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Crystallization of retinol dehydratase from *Spodoptera frugiperda*: improvement of crystal quality by modification by ethylmercury-thiosalicylate

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Retinol dehydratase is a sulfotransferase which is presumed to catalyze the dehydration of its substrate *via* a transient retinyl sulfate intermediate. Crystals (space group $P2_1$, unit-cell parameters $a = 82.05$, $b = 66.61$, $c = 84.90$ Å, $\beta = 111.29^\circ$) are significantly improved by covalent modification of the protein with ethylmercury.

1. Introduction

Retroretinoids are compounds derived from vitamin A in which there has been a shift in the conjugated double-bond system such that the bond which joins the ionone ring to the isoprene tail of the retinoid is a double bond. Retroretinoids have been shown to be physiological active metabolites of retinol in mammalian tissue-culture studies. Both 14-hydroxy retroretinol and anhydroretinol are present in a variety of cell lines, from mammalian to insect (Buck *et al.*, 1991, 1993; Derguini *et al.*, 1995). In the insect *Spodoptera frugiperda* high levels of anhydroretinol are found in pupae; retinol dehydratase, an enzyme which catalyzes the transformation of retinol to anhydroretinol *in vitro*, is expressed during the prepupation and pupation stages of the insect life cycle (Vakiani & Buck, unpublished observation).

Retinol dehydratase (RD) from *S. frugiperda* is a member of the sulfotransferase superfamily of enzymes (Grun *et al.*, 1996) and is presumed to catalyze the dehydration of retinol *via* a transient retinyl sulfate intermediate (Vakiani *et al.*, 1998). The enzyme, a monomer of 351 amino acids (Grun *et al.*, 1996), has 26% sequence identity with estrogen sulfotransferase, for which an X-ray structure has been determined (Kakuta *et al.*, 1997). However, relative to estrogen sulfotransferase, retinol dehydratase contains a 20 amino-acid amino-terminal extension and a 32 amino-acid insertion. We undertook the crystallization of retinol dehydratase to elucidate the structural basis for the observation that this enzyme catalyzes a dehydration reaction while other enzymes of the sulfotransferase superfamily generate sulfonated products.

Crystals which belong to space group $P2_1$ and diffract to 1.9 Å resolution were initially prepared. However, the crystals were highly mosaic in one direction (along the *a* axis) and were of variable quality. The crystals were also sensitive to any type of manipulation, be it transferring to cryo-protectant or mother liquor. We found that crystal quality was significantly improved when crystals were grown in the presence of ethylmercury-thiosalicylate (EMTS) in terms of morphology, fragility and mosaicity. In the refinement of the 2.25 Å resolution structure, the basis for the beneficial effect of EMTS on crystal quality became apparent. It appears that covalent modification by ethylmercury of a single cysteine may shore up an intermolecular packing contact in a crystal lattice in which contacts are sparse.

2. Materials and methods

Protein, purified as described previously (Vakiani *et al.*, 1998), was stored at 203 K and thawed and incubated overnight at 277 K with 3′-phosphoadenosine-5′-phosphate (PAP) and retinol (in dimethylsulfoxide) prior to crystallization. Overnight incubation with cofactor prior to crystallization improved crystal quality, as judged by morphology and size. Conditions which favored crystallization were first identified using the sparse-matrix method (Jancarik & Kim, 1991) as marketed by Hampton Research Products. Precipitants which produced promising results were further optimized by additional buffer, pH, additive and temperature screens. Crystals were grown in hanging drops suspended over 8% polyethylene glycol 3350, 50 m*M* $CaCl_2$, 100 m*M* HEPES pH 6.6–6.8, 0–8% glycerol in the absence and presence of 1 m*M* EMTS. The hanging drops were composed of equal volumes of protein at 20 mg ml^{-1} or one volume of protein and two volumes of well solution. The crystallization experiments were set up over ice and then placed in an 284 K incubator. Crystals usually appeared within 3 d and grew over the course of one week.

For X-ray data collection, the crystal was retrieved from the hanging drop after covering the drop with mineral oil. The crystal was pulled through the oil to remove excess mother liquor and was flash-frozen by plunging into

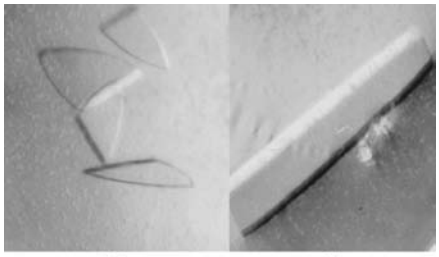

Figure 1
Crystals of retinol dehydratase grown in (*a*) the absence and (*b*) the presence of EMTS.

liquid nitrogen or direct freezing in the nitrogen stream from the cryo-cooling apparatus. Once the drop was pierced to remove crystals, it had to be completely covered with oil or within hours the remaining crystals would no longer be useful.

