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Cadmium-induced crystallization of proteins: II. Crystallization of the *Salmonella typhimurium* histidine-binding protein in complex with L-histidine, L-arginine, or L-lysine

SERGEI TRAKHANOV,[1,2] DAVID I. KREIMER,[1] SEAN PARKIN,[2]
GIOVANNA FERRO-LUZZI AMES,[1] AND BERNHARD RUPP[2]

[1]Department of Molecular and Cellular Biology, Division of Biochemistry and Molecular Biology, University of California, Berkeley, California 94720
[2]Lawrence Livermore National Laboratory, Biology and Biotechnology Research Program, L-452, University of California, Livermore, California 94550

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Abstract

To further investigate favorable effects of divalent cations on the formation of protein crystals, three complexes of *Salmonella typhimurium* histidine-binding protein were crystallized with varying concentrations of cadmium salts. For each of the three histidine-binding protein complexes, cadmium cations were found to promote or improve crystallization. The optimal cadmium concentration is ligand specific and falls within a narrow concentration range. In each case, crystals grown in the presence of cadmium diffract to better than 2.0 Å resolution and belong to the orthorhombic space group $P2_12_12_1$. From our results and from the analysis of cadmium sites in well-refined protein structures, we propose that cadmium addition provides a generally useful technique to modify crystal morphology and to improve diffraction quality.

Keywords: cadmium addition; crystal growth; crystallization; crystal morphology; histidine binding protein; X-ray crystallography

The search for favorable crystallization conditions yielding well-diffracting crystals still remains a mostly empirical, often time-consuming procedure in the course of a crystallographic structure determination (Durbin & Feher, 1996). It has long been recognized that divalent cations in general can facilitate protein crystallization (McPherson, 1982, 1990). In the particular case of cadmium, its use to promote crystallization of ferritin dates back even before the advent of protein crystallography itself. The addition of a 10% $CdSO_4$ solution to horse spleen tissue juice immediately yields cubic ferritin crystals, whose growth can be observed under a cover slide (Michaelis, 1947, and earlier references cited therein).

A first systematic investigation of the influence of divalent cations has been published recently (Trakhanov & Quiocho, 1995). Using the leucine-, isoleucine-, valine-binding protein (LIVBP) in complex with L-leucine as a model system, cadmium cations (Cd^{2+}) were found to exhibit the most profound effect on crystallization of the 10 divalent metal ions examined. A favorable effect of Cd^{2+} has been also observed in the crystallization of several other bacterial periplasmic binding proteins: the histidine-binding protein from *Escherichia coli* in complex with L-histidine (Yao et al., 1994); leucine-, isoleucine-, valine-binding protein in complex with L-isoleucine (S. Trakhanov & F.A. Quiocho, unpubl. results); the leucine-specific-binding protein in complex with L-leucine (Trakhanov & Quiocho, 1995); the dipeptide-binding protein (Nickitenko et al., 1995); and the oligopeptide-binding protein in complex with a tripeptide (A.V. Nickitenko & S. Trakhanov, unpubl. results). We investigate the ability of Cd^{2+} to facilitate the crystallization of another member of the bacterial binding protein family, the histidine-binding protein from *Salmonella typhimurium* (HisJ) in complex with three high-affinity ligands.

HisJ is the water-soluble component of the histidine permease, one of the most extensively characterized traffic ATPases (ABC transporters, Liu et al., 1997). HisJ is located in the periplasm and specifically binds and delivers exogenous amino acids (L-histidine, L-arginine, and L-lysine) to the membrane-bound complex of the permease for subsequent translocation of the ligand into the cytoplasm. HisJ assumes slightly different conformations in complex with each of its ligands, as revealed by difference absorbance and

Reprint requests to: Bernhard Rupp, Lawrence Livermore National Laboratory, Biology and Biotechnology Research Program, L-452, University of California, Livermore, California 94550; e-mail: rupp1@llnl.gov.

fluorescence spectroscopy (Trakhanov et al., 1989; Wolf et al., 1996), time-resolved fluorescence spectroscopy (H. Malak & J.R. Lakowicz, unpubl. obs.), and different recognition by a conformation-specific antibody (Wolf et al., 1994, 1996).

