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Crystallization and Preliminary X-Ray Diffraction Studies of a 40 kDa Calcium Binding Protein Specifically Expressed in Plasmodia of *Physarum polycephalum*[1]

Wakana Iwasaki,* Hiroshi Sasaki,† Akio Nakamura,‡ Kazuhiro Kohama,‡ and Masaru Tanokura*,[2]

**Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences and* †*Biotechnology Research Center, The University of Tokyo, Bunkyo, Tokyo 113-8657; and* ‡*Department of Pharmacology, School of Medicine, Gunma University, Maebashi, Gunma 371-8511*

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A calcium binding protein with a molecular mass of 40 kDa (CBP40), the gene product of plasmodial-specific LAV1-2 of *Physarum polycephalum*, was crystallized in the presence of EDTA. The crystals diffracted X-rays up to a resolution of 3.0 Å. They belonged to the trigonal space group, $P3_221$ (or $P3_121$), with unit cell dimensions of $a=b=64.4$ Å and $c=207.2$ Å. Ca^{2+}-bound crystals were obtained by soaking in a $CaCl_2$ solution, which gave diffraction data of similar quality. The Ca^{2+}-soaked crystals belonged to the same space group as those crystallized in the presence of EDTA with unit cell dimensions of $a=b=64.4$ Å and $c=209.4$ Å.

Key words: crystallization, calcium binding protein, 40 kDa calcium binding protein, *Physarum polycephalum*, X-ray crystallography.

Calcium binding proteins have various functions after their interaction with Ca^{2+}. In the lower eukaryote, *Physarum polycephalum*, a calcium binding protein has been reported to be expressed specifically in plasmodia. Its cDNA was cloned from a plasmodial-specific mRNA and named LAV1-2 (*1, 2*). The protein sequence deduced from LAV1-2 consists of 355 amino acids and contains four EF-hand motifs in the C-terminal region (*3*). The protein has temporarily been named "40 kDa calcium binding protein (CBP40)," and recombinant CBP40 has been overexpressed in *Escherichia coli* (Nakamura *et al.*, unpublished). CBP40 has two main features: higher affinity to Ca^{2+} than other EF-hand proteins such as calmodulin, and self-assembly in the presence of Ca^{2+} ions (Nakamura *et al.*, unpublished). In plasmodial cells, a part of CBP40 is processed by a proteinase and thereby becomes an N-terminal (1-32 residues)-deleted polypeptide named ⊿N32-CBP40. Since ⊿N32CBP40 does not self-assemble even if it binds Ca^{2+} ions (Nakamura *et al.*, unpublished), ⊿N32-CBP40 is useful for elucidating the intramolecular contact of the EF-hand domain with the N-terminal half domain and for gaining an insight into the mechanism underlying the high affinity for Ca^{2+}.

The three-dimensional structures of many EF-hand proteins containing no other domains and EF-hand domain fragments of multidomain proteins have been reported. Few studies have, however, been reported on the intramolecular contact of the EF-hand domain with other domains in multidomain proteins except in the cases of the regulatory domains of scallop myosin (*4, 5*) and BM-40 (also known as SPARC or osteronectin) (*6*). We report here the crystallization and preliminary X-ray crystallographic analysis of ⊿N32CBP40.

Recombinant ⊿N32CBP40 was purified by a modification of the method of Nakamura *et al.* (unpublished). Cultured *E. coli* BL21(DE3) cells were collected, suspended in buffer A containing 20 mM Tris-HCl (pH 7.6) and 13 mM EDTA, and stored at −80°C. The suspension was thawed at room temperature and then subjected to sonication after DTT, PMSF, CHAPS, and TritonX-100 had been added to 20 mM, 0.5 mM, 1%, and 1%, respectively. The addition of detergents and strong sonication were needed for the protein to come into the supernatant. Without detergents, ⊿N32CBP40 was coprecipitated with *E. coli* membranes. After centrifugation, polyethylimine was added to the supernatant to a final concentration of 0.01% in order to precipitate nucleic acids. The supernatant was then subjected to ammonium sulfate fractionation. The precipitate obtained on the addition of ammonium sulfate to 34-65% saturation was dissolved in buffer A and then dialyzed against the same buffer. The resulting solution was applied to a Q-Sepharose Fast Flow column (Pharmacia). The eluted fractions containing ⊿N32CBP40 were applied to a phenyl-Sepharose 6 Fast Flow column (Pharmacia). The fractions comprising the major peak were concentrated and applied to a Superdex 75 FPLC gel filtration column (Pharmacia). Two peaks were obtained. The minor peak eluted earlier was regarded as representing a dimer in

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[2] To whom correspondence should be addressed. Tel: +81-3-5841-5165, Fax: +81-3-5841-8023, E-mail: utanok@mail.ecc.u-tokyo.ac.jp

Abbreviations: CBP40, 40 kDa calcium binding protein; ⊿N32CBP-40, N-terminal 32 residue-truncated CBP40; PMSF, phenylmethylsulfonyl fluoride.

which Cys 283s formed an intermolecular disulfide bond, and the major one eluted later was regarded as the monomer fraction. This was confirmed by SDS/polyacrylamide gel electrophoresis in the presence and absence of 2-mercaptoethanol. The monomer fractions were concentrated in Centriprep-10 ultrafiltration tubes (Amicon) and used for the subsequent crystallization. Purity was analyzed by SDS-PAGE.

