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Ellman's reagent in promoting crystallization and structure determination of *Anabaena* CcbP

Obtaining crystals presented a bottleneck in the structural study of *Anabaena* cyanobacterial Ca^{2+} -binding protein (CcbP). In this report, the promoting effect of Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid); DTNB] on the crystallization of CcbP is described. CcbP contains one free cysteine. A quick and simple oxidation reaction with DTNB blocked the free cysteine in purified CcbP and generated a homogenous monomeric protein for crystallization. The crystal structure of DTNB-modified CcbP was determined by the single-wavelength anomalous diffraction method. Structure analysis indicated that DTNB modification facilitated crystallization of CcbP by inducing polar interactions in the crystal lattice. DTNB-mediated cysteine modification was demonstrated to have little effect on the overall structure and the Ca^{2+} binding of CcbP. Thus, DTNB modification may provide a simple and general approach for protein modification to improve the success of crystallization screening.

1. Introduction

In protein crystallography, a crystallization hit is required for structure determination and paves the way for further structural investigations. However, finding a hit for a particular protein is often a time-consuming and tedious trial-and-error process. Protein crystallization can be hampered by the sensitivity of proteins to the oxidation of cysteines and to aggregation caused by disulfide-bond formation by cysteines. Mutagenesis of cysteine residues (often to serine or alanine residues) has been demonstrated to be useful for the successful crystallization of some proteins (Lenz & Rétey, 1993; Baedeker & Schulz, 2002; Kessler *et al.*, 2004). Different chemical approaches can specifically and rapidly modify cysteines under physiological conditions (Han *et al.*, 1987). Some of these approaches have been successfully applied in crystallization, such as the carboxymethylation of cysteine (Brzozowski *et al.*, 1997).

Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid); DTNB; Ellman, 1959] reacts with cysteines and has often been used in the quantitative determination of sulfhydryl groups contained in peptides (Bulaj *et al.*, 1998). However, it has not commonly been used in protein crystallization. To date, only three cases have been reported. DTNB modification of ArsC (Messens *et al.*, 2004) and of human CLIC2 (Mi *et al.*, 2008) resulted in oxidized forms of the proteins and improved the crystal quality. The human p40 structure was determined as a monomer after the intermolecular disulfide bond had been blocked by DTNB treatment (Yoon *et al.*, 2000).

CcbP is a Ca^{2+} -binding protein (molecular mass 14.7 kDa, isoelectric point of 4.3) from the cyanobacterium *Anabaena* sp. strain PCC7120 without the common Ca^{2+} -binding EF-hand motif (Zhao *et al.*, 2005; Shi *et al.*, 2006). Nuclear magnetic resonance (NMR) structures of CcbP (with and without Ca^{2+} ; PDB entries 2k2v and 2p0p) revealed a novel fold with two Ca^{2+} -binding sites in the protein (Hu *et al.*, 2011). To further understand the Ca^{2+} -binding mechanism of CcbP, we attempted to determine its X-ray crystal structure. DTNB modification of the only cysteine (Cys74) in CcbP facilitated protein crystallization and resulted in crystal structure determination.

2. Materials and methods

2.1. Cloning, expression and purification

Three variants of CcbP were prepared. *Anabaena* CcbP (GenBank ID BAB72967.1) was cloned into a pET15b vector (Novagen, USA) and expressed as an N-terminal His₆-fusion protein in *Escherichia coli* BL21 (DE3) as described previously (Zhao *et al.*, 2005); the recombinant plasmid was from the laboratory of Professor Jindong Zhao at Peking University. Protein expression was induced by 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) when the culture reached an OD₆₀₀ of 0.8 and expression was continued for 3 h at 310 K. The cells were harvested by centrifugation (2000g, 8 min, 277 K) and the cell pellet was resuspended in buffer A (20 mM Tris-HCl pH 7.5, 500 mM NaCl). After sonication, the cell lysate was centrifuged at 58 000g for 45 min. The supernatant was loaded onto a nickel-charged affinity column (5 ml column volume His-Trap HP column, GE Healthcare, USA) which was pre-equilibrated in buffer A. The proteins were extensively washed in buffer A and eluted using buffer A supplemented with 300 mM imidazole. The fusion tag was cleaved by overnight digestion with thrombin (GE Healthcare, USA) (0.5 U thrombin per milligram of target protein) in buffer A at 277 K, leaving an additional three residues (Gly-Ser-His) on the protein. Gel-filtration chromatography (HiLoad Superdex 75, GE Healthcare, USA) was used for further purification in buffer A. Nonreduced and reduced SDS-PAGE assays were applied to check the homogeneity of the purified protein.

