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Crystallization and preliminary X-ray studies of *Pseudomonas putida* histidine ammonium-lyase

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Abstract

Histidine ammonium-lyase from *P. putida* was expressed in *Escherichia coli*, purified to homogeneity, and crystallized by the vapour-diffusion method using polyethylene glycol 3350 as the precipitant. The crystals, which diffract to at least 2.5 Å resolution, exhibit the symmetry of space group $P2_12_12_1$, with unit-cell parameters a = 89.7, b = 138.2 and c = 164.8 Å. The asymmetric unit contains a tetramer, and the crystals have a V_m value of 2.41 Å^3 Da^{-1}.

1. Introduction

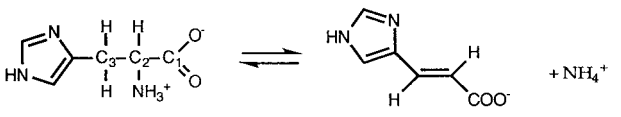
The first step of histidine degradation is catalyzed by histidine ammonium-lyase (histidase; E.C. 4.3.1.3) and results in the production of urocanate and ammonia (Fig. 1). This is a particularly difficult enzymatic reaction since the proton to be abstracted has as high pK_a and since a C—N bond must be broken in the second step of the reaction. A number of amino-acid ammonia lyases catalyze similar reactions, but the members of this family of enzymes have developed different catalytic strategies. It seems reasonable that most amino-acid ammonia lyases should use a pyridoxal cofactor. However, none actually do. Aspartate ammonia lyase requires no co-factor to catalyze the formation of fumarate from aspartate (Falzone *et al.*, 1988). β-methyl aspartase, in contrast, requires a metal ion (Barker *et al.*, 1959). Ornithine cyclodeaminase requires NAD (Costilow & Laylock, 1971).

Histidine ammonia lyase and phenylalanine ammonia lyase appear to use similar mechanisms. Both rely on an electrophilic co-factor whose chemical nature is not completely clear (Smith *et al.*, 1967). This center was postulated to be dehydroalanine by analyzing the products arising from inactivation using a variety of nucleophilic reagents (Wickner, 1969; Givot *et al.*, 1969; Consevage & Phillips, 1985). Dehydroalanine is a rare amino acid. It is known to be present in nisin and subtilin which are small antibiotic peptides (Liu & Hansen, 1990, 1992). However, in larger polypeptides, dehydroalanine has only been found in histidase and phenylalanine ammonium-lyase (Hanson & Havir, 1970; Hodgins, 1971).

In histidase, this unusual electrophilic center is formed autocatalytically and spontaneously from serine. This is not terribly surprising since serine is also the precursor to dehydroalanine in nisin and subtilin (Liu & Hansen, 1990, 1992). Ser143 has been identified as the precursor of dehydroalanine in *P. putida* histidase (Hernandez *et al.*, 1993), and the corresponding Ser254 is the precursor in rat histidase (Taylor & McInnes, 1994). Mutations of this essential Ser to Ala results in an enzyme which cannot use histidine as a substrate (Hernandez & Phillips, 1994; Langer, Reck *et al.*, 1994). Interestingly, when this serine is mutated to a cysteine, the mature enzyme has the same level of activity as wild-type histidase (Langer, Lieber *et al.*, 1994). Presumably, with either Ser or Cys some enzymatic base in histidase abstracts the alpha proton which is followed by the beta elimination of either OH or SH.

Confirmation that Ser143 is the precursor for the electrophilic cofactor came from studies of histidase with L-cysteine in the presence of molecular oxygen. Inactivation of histidase with L-cysteine, a mechanism-based inhibitor, results in the formation of an enzyme derivative which absorbs at 340 nm (Hernandez *et al.*, 1993; Hernandez & Phillips, 1994). After cysteine inactivation electrospray MS/MS has shown that Ser143 contains an unidentified additional mass of 184 Da (Hernandez *et al.*, 1993). It is not clear whether this additional mass is due to some sort of co-factor which would be found in wild-type histidase (presumably attached to the dehydroalanine at residue 143) or whether it is caused by cysteine acting as a suicide inhibitor (Langer, Lieber *et al.*, 1994).

