PROTEINS
STRUCTURE ■ FUNCTION ■ BIOINFORMATICS

STRUCTURE NOTE

Solution structure and calcium binding of protein SSO6904 from the hyperthermophilic archaeon *Sulfolobus solfataricus*

Yingang Feng,* Hongwei Yao, and Jinfeng Wang*

National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China

Key words: solution structure; SSO6904; calcium-binding proteins; NMR spectroscopy; *Sulfolobus*.

INTRODUCTION

Calcium signaling in bacteria and archaea has been believed to be as important as it in eukaryotes for many years, but calcium-binding proteins identified in prokaryotes are much less than those in eukaryotes.[1–3] A calcium-gated potassium channel discovered in the archaeon *Methanobacterium thermoautotrophicum* indicated the importance of calcium in archaea.[4] So far, several calcium-binding proteins, such as pullulanase,[5] MTH1880,[6] Ulilysin,[7] Tk-subtilisin,[8] and M-Crystallin,[9] have been identified from various archaea. Of them only a few structures were solved by X-ray crystallography or NMR spectroscopy. The physiological significance of most of these calcium-binding proteins is still unclear to date.

Sulfolobus solfataricus is a hyperthermophilic archaeon used as a model system for researches on genetics, evolution, and hyperthermophilic mechanism.[10–12] *S. solfataricus* genome encodes about 3000 proteins, in which about 25% proteins are exclusive to *Sulfolobus*.[11] Calcium-binding proteins in *S. solfataricus* were rarely investigated up to now. SSO6904 (UniProt ID: Q97ZE1) is a predicted protein conserved in *Sulfolobales* [Fig. 1(A)] and has no sequence homology to any of other protein families. We determined the solution structure of SSO6904 from *S. solfataricus* P2 and found that it is a helical protein. The tertiary fold of SSO6904 is similar to that of saposin-fold proteins and some calcium-binding proteins. Although SSO6904 has no EF-hand sequence, which is the most common calcium-binding sequence, NMR titration experiments showed that SSO6904 can bind with calcium weakly at its unique binding site.

MATERIALS AND METHODS

Cloning, expression, and purification of SSO6904

The gene of SSO6904 (NCBI RefSeq: NP_342461) was amplified from the genomic library of *S. solfataricus* P2 by polymerase chain reaction (PCR) using a sense primer, 5′-GGGAATTC*CATATG*ATGGCTATGTCAATATTAGAAG-3′ and an antisense primer, 5′-GCCG*CTCGAG*TTTTATAGTAGTTAGT-3′, with *Nde* I and *Xho* I restriction sites, respectively. The PCR product was cloned into a pET30a vector. Expression of this SSO6904 construct was attempted in *Escherichia coli*. However, no expression of the recombinant SSO6904 was obtained. We noticed that this SSO6904 construct has Met-Met-Ala as the first three N-terminal amino acid residues, which do not exist in the homologues. More-

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: National Natural Science Foundation of China; Grant numbers: NNSFC 30270301, NNSFC 30770434.

*Correspondence to: Jinfeng Wang, Institute of Biophysics, Chinese Academy of Sciences, Datun Road 15, Beijing 100101, China. E-mail: jfw@sun5.ibp.ac.cn or Yingang Feng, Institute of Biophysics, Chinese Academy of Sciences, Datun Road 15, Beijing 100101, China. E-mail: fyg@moon.ibp.ac.cn

Received 29 May 2009; Revised 30 July 2009; Accepted 31 July 2009
Published online 17 August 2009 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/prot.22580

