Crystal structure of p-hydroxybenzoate hydroxylase

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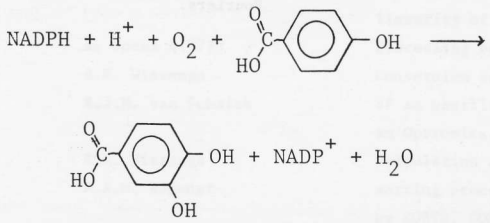
SUMMARY

This thesis deals with the crystal structure determination of the complex of p-hydroxybenzoate hydroxylase (PHBH) and its substrate, p-hydroxybenzoate by X-ray crystallographic methods at a nominal resolution of 2.5 Å.

p-hydroxybenzoate hydroxylase.

PHBH is an intracellular bacterial flavo-enzyme. It catalyzes the hydroxylation of p-hydroxybenzoic acid into 3,4-dihydroxybenzoic acid:



The reaction is catalyzed via a number of separate steps. The reduction of FAD occurs only after the enzyme-substrate complex has been formed. The reaction of O_2 with $FADH_2$ occurs only after $NADP^+$ has been realeased from the reduced enzyme. In the Introduction of this thesis the various steps are discussed in more detail.

The enzyme exists in solution as a dimer; the molecular weight of one subunit is 43 000. No S-S bridges have been demonstrated.

The crystals.

PHBH-crystals grow in 0.1 M potassium phosphate buffer, pH = 7.5, with 1 mM p-hydroxybenzoate. Ammonium sulphate is added to 39% saturation and 0.002 mM FAD, 0.15 mM EDTA and 0.1 mM reduced

glutathion are present as stabilizing compounds. The space group is $C222_1$ with cell dimensions of a = 71.7 Å, b = 146.4 Å and c = 88.6 Å. The asymmetric unit contains one subunit. It has been shown that the PHBH crystals exhibit a residual enzymatic activity in their mother liquor.

Data collection.

The diffraction data was collected according to the oscillation method. In this method the reflections are recorded on a flat film, perpendicular to the X-ray beam, while the crystal is rotated or oscillated over a small angle about an axis, which is perpendicular to the X-ray beam and which is parallel to the film. A great number of reflections are recorded on a film only partially ("partials").
An important feature of this oscillation method is, that the intensities of reflections which are recorded partially on two contiguous films can be obtained by adding the intensities of the two corresponding partials.

It is known that the optical density of an exposed film is only up to a certain value linearly related to the intensity of the incident X-ray beam. This non-linearity was corrected for with a calibration curve.

Various calculations have been carried out to check the reliability of the amplitudes calculated from combined partials. The results show that these amplitudes do not systematically deviate from the amplitudes derived from data which were recorded on one film. The variance of the combined partials is only slightly larger than the variance of the fully recorded data.

No absorbtion correction has been applied. This would have been an improvement because various observations suggest that systematic variations in the amplitudes are introduced e.g. due to the absorbtion by the glass capillary.

23 crystals have been used for the collection, with the oscillation camera, of four data sets. The data of different film packs which together form one data set were scaled with respect to each other according to the refinement procedure of Hamilton, Rollett and Sparks by refining a relative scale factor and a relative temperature factor.
The innermost reflections, with a resolution between 73.2 Å and 23 Å were obtained by taking precession photographs.

Refinement of the heavy atom parameters and determination of the phase angles.

The protein phase angles were obtained by the method of multiple isomorphous replacement and anomalous scattering (MIRAS). The three heavy atom derivatives contained p-chloromercury(II)-benzoate, ethylmercury(II)phosphate and sodium gold(I)dicyanide respectively. The heavy atom parameters were initially refined by the Rossmann procedure and subsequently by the lack-of-closure error method.
The size of the anomalous differences and the size of the error in the amplitudes are approximately equal. However, by calculating Kraut-Fouriers from data in different ranges of increasing resolution it could be shown that the anomalous differences of the heavy atom derivative data sets contained useful information even at a resolution better than 3.0 Å.
The final electron density map was calculated with 13221 reflections, which is 80% of the maximal possible number of reflections in the asymmetric part of the 2.5 Å resolution sphere. 65% of the possible data between 2.8 Å and 2.5 Å was included in the final calculations. The Fourier was calculated using the "best" phases. The mean figure-of-merit was 0-74.

The structure of PHBH.

The boundaries of the subunit could be traced unambiguously in the electron density map. The FAD could be recognized easily. The course of the main chain was clear in most regions and all

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electron density is used in the interpretation of the map. The 60 amino acids at the N-terminus from which sequence information is available do fit in the electron density. However, it cannot be excluded that in a few regions elsewhere the main chain connectivity will turn out to be different from the present interpretation. Therefore only a tentative numbering scheme can be presented. Also the hydrogen bonds have not yet been assigned.
A Kendrew-Watson polyalanine model consisting of 388 amino acids has been built in a "Richards-box".

The subunit has approximately the shape of a rectangular prism with dimensions of 70 x 50 x 45 $Å^3$. Near a crystallographic twofold axis numerous interactions between neighbouring subunits indicate the intersubunit contact region of one molecule.

The folding of the polypeptide chain has resulted in a structure in which Domain II and Domain III can be recognized rather unambiguously. Three, non-adjacent parts of the sequence form Domain I.

16% of the residues occur in one parallel and three antiparallel β-sheets. 26% of the amino acids form eight helices; the two longest helices consist of 5.5 and 7 turns respectively.

FAD is bound in an extended conformation. The AMP moiety binds near a βαβ folding unit in an equivalent manner to that in lactate dehydrogenase.
The isoalloxazine ring presumably points with its dimethylbenzene end to the solvent.
The N(5)-edge of the isoalloxazine ring points to the substrate molecule, which is bound deep inside the subunit. A conformational change seems to be necessary before the substrate can dissociate from the subunit. This is in agreement with the observation that the cell dimensions change when crystals are soaked in mother liquor without substrate.

The binding site for NADPH and O_2 has not yet been established. It is postulated that both the pyridine-moiety of NADPH and the O_2 molecule bind in the same small pocket near the isoalloxazine ring. This pocket is accessible via a large groove, which divides the subunit in two lobes.