Single Crystals of Cadmium, Zinc Metallothionein*

(Received for publication, December 6, 1982)

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Single crystals have been grown of Cd,Zn metallothionein isoform II from rat liver. The space group is P4212 (P43212) with unit cell dimensions a = b = 31.0 Å and c = 120.0 Å, and one molecule in the crystallographic asymmetric unit. The crystals are square bipyramids elongated on the tetragonal c-axis and are grown by repetitive seeding. The crystals are suitable for high resolution structure analysis. Assays of dissolved crystals show that the crystals have the same Cd and Zn content and amino acid composition as the native, as-isolated protein.

Metallothionein is a low molecular weight protein with the ability to bind a variety of metals, commonly Zn, Cd, and Cu (1). The protein was initially isolated and characterized from mammalian kidney and liver (2-4). Primary structures are known for human (5), equine (6), mouse (7), crab (8), and Neurospora (9) metallothioneins. These sequences are strongly homologous, contain 57-61 residues, are rich in cysteine, lysine and serine, and are devoid in aromatic amino acids. The mammalian proteins contain 20 conserved cysteine residues. Coordination of the metals by cysteine is tetrahedral (1). 113Cd NMR of rabbit liver Cd,Zn metallothionein demonstrates that the metals are arranged in polyamino clusters (10). Using homonuclear decoupling, the 113Cd NMR spectra have been assigned in terms of 4-metal and 3-metal cluster/molecule in the rabbit liver protein (11). 113Cd NMR experiments have shown that the Cu,Cd metallothionein from calf liver contains 3Cu and 4Cd clusters (12), while the protein from crab contains two 3Cd clusters (13).

The structure of the mammalian protein has been found to consist of two domains (14). Rat liver Cd,Zn metallothionein isoform I cleaved at lysine 30 by subtilisin yields a fragment (α1), which contains the 32 residues of the carboxyl-terminal half of the molecule and 4Cd; a similar peptide (α2) is obtained with isoform II (14). 113Cd NMR of α1 reveals four Cd sites as previously assigned to the 4-metal cluster (15). Therefore, the carboxyl-terminal 11 cysteines coordinate to a 4Cd cluster, modeled with 5 terminal and 6 bridging thiolute ligands, while the NH-terminal 9 cysteines coordinate to a 3-metal cluster, modeled with 6 terminal and 3 bridging thiolutes (11, 14).

The more labile 3-metal cluster contains both Cd and Zn (14).

In this paper, we report preliminary crystallographic data for single crystals of the Cd,Zn metallothionein isoform II from rat liver. This protein contains 61 amino acids and on the average 5Cd and 2Zn in the 7 sites of the molecule. The primary structure of the NH2-terminal 25 residues is closely homologous to mouse metallothionein isoform II (7); the amino acid composition is also very similar to the mouse isoform II protein.

EXPERIMENTAL PROCEDURES

Cd,Zn MT II was prepared from rat liver as previously described (16, 17), except that the material was passed through a second DEAE-cellulose column in order to purify. Fractions from the final Sephadex G-25 column at 0.005 M potassium phosphate, pH 7.8, were pooled and lyophilized. Each sample showed a single band on non-denaturing polyacrylamide electrophoresis gels. Cd and Zn were determined by atomic absorption spectroscopy. Based on quantitative amino acid analysis, the metal content was determined to be 4.9-6.0 mol of Cd and 2.5-1.0 mol of Zn/mol of protein in six samples used for crystallization. The amino acid analysis also detected no leucine, histidine, arginine, tyrosine, or phenylalanine.

Lyophilized samples are dissolved to make a solution 10 mg/ml in protein containing 1.0 M sodium formate and 0.2 M potassium phosphate at pH 7.5. The solution is equilibrated in 5- to 10-μl volumes against 1.0-ml volumes of 5.0 M sodium formate, pH 7.5, using vapor diffusion and hanging drops. Within 1 day at 22 °C or 3 days at 2 °C, several dozen single crystals appear. The larger crystals are used as seeds and transferred into pre-equilibrated droplets of fresh protein solution just prior to the onset of nucleation. Repetition of the seeding procedure (18) affords large single crystals. The crystals are square bipyramidal, morphologically single, and highly reflective.

Variation of the concentrations of sodium formate and potassium phosphate in the droplet leads to more rapid nucleation, where literally hundreds of crystals are observed per droplet, or to oiling out or precipitation of the protein. The results of approximately 650 hanging drop experiments are summarized in a phase diagram (Fig. 1), which is used as a guide for repetitive seeding. Crystals have been grown with protein from each of the six preparations.

In addition to the requirement for formate and phosphate salts in the correct concentrations, it appears that Cd,Zn MT II is unique in its ability to crystallize favorably. Systematic crystallization surveys with the following thionines were unsuccessful: Cd,Zn MT I, Zn MT II, and the α fragments of Cd,Zn MT I and II (14), all from rat liver, and Cu,Cd MT II from calf liver provided by I. M. Armitage (Yale University) (12). However, small needle crystals were obtained of Cd,Zn MT I and II and the α peptide, using polyethylene glycols 4000 and 6000 at low ionic strength. Needles were also grown of the α fragment and Cu,Cd MT II using sodium formate.