Structures of HisJ complexes will also provide a unique opportunity for structural comparative studies. The high-resolution structures (1.7–1.9 Å) of the complexes of lysine-, arginine-, ornithine-binding protein (LAO) in the apo form and with four ligands, L-lysine, L-arginine, L-ornithine, and L-histidine, have been solved (Oh et al., 1993). LAO shares 70% sequence identity with HisJ and is functionally closely related, but the structure of a HisJ complex is only available at a lower resolution of 2.5 Å (Oh et al., 1994). It is of great interest therefore to obtain crystals of HisJ in complex with other ligands at high resolution.

Results and discussion

Crystallization

The search for crystallization conditions was performed in two steps. First, the concentrations of Cd^{2+} and PEG-400[3] were screened widely while the initial volume of a crystallization drop was kept constant (4 μL of the stock protein solution were mixed on a siliconized cover slip with 4 μL of the well solution). In a second step, the best conditions found in the initial PEG/Cd^{2+} screen [well solutions containing 22–25% (v/v) PEG-400, 50 mM Bis-tris propane at pH 8.5, and 20 mM $CdSO_4$] were used as a starting point for optimization of the drop size and the cadmium concentration.

The cadmium concentration was varied down the columns of a Linbro matrix by adding another 10–100 μL of 1 M $Cd(OAc)_2$ to the well, while, along the rows, the drop size and the ratio of well solution to protein stock was varied as follows: 5 μL of protein stock solution were mixed on a cover slide with increasing amounts of well solution (2–7 μL in 1-μL increment). This setup effectively screened for unique crystallization conditions in each of the 24 protein drops in the crystallization plate.

The nature of the anion in the cadmium salt appears to have no effect on the crystallization. Similar results were obtained with either $CdSO_4$ alone, or a mixture of $CdSO_4$ and $Cd(OAc)_2$. In experiments with LIVBP, we achieved a similar improvement in crystal morphology using CdI_2 or $CdCl_2$. The optimal Cd^{2+} concentrations were determined individually for each of the three HisJ complexes. In the course of the final optimization, glycerol was screened in an effort to improve cryo-protection. However, an increase in glycerol concentration above 5% had a detrimental effect on the crystal morphology (yielding thin plates), especially for the L-arginine and L-lysine complexes. Therefore, 5% glycerol was used in all trials for these two complexes.

The HisJ complex with L-histidine crystallized from a solution containing 1 mM L-histidine, 25% PEG-400, 20 mM $CdSO_4$, 50 mM Bis-tris propane, pH 8.5, and 5–15% glycerol within 2–3 days. In the presence of 15% glycerol, the crystals grew as plates with average dimensions of $0.5 \times 0.4 \times 0.08$ mm^3 (Fig. 1A). Their morphology changed favorably to rectangular rods with a decrease in glycerol concentration to 5% and an increase in the initial concentration of Cd^{2+} in the protein drop to 35–40 mM (Fig. 1B). These crystals, with average dimensions $0.3 \times 0.3 \times 1.0$ mm^3, were used for data collection.

The HisJ–L-arginine complex (Fig. 1C) crystallized from a solution of 25% PEG-400, 50 mM Bis-tris propane, pH 8.5, 30 mM Cd^{2+}, and 5% glycerol within 2–3 days. The crystals formed long thick prisms with average dimensions of $0.3 \times 0.3 \times 0.7$ mm^3. For the complex of HisJ with L-lysine, the best results were obtained in the presence of an initial concentration of 17.5 mM Cd^{2+} in the protein drop (Fig. 1D). The crystals grew in the shape of single prisms to their final size $0.3 \times 0.3 \times 0.8$ mm^3 in 3–4 days at room temperature. Two HisJ–L-lysine crystals were used to collect a data set complete to 1.8 Å resolution.