To prepare a variant of CcbP without the cysteine, CcbP-C74A was constructed. The coding sequence was generated by overlapping polymerase chain reaction (PCR) using mutagenic oligonucleotides and was inserted into a pET28a vector (Novagen, USA) at the *Bam*HI and *Xho*I restriction sites. The recombinant vector was transformed into *E. coli* BL21 (DE3). CcbP-C74A protein was expressed and purified using the same protocol as used for wild-type CcbP.

For the production of selenomethionine-labelled (SeMet) CcbP, expression was carried out in minimal medium (M9) with incorporation of selenomethionine as described by Van Duyne *et al.* (1993). Expression was induced by 0.5 mM IPTG. Cultivation was continued at 291 K for 20 h.

2.2. Preparation of wild-type and SeMet DTNB-modified CcbP

Wild-type DTNB-modified CcbP was prepared using a four-step procedure: purification by nickel-affinity chromatography, thrombin digestion, DTNB modification and purification by size-exclusion chromatography. DTNB modification was performed as follows. The reaction buffer for DTNB modification was buffer A and the protein concentration was 2 mM. Dithiothreitol (DTT) was added to the protein solution to a final concentration of 0.5 mM. The mixture was incubated for about 10 min on ice. DTNB powder (Sigma-Aldrich, USA) was then added to a final concentration of 3 mM while stirring, followed by a further 10–20 min incubation on ice. The modified protein was further purified on a HiLoad Superdex 75 column in buffer A.

SeMet DTNB-modified CcbP was produced using the same procedure as used for the wild-type DTNB-modified CcbP protein.

2.3. Crystallization and data collection

Wild-type CcbP, CcbP supplemented with 10 mM DTT, DTNB-modified CcbP and CcbP-C74A were concentrated to 25 mg ml⁻¹ in two different buffers, buffer A and buffer C (20 mM Tris-HCl pH 7.5, 150 mM NaCl), and subjected to crystallization screening. About 600

crystallization conditions including Crystal Screen, Crystal Screen 2, Natrix, Index (Hampton Research, USA), Wizard I, Wizard II, Wizard III (Jena Bioscience, Germany), BioXtal crystallization screen (BCR) and Sodium Cacodylate BioXtal crystallization screen (CAD-BCR) (XtalQuest, China) were initially screened in 48-well sitting-drop plates (XtalQuest, China) at 277 and 289 K. Crystallization drops were set up manually by mixing 1 μ l protein solution with 1 μ l crystallization solution. The crystallization condition that produced the crystals of DTNB-modified CcbP was optimized using the hanging-drop vapour-diffusion method. The optimized drops consisted of 2.5 μ l DTNB-modified CcbP (25 mg ml⁻¹ in buffer A) and 2.5 μ l reservoir solution (3 M sodium malonate pH 7.0) equilibrated against 500 μ l reservoir solution at 277 K. SeMet-labelled crystals of DTNB-modified CcbP were obtained using the same protocol as that used for the native crystals.