Diffraction data were collected on an R-AXIS IV image plate mounted on a Rigaku RU-200 rotating-anode generator. For optimal resolution, 1° frames require a 30 min exposure. Data were processed and indexed with *DENZO* and *SCALEPACK* (Minor, 1993).

The retinol dehydratase C258S mutant expression construct was synthesized by PCR-based site-directed mutagenesis (QuickChange; Stratagene, La Jolla, CA, USA) using wild-type pET19b RD as a template and the oligonucleotide primers 5′-GGACTTACCAGGCTCCATTGCACGT-ATCGCTGAC-3′ and 5′-GTCAGCGATA-CGTGCAATGGAGCCTGGTAAGTCC-3′. Subsequently, the C279S mutation was introduced using oligonucleotide primers 5′-GGAACAAATTCAGCGCCTCAGCGAA-CACCTGAA-3′ and 5′-TTCAGGTGT-TCGCTGAGGCGCTGAATTTGTTCC-3′. The nucleotide sequence of the C258S:C279S double mutant was verified using the DNA-sequencing facility of Cornell University. Dehydratase proteins were expressed in *Escherichia coli* strain BL21(DE3) and purified as described previously (Vakiani *et al.*, 1998).

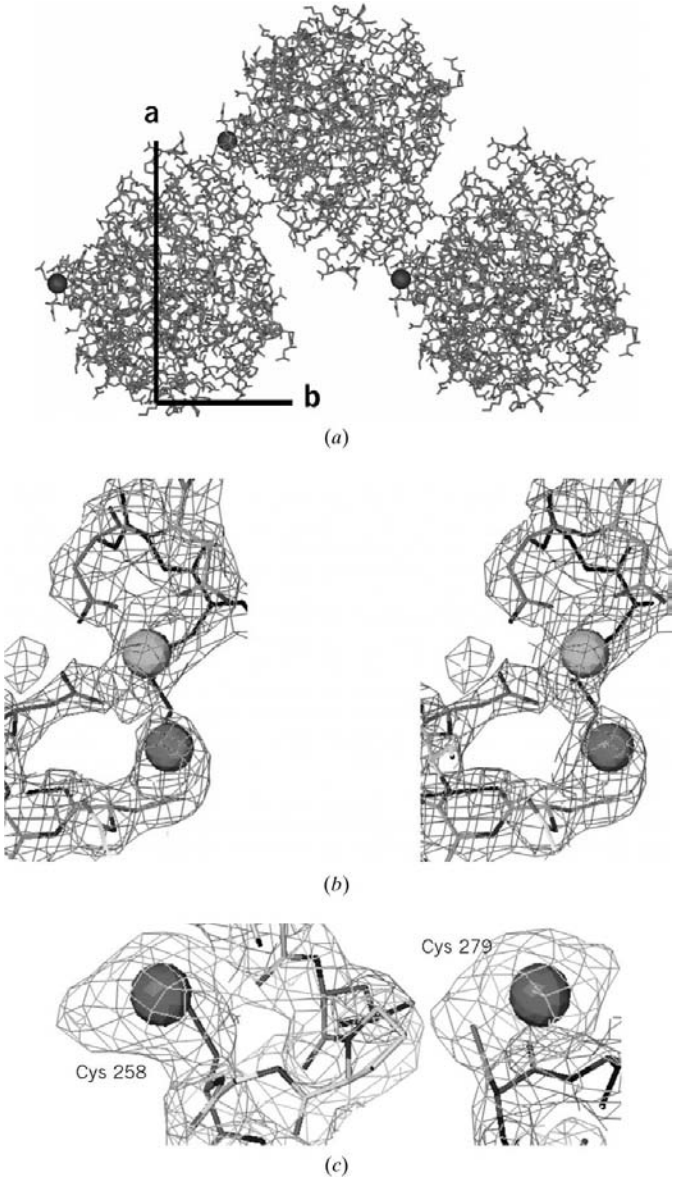

Figure 2
Cys318 is positioned in the proximity of a crystal packing contact which appears to involve an interaction with aspartate residues from neighboring molecules and Ca^{2+}, provided in the crystallization conditions. (*a*) The context of this interaction is illustrated. Only one of the two molecules of the asymmetric unit is shown, along with its symmetry mates. The sphere marks the position of the Hg atom. (*b*) Detail of the Ca^{2+} binding site, in which the large dark gray sphere indicates Hg and the light gray calcium. The chelating amino acids are (clockwise from left to right) Asp321, Asn121 and Asp122. The latter two amino acids are from a symmetry mate. (*c*) Electron density of ethylmercury-modified Cys258 and Cys279 in one of the two molecules of the asymmetric unit.

