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NaCl-activated nucleoside diphosphate kinase from extremely halophilic archaeon, *Halobacterium salinarum*, maintains native conformation without salt

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Abstract Enzymes from extremely halophilic archaea are readily denatured in the absence of a high salt concentration. However, we have observed here that a nucleoside diphosphate kinase prepared from *Halobacterium salinarum* was active and stable in the absence of salt, though it has the amino acid composition characteristic of halophilic enzymes. Recombinant nucleoside diphosphate kinase expressed in *Escherichia coli* requires salt for activation in vitro, but once it acquires the proper folding, it no longer requires the presence of salts for its activity and stability.

Key words: Halophilic; Salt; Nucleoside diphosphate kinase; Cloning; *ndk*; *Halobacterium*

1. Introduction

Extremely halophilic archaea require more than 2.5 M NaCl for growth, and accumulate compatible concentrations of solute inside their cells: *Halobacterium salinarum* (formerly called *H. cutirubrum*) accumulates 0.8 M Na^+ and 5.32 M K^+ in its cells [1]. Thus, enzymes from extremely halophilic archaea always require at least 1 M salt for stability, and all, with a few exceptions, require salt for their activity [1–3]. There appear to be two possible reasons why they are unstable in low salt buffer [2–4]. One is the large number of acidic residues on the surface of halophilic proteins, resulting in electrostatic repulsion which favors the unfolded state. The other is an inherent weak hydrophobicity. It has been proposed that salts exert charge screening, reducing electrostatic repulsion and enhancing hydrophobic interaction, favoring a compact folded structure of halophilic proteins [1–4]. In addition, recently other mechanisms have been proposed: the effect of salt on the dielectric constant of water [5] and specific binding, rather than charge screening, of NaCl to the native protein with a low affinity [6]. Both effects confer stabilization to halophilic proteins at high salt concentrations.

We are interested in the mechanisms of protein folding under intracellular conditions of an unusually high ionic strength in halophilic archaea. We have studied the halophilic and non-halophilic molecular chaperones [7–9], and identified and characterized DnaK from the extremely halophilic archaeon *H. salinarum* [9]. This haloarchaeal DnaK requires the presence of more than 2 M NaCl to bind ATP resin. During this study, we found that one protein, with an apparent molecular mass of 24 kDa on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, 12% gel), bound to the ATP column without salt, suggesting that this protein is active without salt.

In the present paper, we describe the salt requirement of this protein, the molecular cloning and the expression in *Escherichia coli* of its gene, and the characterization of activation of this apparent 24 kDa protein, identified as a haloarchaeal nucleoside diphosphate kinase (NDK).

2. Materials and methods

2.1. Bacterial strains and culture

H. salinarum (gift of H. Onishi) [10] was grown in SGC medium [11] containing 4 M NaCl at 37°C for 3 days. For expression of the *ndk* gene, *E. coli* BL21(DE3) was used in LB-ampicillin.

2.2. Cloning of the ndk gene from H. salinarum and construction of expression vector pETHsndk

The chromosomal DNA of *H. salinarum* was prepared according to Mevarech [12]. In order to isolate the DNA fragment encoding the amino-terminal 41 amino acids of NDK from *H. salinarum* (HsNDK), a forward mixed primer, 5′-ATGACSGACCACGACGA-3′, encoding the 1st to 6th amino acids and a backward mixed primer, 5′-GAASCCSGCSCCSACCATYTT-3′, encoding the 35th to 41st amino acids of the amino-terminus, were used for polymerase chain reaction (PCR) with *H. salinarum* chromosomal DNA as a template. Southern and colony hybridizations using this amplified fragment as a probe were performed with an enhanced chemiluminescence direct labeling kit (Amersham-Pharmacia). The nucleotide sequence of the *ndk* region has been deposited in the DDBJ/EMBL/GenBank databases with the accession number AB036344 (submitted on December 24, 1999). An online database search was carried out using 'GenomeNet Database Service' operated by the Institute for Chemical Research, Kyoto University (http://www.genome.ad.jp/) with the programs of BLASTP [13] and FASTA [14].