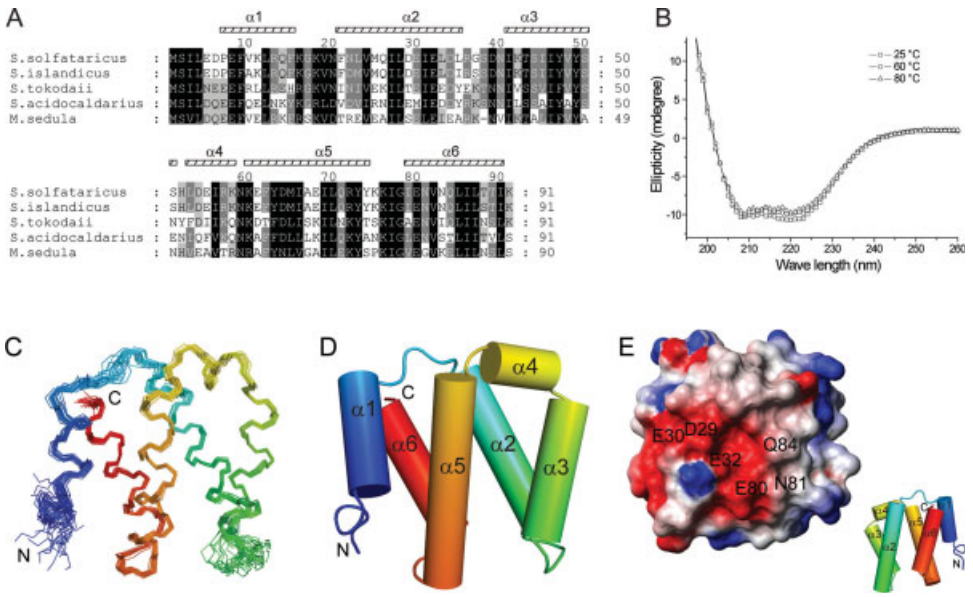

Figure 1

Structure of SSO6904. (**A**) Sequences of SSO6904 and homologues. Secondary structures of SSO6904 are indicated on the top of the sequences. (**B**) Far-UV circular dichroism spectra of SSO6904 at the temperatures of 25 (square), 60 (circle), and 80 (triangle)°C. (**C**) Superposition of the 20 conformers of SSO6904. The structures were colored as a rainbow from the N-terminus to the C-terminus. (**D**) Cartoon representation of SSO6904 structure. Helices are shown as cylinders. The molecular orientation and color scheme are same as Figure C. (**E**) The electrostatic surface of SSO6904. Red, negatively charged; blue, positively charged; white, neutral. A cartoon representation with same color scheme of Figure D was also shown to indicate the molecular orientation. For clarity, the C-terminal His-tag was not shown in Figures C, D, and E.

over, in the *Sulfolobus* database (http://www.sulfolobus.org/) which includes an updated version of *S. solfataricus* P2 genome sequence,[13] there are no such three residues (MMA) at the N-terminus of the amino acid sequence of SSO6904. Therefore, the region coding 4–94 amino acid residues of NP_342461 was amplified by PCR using a new pair of primers: forward 5′-CTG*CATATG*TCAATATAGAAGATCCAG-3′ and reverse 5′-GCCG*CTCGAG*TTTTATAGTAGTTAGTATC-3′. The amplified PCR product was subcloned into a pET30a vector, forming a vector pET30a-SSO6904. The protein product of this construct contains an additional C-terminal His-tag (LEHHHHHH) to the original gene product. The recombinant protein was successfully overexpressed and purified using this construct. In this article, the sequence of SSO6904 is numbered according to the *Sulfolobus* database.

The vector pET30a-SSO6904 was transformed into *E. coli* Rosetta(DE3)pLysS. The recombinant SSO6904 was expressed in M9 minimal medium at 37°C. When the cell density reached an OD_{600} of about 0.8, the protein expression was induced with 1.0 m*M* isopropyl-1-thio-β-D-galactopyranoside for 4 h at 37°C. The cells were harvested by centrifuging at 4000 rpm, 4°C for 30 min. The harvested cell pellet was resuspended in 50 m*M* Tris-HCl, pH 8.0, 500 m*M* NaCl, and then frozen at −20°C overnight. The resuspended cell pellet was thawed and lysed by sonication. Thereafter, the target protein was purified via a Chelating Sepharose Fast Flow (GE Healthcare) column and eluted with 500 m*M* imidazole, and the eluate containing the recombinant SSO6904 was collected and concentrated to 2 mL using Amicon Ultra-15 centrifugal filter units (5 kDa NMWL) (Millipore). Then, the protein was further purified using a Superdex 75 (GE Healthcare) gel filtration column pre-equilibrated in 50 m*M* potassium phosphate buffer and 100 m*M* KCl at pH 7.2. The fractions containing the recombinant SSO6904 were collected and concentrated. Protein concentration was determined by the UV absorption at 280 nm using a theoretical molar extinction coefficient 6400 M^{-1} cm^{-1}.

