FOR THE RECORD

Crystallization of recombinant human heme oxygenase-1

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Abstract: Heme oxygenase catalyzes the NADPH, O₂, and cytochrome P450 reductase dependent oxidation of heme to biliverdin and carbon monoxide. One of two primary isozymes, HO-1, is anchored to the endoplasmic reticulum membrane via a stretch of hydrophobic residues at the C-terminus. While full-length human HO-1 consists of 288 residues, a truncated version with residues 1–265 has been expressed as a soluble active enzyme in *Escherichia coli*. The recombinant enzyme crystallized from ammonium sulfate solutions but the crystals were not of sufficient quality for diffraction studies. SDS gel analysis indicated that the protein had undergone proteolytic degradation. An increase in the use of protease inhibitors during purification eliminated proteolysis, but the intact protein did not crystallize. N-terminal sequencing and mass spectral analysis of dissolved crystals indicated that the protein had degraded to two major species consisting of residues 1–226 and 1–237. Expression of the 1–226 and 1–233 versions of human HO-1 provided active enzyme that crystallizes in a form suitable for diffraction studies. These crystals belong to space group P2₁, with unit cell dimensions $a = 79.3 \text{ \AA}$, $b = 56.3 \text{ \AA}$, $c = 112.8 \text{ \AA}$, and $\beta = 101.5^\circ$.

Keywords: crystallization; heme oxygenase; proteolysis; X-ray diffraction

Two main isoforms of heme oxygenase (HO), HO-1 and HO-2, catalyze the NADPH-dependent oxidation of heme to biliverdin (Tenhunen et al., 1969; Fig. 1). Inducible HO-1 is membrane bound and is found in greatest abundance in the liver and spleen (Yoshida & Kikuchi, 1978, 1979) while the constitutive HO-2 is found in the testes and brain (Trakshel et al., 1986). Being part of the heme degradation pathway, HO is a key enzyme in ridding the system of heme, a potentially toxic oxidant. Recently, it has been suggested that the second product of the HO reaction, the toxic gas carbon monoxide, serves as a messenger molecule in a manner similar to nitric oxide (Maines, 1993; Zakhary et al., 1997).

In addition to the importance of HO in maintaining heme homeostasis and its possible involvement in signaling pathways, there is considerable interest in the mechanism of HO catalysis. HO is similar in its reaction to cytochromes P450 with the unusual feature that the cofactor for the reaction, heme, is also the substrate. Also, like P450, the source of electrons, NADPH, is funneled to the heme group via a diflavin cytochrome P450 reductase (Schacter et al., 1972). Nevertheless, HO appears not to have a Cys residue coordinated to the heme iron as in P450, but rather a His (Ito-Maki et al., 1995; Wilks et al., 1996) as in the globins and peroxidases.

Much of the information available is on HO-1. Human HO-1 is a single polypeptide of 288 residues and approximately 33,000 Da, and is anchored to the endoplasmic reticulum membrane via a C-terminal hydrophobic tail (Yoshida & Sato, 1989). Wilks et al. (1995) have developed an *Escherichia coli* expression system for a soluble version of human HO-1, which is missing the last 23-residue membrane-binding segment. This 265-residue recombinant HO-1 (Δ^{265}) is fully active, and the availability of the enzyme has stimulated structure/function studies. Here we report crystallization of shortened forms of recombinant human HO-1 as the apo-enzyme and as a complex with the substrate, heme, and with the product, biliverdin.

Results and discussion Crystals were grown from early samples of soluble HO-1 from the Δ^{265} expression system. The quality and reproducibility of the crystals were poor. SDS-polyacrylamide gels of the protein showed double bands of smaller than expected size (not shown), leading to the suspicion of proteolytic degradation. Alterations were made to the published purification scheme (Wilks & Ortiz de Montellano, 1993) to improve the sample quality, with the chief change being an increase in the amount and frequency of the use of protease inhibitor, PMSF, during purification. SDS-polyacrylamide gels verified the purity and stability of the new Δ^{265} samples (not shown). The crystallization studies were resumed, but the full-length Δ^{265} would not crystallize. These results suggest that a proteolytically clipped fragment or fragments must have been responsible for the crystallization observed earlier.

To determine where proteolysis had occurred, crystals were dissolved and subjected to N-terminal peptide sequencing and electrospray mass spectrometry. The N-terminal analysis gave the

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Fig. 1. The reaction catalyzed by heme oxygenase.

weight 28,489. Based on the sequencing and mass spectrometry results, we hypothesized that the 26,141 and/or 27,303 molecular weight versions of HO-1 had crystallized. Therefore, recombinant expression systems were prepared to produce the 1–226 (Δ^{226}) and the 1–233 (Δ^{233}) forms of HO-1 (Wilks et al., 1998). These truncated versions of HO-1 bind heme and biliverdin and retain about half of the catalytic activity of Δ^{265} when coupled with the reductase which supplies electrons (Wilks et al., 1998).