Crystals of DTNB-modified CcbP appeared within a week and were flash-cooled directly in liquid nitrogen without any cryoprotectant (Holyoak *et al.*, 2003). Diffraction data were collected from these crystals on beamline BL5A at KEK (The High Energy Accelerator Research Organization), Photon Factory (PF), Tsukuba, Japan. The wavelength was set to 0.9790 Å and the crystal-to-detector distance was set to 200 mm. Single-wavelength anomalous diffraction (SAD) data were collected from SeMet-labelled crystals of DTNB-modified CcbP on beamline 3W1A at BSRF (Beijing Synchrotron Radiation Facility, People's Republic of China). The wavelength was set to 0.9703 Å and the crystal-to-detector distance was 200 mm. All

Figure 1
SDS-PAGE gel and representative crystallization drops of CcbP. (a) SDS-PAGE gel of CcbP with or without DTNB modification. Purified fresh CcbP without DTNB modification is indicated by a circle and CcbP without DTNB modification exposed to air for a few days is indicated by filled circles, while DTNB-modified CcbP is indicated by an asterisk. The reduced sample contained 10 mM DTT. (b) Clear crystallization drop of CcbP without DTNB modification. (c) Crystals of DTNB-modified protein.

Table 1

Data-collection and refinement statistics for DTNB-modified CcbP.

Values in parentheses are for the highest resolution shell.

	CcbP	SeMet CcbP
Data collection		
Beamline	BL5A, PF	3W1A, BSRF
Wavelength (Å)	0.9790	0.9703
Resolution (Å)	50.00–2.00 (2.12–2.00)	20.00–2.65 (2.81–2.65)
No. of molecules in asymmetric unit	1	1
Space group	<i>P</i> 3 ₁ 21	<i>P</i> 3 ₁ 21
Unit-cell parameters (Å, °)	<i>a</i> = <i>b</i> = 50.74, <i>c</i> = 150.77, α = β = 90, γ = 120	<i>a</i> = <i>b</i> = 52.09, <i>c</i> = 153.63, α = β = 90, γ = 120
No. of unique reflections	15727	12158
Completeness (%)	98.6 (94.5)	90.3 (81.2)
Average <i>I</i> /σ(<i>I</i>)	29.75 (5.0)	26.8 (7.6)
<i>R</i> _{merge} [†] (%)	4.8 (46.3)	5.9 (33.4)
Refinement statistics		
<i>R</i> _{cryst} [‡] (%)	20.71	
<i>R</i> _{free} (%)	24.17	
R.m.s.d. bond lengths (Å)	0.007	
R.m.s.d. bond angles (°)	1.00	
Mean <i>B</i> factor (Å ²)	46.92	
Ramachandran plot§		
Favoured (%)	92.9	
Allowed (%)	7.1	
Disallowed (%)	0	

[†] $R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$. [‡] $R_{\text{cryst}} = \frac{\sum_{hkl} |F_{\text{obs}}| - |F_{\text{calc}}|}{\sum_{hkl} |F_{\text{obs}}|}$. [§] Calculated with *PROCHECK* (Laskowski *et al.*, 1993).

of the data were processed with *HKL-2000* (Otwinowski & Minor, 1997). The statistics of data collection and processing are summarized in Table 1.

2.4. Structure determination

The SeMet SAD data set was used to determine the structure of DTNB-modified CcbP. All five Se sites were located using *SHELXD* (Sheldrick, 2008). Heavy-atom parameter refinement, initial phase

generation and model building were carried out using *phenix.autosol* (Adams *et al.*, 2010). Further model building was performed manually using *Coot* (Emsley *et al.*, 2010). The structure was refined with *PHENIX* (Adams *et al.*, 2010) against the native data set to 2.0 Å resolution. The statistics of data collection, structure determination and refinement are summarized in Table 1.

2.5. Isothermal titration calorimetry (ITC) assay

The Ca²⁺-binding affinity of purified CcbP protein with and without DTNB modification was measured using a MicroCal ITC200 (GE Healthcare, USA) at 298 K. For CcbP without DTNB modification, the protein was dialyzed against 20 mM Tris–HCl pH 8.5, 10 mM DTT. The CaCl₂ solution (4 mM stock solution) used for titration in the ITC assay was also prepared in 20 mM Tris–HCl pH 8.5, 10 mM DTT. A total of 40 µl CaCl₂ stock solution was titrated into 200 µl protein solution (100 µM) over 20 injections at 298 K. The spacing time between injections was set to 4 min. The reference power was 5 µCal s^{−1} (21 µJ s^{−1}). For Ca²⁺-binding studies of DTNB-modified CcbP the experiment was performed using the same protocol as for the wild-type CcbP, except that the concentration of the stock CaCl₂ solution for titration was 3 mM. All data were analyzed using the program *Origin 7.0* (MicroCal, USA) with the CaCl₂ titration against buffer reference data subtracted.