The crystallization conditions were screened with the hanging drop vapor diffusion method using crystallization kits (Hampton Research) at 5 and 25°C. An 8 μl hanging drop resting on a coverslip was equilibrated with 1 ml of a reservoir solution. Crystals suitable for X-ray analysis were obtained by mixing 4 μl of the protein solution (74 mg/ml in 10 mM Tris-HCl, pH 7.6), and 4 μl of the reservoir solution consisting of 1.4-1.8 M ammonium sulfate, 1 mM DTT, 10 mM EDTA, 0.02% NaN_3, and 0.1 M HEPES-NaOH (pH 6.4-7.6) at 25°C. Crystals of the Ca^{2+}-bound form were prepared by soaking crystals obtained in the presence of EDTA in a 2 mM $CaCl_2$ solution for 4 days. For cryo-measurements, crystals were soaked in a 30% glycerol solution for 40 min. A preliminary diffraction study was carried out using a Rigaku imaging-plate detector system, R-AXIS IIc, with double mirror-focused CuKα radiation from a Rigaku RU-200R X-ray generator. For data collection, the Weissenberg cameras for macromolecular crystallography with imaging plates installed in beamlines BL6A and BL6B of the Photon Factory (High Energy Accelerator Research Organization, KEK, Tsukuba) and SPring-8 (Japan Synchrotron Radiation Research Institute, JASRI, Harima) were used.

Hexagonal prismatic crystals with dimensions of 0.4 mm in diameter and 0.2 mm in thickness appeared within a few weeks (Fig. 1). The crystals prepared in the presence of EDTA diffracted X-rays to 3.0 Å resolution at room temperature, and to 2.7 Å at 100 K. The data set taken at room temperature had an overall R_{merge} value of 6.4% with 91.7% completeness, the corresponding values for the outermost shell (3.10-2.99 Å) being 33.7 and 96.2%, respectively. The relatively low value of overall completeness as compared with the value for the outermost shell may be due to the contribution of the low value (87.9%) for the innermost shell (larger than 11.11 Å). The crystals belonged to the trigonal space group, $P3_221$ (or $P3_121$), with unit cell dimensions of $a=b=64.4$ Å and $c=207.2$ Å. Assuming one molecule per asymmetric unit, the crystal volume per unit of protein mass is $V_M=3.37$ Å^3/Da, which corresponds to a solvent content of 63.5% (7).

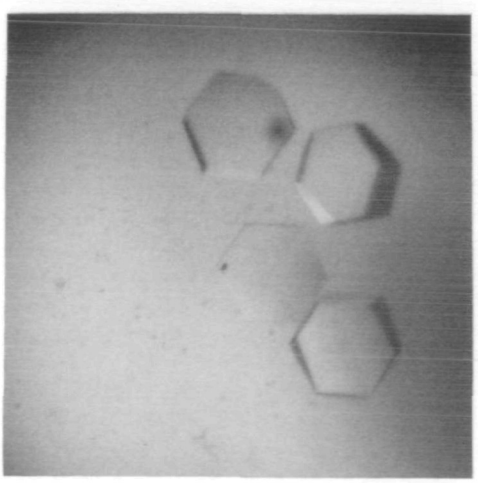

Fig. 1. **Typical crystals of ⊿N32CBP40.** The approximate dimensions are 0.4 mm in diameter and 0.2 mm in thickness.

In a similar way, data sets for Ca^{2+}-soaked crystals were collected up to 3.1 Å resolution at room temperature. This data set had an overall R_{merge} value of 9.7% with 97.1% completeness, the corresponding values for the outermost shell (3.23-3.12 Å) being 34.2 and 99.6%, respectively. The Ca^{2+}-soaked crystals of ⊿N32CBP40 belonged to the same space group ($P3_221$ or $P3_121$) as those obtained in the presence of EDTA, and had unit cell dimensions of $a=b=64.4$ Å and $c=209.4$ Å. The assumption of one subunit per asymmetric unit leads to a V_M value of 3.38 Å^3/Da, corresponding to a solvent content of 63.6% (7). The crystallographic parameters are very similar to those of the crystals obtained in the presence of EDTA. This is consistent with the observation that the far-ultraviolet circular dichroism spectrum of ⊿N32CBP40 is not so different in the presence of EDTA from that in the presence of Ca^{2+} (Iwasaki *et al.*, unpublished). The addition of detergents or organic solvents did not give crystals of higher-resolution. It has been reported that deliberately induced dehydration results in crystals of reduced unit cells, the best-ordered of which often show diffraction to a higher resolution (*8, 9*). In view of these reports, the pregrown crystals were soaked in a solution containing a higher concentration (3-4 M) of ammonium sulfate than that in the reservoir solution. The unit cell volume was in fact reduced by this treatment, which suggested that dehydration did occur, but the resolution of the diffraction data became worse. The fraction containing intermolecular disulfide-bonded dimers was also subjected to crystallization in a similar way to the monomer fraction, but it diffracted X-rays very weakly. We are now under way to determine the structure of ⊿N32CBP40 using multiple isomorphous replacement methods.

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