The cloned *ndk* gene was amplified by PCR using forward primer 5′-CCCATATGACCGATCACGACGAGCC-3′ which encodes an *Nde*I site following the amino-terminal Met to 6th Glu, and backward primer 5′-CCGGATCCTCAGTCGTGGTCTGCGAGGT-3′ which

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Abbreviations: NDK, nucleoside diphosphate kinase; HsNDK, NDK from *Halobacterium salinarum*; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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encodes 157th Leu to COOH-terminal Asp followed by a *Bam*HI site. This amplified fragment was cloned into pET3a to construct pETHsndk.

2.3. ATP affinity column chromatography

H. salinarum cells were disrupted by sonication in E buffer (50 mM Tris–HCl buffer, pH 8.0, and 2 mM $MgCl_2$) with or without NaCl, and centrifuged at 14 000 rpm for 20 min. The supernatant was applied to an ATP-agarose column (Sigma A2767), and eluted with 3 mM ATP in E buffer with and without NaCl.

2.4. Enzyme assay of HsNDK

The NDK activity in the presence of a high concentration of salt was measured by a two-step assay. A reaction mixture (100 μl) containing 5 mM TDP, 10 mM ATP, 25 mM $MgCl_2$, 50 mM Tris–HCl buffer, pH 8.0, enzyme (below 0.1 U), and 0–4 M NaCl was incubated at 30°C for 1–6 min, and the enzyme reaction was terminated with addition of 10% trichloroacetic acid. The amount of ADP produced in the first reaction mixture (100 μl) was measured by an enzyme-coupling method [15]: it was measured in the second reaction mixture (1 ml) containing 3 mM phosphoenolpyruvate, 0.3 mM NADH, 25 mM $MgCl_2$, 0.1 M KCl, 2.6 U pyruvate kinase, 11 U lactate dehydrogenase, and 100 μl of first reaction mixture after adjustment of the pH to 8.0. 1 U was defined as the activity which forms 1 μmol product/min. The presence of 0.4 M NaCl derived from the first reaction did not affect the second assay. Commercial pyruvate kinase was passed through an ATP-agarose column twice to remove NDK activity.

2.5. Other methods

The amount of protein was measured as described by Lowry et al.[16]. The amount of protein in the band stained with Coomassie brilliant blue after SDS–PAGE was also measured using NIH Image software with purified HsNDK as the standard. To raise the antibody, HsNDK purified on an ATP affinity column was injected into a white rabbit with complete Freund's adjuvant. Protein, blotted onto Problot membranes (Applied Biosystems), was used for amino-terminal amino acid sequence analysis.

3. Results and discussion

3.1. HsNDK is active and stable without salt

We reported previously that the molecular chaperone DnaK from the extremely halophilic archaeon *H. salinarum*, required the presence of more than 2 M NaCl to bind an ATP-agarose affinity column [9]. Here, we carried out chromatography for the crude homogenate of *H. salinarum* in the presence of 0, 0.2, 2.0 and 4.0 M NaCl in E buffer. As shown in Fig. 1, DnaK protein bound to the ATP column with more than 2 M NaCl, while one distinct protein, with an apparent molecular mass of 24 kDa on 12% SDS–PAGE, bound to ATP resin in the absence of salt. To identify this protein, the amino-terminal amino acid sequence was determined: it was found to be a mixture of MTDHDERTFVMVKPDGVQRGLI and a sequence starting with the second residue, T. This amino-terminal sequence shows high similarity with that of NDK from eukaryote and archaea such as *Archaeoglobus fulgidus* (94% identity), soybean (83%), *Drosophila melanogaster* (72%), and *Xenopus laevis* (70%) (BLASTP in GenomeNet Database). Indeed, this protein, eluted from ATP-agarose gel with 50 mM Tris–HCl buffer, pH 8.0, 2 mM $MgCl_2$ and 3 mM ATP, showed a specific activity for NDK of 250 ~ 300 U/mg protein: the specific activities of the purified NDK (Fig. 1, lane 1) were slightly varied in different NDK preparations most likely depending on the protein concentration of NDK in preparations. We concluded that this protein is HsNDK. To clarify the salt requirement for its stability in storage, the NDK fraction in Fig. 1, lane 1, was dialyzed against E buffer overnight. The dialyzed HsNDK showed the same activity as that before dialysis and it retained almost the same specific activity after one month storage at −20°C, demonstrating that this enzyme is active and stable in the absence of salt. This is an exceptional property among the enzymes which have been isolated from extremely halophilic archaea, since most, if not all, enzymes reported to date require the presence of at least 1 M salt for their stability. We then examined the effects of the NaCl concentration in the reaction mixture on the NDK activity. As shown in Fig. 2, HsNDK showed only slightly higher, 1.3-fold, activity in the presence of 2.0 M NaCl than in the absence of salt, and showed almost the same activity in the presence of 4 M NaCl as in the absence of salt, demonstrating that this halo-