Both the Δ^{226} and Δ^{233} forms of HO-1 were purified using the same procedure as for Δ^{265} , and both forms crystallize readily with the same morphology. Crystals were grown of the apo-enzyme and of the enzyme reconstituted with either heme or biliverdin (Fig. 2). All forms grow under the same conditions and have the same space group and unit cell dimensions. Crystals grew as plates in clusters, with the occasional appearance of a single plate. Single plates were removed from clusters if necessary for data collection.

The best diffracting crystal to date was grown from apo HO-1 Δ^{226} ; 125,466 observations of 32,681 unique reflections were processed over the resolution range 20.0–2.5 Å to give a merging *R* factor of 0.083. Completeness for the dataset is 97.4%, with 94.9% in the outermost shell (2.54–2.50 Å). The *I*/ σ *I* ratio for the outermost shell is 1.9. The space group is monoclinic P2₁, with *a* = 79.3 Å, *b* = 56.3 Å, *c* = 112.8 Å, and β = 101.5°. The current best data from hemo HO-1 were collected from a crystal grown from Δ^{233} ; 95,272 observations of 25,583 unique reflections were processed in the resolution range 20.0–2.75 Å to a merging *R* factor of 0.080. Completeness is 97.9%; 98.0% for the outermost shell

Fig. 2. Photos of various HO-1 crystals. **A:** Early crystal clusters grown with a holo-enzyme version of proteolytically degraded Δ^{265} . **B:** Δ^{226} in the apo form; the crystal depicted is 1.5×2.0 mm, with a depth of ~ 0.1 mm. **C:** Δ^{233} with bound substrate heme, ~ 1.5 mm long. **D:** Δ^{233} with bound product biliverdin, ~ 1.0 mm long for the largest single crystals.

(2.80–2.75 Å). The $I/\sigma I$ ratio for the outermost shell is 2.8. Data were also collected and processed from Δ^{233} with biliverdin bound; 90,769 observations of 26,971 unique reflections were processed over the resolution range 20.0–2.70 Å to a merging R -factor of 0.095. Completeness is 99.6%; 100.0% for the outermost shell (2.80–2.75 Å). The $I/\sigma I$ ratio for the outermost shell is 2.0.

Materials and methods: Purification: Design and construction of expression systems are described elsewhere (Wilks et al., 1998). Purification of various forms of human recombinant HO-1 was based on the procedures of Wilks and Ortiz de Montellano (1993) for rat HO-1 with modifications. Briefly, DH5 α cells containing one of the expression plasmids for human HO-1 were grown in LB media with 100 mg/L of ampicillin and induced with 1 mM of IPTG. After 18 h of expression, the cells were harvested, suspended in 50 mM Tris, 2 mM EDTA, and lysed in a French press. Ammonium sulfate (250 mM) and 0.35% polyethyleneimine were added followed by centrifugation. PMSF (1 mM) was added before lysis and after the polyethyleneimine precipitation, 2 mM was added just after lysis. Up to this point the protein is green indicating that biliverdin is bound to the enzyme due to the conversion of endogenous *E. coli* heme to biliverdin by the expressed HO-1. The 40–70% saturated ammonium sulfate fraction was re-suspended in 10 mM diaminopropane pH 9.0 and run on a QAE anion exchange column with a gradient of 0–250 mM potassium chloride. The green biliverdin adheres to the column and the apo-enzyme is eluted. Fractions containing HO-1 were pooled, combined with a 2:1 molar ratio of hemin if appropriate, and dialyzed into 20 mM potassium phosphate, pH 7.4. Following heme incorporation, HO-1 was run over a DEAE anion exchange column to remove excess heme.

Crystallization: Crystallization screening was carried out using hanging drop or sitting drop vapor diffusion. Protein stock consisted of 35 mg/mL protein, sometimes with hemin or biliverdin added to a molar ratio of 1:1 to 2:1 just prior to crystallization trials. Well solutions of 1 mL contained 2.08 M ammonium sulfate, 0.1 M Hepes pH 7.5 and 0.9% 1,6-hexanediol. Drops consisted of 5 μ L of protein stock and 5 μ L of well solution.

Peptide sequencing: Automated N-terminal protein sequencing of a dissolved crystal was performed on a Hewlett-Packard model G1005A. The results were consistent with a virtually intact N-terminus of the recombinant HO-1, with the main sequence recovered in high yield being Met-Glu-Arg-Pro-Gln-. In approximately 5% of the material the N-terminal Met residue was missing.

Mass spectrometry: Electrospray mass spectrometry was carried out at the City of Hope. Samples were run on a C18 reverse-phase

column connected online to the mass spectrometer. Scans were run every 3 s in a mass to charge range of 50 to 2,000.

X-ray diffraction: Single crystals were mounted at room temperature on a Rigaku RU300 X-ray generator with mirror optics, a single circle goniostat and a Rigaku R-axis IV detector. Oscillations of 1° were collected. Data were integrated and scaled with Denzo and Scalepack (Otwinowski, 1993).

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