In addition to questions about the chemical nature of the electrophilic co-factor, there are now questions about the role of the co-factor. For a number of years, it was postulated that the co-factor formed a covalent bond to the departing ammonia molecule (Furuta *et al.*, 1990; 1992). However, recent studies with histidine mutants that are unable to form a dehydroalanine moiety (S143A and S143T) suggest a very different role for the cofactor (Langer *et al.*, 1995). In these mutants, 5′-nitrohistidine is still a substrate. This suggests that the role of the cofactor is to form a covalent bond (and hence an electron sink) to the 5′ position of the imidazole ring during catalysis. This reaction would make the H atom attached to C3 considerably more acidic. In the histidase mutants which cannot form dehydroalanine, the 5′-nitro group serves the same role as the cofactor. Clearly, the crystal structure of histidase would explain the structure of the electrophilic cofactor and would increase our understanding of the chemical mechanism for both histidase and phenylalanine ammonia-lyase. Here we report the characterization of crystals of histidase from *P. putida*. Histidase from *P. putida* is active as a tetramer with identical subunits of molecular weight approximately 53 000 kDa. Each subunit has 510 amino acids (Consevage & Phillips, 1985, 1990).



histidine urocanate

Fig. 1. The reaction catalyzed by histidase.

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Table 1. *Summary of diffraction data from the native histidase crystals*

All data were taken from the output of *SCALEPACK*. The R_{merge} is reported on intensities. Observations lists the number of times that a reflection was observed.

					Observations						
Lower resolution	Upper resolution	Average intensity	Average error	R_{merge}	0	1	2	3	4	5+	Total
100.00	8.68	2217.1	118.7	0.038	404	312	354	407	291	131	1495
8.68	6.89	1559.9	94.5	0.041	284	394	450	405	192	54	1495
6.89	6.02	694.1	56.8	0.066	266	396	536	374	159	44	1509
6.02	5.47	701.4	62.2	0.072	295	404	517	340	157	44	1462
5.47	5.08	884.6	76.8	0.076	288	431	481	323	145	61	1441
5.08	4.78	1103.8	91.5	0.070	341	377	488	312	147	59	1383
4.78	4.54	1318.5	110.2	0.073	313	446	477	297	149	58	1427
4.54	4.34	1428.2	119.8	0.079	330	424	457	283	155	61	1380
4.34	4.18	1259.9	115.1	0.085	338	409	460	296	144	79	1388
4.18	4.03	1182.7	117.0	0.091	342	428	439	294	134	81	1376
4.03	3.91	1187.7	120.1	0.092	337	426	440	295	139	70	1370
3.91	3.79	1141.1	119.0	0.096	348	448	417	286	142	80	1373
3.79	3.69	950.5	112.2	0.114	357	458	403	242	147	93	1343
3.69	3.60	849.2	107.0	0.120	357	416	424	263	149	94	1346
3.60	3.52	794.6	103.5	0.120	370	415	413	274	150	94	1346

2. Materials and methods

The histidine ammonium-lyase *hutH* gene from *P. putida* PRS1 (ATCC 12633) was previously cloned into a high-expression vector pPL (Pharmacia) under the control of the lambda *PL* promoter (Hu & Phillips, 1988). The resulting plasmid (pLH11) was maintained in *E. coli* strain N4830, a lambda lysogen containing a temperature-sensitive *cI* repressor which controls transcription from the *PL* promoter. Histidase was purified to electrophoretic homogeneity by ammonium sulfate precipitation, rapid heat shock, and ion-exchange chromatography as described elsewhere (Hernandez & Phillips, 1993).

