FEBS Letters 352 (1994) 137–140

FEBS 14549

Modification of a metal ligand in carbonic anhydrase: crystal structure of $His^{94} \rightarrow Glu$ human isozyme II

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Received 9 August 1994

Abstract One of the zinc ligands in human carbonic anhydrase II, His^{94}, has been replaced with glutamic acid by site-directed mutagenesis. The mutation leads to a less stable zinc binding site and to significant non-local perturbations of the protein structure. The crystals are composed of a mixture of holo- and apoenzyme, and the side chain of Glu^{94} has two conformations. In the holoenzyme, Glu^{94} coordinates to the metal ion and is hydrogen bonded to Gln^{92}. In the apo form, Glu^{94} is hydrogen bonded to Asn^{67}. The mutation has resulted in a 500-fold decrease of the catalyzed rate of CO_2 hydration (k_{cat}/K_m).

Key words: Carbonic anhydrase; Site-specific mutagenesis; X-ray crystallography; Zinc coordination

1. Introduction

The catalytically essential zinc ion in carbonic anhydrase is firmly coordinated to the imidazole rings of three conserved histidine residues, His^{94}, His^{96} and His^{119}, which are located on two adjacent, antiparallel β-strands. These ligands have very low crystallographic temperature factors and they undergo minimal perturbations upon binding of substrates or inhibitors or upon mutations at various positions in the active site [1–3]. These observations indicate that the zinc center is a stable and rather rigid entity and that its integrity is a prerequisite for proper catalytic function.

To test the effects of altering the metal center, we have used site-specific mutagenesis to replace one of the zinc ligands with a glutamate residue which is a common ligand in zinc-containing enzymes. We selected His^{94} because it has a considerably larger surface accessibility than His^{96} and His^{119} and, therefore, this position might accommodate changes without severe alterations of the protein structure.

2. Materials and methods

In vitro site-directed mutagenesis was performed with the MutaGene system (Bio-Rad), which is based on the method of Kunkel [4]. Mutagenesis was performed on the single-stranded form of the expression plasmid pACA, which was constructed in the laboratory of Dr. C.A. Fierke, Duke University, NC [5]. $His^{94} \rightarrow Glu$ human carbonic anhydrase II was produced after transformation of the *E. coli* strain BL21/DE3 [6] with the mutant plasmid. The cell growth conditions described by Forsman et al. [7] were used except that $ZnSO_4$ was added to a final concentration of 0.5 mM and that the cells were grown at room temperature. The mutant enzyme was purified by ion-exchange chromatography on S-Sepharose Fast Flow (Pharmacia), which was equilibrated with 20 mM MES-NaOH, pH 6.2. The enzyme was eluted by step-wise increases of the $(NH_4)_2SO_4$ concentration and subsequently dialyzed against 50 mM Tris-sulfate at pH 8.5 for 4 days.

Initial rates of CO_2 hydration were measured in a Hi-Tech stopped-flow apparatus at pH 8.8 and 25°C by the changing pH-indicator method [8,9]. The buffer-indicator pair was TAPS-NaOH with metacresol purple monitored at 578 nm. $ZnSO_4$ was added to the buffer in a 1:1 enzyme/Zn^{2+} ratio. The ionic strength was maintained at 0.1 M by the addition of Na_2SO_4. Proper folding of the mutant enzyme was checked by recording circular dichroism spectra between 200 nm and 320 nm in a Jasco-720 spectropolarimeter. A Shimazu RF-500 spectrofluorimeter was used to investigate the binding of the fluorescent inhibitor dansylamide (5-dimethylaminonaphthalene-1-sulfonamide).

Crystals of the mutant enzyme were grown by the hanging drop method using 2.3 M $(NH_4)_2SO_4$ in 50 mM Tris-H_2SO_4, pH 8.5, containing methyl mercuric chloride in a 1:4 molar enzyme/CH_3HgCl ratio to prevent formation of intermolecular disulfide bonds [10]. X-ray intensity data were collected using a Siemens (Xentronics) multiwire area detector mounted on a Rigaku RU200HB rotating anode generator (Cu K_α radiation, 0.3 × 0.3 focal spot, graphite monochromator). The detector was placed 8.0 cm from the crystal. The Siemens 3-axis goniostat with a fixed $\chi = 45°$ was used with $2\theta = 15°$. The crystal was rotated 180° about ω with a step size of 0.30° and an exposure time of 150 s per frame. The total data were collected from three crystals during about 36 h. The reflection data were integrated, reduced, merged and scaled using the Xengen program suite [11]. We measured 24283 intensities corresponding to 13924 independent reflections to 2.1 Å resolution (89% of the available reflections). R_m was 7.5%. A table with statistics of data collection and refinement is available upon request.

