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The Sensitivity of the Synchrotron Laue Method to Small Structural Changes: Binding Studies of Human Carbonic Anhydrase II (HCAII)

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Abstract

Human carbonic anhydrase II has been studied using Laue crystallography and short exposure times (3–20 s) at the Daresbury Synchrotron Radiation Source (SRS). Two types of crystals were investigated, the enzyme in complex with the inhibitor bisulfite and the enzyme at pH 6.0. A bisulfite ion and a water molecule, respectively, are bound with tetrahedral coordination to the zinc ion. These results have been subsequently confirmed by monochromatic methods. Synchrotron Laue crystallography is evidently able to characterize minor structural differences.

Introduction

The zinc enzyme carbonic anhydrase is widely spread among living organisms and at least seven genetically distinct isoenzymes are known in mammals (Tashian, 1989). The isoenzyme II is a high activity form, the structure of which is known (Liljas *et al.*, 1972) and well refined (Eriksson, Jones & Liljas, 1988). The enzyme is inhibited by a number of inorganic ions as well as by sulfonamides (Lindskog *et al.*, 1984).

Extensive spectroscopic investigations of Co^{2+}-substituted carbonic anhydrases and crystallographic studies of the native enzyme have indicated that the coordination of the metal varies depending on the pH and the type of ligand (Lindskog, 1963; Bertini & Luchinat, 1982; Eriksson, Jones & Liljas, 1988; Vidgren, Liljas & Walker, 1990). This is in agreement with EXAFS studies of the Co^{2+} enzyme (Yachandra, Powers & Spiro, 1983). The cobalt electronic absorption spectrum of the enzyme when inhibited by thiocyanate is interpreted as originating from a penta-coordinated metal (Bertini & Luchinat, 1982). This has been confirmed by crystallographic methods on the zinc enzyme (Eriksson, Kylsten, Jones & Liljas, 1988). In addition to the three histidine ligands the inhibitor occupies a fourth and a water molecule a fifth coordination position.

We wanted to extend these studies of penta-coordinated zinc in carbonic anhydrase in order to further elucidate the structure and function of the enzyme. At the same time we wanted to test the potential of Laue crystallography to observe small details in electron density which is a prerequisite to time-resolved protein crystallography. In contrast to the spectroscopic observations of the Co^{2+} enzyme we have observed that the zinc ion of the crystallized enzyme is tetra-coordinated in complex with bisulfite as well as at pH 6.0 where a water molecule is situated at the fourth coordination site. Interestingly, EXAFS studies of Zn^{2+} carbonic anhydrase found that the average coordination number seems independent of pH, or of inhibitor binding, and is judged to be four (Yachandra *et al.*, 1983).

Experimental

Crystals of human carbonic anhydrase II with space group $P2_1$, cell parameters $a = 42.7$, $b = 41.7$, $c = 73.0$ Å, $\beta = 104.6°$, were grown, complexed with Hg^{2+}, in 2.3 M $(NH_4)_2SO_4$ at pH 8.5. Soaking experiments with the crystals are summarized in Table 1. Diffraction data were collected at 293 K on experimental stations 9.6 and 9.7 at the Daresbury Synchrotron Radiation Source (SRS), using polychromatic (Laue) radiation in the interval 0.40–2.6 Å on stations 9.6 and 9.7. Data were extended to a resolution of 2.2 Å and were sampled with a completeness of 55–70% (Table 2).

The crystals were elongated plates in morphology and were mounted with the a axis approximately

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Table 1. *Soaking conditions for the forms investigated*

6m*M* β-mercaptoethanol was added to all buffers to extract Hg^{2+}. The soaking time was 24 h at 293 K.