Circular dichroism spectroscopy

The sample for circular dichroism (CD) measurements contained 0.3 mg/mL recombinant SSO6904 in 50 m*M* potassium phosphate buffer at pH 7.2. The Far-UV CD spectra were recorded on a PiStar spectropolarimeter (Applied Photophysics).

NMR spectroscopy

Uniformly ^{15}N- and $^{15}N/^{13}C$-labeled proteins were obtained by growth in M9 minimal media containing

$^{15}NH_4Cl$ and [^{13}C]-glucose as the sole nitrogen and carbon sources, respectively. NMR samples consisted of 1–1.5 m*M* recombinant SSO6904 proteins in 90% H_2O/10% D_2O containing 50 m*M* potassium phosphate buffer (pH 7.2), 100 m*M* KCl, 0.02%(w/v) NaN_3, 0.02%(w/v) sodium 2,2-dimethylsilapentane-5-sulfonate (DSS), 50 m*M* arginine, and 50 m*M* glutamic acid for improving the solubility and long-term stability.[14] In this study, the NMR buffer (pH 7.2) was used to prepare the sample because the theoretical pI of SSO6904 is about 6.4 and the intracellular pH of *S. solfataricus* is maintained at about 6.5, although the optimal growth of *Sulfolobus* species occurs at pH 2–3.[15]

All NMR experiments were performed at 310 K on a Bruker DMX 600 spectrometer equipped with a z-gradient triple-resonance cryoprobe. Backbone and side-chain resonance assignments were derived from 2D ^{1}H-^{15}N HSQC and ^{1}H-^{13}C HSQC, 3D HNCA, HNCACB, CBCA(CO)NH, HNCO, HN(CA)CO, HBHA(CBCA)(CO)NH, HBHA(CBCA)NH, and (H)CCH-TOCSY. NOE distance constraints were derived from 3D ^{1}H-^{15}N NOESY-HSQC spectrum and two 3D ^{1}H-^{13}C NOESY-HSQC spectra for aliphatic and aromatic regions. The mixing times for ^{13}C-^{13}C TOCSY and ^{1}H-^{1}H NOESY experiments were 12 and 120 ms, respectively. All NMR spectra were processed and analyzed using the software Felix (Accelrys). Proton chemical shifts were referenced to the internal DSS, and ^{15}N and ^{13}C chemical shifts were referenced indirectly.[16]

Structure calculation

Initial structures of SSO6904 were generated using CANDID module of CYANA.[17] Backbone dihedral angle restraints incorporated into the structural calculation were obtained using TALOS.[18] Hydrogen-bond restraints were applied according to the regular secondary structure patterns. The NOE assignments generated by CANDID were checked manually, and the structures were refined in explicit water[19] using CNS[20] and RECOORDScript.[21] A family of 100 structures was generated by CNS, and the 50 structures with the lowest energies were subjected to the refinement in explicit water, from which a final set of 20 structures with the lowest energies was selected for the final analysis. The quality of the determined structures was analyzed using MOLMOL[22] and PROCHECK-NMR.[23] MOLMOL and PyMol (DeLano Scientific LLC) were used for the visualization of the structures. Structural similarity was searched using Dali server[24] and SSM server.[25]

The chemical shifts of SSO6904 were deposited in the BioMagResBank database under an accession number 16320. The atomic coordinates for SSO6904 and all restraints were deposited in the Protein Data Bank database with the accession code 2KJG.