3. Results and discussion

3.1. CcbP crystallization success using DTNB-modified protein

Like other Ca²⁺-binding proteins such as calmodulin, CcbP displays a mobility shift in a Ca²⁺-dependent gel-shift assay (Zhao *et al.*, 2005). The apparent molecular weight of CcbP on the SDS–PAGE is larger than its theoretical molecular weight (Fig. 1*a*).

Figure 2

Two perpendicular views of the overall CcbP structure. All structural figures were generated using *PyMOL* (DeLano, 2002). CcbP contains an α-helical subdomain (orange) and a β-barrel subdomain (cyan) and two short 3₁₀-helices (yellow).

The purified protein ran as a monomer on a nonreduced SDS-PAGE (Fig. 1*a*). A weak dimer band appeared on the nonreduced SDS-PAGE after exposure of the purified protein to air in an open test tube for 2 d at 277 K. The dimeric protein could be reduced to the monomeric form by adding 10 mM DTT (Fig. 1*a*). The nonreduced and reduced SDS-PAGE assays suggested that the free Cys74 residues could dimerize, leading to a mixture of dimers and monomers in the protein solution.

Wild-type CcbP protein was screened using about 600 crystallization conditions. However, no hits were identified. To produce homogeneous monomeric CcbP for the next rounds of crystallization, three approaches were used: treatment of CcbP with 10 mM DTT, mutation of Cys74 to alanine and DTNB modification. Although treatment with DTT or the mutation of Cys74 to alanine did generate homogeneous monomeric proteins, these two proteins still failed to yield any crystals after a wide range of screening (about 600

Figure 3

MNB bound to Cys74 of CcbP and structure comparison of CcbP. (a) A close-up view of MNB bound to Cys74. MNB interacts with Glu26, Asp71 (grey) and Lys49 in the symmetry-related molecule (pink). The $|F_o - F_c|$ electron-density map of bound MNB is contoured at 2.5σ . The $|2F_o - F_c|$ electron-density map of Cys74 is contoured at 2.0σ . (b) Overlay of our CcbP crystal structure with the Ca^{2+} -free CcbP solution structure (PDB entry 2p0p). (c) Overlay of our CcbP crystal structure with the Ca^{2+} -bound CcbP solution structure (PDB entry 2k2v). The Ca^{2+} ion is shown as a small purple sphere.

conditions). Surprisingly, on using the DTNB-modified monomeric protein for crystallization screening crystals suitable for data collection were obtained within one week in one condition from the sparse-matrix screening (Figs. 1*b* and 1*c*).

3.2. Structure description of DTNB-modified CcbP

The monomeric structure of DTNB-modified CcbP was determined and refined to 2.0 Å resolution using the SeMet-based SAD method (Table 1). The final model contained the entire 126 residues with three disordered residues (residues 87, 88 and 89). The overall structure comprises an α -helical subdomain and a β -barrel subdomain. Three α -helices, the N-terminal part of α 1 (residues 8–17), α 2 (residues 25–39) and the C-terminal part of α 3 (residues 107–122), assemble into the α -helical subdomain with α 1 almost perpendicular to the other two helices. The β -barrel subdomain sits beside the α -helical subdomain. It consists of five antiparallel β -strands: residues 44–52 (β 1), residues 57–68 (β 2), residues 79–84 (β 3), residues 91–96 (β 4) and residues 100–103 (β 5). One short 3_{10} -helix (η 1) links the β 2 and β 3 strands and a second 3_{10} -helix (η 2) links the β 4 and β 5 strands (Fig. 2*a*). No Ca^{2+} ions were observed in the crystal structure.