Derivative	Inhibitor concentration (m*M*)	Preciptant	pH
HSO_3^-	300	2.4 *M* $(NH_4)_2SO_4$	7.3
pH 6	-	2.4 *M* $(NH_4)_2SO_4$	6.0 (80 m*M* citrate buffer)

along the capillary. In this orientation a total rotation of 90° of the crystal yields a complete pass through the unique portion of reciprocal space for this space group. The angular interval used between each exposure was 10–12.5° and the crystal-to-film distance was 95 mm. At this distance, a film of 59 mm radius subtends an acceptance angle $2\theta_{acc}$ with respect to the direct beam of 31.8°. For a resolution limit, d_{min}, of 2.2 Å and a maximum wavelength, λ_{max}, of 2.6 Å the maximum Bragg angle possible is $\theta_{max} = \sin^{-1}\lambda_{max}/2d_{min} = 36.2°$ (*i.e.* $> 2\theta_{acc}$). Hence, there is an angular truncation of the Laue pattern (Cruickshank, Helliwell & Moffat, 1987), *i.e.* there are diffracted spots extending beyond the edge of the film. The choice of 95 mm, rather than a shorter crystal-to-film distance, was to reduce the proportion of spatially overlapping spots (Helliwell, 1985). Inevitably there is a certain proportion of recorded spots which contain energy overlaps. This has the effect that in the resolution band of $2d_{min}$ (4.4 Å here) to ∞, there are only a few single-component Laue spots (Cruickshank *et al.*, 1987). For the purposes of refinement and $(F_o - F_c)$ maps the completeness of the data between d_{min} and $2d_{min}$ is of greatest interest, whereas for $(2F_o - F_c)$ maps it is the overall completeness that is of interest; Table 2 gives both values.

Data were integrated and scaled using the Laue processing program suite developed at Daresbury (Helliwell, Habash *et al.*, 1989). Structure factors were calculated using the *PROTEIN* program (Steigemann, 1974) with the coordinates of the native enzyme (Eriksson, Jones & Liljas, 1988) used as the initial phasing model. Four water molecules (see Fig. 1 and Table 3) in the immediate environment of the zinc ion were removed to yield an unbiased electron density. $(F_o - F_c)$ and $(2F_o - F_c)$ electron density maps were calculated and examined on an Evans & Sutherland PS390 computer-graphics system utilizing the program *FRODO* (Jones 1978, 1982). Water molecules originally deleted from the active site were reinserted as their electron density appeared in the electron density maps, and the HSO_3^- inhibitor was inserted as suggested by the electron density maps. Several passes of least-squares constrained refinement were performed using *PROLSQ* (Hendrickson & Konnert, 1980) interspersed with examination of subsequently calculated electron density maps using the computer-graphics system. In the refinement, the atomic temperature factors of the native structure were kept owing to the limited amount of data at 2.2 Å resolution and a completeness of 55–70%. The crystallographic *R* factor in this way decreased to about 19%. The results of the refinement are summarized in Table 3.*

Results and discussion

The low initial *R* values (Table 3) and the low level of noise (Table 4) in the electron density maps suggest that the molecule undergoes only slight changes outside the active site and that the Laue method is capable of showing fine structural details. The $(F_o - F_c)$ maps adequately reveal the absence or presence of water molecules and ligands, while the $(2F_o - F_c)$ maps lack density on some points of both the main chain as well as on some long side chains. This is most certainly owing to the incompleteness of the data sets in the respective resolution bands, the $(2F_o - F_c)$ maps being particularly vulnerable, in terms of connectivity, to missing low-resolution data. This has been examined using monochromatic data in the case of concanavalin-A (Wan, 1990).

* Atomic coordinates and structure factors have been deposited with the Protein Data Bank, Brookhaven National Laboratory (References: coordinates for HSO_3^-: 3CAC; for pH 6: 4CAC; structure factors for HSO_3^-: R3CACSF; for pH 6: R4CACSF) and are available in machine-readable form from the Protein Data Bank at Brookhaven. The data have also been deposited with the British Library Document Supply Centre as Supplementary Publication No. SUP 37060 (as microfiche). Free copies may be obtained through The Technical Editor, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England.