Crystals were dissolved and assayed in order to compare their metal content to the as-isolated native protein in low ionic strength solution. Two samples were prepared from the crystals. For each, single crystals were washed with 7.0 M sodium formate, collected by centrifugation, and dissolved in 0.05 M potassium phosphate, pH 7.8. For both samples, the metal ion content was determined by atomic absorption spectroscopy and protein was determined by quantitative amino acid analysis. The data (Table I) indicate strongly that the crystals contain native Cd,Zn MT II with...
CRYSTALLIZATION OF Cd,Zn MT II

a full complement of metal ions. Amino acid analysis of the dissolved crystals also showed the same content of lysine, aspartic acid, threonine, glutamic acid, proline, glycine, alanine, and valine as the starting samples. Desalted solutions of dissolved crystals also have the same UV absorption spectrum as the as-isolated protein with a distinct shoulder at 250 nm. Further, a fresh protein solution incubated in 1.6 M sodium formate, 0.2 M potassium phosphate, pH 7.5, showed no change in its 250-nm absorption versus 1.6 M sodium formate after 21 days.

From measuring the volume of several droplets following equilibration and after the onset of nucleation, the protein crystallizes at ~2.0 M sodium formate, ~0.4 M potassium phosphate. As a further test of these conditions, the $^{197}$Cd NMR spectra of rabbit kidney MT II in 0.8, 1.2, and 1.6 M sodium formate solutions were recorded. The spectra showed no indication of signal loss or line broadening due to facilitated exchange, i.e., displacement of Cd from the protein.2

The density of 3 crystals, maximum dimensions 0.3 mm, was measured using a linear density gradient (19) formed by pyridine and chloroform. The crystals were transferred directly from the mother liquor to the gradient with a looped metal wire, as for seeding experiments. The gradient was calibrated with sodium formate solutions. For the 3 crystals, the crystal density is 1.29 ± 0.02 g/cm3.

For X-ray experiments, crystals were mounted in 0.7-mm glass capillaries using 7.0 M sodium formate, 0.2 M potassium phosphate, pH 7.5, as a synthetic mother liquor.

RESULTS

Fig. 2 shows the 0kl zone of the diffraction pattern for crystals of Cd,Zn metallothionein isoform II. Reflections are absent for 0kl, l ≠ 4n, and 0k0, k ≠ 2n. The diffraction symmetry is mm for the 0kl, h0l and hkl zones. The intensity distribution for the 0kl and hkl zones is identical, where these films are obtained from the same crystal rotated 90°. The hkl zone shows 4-mm symmetry. For the hkl and hkl zones, reflections are absent for h0l, h ≠ 2n. Therefore, the Laue group is 4/mmm and the space group is $P4_12_12_1$, or its enantiomer, $P4_12_2_2$. Unit cell dimensions measured from precession photographs are $a = b = 31.0$ Å and $c = 120.0$ Å. The crystal morphology is a square bipyramid elongated on the tetragonal c-axis.

The crystal density may be calculated from the unit cell parameters (Table II) by assuming the density of the mother liquor is 1.08 g/cm3 (2.0 M sodium formate), and by letting $r = 0.64$ ml/g (1). For one molecule in the asymmetric unit, the calculated density is 1.31 g/cm3, in agreement with the observed value of 1.29 ± 0.02 g/cm3. For two molecules in the asymmetric unit, the calculated density is 1.55 g/cm3, well outside the experimental error. If the solvent content of the crystals is taken as pure water, the calculated density is 1.29 g/cm3. Therefore, the assumed salt content of the mother liquor does not significantly affect the calculated value of the crystal density.

The diffraction pattern shown in Fig. 2 has been recorded from four crystals, and crystals have been grown using six separate preparations of the protein. Reflections are uni-
Crystals of Metallothionein

TABLE II

Crystal data for Cd,Zn metallothionein isoform II

<table>
<thead>
<tr>
<th>Crystal system</th>
<th>Tetragonal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P4_2_2_2 or P4_2_2</td>
</tr>
<tr>
<td>Unit cell dimensions</td>
<td>a = 31.0 Å, b = 31.0 Å, c = 120.0 Å, α = β = γ = 90°</td>
</tr>
<tr>
<td>Unit cell volume</td>
<td>115,300 Å³</td>
</tr>
<tr>
<td>Molecules/unit cell</td>
<td>8</td>
</tr>
<tr>
<td>Molecules/asymmetric unit</td>
<td>1</td>
</tr>
<tr>
<td>Observed density</td>
<td>1.29 g/cm³</td>
</tr>
<tr>
<td>Calculated density</td>
<td>1.31 g/cm³</td>
</tr>
<tr>
<td>Matthew's coefficient</td>
<td>2.2 Å³/dalton¹</td>
</tr>
<tr>
<td>Solvent fraction</td>
<td>0.51</td>
</tr>
<tr>
<td>Typical size of crystals</td>
<td>0.2 × 0.2 × 0.7 mm</td>
</tr>
</tbody>
</table>

¹ Ref. 20.
² For M₆ = 6500.
³ For ρ = 0.64 ml/g.

formly observed to 2.2 Å in precession photographs using as an x-ray source a standard focus sealed tube operated at 35 kV, 15 mA. It is anticipated that even higher resolution data will be obtained from larger crystals or with a more intense x-ray source. The crystals are stable in the x-ray beam; the diffraction pattern shown in Fig. 2 is still observed after 5 days of exposure at 2 °C. The crystals, therefore, are suitable for a high resolution structure determination. For CuKα radiation (λ = 1.5418 Å), the anomalous scattering factors (in electrons) for cadmium are f' = −0.6 and f" = 5.0. Assuming 5 Cd/molecule, and disregarding Zn and S, the expected average Bijvoet difference is 11%. This value compares favorably with that for the protein crambin, which has been solved using the anomalous scattering from sulfur in the native crystals (21). A precession photograph of the 1kl zone of a Cd,Zn MT II crystal shows apparent mm (2m) symmetry, consistent with the Laue group, but large Bijvoet differences are not immediately obvious in this film.

Acknowledgments—We thank I. M. Armitage for stimulating discussions. Technical assistance in the preparation of the protein was provided by K. A. Miklossy and K. Nielsen.

REFERENCES

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