3.3. MNB-induced interactions in the crystal lattice of CcbP

After solving the crystal structure of DTNB-modified CcbP, we built an MNB (5-mercapto-2-nitrobenzoic acid) moiety into the CcbP model according to the $|F_o - F_c|$ electron-density map (Fig. 3*a*). MNB is the product of the DTNB-modification reaction. The structure revealed that MNB was involved in polar interactions in the crystal lattice of CcbP. DTNB reacted with Cys74 of CcbP, resulting in MNB being covalently bound to Cys74 through a disulfide bond. A salt bridge is formed between the nitro group of MNB and the ϵ -amino group of Lys49 in a symmetry-related CcbP molecule. The nitro group of MNB is hydrogen bonded to the carboxyl group of Glu26, and the carboxyl group of MNB forms a hydrogen bond to the carboxyl group of Asp71 (Fig. 3*a*). These interactions facilitate the crystal packing of DTNB-modified CcbP. Although the DTT-reduced CcbP and the C74A mutant were monomeric, they failed to crystallize despite extensive screening. It appears that the intermolecular contacts provided by MNB were more important for the success of CcbP crystallization than its role in ensuring a stable monomeric form of the protein. Therefore, the polar interactions formed by MNB in the crystal lattice reveal a new mechanism for aiding crystallization by DTNB-mediated cysteine modification.

Figure 4
 Ca^{2+} -binding assay of CcbP. (a) Trace of calorimetric titration of CaCl_2 into CcbP without DTNB modification (upper panel) and integrated binding isotherm (lower panel). The integrated binding isotherm was fitted using the two-sites binding model with $N_1 = 1$ (fixed), $K_1 = 29\,100 \pm 9200\text{ M}^{-1}$, $\Delta H_1 = -3.52 \pm 0.94\text{ kcal mol}^{-1}$, $N_2 = 1$ (fixed), $K_2 = 486 \pm 87\text{ M}^{-1}$, $\Delta H_2 = -3.21 \pm 0.31\text{ kcal mol}^{-1}$. N is the stoichiometry of binding, K is the Ca^{2+} -binding association constant ($1/K_d$) and ΔH is the heat change per mole. (b) Ca^{2+} binding of DTNB-modified CcbP. The integrated binding isotherm was fitted using the two-sites binding model with $N_1 = 1$ (fixed), $K_1 = 11\,400 \pm 3500\text{ M}^{-1}$, $\Delta H_1 = -1.56 \pm 0.98\text{ kcal mol}^{-1}$, $N_2 = 1$ (fixed), $K_2 = 579 \pm 24\text{ M}^{-1}$, $\Delta H_2 = -3.25 \pm 0.3\text{ kcal mol}^{-1}$. 1 cal = 4.186 J.

3.4. DTNB modification has little effect on the overall structure or the Ca²⁺ binding of CcbP

A structure comparison (Holm & Sander, 1993) between the crystal structure determined here and the solution structure of CcbP showed good overall agreement. For Ca²⁺-free CcbP (PDB entry 2p0p; Hu *et al.*, 2011), the r.m.s.d. for 123 aligned C α atoms was 2.3 Å, with a Z-score (a measure of structure similarity) of 18.6. For Ca²⁺-bound CcbP (PDB entry 2k2v; Hu *et al.*, 2011), the r.m.s.d. for 123 aligned C α atoms is 2.2 Å, with a Z-score of 17.9. DTNB modification thus shows little effect on the overall CcbP structure.

To investigate whether DTNB modification influences CcbP Ca²⁺ binding, Ca²⁺-binding experiments of CcbP with or without DTNB modification were performed by ITC. Wild-type CcbP bound two Ca²⁺ ions with a first K_d value of $34 \pm 4 \mu\text{M}$ and a second K_d value in the millimolar range (Fig. 4a). The Ca²⁺-binding affinity of the DTNB-modified protein is quite similar to that of wild-type CcbP (Fig. 4b), implying that DTNB modification has very little effect on CcbP Ca²⁺ binding.

