 7.5), 2 mM $MgCl_2$ containing 0.2 M NaCl. The 150–250 mM imidazole fractions were collected. To confirm the purity, 10% SDS-PAGE was carried out according to Laemmli [7]. The amount of protein was measured using BCA method as described by Smith et al. [8].

2.3. Thrombin digestion of HisD58GD63G and HisG114D to remove His-tag sequence

Purified HisD58GD63G and HisG114D (1 mg) were dialyzed against E buffer containing 0.2 M NaCl and digested by 100 U thrombin (ITOHAM, Japan) at 18 °C overnight. This reaction was stopped by the addition of 0.5 mM phenylmethane–sulfonyl fluoride.

2.4. Thermal stability of NDK and HisNDK

NDK and HisNDK (0.1 mg/ml) were dialyzed against E buffer containing 0–3.8 M NaCl and heated at different temperatures between 20 and 90 °C for 5 min. After heating, the samples were cooled on ice for 5 min and assayed for enzymatic activity.

2.5. Refolding assay of NDK mutants in 3 M NaCl

NDK mutants (0.37 mg/ml), dialyzed against 50 mM Tris–HCl (pH7.5), were heated at 90 °C for 5 min, set on ice for 5 min and then 0.1 volume was added to refolding buffer, 50 mM Tris–HCl buffer containing 3 M NaCl. The sample was kept at 4 °C and enzymatic activity was assayed as described previously [4].

2.6. CD spectra and thermal melting of HisG114D

CD measurements were carried out on a Jasco J820Q4 spectropolarimeter equipped with a Peltier cell holder and a PTC-423L temperature controller. A 0.1 cm cell was used throughout the experiments. The protein concentration was 0.2 mg/ml for all the CD measurements. For wavelength scans, a scan rate of 10 nm/min was used at a time constant of 4 s and 5 scans were averaged. The solvent spectrum was subtracted from the sample spectrum and the CD signal after subtraction was converted to mean residue ellipticity. 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.

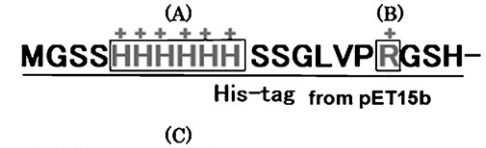
2.7. Others

Native-PAGE was carried out using 14% polyacrylamide gel without SDS at 4 °C according to Laemmli [7] and the gel was stained with Coomassie brilliant blue.

3. Results and discussion

3.1. Expression and purification of NDK mutants

We have successfully expressed all HisNDK mutants as well as the NDK and HisNDK (Fig. 1, Table 1) in *E. coli* BL21 Star (DE3). They were all expressed in soluble form at 37 °C in *E. coli* cytoplasm. After centrifugation, the soluble fraction was applied to ATP-agarose column in 0 or 4 M NaCl. Bound NDK mutant was eluted with 3 mM ATP in 0 or 4 M NaCl. Specific activity of purified NDK mutants in 4 M NaCl, i.e., HisGNDK, SerNDK, SerGNDK, HisD112S, HisD58G, HisD63G and HisD58GD63G (Fig. 1) was 293,



protein	(A)	(B)	(C)	specific activity (U/mg)
NDK (wild type)	–	–	○	347
HisNDK	His	Arg	○	264
HisGNDK	His	Gly	○	293
SerNDK	Ser	Arg	○	226
SerGNDK	Ser	Gly	○	249
HisD112S	His	Arg	Asp112–>Ser	220
HisD58G	His	Arg	Asp58–>Gly	254
HisD63G	His	Arg	Asp63–>Gly	319
HisD58GD63G	His	Arg	Asp58–>Gly /Asp63–>Gly	256
HisG114D	His	Arg	Gly114–>Asp	26

Fig. 1. Structure of His-tag sequence and NDK N-terminal amino acid. Locations of Hexa-His (A) and Arg (B) in His-tag from pET15b vector were presented by gray. Hyphen presented between His-tag sequence and NDK (C). Table shows the nomenclature, mutations and specific activity of each NDK mutant.