Initial crystallization conditions were screened using the sparse-matrix method of Jancarik & Kim (1991). Crystallization was achieved at 294 K by the hanging-drop vapor-diffusion technique with a histidase concentration of 6 mg ml^{-1} and reservoir buffer containing dithiothreitol, sodium acetate (pH 4.6) and polyethylene glycol 3350.

These initial crystals were usually long rectangular prisms. At each end of the prism there was an invagination, as if a wedge had been removed. Furthermore, these crystals often grew in parallel or fanned bunches that made individual crystals difficult to separate. Efforts to eliminate the hollowness and to promote better growth on the ends of the crystals included microseeding with both crushed and uncrushed seed stock. However, these experiments resulting in crystals that were smaller, thin and needle like.

Attempts to optimize crystallization conditions showed that the enzyme is very sensitive to changes in pH. Crystals do not form if the pH is above 5 or below 4. However, between pH 4 and 5, a number of buffers will support crystal growth. These include sodium formate, ammonium acetate and sodium succinate. Crystals will not grow at pH 4.6 using HOMOPIPES as the buffer.

Other attempts at optimization included both varying the protein concentration and trying a number of additives at low concentrations. The additives tested include zinc chloride, isopropanol, β-octylglucoside, and the inhibitors phosphanohistidine and hydroxylamine. Crystals grew in the presence of all of these additives, but they had the same morphology as wild-type crystals. The enzyme does not crystallize at 277 K.

In the current conditions for crystallization, the 1 ml reservoir contains 1 m*M* DTT, 90–120 m*M* sodium acetate (pH 4.6) and 4–10%(*w*/*v*) PEG 3350. Protein samples (5 µl) at a concentration of 6 mg ml^{-1} were mixed with equal amounts of reservoir solution and allowed to equilibrate. Protein concentration was determined using the Pierce assay with BSA as the standard. Within 4–6 d, large crystals grew to 2 × 0.2 × 0.2 mm. Precipitation usually precedes crystallization, but crystals have been observed in otherwise clear drops.

For X-ray analysis, crystals were mounted in thin-walled quartz capillaries. A native data set to 2.5 Å resolution was collected at room temperature using a Rigaku R-AXIS II imaging-plate detector with a Rigaku RU200 rotating-anode generator operating at 50 kV and 100 mA and equipped with a graphite monochromator. Data were collected with 1 and 1.5° oscillation frames, processed with *DENZO*, and scaled with *SCALEPACK* (Otwinowski & Minor, 1997). Screened X-ray precession photographs were recorded on a Charles Supper precession camera using Ni-filtered Cu $K\alpha$ radiation from an Enraf–Nonius X-ray generator operating at 40 kV and 30 mA.

3. Results and discussion

Single crystals of histidase ammonium lyase diffract to 2.5 Å resolution on a conventional X-ray source. Data reduction by the auto-indexing routine in *DENZO* indicate an orthorhombic space group with cell dimensions a = 89.4, b = 138.0 and c = 164.9 Å. The systematic absences in the data set are consistent with the $P2_12_12_1$ space group. This tentative space-group assignment was confirmed using precision photographs.

A native data set was collected from two different crystals at room temperature. The overall merging R factor (on intensities) was 8.3% to 3.2 Å resolution. Data were observed out to 2.5 Å, but the low number of observations for this high-resolution data resulted in much higher R factors. The current data set is 80% complete to 3.2 Å and contains 27 769 reflections (Table 1).

Assuming four subunits per asymmetric unit, the specific volume of the protein is 2.41 Å^3 Da^{-1}, which corresponds to 49.0% solvent content. This density is well within the range normally found for proteins (Matthews, 1968). Assuming

either three or five subunits in the asymmetric unit leads to unreasonable packing densities.

A heavy-atom derivative screen is now under way. $Pb(NO_3)_2$, $MeHg(Cl)_2$, and $AuCl_3$ all cause the crystals to crack. Conditions are now being optimized for these heavy atoms and for others to produce useful derivatives.

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