3.5. Advantages of DTNB modification for protein crystallization

DTNB modification has several advantages in increasing the crystallization success rate for proteins. (i) It can be rapidly carried out and easily monitored. Monitoring the DTNB modification is convenient as MNB has an absorption maximum at 412 nm giving a yellow colour, while DTNB has an absorption maximum at 324 nm. (ii) DTNB modification can prevent unwanted intramolecular and intermolecular disulfide-bond formation between cysteine residues. The DTNB modification is stable for extended periods unless extra reducing agent is added. (iii) The polar interactions between the MNB moiety and residues in a crystal lattice may strengthen the crystal packing to help protein crystallization, as demonstrated in this report. (iv) In our case, DTNB modification did not affect the overall structure or Ca²⁺ binding and it made the crystallization of CcbP possible. We therefore suggest that modification of cysteine residues in proteins by Ellman's reagent DTNB can be used as an easy and efficient method for aiding protein crystallization and thus X-ray structure studies in general.

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References

- Adams, P. D. *et al.* (2010). *Acta Cryst.* **D66**, 213–221.
- Baedecker, M. & Schulz, G. E. (2002). *Structure*, **10**, 61–67.
- Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engström, O., Ohman, L., Greene, G. L., Gustafsson, J. A. & Carlquist, M. (1997). *Nature (London)*, **389**, 753–758.
- Bulaj, G., Kortemme, T. & Goldenberg, D. P. (1998). *Biochemistry*, **37**, 8965–8972.
- DeLano, W. L. (2002). *PyMOL*. <http://www.pymol.org>.
- Ellman, G. L. (1959). *Arch. Biochem. Biophys.* **82**, 70–77.
- Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. (2010). *Acta Cryst.* **D66**, 486–501.
- Han, K. K., Delacourte, A. & Hemon, B. (1987). *Comp. Biochem. Physiol. B*, **88**, 1057–1065.
- Holm, L. & Sander, C. (1993). *J. Mol. Biol.* **233**, 123–138.
- Holyoak, T., Fenn, T. D., Wilson, M. A., Moulin, A. G., Ringe, D. & Petsko, G. A. (2003). *Acta Cryst.* **D59**, 2356–2358.
- Hu, Y., Zhang, X., Shi, Y., Zhou, Y., Zhang, W., Su, X.-D., Xia, B., Zhao, J. & Jin, C. (2011). *J. Biol. Chem.* **286**, 12381–12388.
- Kessler, D., Rétey, J. & Schulz, G. E. (2004). *J. Mol. Biol.* **342**, 183–194.
- Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (1993). *J. Appl. Cryst.* **26**, 283–291.
- Lenz, M. & Rétey, J. (1993). *Eur. J. Biochem.* **217**, 429–434.
- Messens, J., Van Molle, I., Vanhaesebrouck, P., Van Belle, K., Wahni, K., Martins, J. C., Wyns, L. & Loris, R. (2004). *Acta Cryst.* **D60**, 1180–1184.
- Mi, W., Li, L. & Su, X.-D. (2008). *Biochem. Biophys. Res. Commun.* **368**, 919–922.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Sheldrick, G. M. (2008). *Acta Cryst.* **A64**, 112–122.
- Shi, Y., Zhao, W., Zhang, W., Ye, Z. & Zhao, J. (2006). *Proc. Natl Acad. Sci. USA*, **103**, 11334–11339.
- Van Duyne, G. D., Standaert, R. F., Karplus, P. A., Schreiber, S. L. & Clardy, J. (1993). *J. Mol. Biol.* **229**, 105–124.
- Yoon, C., Johnston, S. C., Tang, J., Stahl, M., Tobin, J. F. & Somers, W. S. (2000). *EMBO J.* **19**, 3530–3541.
- Zhao, Y., Shi, Y., Zhao, W., Huang, X., Wang, D., Brown, N., Brand, J. & Zhao, J. (2005). *Proc. Natl Acad. Sci. USA*, **102**, 5744–5748.