Crystallographic analysis

Due to the high PEG content and the glycerol present in the mother liquor, the crystals could be flash-cooled directly in liquid nitrogen and were transferred with cryo-tongs into the cold nitrogen stream of a modified Siemens LT-2 low-temperature apparatus attached to a ADSC dual multi-wire detector system.[4] All three HisJ complexes investigated in this study crystallize in the orthorhombic space group $P2_12_12_1$ (Table 1).

The structure of the unliganded *E. coli* histidine-binding protein (Yao et al., 1994), determined at 1.9 Å resolution, was selected as an initial phasing model for molecular replacement. The molecular replacement solution was unambiguous. The model structures for each of the three HisJ complexes are currently being refined. There is also an effort underway to crystallize complexes of HisJ with each of two low-affinity ligands, L-ornithine and D-histidine (K_D ca. 1 mM).

The role of cadmium

The analysis of a number of high-resolution structures of proteins crystallized employing cadmium ions, namely the histidine-binding protein from *E. coli* (Yao et al., 1994), the dipeptide-binding protein (Nickitenko et al., 1995), and the leucine-binding proteins (S. Trakhanov & F.A. Quiocho, unpubl. data), provides insight into the role of cadmium in crystal formation. Cadmium atoms are positioned at the interface between two neighboring protein molecules and coordinated by two carboxyl groups of glutamic or aspartic acid side chains each belonging to one of the two molecules. This coordination is often supplemented by coordination bonds formed with carbonyl groups of the polypeptide backbone and/or with a water molecule.

Similar cadmium coordinations at intermolecular contacts are present in crystals of ferritin. In native cubic horse spleen ferritin (Granier et al., 1997), two cadmium ions occupy special positions on a threefold axis and are coordinated by three glutamine or three aspartic acid residues, respectively. A third Cd^{2+} occupies a twofold axis and is coordinated by Gln and Asp from different molecules. A complex arrangement involving partially occupied Cd sites has been observed in a crystal of cubic L-chain horse spleen ferritin (Hempstead et al., 1997), where a His side chain is involved in the Cd coordination in addition to Glu and Asp.

Whether the imidazole group of a histidine side chain is involved in Cd coordination depends upon the protonation status of

[3]PEG-400 has the additional advantage of serving as a cryoprotectant during the flash-cooling of the crystals for data collection at near liquid nitrogen temperature (Parkin, 1997).

[4]Supplemental information about equipment and cryo-procedures used can be found on our web site: www-structure.llnl.gov.