Calcium titration

A Ca^{2+} titration of ^{15}N-labeled SSO6904 was performed using 2D ^{1}H-^{15}N HSQC spectra. The samples containing 0.4 m*M* ^{15}N-labeled SSO6904 used for the titration were dialyzed extensively for removing the residual calcium from the sample solution. The dialysis was made first against the sample buffer (20 m*M* Tris-HCl, 100 m*M* KCl, pH 7.2) containing 5 m*M* EDTA and 5 m*M* EGTA, and subsequently against the sample buffer without EDTA/EGTA. For each titration point, a small volume of the stock $CaCl_2$ solution was added into the sample to an appropriate ratio. The dissociation constant K_D of Ca^{2+} with SSO6904 was estimated by fitting the chemical shift changes during the titration as previously described.[26]

RESULTS AND DISCUSSION

SSO6904 is an all-helix protein with high stability

The CD spectra of SSO6904 at 25, 60, and 80°C are similar [Fig. 1(B)], indicating that SSO6904 is a thermostable protein with abundant helical secondary structures. The high thermal stability of SSO6904 is expected because the protein is from the hyperthermophilic archaeon *S. solfataricus*.

The structure of SSO6904 was determined using heteronuclear NMR spectroscopy. A superposition of the final 20 lowest energy structures of SSO6904 is shown in Figure 1(C) and a cartoon representation of the lowest energy structure is depicted in Figure 1(D). The experimental restraints for structure calculations and structural statistics for the SSO6904 ensemble are summarized in Table I.

SSO6904 shows an all-helix structure comprising six α-helices (α1:7-15, α2:21-35, α3:41-51, α4:53-58, α5:60-74, and α6:79-90) [Fig. 1(D)]. Helices α2 and α3 run antiparallel to helices α6 and α5 at angles of ∼172° and ∼158° between the α-helix axes, respectively, whereas helices α2 and α6 pack against helices α3 and α5 at angles of ∼140° and ∼147° between the α-helix axes, respectively. The relatively short helix α4 packs almost perpendicularly to helices α3 and α5 (102° and 95° between the α-helix axes, respectively) and the N-terminal helix α1 packs on the side of helices α5 and α6 facing opposite to helices α2 and α3. The side chains of hydrophobic residues I3, F9, L12, V19, F21, V24, M25, I27, L28, I31, L35, I41, I45, V48, Y49, L53, I56, F62, Y63, M65, I66, A67, I69, L70, Y73, Y74, I77, I79, V82, L85, I86, L87, T89, and I90 of the six α-helices build a tightly packed hydrophobic core, and most of these residues are conserved in the homologues of SSO6904. The large number of hydrophobic residues (more than 1/3, i.e., 34 of total 91 residues) may account for the high thermal stability of SSO6904, because hydrophobic interactions

Table I
Experimental Constraints and Structural Statistics for the 20 Lowest Energy Structures of SSO6904

Parameter	Value
Number of distance restraints	
Total NOEs	3208
Intraresidual NOEs	1193
Sequential NOEs	652
Medium-range NOEs	559
Long-range NOEs	420
Ambiguous NOEs	384
Hydrogen bonds	53
Number of dihedral angle restraints	
ϕ angle	71
ψ angle	71
PROCHECK_NMR Ramachandran map analysis	
Most favored regions	86.6%
Additional allowed regions	10.4%
Generously allowed regions	1.5%
Disallowed regions	1.5%
RMSD from mean (secondary structure residues[a]) (Å)	
Backbone heavy atoms	0.32 ± 0.07
All heavy atoms	0.76 ± 0.05
RMSD from experimental restraints	
Distance restraints (Å)	0.0062 ± 0.0009
Dihedral angle restraints (°)	0.28 ± 0.05
RMSD from ideal geometry	
Bond lengths (Å)	0.0087 ± 0.0003
Bond angles (°)	1.12 ± 0.03
Impropers (°)	1.28 ± 0.07
CNS energies	
Lennard–Jones (kcal/mol)	−492 ± 12
Electrostatic (kcal/mol)	−4405 ± 59