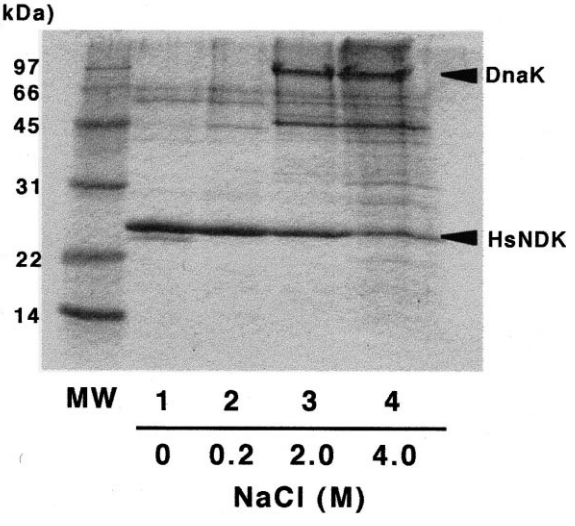

Fig. 1. SDS–PAGE of *H. salinarum* proteins bound to ATP columns in the presence and absence of NaCl. *H. salinarum* cells were disrupted in E buffer containing 0 (lane 1), 0.2 (lane 2), 2.0 (lane 3) or 4.0 (lane 4) M NaCl. Coomassie brilliant blue stained bands appearing at around 66 kDa in all lanes were not protein, but contaminating substances related to the sample buffer of SDS–PAGE. DnaK, DnaK from *H. salinarum*; HsNDK, nucleoside diphosphate kinase from *H. salinarum*. MW, molecular weight standard.

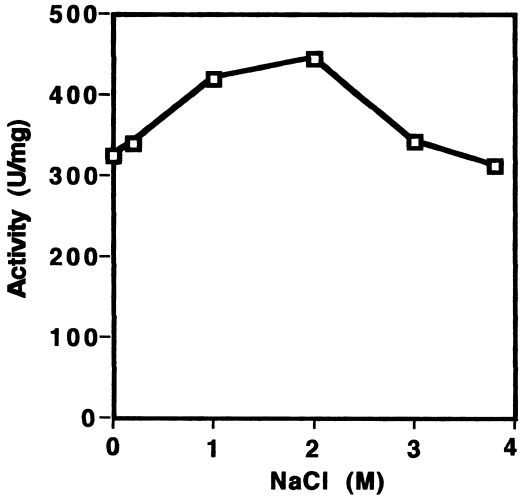

Fig. 2. Effects of NaCl concentration on the activity of HsNDK. HsNDK activity was assayed in the presence of 0, 0.2, 1.0, 2.0, 3.0 and 3.8 M NaCl.

archaeal enzyme can function in the presence of high salt concentrations, but does not require salt for its activity. This observation is also important since it has been demonstrated that a few haloarchaeal enzymes which do not require salt for their activities were strongly inhibited in the presence of high salt concentrations [17,18].