3. Results and discussion

Crystals of retinol dehydratase were grown in hanging-drop vapor-diffusion experiments in which 20 mg ml^{-1} protein was mixed with one or two volumes of 8% PEG 3350, 50 m*M* $CaCl_2$, 100 m*M* HEPES pH 6.6–6.8. The crystals are large, but generally very thin in one dimension (∼0.8 × 0.6 × 0.05 mm). Transfer to cryoprotectant often resulted in fractured crystals and thus it was determined that up to 8% glycerol could be included in the crystallization solutions so that no manipulation other than a direct plunge into liquid nitrogen or a nitrogen stream was necessary. The crystals are monoclinic (unit-cell parameters a = 82.05, b = 66.61, c = 84.90 Å, β = 111.29°) and display systematic absences in the diffraction data consistent with a screw axis (space group $P2_1$). There are two monomers in the asymmetric unit. Although diffraction to 1.9 Å was readily observed, the crystals are highly mosaic in the direction of the a axis and often appear as stacks of thin plates in this direction. An extensive search of additives to improve crystal quality yielded no new conditions.

Furthermore, in the course of the structure determination it was observed that the fragile nature of the crystals made it impossible to soak crystals in heavy-atom solutions in order to search for derivatives for multiple isomorphous replacement data sets. Although the selenomethionine form of retinol dehydratase was prepared and crystallized, the poor quality of the diffraction data made it useless for multiwavelength

anomalous dispersion phasing. Consequently, potential heavy-atom derivatives were screened by setting up crystallization experiments in the presence of 1 m*M* heavy atom. Trimethyllead acetate, $KAu(CN)_2$, ethylmercurythiosalicylate (EMTS) and tetracyanoplatinate were some of the compounds tried. Crystals grew in the presence of EMTS, trimethyllead acetate and $KAu(CN)_2$. Much to our delight, we found that crystals grown in the presence of 1 m*M* EMTS were larger and had much sharper faces than those seen in the absence of EMTS (Fig. 1). Furthermore, the mosaicity was improved significantly. Data from our best native crystal was obtained to 2.7 Å resolution. The R_{sym} for the data was 11% and the mosaicity refined to 1.4–2.7°. In contrast, diffraction data to 2.25 Å resolution was obtained from the crystal grown in the presence of EMTS, which had an R_{sym} of 6% and a mosaicity between 0.8 and 1.4°.

Our first interpretation of the observation of improved crystal morphology and diffraction data from crystals grown in the presence of EMTS was that the poor quality of the native crystal is a result of a heterogeneous mixture in which either intra- or intermolecular disulfide bonds form which preclude the formation of a highly ordered lattice. Oscillation images taken of crystals at room temperature in capillary mounts confirmed the problem with mosaicity was not a result of poor freezes for cryo-data collection.

Despite the high mosaicity of the native data, we were able to obtain a preliminary molecular-replacement solution with a polyalanine estrogen sulfotransferase as a search model and to locate the Hg atoms by difference Fourier. [The details of the structure solution (ultimately solved by single isomorphous replacement with anomalous scattering) and its interpretation will be reported elsewhere.] The positions of the heavy atoms were consistent with the position of the six cysteines in the asymmetric unit, corresponding to three Cys per monomer. Although no possibility of intermolecular disulfide-bond formation was apparent with this solution, cysteines 258 and 279 were judged close enough in the preliminary model to allow intramolecular disulfide bond formation. These cysteines are on the surface, close to a packing contact, and formation of a disulfide bond between residues Cys258 and Cys279 might result in a displacement of packing contact residues. The double mutant C258S:C279S was prepared and has native activity. Furthermore, crystals of the mutant protein grew readily under our standard conditions. However, these crystals were no less mosaic than the native crystals. Addition of EMTS to the crystallization drops for the double-mutant protein also resulted in apparently better quality crystals.

Since we were unable to improve the native crystal quality, our final model is that of the protein in the presence of EMTS. All three thiols are on the surface, removed from the retinol or cofactor-binding site. In the refined model, Cys258 and Cys279 have clear electron density consistent with covalent modification by ethylmercury. For Cys318, only a high peak of electron density for the Hg atom is apparent. The ethyl group is disordered. It is modification of Cys318 which is critical for crystal quality and the basis for this is illustrated in Fig. 2. The protein is modified at a site of an intermolecular packing contact. Cys318 is in proximity to Asp321 and Asp122 of a symmetry mate which, as indicated by the presence of a high peak of electron density and an absolute requirement for Ca^{2+} in the crystallization set-up, chelate Ca^{2+}. The presence of the bulky ethylmercury group on Cys318 further restricts the movement of the chelating residues and buttresses the packing contact.

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