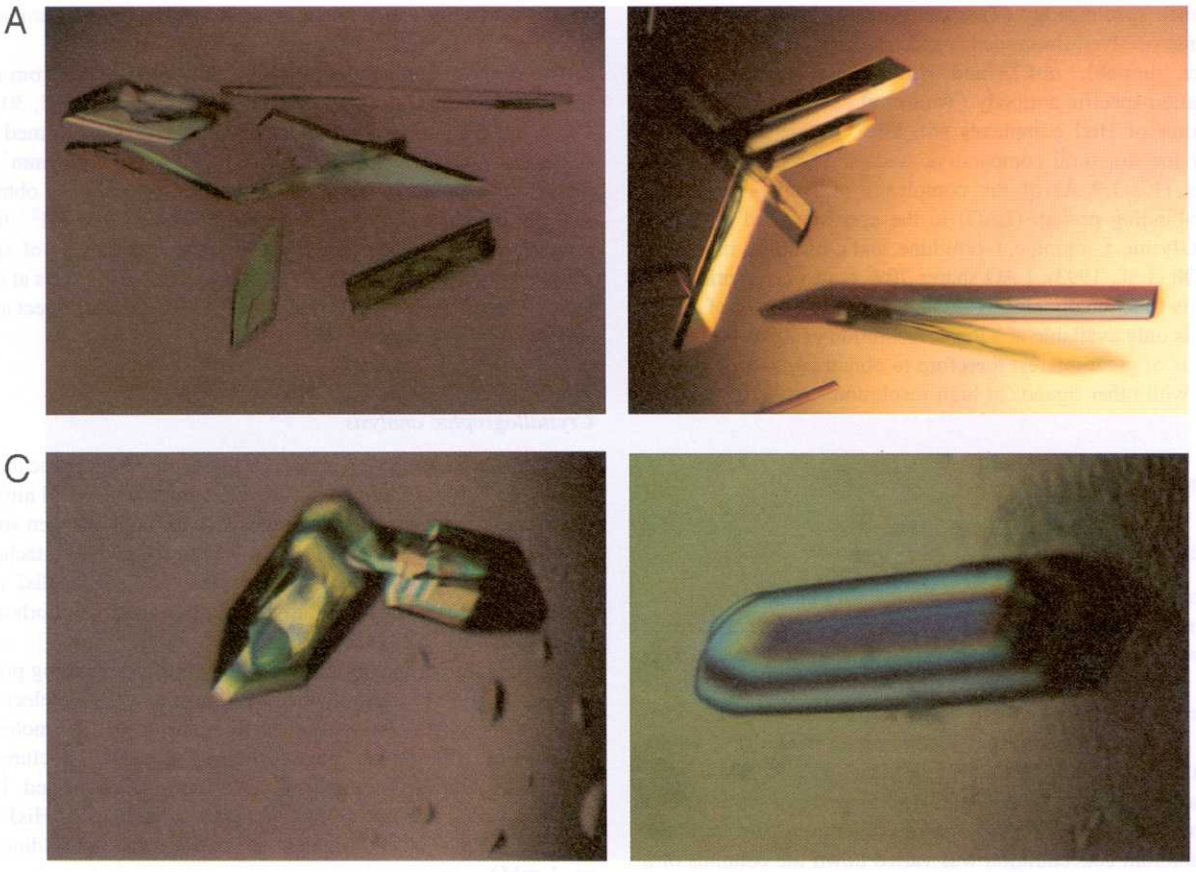

Fig. 1. Crystals of histidine-binding protein from *S. typhimurium* in complex with L-histidine (**A**,**B**), L-arginine (**C**), and L-lysine (**D**); magnification 40× (A,B,C) or 60× (D). Concentration of glycerol in the well solution is 5% (B, C, D) and 15% (A).

the histidine. In the HisJ–His complex, histidine-129 is essentially deprotonated at pH 8.5, thus providing the coordination bond for cadmium (Fig. 2), whereas, at pH below 6, protonation of the imidazole group does not allow the formation of a coordination bond. The pH dependence of the formation of a coordination bond with cadmium thus can provide an additional means to modify crystal morphology. Because none of the interactions between the amino acid residues and the cadmium ions are unique to HisJ and related complexes, the formation of intermolecular bridges by cadmium atoms should be of general use in protein crystallization.

Conclusions

The addition of cadmium can provide an advantage in growing well-diffracting crystals of a variety of proteins in a form suitable for high-resolution crystallographic studies. The HisJ complexes

Table 1. *Unit cell dimensions and diffraction limits of HisJ in complex with L-histidine, L-arginine, and L-lysine at 125 K and room temperature data for crystals of S. typhimurium HisJ–L-histidine grown in the absence of any cadmium (Kang et al., 1992)*[a]

Ligand	Optimal initial $[Cd^{2+}]$ in drop (mM)	HisJ unit cell dimensions (Å) Space group $P2_12_12_1$			Resolution limit (Å)
		a	*b*	*c*	
L-Histidine	None	39.26	66.17	88.33	2.5
L-Histidine	35–40	39.52	40.17	154.01	1.7
L-Arginine	15–17	39.37	40.43	154.52	1.9
L-Lysine	17–18	39.24	40.57	154.20	2.0

[a]Despite the use of a synchrotron source for data collection (Oh et al., 1994), the latter crystals diffracted to considerably lower resolution (2.5 Å).