3.2. Cloning of ndk from H. salinarum

We sequenced 41 amino acid residues at the amino-terminus of HsNDK, and attempted to isolate a PCR fragment corresponding to this region. A DNA fragment of ~120 bp was amplified as described in Section 2, and its nucleotide sequence was found to contain the same sequence deduced from the determined amino-terminal amino acid sequence of HsNDK. Using the peroxidase-labeled fragment as a probe, *H. salinarum* chromosomal DNA was analyzed by Southern hybridization. Several restriction fragments showed a single hybridized band, and *Kpn*I-digested chromosomal fragments of around 9 kb, cut out from the agarose gel, were subcloned to *Kpn*I-digested pUC19. The transformant containing the *ndk* gene was screened by colony hybridization using the above PCR fragment as a probe. The nucleotide sequence of the isolated positive clone revealed that this clone contained the *ndk* gene and at least three open reading frames upstream of it (deposited as AB036344). The deduced amino acid sequence of the *ndk* gene shows high similarity with that of the NDK from *A. fulgidus* (58.9% identity), *Bacillus subtilis* (57.7%), *Synechocystis* sp. (56.7%) and *Staphylococcus aureus* (53.3%) (FASTA). HsNDK contains a significantly large number of acidic amino acid residues (23.0 mol%) compared with that from *A. fulgidus* (16.6%), *B. subtilis* (13.4%), *Synechocystis* sp. (13.4%), and *S. aureus* (14.1%). The high content of acidic residues is a typical characteristic of halophilic proteins [1–3]. This might be the reason why 18 kDa HsNDK shows anomalous mobility on SDS–PAGE (Fig. 1) as has been reported in *H. salinarum* DnaK protein [9]. These results clearly indicate that HsNDK is unique in that it is active and stable without salt, though it conserves the general characteristics found in the primary structure of halophilic enzymes.

3.3. Expression of the Hsndk gene in E. coli

We attempted to clarify whether a high concentration of salt is required for post-translational processes in HsNDK. To examine the possibility that high salt might affect the folding and/or assembly of HsNDK, we attempted the heterologous expression of the *Hsndk* gene in non-halophilic bacteria,

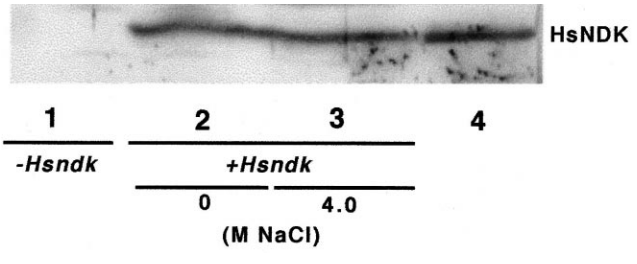

Fig. 3. Western blotting of recombinant HsNDK expressed in *E. coli*. Crude homogenates of *E. coli* with or without the *Hsndk* gene were stained with anti-HsNDK antiserum. Lane 1, *E. coli* harboring pET3a; lanes 2 and 3, *E. coli* containing pETHsNDK. Homogenates were prepared with (lane 3) or without (lane 2) 4 M NaCl; lane 4, HsNDK purified from *H. salinarum*.

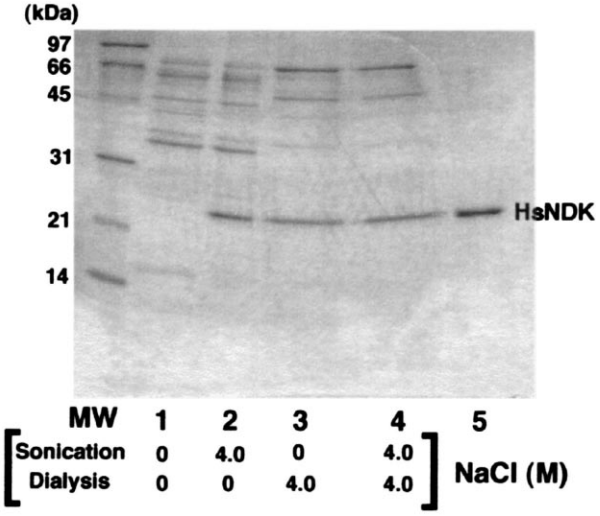

Fig. 4. ATP affinity column chromatography for recombinant HsNDK refolded in vitro. Samples, in lanes 1, 2, 3, and 4 corresponding to those of rows 1, 2, 3 and 4 of Table 1, were applied to an ATP column equilibrated with E buffer containing 0 M (for samples in lanes 1 and 2) and 4 M (for samples in lanes 3 and 4). Bound fractions were subjected to SDS–PAGE. Lane 5, HsNDK purified from *H. salinarum*. MW, molecular weight standard.