[a]Secondary structure residues: 7–15,21–35,41–51,53–58,60–74,79–90.

are one of the determinants of hyperthermostability.[27,28] A number of salt bridges (E5-K76, D6-R72, E8-K11, E32-R36, D54-R57, E55-K58, K60-E61, and E68-R72) were identified on the protein surface using distance cutoff 6.0 Å between side-chain oxygen atoms and nitrogen atoms of negatively and positively charged residues, respectively. These salt bridges may also contribute to the high thermal stability of the protein. However, most of them are not conserved between SSO6904 and the other proteins in the same family.

The electrostatic surface of SSO6904 is shown in Figure 1(E). Interestingly, the negatively charged residues D29, E30, E32, and E80 are clustered on the protein surface. As is known, the clustered negatively or positively charged residues can be a structural region for interactions with charged molecules such as cations, anions, and nucleic acids. Therefore, the negatively charged region on SSO6904 surface may be a potential binding site for cations or positively charged proteins (see the discussion later).

Calcium binding of SSO6904

Because SSO6904 has no sequence homology with other known protein families, the structure homology of SSO6904 was then searched using Dali[24] and SSM.[25] The highest scored structure homologues are eukaryotic saposin-fold proteins [human saposin C, Dali Z-Score

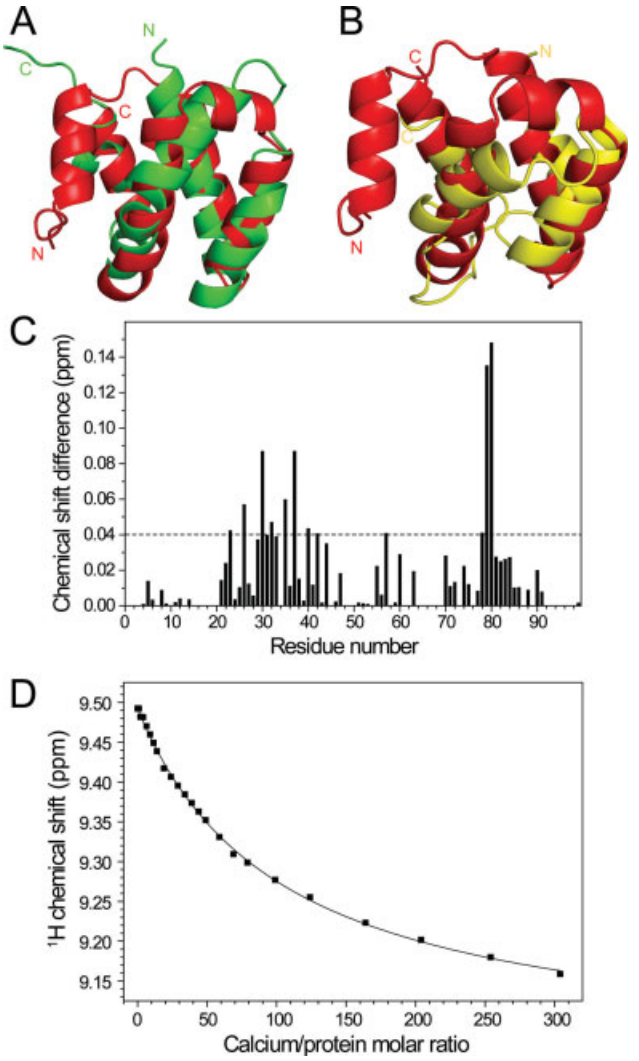

Figure 2
Calcium binding of SSO6904. (**A**) Structural alignment between SSO6904 (red) and human saposin C (PDB 1M12) (green). (**B**) Structural alignment between SSO6904 (red) and human calmodulin-like skin protein C-terminal domain (PDB 2B1U) (yellow). (**C**) The chemical shift differences between calcium-free SSO6904 and SSO6904 in the presence of 9.8 m*M* $CaCl_2$. (**D**) Curve fitting of E80 ^1H_N chemical shifts using the following equation:

$$\varpi_{obs} = \varpi_0 + \Delta_{max} \times \left(\frac{([P]_0 + [P]_0 \cdot x + K_D) - \sqrt{([P]_0 + [P]_0 \cdot x + K_D)^2 - 4[P]_0 \cdot ([P]_0 \cdot x)}}{2 \cdot [P]_0} \right),$$

where ω_{obs} is the observed E80 ^1H_N chemical shift at the particular $CaCl_2$ concentration, ω_0 is the E80 ^1H_N chemical shift in the absence of $CaCl_2$, Δ_{max} is the E80 ^1H_N chemical shift difference between calcium-free and calcium-saturated SSO6904, $[P]_0$ is the initial protein concentration of SSO6904, x is the molar ratio of $CaCl_2$ to SSO6904 and K_D is the dissociation constant.

5.8, RMSD 3.5 Å, Figure 2(A)], which are membrane-interacting glycoproteins required for the hydrolysis of certain sphingolipids by specific lysosomal hydrolases.[29,30] The six cysteine residues forming three disulfide bonds of saposin fold are not found in SSO6904, suggesting that SSO6904 may not be a typical saposin-like protein. Interestingly, SSO6904 has also structure homology with a number of calcium-binding proteins, such as human calmodulin-like skin protein C-terminal domain [PDB 2B1U, Dali Z-Score 3.0, RMSD 2.9 Å, Fig. 2(B)], calcyclin (PDB 1A03, Dali Z-Score 2.4, RMSD 3.6 Å), calcium vector protein (PDB 1J7Q, Dali Z-Score 2.2, RMSD 3.0 Å). All these calcium-binding proteins have EF-hand motifs, which bind to calcium ions. SSO6904 has no EF-hand motif.[31] However, the negatively charged surface region of SSO6904 found in the previous section may be a site interacting with a cation such as Ca^{2+}.

To detect whether SSO6904 can bind with calcium, calcium titrations were performed and the chemical shifts of cross peaks in the 2D ^{1}H-^{15}N HSQC spectra of SSO6904 were used as probes to detect the calcium-binding site. A number of cross peaks showed significant shifts during the titration, in which the cross peak of E80 shifted most significantly [Fig. 2(C) and Supporting Information Fig. S1]. The dissociation constant K_D estimated by the fitting of the chemical shift changes is 0.0205 $\pm$ 0.0005 M [Fig. 2(D)], indicating a very weak calcium binding, which is similar to the calcium binding of some known calcium-binding proteins.[26,32] The calcium-binding site was identified by mapping the chemical shift changes on the structure of SSO6904. As is shown in Figure 1(E) and Supporting Information Figure S2, of the residues showing chemical shift changes D29, E30, E32, E80, N81, and Q84 clustered together on the surface of SSO6904 forming a negatively charged pocket. The oxygen atoms of the carboxyl groups of D29, E30, E32, and E80 and the side-chain carbonyl oxygen atoms of N81 and Q84 may form a Ca^{2+} coordination, which may be a potential calcium-binding site of the protein.

The results of this study indicated that SSO6904 has the similar fold but the different calcium-binding site compared with the eukaryotic calcium-binding proteins, although there is no sequence homology between SSO6904 and the eukaryotic calcium-binding proteins. Because the calcium-binding affinity of SSO6904 is very weak, the physiological significance of the calcium binding needs further investigation *in vivo*. In addition, SSO6904 also shows very weak affinity for some other cations such as Mg^{2+} and Zn^{2+} *in vitro* (data not shown). In the genome, the gene of SSO6904 is adjacent with some genes of metabolism-related enzymes (SSO0976: uridylate kinase; SSO0975: 3-oxoacyl-[acyl-carrier-protein] reductase; SSO0974: 3-hydroxyisobutyrate dehydrogenase; SSO0972: pseudouridine synthase; SSO0977: 2-isopropylmalate synthase 1; SSO0978: ribose 5-phosphate isomerase; SSO0981: pyruvate kinase), a leucine tRNA gene (SSOt41), and a gene of a chromatin protein Cren7 (SSO6901) discovered recently.[33] Whether the calcium binding of SSO6904 has relationship with these genes is interesting and should be explored in future.