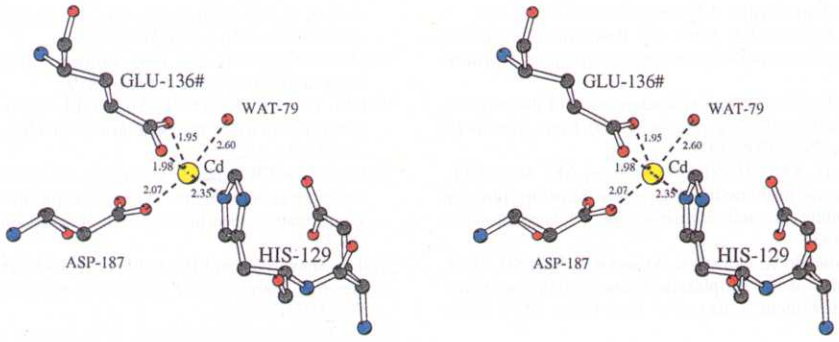

Fig. 2. Parallel stereo view of Cd coordination at an intermolecular contact region in the HisJ–His complex in partially refined structure. Cd is coordinated between two molecules by two acidic residues (Asp-187 of molecule 1, Glu-136# from molecule 2), as well as His-129 from molecule 1, and a water molecule, Wat 79. This figure was generated using MOLSCRIPT (Kraulis, 1991).

reported in this work provide three examples of the improvement in crystal quality obtained from the addition of Cd^{2+}. From the analysis of refined structures, we have strong evidence that the propensity of cadmium cations to form coordination bonds enables the development of cadmium bridges across the interfaces of protein molecules. Side groups of various amino acids, water molecules, and carbonyl groups of the backbone chain can serve as coordination partners. The unique pattern of the negative charge distribution on the surface of any given protein molecule will necessitate the variation of the cadmium concentration to create the conditions essential for Cd^{2+} coordination and crystal formation. Cadmium concentrations can range from as low as 1 mM [for the leucine-, isoleucine-, valine-binding protein (Trakhanov & Quiocho, 1995)] to as high as 200 mM [for the histidine-binding protein from *E. coli* (Yao et al., 1994)].

It is conceivable that substantial changes could be induced by cadmium binding, especially at high concentrations. So far, we have no evidence of significant changes in the three-dimensional structure of the leucine-, isoleucine-, valine-binding protein solved with cadmium compared to these structures without Cd^{2+} (S. Trakhanov, unpubl. results). In addition, the analysis of seven cadmium-binding sites in the 1.9 Å crystal structure of the histidine-binding protein from *E. coli* has confirmed that all Cd sites are located at the molecular interface and not inside the protein, regardless of the high Cd concentration used (Yao et al., 1994). Additional structural details should become available when the refinements of our HisJ–ligand complexes are completed.

Materials and methods

Purification of HisJ

HisJ was extracted from *S. typhimurium* periplasm by osmotic shock, and purified by two-step ammonium sulfate precipitation and DEAE-HPLC (Nikaido & Ames, 1992) on a Bio-Gel DEAE-5-PW column (Bio-Rad Laboratories, Richmond, California). The flow rate was 5 mL/min, and approximately 15 mg of 85% pure HisJ in 5 mM Tris-HCl buffer, pH 8.3, were injected. The column was washed for 1 h with the same buffer prior to elution with a NaCl gradient from 0 to 30 mM. This procedure yields highly purified HisJ essentially free of endogenous ligands, as confirmed by fluorescent titration (Wolf et al., 1995, 1996). Unliganded HisJ was concentrated to 75 mg/mL in 5 mM Tris-HCl buffer, pH 8.3, using Centricon-10 concentrators (Amicon, Inc., Beverly, Massachusetts), yielding stock protein solution. Protein stock stored on ice for up to two months retained its full binding activity and its ability to produce crystals.

Crystallization

A fivefold dilution of the protein stock was prepared with 10 mM Tris-HCl buffer, pH 8.0, to which 1 mM L-histidine, 1 mM L-arginine, or 5 mM L-lysine were added. Crystals were grown at room temperature using the hanging drop vapor diffusion method (McPherson, 1982) with 1-mL well volume and drops of varying sizes (7–12 μL). The drops consisted of 5 μL of protein stock and 2–7 μL of well solution.

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