E. coli. *E. coli* BL21(DE3) cells harboring pETHsNDK were disrupted in E buffer by sonication and the crude homogenate was subjected to SDS–PAGE. As shown in Fig. 3, lane 2, a band whose mobility was the same as that of HsNDK purified from *H. salinarum* was detected by Western blotting, demonstrating that HsNDK was expressed in *E. coli* transformants. However, this recombinant HsNDK did not bind to the ATP column (data not shown). We then prepared crude homogenates in the presence and absence of 4 M NaCl, and these two crude homogenates, with and without 4 M NaCl, were dialyzed overnight against E buffer containing 0 or 4 M NaCl. All four dialyzed samples thus obtained were applied to an ATP-agarose column to examine the binding of recombinant HsNDK. As shown in Fig. 4, recombinant HsNDK did not bind to ATP resin when it was prepared and dialyzed in the absence of NaCl (lane 1). It was confirmed in separate experiments that an active non-recombinant HsNDK readily binds to the ATP column, but the heat- or acid-denatured inactive form does not (data not shown). In contrast, the recombinant HsNDK prepared and dialyzed in the presence of 4 M NaCl could bind to ATP resin (lane 4). The HsNDK prepared in 0 M NaCl and dialyzed against 4 M NaCl (lane 3), and that prepared in 4 M NaCl and dialyzed against 0 M NaCl (lane 2), also bound to the ATP column with almost the same efficiency as the HsNDK prepared and dialyzed in the presence of 4 M NaCl (lane 4). This result suggests that salt is required for the activation, most likely for folding and/or assembly, of recombinant HsNDK in vitro, but not once is the proper conformation acquired. Amino-terminal sequence analysis of the protein band, shown as HsNDK in Fig. 4, confirmed that it was the recombinant HsNDK. Only this band was immunostained with anti-HsNDK antiserum (not shown). Recombinant malate dehydrogenase from *Haloarcula marismortui* was reported to be expressed in an inactive form in *E. coli*, and activation of the enzyme was obtained by increasing the salt concentration to 3 M NaCl. This activated

Table 1
In vitro refolding of HsNDK expressed in *E. coli*

Row No.	NaCl (M)		Total activity (U/50 ml culture)			Total HsNDK protein (μg/50 ml culture)	Specific activity (U/mg)
	sonication	dialysis	+*Hsndk* (a)	−*Hsndk* (b)	(a)–(b)		
1	0	0	0.91	0.36	0.55	nd	nd
2	4.0	0	5.57	0.69	4.88	20.2	241.8
3	0	4.0	5.06	0.08	4.98	27.5	181.2
4	4.0	4.0	6.72	0.27	6.45	25.8	250.3

E. coli cells with or without *Hsndk* gene were disrupted and dialyzed in the presence or absence of 4 M NaCl. nd, not determined. The amount of HsNDK was determined using NIH Image software as described in Section 2.

enzyme was shown to be indistinguishable from the native enzyme isolated from *H. marismortui*. Thus, it was unstable and inactivated by decreasing the salt concentration to less than 2.5 M NaCl [19].

We further examined the enzymatic activity of the above NDK samples. As shown in Table 1, the NDK sample exposed to high salt (rows 2–4) exhibited a level of NDK activity comparable to that of non-recombinant HsNDK. We attempted to use an *E. coli ndk*-null mutant (provided by M. Inouye) for the expression of HsNDK. However, the expression of HsNDK in this null mutant was low for unknown reasons. Therefore, we used BL21(DE3) cells (ndk^+) as a host, and measured NDK activity in the presence of 4 M NaCl to repress the activity of *E. coli* NDK which might be fractionated in the sample preparation. It was confirmed in separate experiments that *E. coli* NDK was repressed under these assay conditions. In rows 2–4, more than 90% of recombinant HsNDK was recovered in the fraction eluted from the ATP column, whereas, almost all the NDK in row 1 was found in the flow-through fraction of the column as estimated by Western blotting (data not shown). The fact that the enzyme activities in Table 1 and the binding efficiencies for the ATP column (Fig. 4) of HsNDK changed in parallel demonstrates that the recombinant HsNDK refolded properly in the presence of high salt. Since the mobilities on SDS–PAGE of HsNDK prepared from *H. salinarum* and NDK from *E. coli* with or without activity were indistinguishable, the post-translational modification of protein seems less likely. However, the possibility of salt-dependent post-translational modification of HsNDK cannot be fully ruled out.