ACKNOWLEDGMENTS

The authors thank Prof. Li Huang and Miss Li Guo, Institute of Microbiology, Chinese Academy of Sciences, for kindly providing the genomic DNA of *Sulfolobus solfataricus* P2.

REFERENCES

1. Michiels J, Xi C, Verhaert J, Vanderleyden J. The functions of Ca^{2+} in bacteria: a role for EF-hand proteins? Trends Microbiol 2002;10: 87–93.
2. Dominguez DC. Calcium signalling in bacteria. Mol Microbiol 2004;54:291–297.
3. Permyakov EA, Kretsinger RH. Cell signaling, beyond cytosolic calcium in eukaryotes. J Inorg Biochem 2009;103:77–86.
4. Jiang YX, Lee A, Chen JY, Cadene M, Chait BT, MacKinnon R. Crystal structure and mechanism of a calcium-gated potassium channel. Nature 2002;417:515–522.
5. Schwerdtfeger RM, Chiaraluce R, Consalvi V, Scandurra R, Antranikian G. Stability, refolding and Ca^{2+} binding of pullulanase from the hyperthermophilic archaeon *Pyrococcus woesei*. Eur J Biochem 1999;264:479–487.
6. Lee CH, Jung JW, Yee A, Arrowsmith CH, Lee W. Solution structure of a novel calcium binding protein. MTH1880, from Methanobacterium thermoautotrophicum Protein Sci 2004;13:1148–1154.
7. Tallant C, Garcia-Castellanos R, Seco J, Baumann U, Gomis-Ruth FX. Molecular analysis of ulilysin, the structural prototype of a new family of metzincin metalloproteases. J Biol Chem 2006;281:17920–17928.
8. Tanaka SI, Saito K, Chon H, Matsumura H, Koga Y, Takano K, Kanaya S. Crystal structure of unautoprocessed precursor of subtilisin from a hyperthermophilic Archaeon—evidence for Ca^{2+}-induced folding. J Biol Chem 2007;282:8246–8255.
9. Barnwal RP, Jobby MK, Devi KM, Sharma Y, Chary KVR. Solution structure and calcium-binding properties of M-crystallin. A primordial beta(Y)-crystallin from archaea. J Mol Biol 2009;386:675–689.
10. Ciaramella M, Pisani FM, Rossi M. Molecular biology of extremophiles: recent progress on the hyperthermophilic archaeon *Sulfolobus*. Antonie Van Leeuwenhoek 2002;81:85–97.
11. She Q, Singh RK, Confalonieri F, Zivanovic Y, Allard G, Awayez MJ, Chan-Weiher CCY, Clausen IG, Curtis BA, De Moors A, Erauso G, Fletcher C, Gordon PMK, Heikamp-de Jong I, Jeffries AC, Kozera CJ, Medina N, Peng X, Thi-Ngoc HP, Redder P, Schenk ME, Theriault C, Tolstrup N, Charlebois RL, Doolittle WF, Duguet M, Gaasterland T, Garrett RA, Ragan MA, Sensen CW, Van der Oost J. The complete genome of the crenarchaeon *Sulfolobus solfataricus* P2. Proc Natl Acad Sci USA 2001;98:7835–7840.
12. Bernander R. The cell cycle of *Sulfolobus*. Mol Microbiol 2007;66: 557–562.
13. Brugger K. The *Sulfolobus* database. Nucleic Acids Res 2007;35: D413–D415.
14. Golovanov AP, Hautbergue GM, Wilson SA, Lian LY. A simple method for improving protein solubility and long-term stability. J Am Chem Soc 2004;126:8933–8939.
15. Baker-Austin C, Dopson M. Life in acid: pH homeostasis in acidophiles. Trends Microbiol 2007;15:165–171.