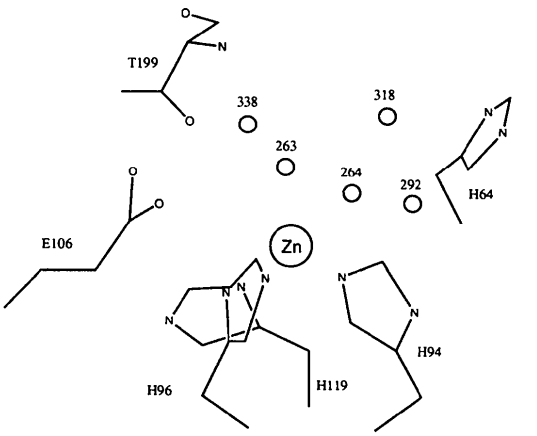

Fig. 1. The active site of carbonic anhydrase with the zinc ion bound to three histidyl residues (94, 96 and 119). Water molecule 263 is situated in a tetrahedral coordination position at the metal and hydrogen bonded to Thr199 which in turn is hydrogen bonded to Glu106. His64 and a number of water molecules in the active site are also shown.

Table 2. *Laue data collection summary*

Data were collected at 293 K.

Derivative	Date data recorded	Station	SRS current (mA)	Exposure time/ pack (s)	No. of packs	No. of crystals	No. of unique data	Completeness of data (%) All	2.2–4.4 Å[a]	>4.4 Å	R_m (%)
HSO_3^-	Sept. 88[b]	SRS 9.7	200	3	16[c]	2	9320	67	81	16	9.4
pH 6	Nov. 88[d]	SRS 9.6	17	20	7	1	7629	55	66	16	7.5

Notes: (*a*) $d_{min} - 2d_{min}$. (*b*) Unfocused beam $0.4 < \lambda < 2.6$ Å. (*c*) Vertically focused beam $0.4 < \lambda < 2.6$ Å. (*d*) Two data sets were merged.

Table 3. *Summary of refinement parameters*

(*a*) *R* values and structural results

Derivative	*R* factor (%) Initial	Final	Waters deleted Initially	Finally	Coordination of Zn
HSO_3^-	23.6	18.9	263, 292, 318, 338	263, 338	4
pH 6	23.4	18.7	263, 292, 318, 338	292	4

(*b*) R.m.s. deviations from standard geometry

Derivative	Bond distances (Å)	Angle distances (Å)	Fixed dihedral distances (Å)	Out of plane restraints (Å^2)	Chiral volume deviation (Å^3)	Coordinate shifts (Å)
HSO_3^-	0.022	0.037	0.035	0.009	0.132	0.013
pH 6	0.019	0.034	0.032	0.008	0.118	0.012

Table 4. *Peak heights in the* $(F_o - F_c)$ *difference electron density maps*

Derivative	Signal peak (σ*)	Highest noise peak (σ)	Signal/ highest noise
HSO_3^-	12.0	4.9	2.45
pH 6	6.5	4.5	1.44

* The value σ is the r.m.s. value of the electron density averaged over the whole map.

The quality of $(2F_o - F_c)$ maps would be improved if the low-resolution data were more complete. A finer angular interval than 10° (used in this study) could be used, *e.g.* about 3°, which could be performed in conjunction with a narrower bandpass at the cost of a considerable increase in data-collection time. Apart from geometric effects, low-resolution data are being lost owing to strong spots saturating the films. Such effects can be avoided by using a larger number of films in the cassette or by using a detector with a better dynamic range (*e.g.* image plate or storage phosphor). Likewise, weak reflections at high resolution are lost owing to the high background of the films. This part of the data can also be better recorded with an image plate.

HSO_3^-: The HSO_3^- ion was modelled into electron density (Fig. 2) so that one of the oxygens coordinates to the zinc ion (Zn—O distance 2.0 Å) and is hydrogen bonded to Oγ of Thr199 (O—O distance 2.73 Å). One of the other oxygens is hydrogen bonded to the peptide nitrogen on Thr199 (N—O distance 2.6 Å). The zinc ion has a tetrahedral coordination. As follows from Table 3, no water molecules except 263 and 338, which are replaced by the inhibitor, are absent from the active site. The resolution and the amount of data do not permit us to detail the extension of density from the bisulfite oxygen bound to the zinc ion. The coordination around the zinc has subsequently been confirmed by a monochromatic data set (Håkansson, Carlsson, Svensson & Liljas, 1992).