In general, enzymes from halophilic archaea are unstable in low salt buffer, becoming stable in the presence of at least 1 M salt [2,3]. Unlike typical halophilic enzymes, HsNDK does not require the assistance of salts to maintain its conformation once acquired in their presence, suggesting either that the charge effect is weak or that the hydrophobic core structure is strong, or both. The net negative charge of HsNDK, i.e. the residue number of acidic minus basic amino acids, is in the lower range among the halophilic proteins [2]. Regardless of the mechanism of salt-induced stabilization described above, the weaker charge effect (or few specific salt binding sites) may make a high salt concentration unnecessary for maintaining active conformation. Mevarech et al. [12] suggested that a high content of negative charges confers flexibility and solubility on halophilic proteins in the presence of concentrated salt, since without a high charge content such high salt concentration would render protein molecules either too rigid and compact to function or less soluble. In this sense, the decrease in excess negative charges may cause HsNDK decreased flexibility.

Although high salt is not required for maintaining the folded structure and hence activity of HsNDK, it was essential to activate the protein since the recombinant HsNDK prepared without high salt was inactive. Only when exposed to high salt, did the protein show binding to the ATP column and enzyme activity. It appears most likely that the protein is trapped as an inactive conformation, at least partially denatured and/or not fully assembled, in the absence of high salt and the addition of high salt converts this intermediate structure into the folded and fully assembled conformation.

During this study, the isolation and partial characterization of a NDK from the haloalkaliphilic archaeon *Natronobacterium magadii* was reported [20]. It was purified and determined to form a hexamer. About 70% of reported amino-terminal sequences of *N. magadii* NDK were identical to that of HsNDK. Regarding stability to protease treatment, *N. magadii* NDK denatures at a low salt concentration. On this point, HsNDK is distinct from *N. magadii* NDK.

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References

[1] Kushner, D.J. (1978) in: Microbial Life in Extreme Environments (Kushuner, D.J., Ed.), pp. 317–368, Academic Press, London.
[2] Lanyi, J.K. (1974) Bacteriol. Rev. 38, 272–290.
[3] Kushner, D.J. (1985) in: The Bacteria, Vol. VIII, pp. 171–214, Academic Press, London.
[4] Danson, M.J. and Hough, D.W. (1997) Comp. Biochem. Physiol. 117A, 307–312.
[5] Elcock, A.H. and McCammon, J.A. (1998) J. Mol. Biol. 280, 731–748.
[6] Mevarech, M., Frolow, F. and Gloss, L.M. (2000) Biophys. Chem. 86, 155–164.
[7] Tokunaga, M., Kawamura, A. and Kohno, K. (1992) J. Biol. Chem. 267, 17553–17559.
[8] Tokunaga, M., Miyawaki, H., Shiraishi, Y. and Tokunaga, H. (1997) FEMS Microbiol. Lett. 152, 321–326.
[9] Tokunaga, H., Hara, S., Arakawa, T., Ishibashi, M., Gupta, R.S. and Tokunaga, M. (1999) J. Protein Chem. 18, 837–844.
[10] Onishi, H., McCance, M.E. and Gibbons, N.E. (1965) Can. J. Microbiol. 11, 365–373.
[11] Sehgal, S.N. and Gibbons, N.E. (1960) Can. J. Microbiol. 6, 165–169.
[12] Mevarech, M., Hirsch-Twizer, S., Goldman, S., Yakobson, E., Eisenberg, H. and Dennis, P.P. (1989) J. Bacteriol. 171, 3479–3485.
[13] Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang,

Z., Miller, W. and Lipman, D.J. (1997) Nucleic Acids Res. 25, 3389–3402.
[14] Pearson, W.R. (1988) Proc. Natl. Acad. Sci. USA 85, 2444–2448.
[15] Agarwal, R.P. and Parks Jr., R.E. (1971) J. Biol. Chem. 246, 2258–2264.
[16] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[17] Louis, B.G. and Fitt, P.S. (1972) Biochem. J. 127, 69–80.
[18] Pugh, E.L., Wassef, M.K. and Kates, M. (1971) Can. J. Biochem. 49, 953–958.
[19] Cendrin, F., Chroboczek, J., Zaccai, G., Eisenberg, H. and Mevarech, M. (1993) Biochemistry 32, 4308–4313.
[20] Polosina, Y.Y., Jarrell, K.F., Fedorov, O.V. and Kostyukova, A.S. (1998) Extremophiles 2, 333–338.