16. Markley JL, Bax A, Arata Y, Hilbers CW, Kaptein R, Sykes BD, Wright PE, Wuthrich K. Recommendations for the presentation of NMR structures of proteins and nucleic acids—(IUPAC Recommendations 1998). Pure Appl Chem 1998;70:117–142.
17. Herrmann T, Guntert P, Wuthrich K. Protein NMR structure determination with automated NOE assignment using the new software CANDID and the torsion angle dynamics algorithm DYANA. J Mol Biol 2002;319:209–227.
18. Cornilescu G, Delaglio F, Bax A. Protein backbone angle restraints from searching a database for chemical shift and sequence homology. J Biomol NMR 1999;13:289–302.
19. Linge JP, Williams MA, Spronk CA, Bonvin AM, Nilges M. Refinement of protein structures in explicit solvent. Proteins 2003;50:496–506.
20. Brunger AT, Adams PD, Clore GM, DeLano WL, Gros P, Grosse-Kunstleve RW, Jiang JS, Kuszewski J, Nilges M, Pannu NS, Read RJ, Rice LM, Simonson T, Warren GL. Crystallography & NMR system: a new software suite for macromolecular structure determination. Acta Crystallogr D Biol Crystallogr 1998;54:905–921.
21. Nederveen AJ, Doreleijers JF, Vranken W, Miller Z, Spronk CAEM, Nabuurs SB, Guntert P, Livny M, Markley JL, Nilges M, Ulrich EL, Kaptein R, Bonvin AMJJ. RECOORD: a recalculated coordinate database of 500+proteins from the PDB using restraints from the BioMagResBank. Proteins 2005;59:662–672.
22. Koradi R, Billeter M, Wuthrich K. MOLMOL: a program for display and analysis of macromolecular structures. J Mol Graph 1996; 14:51–55.
23. Laskowski RA, Rullmann JAC, MacArthur MW, Kaptein R, Thornton JM. AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. J Biomol NMR 1996; 8:477–486.
24. Holm L, Sander C. Protein structure comparison by alignment of distance matrices. J Mol Biol 1993;233:123–138.
25. Krissinel E, Henrick K. Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. Acta Crystallogr D Biol Crystallogr 2004;60:2256–2268.
26. Feng YG, Liu DS, Yao HW, Wang JF. Solution structure and mapping of a very weak calcium-binding site of human translationally controlled tumor protein by NMR. Arch Biochem Biophys 2007; 467:48–57.
27. Haney PJ, Stees M, Konisky J. Analysis of thermal stabilizing interactions in mesophilic and thermophilic adenylate kinases from the genus *Methanococcus*. J Biol Chem 1999;274:28453–28458.
28. Fang XY, Cui Q, Tong YF, Feng YG, Shan L, Huang L, Wang JF. A Stabilizing alpha/beta-hydrophobic core greatly contributes to hyperthermostability of archaeal [P62A]Ssh10b. Biochemistry 2008; 47:11212–11221.
29. Kishimoto Y, Hiraiwa M, Obrien JS. Saposins: structure, function, distribution, and molecular genetics. J Lipid Res 1992;33:1255–1267.
30. Bruhn H. A short guided tour through functional and structural features of saposin-like proteins. Biochem J 2005;389:249–257.
31. Zhou Y, Yang W, Kirberger M, Lee HW, Ayalasomayajula G, Yang JJ. Prediction of EF-hand calcium-binding proteins and analysis of bacterial EF-hand proteins. Proteins 2006;65:643–655.
32. Kidd RD, Yennawar HP, Sears P, Wong CH, Farber GK. A weak calcium binding site in subtilisin BPN′ has a dramatic effect on protein stability. J Am Chem Soc 1996;118:1645–1650.
33. Guo L, Feng YG, Zhang ZF, Yao HW, Luo YM, Wang JF, Huang L. Biochemical and structural characterization of Cren7, a novel chromatin protein conserved among Crenarchaea. Nucleic Acids Res 2008;36:1129–1137.