pH 6: The zinc ion has the same coordination as at pH 8.5 (Fig. 3). Only one water molecule (263) is seen at the zinc and in the normal ligand orientation. Water 292 (about 4 Å from the zinc ion) is absent,

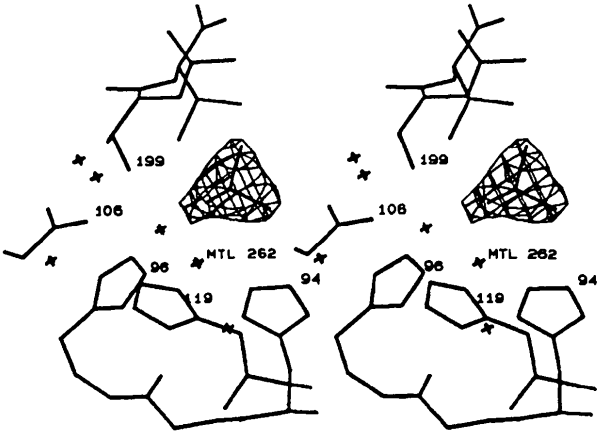

Fig. 2. The bisulfite ion bound at the zinc ion shown with its electron density calculated as an omit map. The ion is bound by only one of its oxygens to the zinc according to the electron density map. The electron density is based on Laue data. The contour level was 0.37 e Å^{-3}.

however, from the electron density maps. The Laue results were subsequently checked by a monochromatic study (Håkansson *et al.*, 1992). The coordination around the zinc was confirmed. Water 292 was visible, however, in contrast to the Laue results.

The spectroscopic investigations of carbonic anhydrases do not contradict the present findings. The electronic absorption spectra of Co^{2+}-carbonic anhydrase shows a variation related to pH or type of bound inhibitor (Bertini & Luchinat, 1982). Thus at low pH, or in complex with bisulfite, the Co^{2+} enzyme gives a spectrum with penta-coordinated characteristics. EXAFS spectra of Co^{2+}-carbonic anhydrase show a similar variation in ligand number (Yachandra *et al.*, 1983). The EXAFS spectra of the Zn^{2+}-carbonic anhydrase on the other hand indicate very little variation in the number of ligands. The slight variations in the *K*-edge spectrum when changing the pH cannot be interpreted in terms of a change in coordination number but rather in the change of charge of the ligand. Short of penta-coordinated model compounds and with little variation in the *K*-edge spectra, Yachandra *et al.* (1983) conclude that all forms of Zn^{2+}-carbonic anhydrase appear tetrahedrally coordinated. The present results obtained from Laue crystallography are in agreement with EXAFS studies on the zinc enzyme. It is evident that X-ray crystallography on the Zn^{2+}- and Co^{2+}-carbonic anhydrases can give further insight into the variations in coordination and into the interpretation of the spectroscopic results. Such work is in progress, including the investigation with monochromatic radiation of the complexes reported here.

It has been suggested (Eriksson, Kylsten *et al.*, 1988; Lindahl *et al.*, 1990) that only protonated ligands are able to replace water 263 (hydroxyl at higher pH) at its original position. This is owing to the fact that Glu106 is hydrogen bonded to Thr199 (2.4 Å). Glu106 is expected to be charged and therefore only able to accept a hydrogen bond from Oγ of Thr199. Hence, an atom bound to the tetrahedral position of the zinc must be able to function as a hydrogen-bond donor. Unprotonated groups binding to the zinc such as SCN^- must therefore be displaced to the van der Waals interaction radius from the Oγ of Thr199, thereby giving room for a fifth ligand. The current results are consistent with this hypothesis.

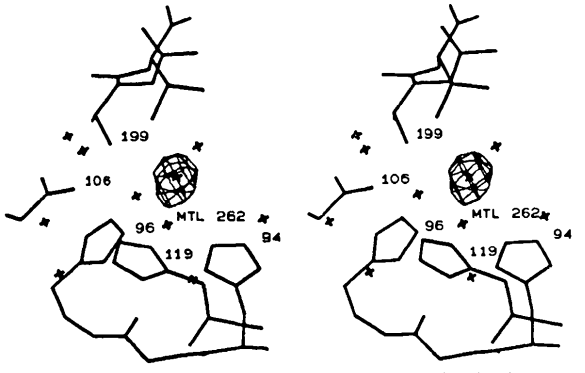

Fig. 3. The active-site region at pH 6. Only one density is seen at the zinc ion corresponding to a water molecule in the tetrahedral position. The electron density is based on Laue data. The contour level was 0.37 e $Å^{-3}$.

These studies show that synchrotron Laue photography of protein crystals can accurately reveal the details of small structural changes, *e.g.* a single water molecule or one HSO_3^- in 30 000 Daltons of protein. An earlier study on a small-molecule crystal showed that the Laue method was able to reveal all the hydrogen atoms in that structure (Helliwell, Gomez de Anderez, Habash, Dodson & Helliwell, 1989).

Clearly there is scope for improvement of the Laue method. Firstly, a more homogenous sampling of reciprocal space, particularly the low-resolution data, would be beneficial. There has been some success in deconvoluting doublet harmonic overlapped spots (Helliwell, Habash *et al.*, 1989; Helliwell, Harrop *et al.*, 1989) but triplet and higher harmonics have not been deconvoluted with sufficiently reliable results so far. Second, the weaker spots, particularly at higher resolution, are adversely affected by background. The implementation of a toast rack, a three-dimensional arrangement of films (Helliwell, 1991), can lead to peak to background improvements for those Laue data recorded on the rear films (Weisgerber & Helliwell, 1992).

The main advantage of the synchrotron Laue method lies in kinetic studies, examining small crystals or perturbation studies, rather than routine structure solving, where monochromatic methods are appropriate.

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Solid-State Conformations of Aminosuccinyl Peptides: Structure of *tert*-Butyloxycarbonyl-L-prolyl-L-aminosuccinyl-glycyl-L-alanine Methyl Ester (Boc-L-Pro-L-Asu-Gly-L-Ala-OMe). A Case of Pseudo-Translational Symmetry

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Abstract

$C_{20}H_{30}N_4O_8$, $M_r = 454.48$, monoclinic, $P2_1$, $a = 13.411$ (2), $b = 12.592$ (2), $c = 14.710$ (1) Å, $\beta = 104.30$ (1)°, $V = 2407$ (6) Å^3, $Z = 4$, $D_x = 1.254$ Mg m^{-3}, λ(Cu $K\alpha$) = 1.5418 Å, $\mu = 0.783$ mm^{-1}, $F(000) = 968$, room temperature, final $R = 0.086$, $wR = 0.080$ for 4055 observed reflections. The title compound is a model for the intermediate formed in the deamidation reaction of porcine adrenocorticotropin hormone. The structure presents a pseudo-translational symmetry and was solved by using a modified version of the *SIR*88 package. In the refinement, few stereochemical restraints were needed to handle the static disorder shown by the C-terminal fragment of one molecule in the asymmetric unit. The conformation of the two independent molecules is almost identical and is a II′ β-bend, stabilized by an intramolecular hydrogen bond. In the crystal, screw-related molecules are linked by hydrogen bonds. The two molecules in the independent unit are related by the translation vector $\mathbf{u} = 0.4962\ (2)\mathbf{a} + 0.7310\ (2)\mathbf{b} + 0.5075\ (2)\mathbf{c}$.

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Introduction

In protein chemistry the non-enzymatic deamidation of the asparaginyl side chain is a well-characterized process, which transforms specific asparagines into aspartyl and isoaspartyl residues (Clarke, 1987). In particular, porcine adrenocorticotropin hormone (ACTH) has been shown to deamidate easily at Asn in position 25 (Graf, Bajusz, Patthy, Barart & Cseh, 1971). During the deamidation process an aminosuccinyl (Asu) residue is formed as an intermediate.