All calculations were done on a VAX workstation 3100. $2|F_o|-|F_c|$ and $|F_o|-|F_c|$ electron density maps were calculated with the CCP4 program package [12] and inspected at several stages of the work on the ESV and Evans & Sutherland PS390 stations using the programs FRODO [13,14] and O [15]. The least squares refinement [16] was carried out with the program PROFFT [17] refining both atomic positions and individual temperature factors. The starting model for the refinement was the refined structure of human carbonic anhydrase II at 1.54 Å resolution [3] without the side chain of residue 94 and 11 of the active site water molecules. The side chain of Glu^{94} was subsequently built into the model based on the electron density maps.

3. Results

After extensive dialysis against water the $His^{94} \rightarrow Glu$ mutant contained 0.37 mol of zinc per mol of enzyme indicating a rather weak binding of the metal ion. In the presence of one added equivalent of Zn^{2+} (17 μM) we obained the following steady-state kinetic parameters for the catalyzed CO_2 hydration in 50 mM TAPS-sulfate buffer at pH 8.8 and 25°C: $k_{cat} = 500\ s^{-1}$, $K_m = 2.2$ mM, $k_{cat}/K_m = 2.3 \times 10^5\ M^{-1} \cdot s^{-1}$. The corresponding values for the unmodified enzyme are $k_{cat} = 1 \times 10^6\ s^{-1}$, $K_m = 10$ mM, $k_{cat}/K_m = 1 \times 10^8\ M^{-1} \cdot s^{-1}$ [18]. When 5 μM of the mutant enzyme was added to 10 μM of the fluorescent inhibitor, dansylamide, at pH 7.5, no change of the emission spectrum attributable to the formation of an enzyme–dansylamide

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SSDI 0014-5793(94)00936-8

complex could be observed. Addition of 50 mM Zn^{2+} did not alter the spectra. These results indicate $K_d > 200$ μM for the dansylamide complex of the mutant enzyme, while the wild-type enzyme has a K_d value of about 1 μM under similar conditions.

The crystal structure was refined at 2.1 Å resolution to a final R value of 15.2%. Significant deviations from the structure of wild-type enzyme were observed. Thus, the root-mean square deviation was 0.27 Å for Cα atoms, 0.42 Å for all atoms and 0.53 Å for side chain atoms. The Cα atom of residue 94 has moved 0.7 Å compared to its position in wild-type enzyme. The zinc ion was refined to half occupancy with a B factor of 24.8 $Å^2$. It shows a well-defined electron density in the $2|F_o|-|F_c|$ map after refinement. Apparently, the crystals contain approximately equal proportions of holoenzyme and zinc-free apoenzyme. A high B factor of 23.0 $Å^2$ resulted for the zinc-bound water molecule (half occupancy, 2.5 Å from the zinc ion) without bond restraints between it and the zinc ion during refinement. A quite small electron density (contour level of 0.7 σ) for the zinc water was seen in the $2|F_o|-|F_c|$ map, and this probably reflects a very high mobility.

Two different orientations of the side chain of residue 94 were observed (Fig. 1). Each orientation had half occupancy and the B factors were 11 and 15 $Å^2$, respectively. In one orientation Glu^{94} is coordinated to the zinc ion. In the other orientation, presumably representing the apoenzyme, two solvent molecules, Wat^{292} and Wat^{369}, are displaced by the carboxyl oxygens of Glu^{94}. In the wild-type structure, both of these water molecules are located within 4 Å of the side chain of His^{94} and participate in the hydrogen-bonded network connecting the zinc-bound water molecule and His^{64}, which functions as a proton shuttle group during catalysis [1]. The zinc ion has moved 0.6 Å from its position in the wild-type enzyme. The zinc water (Wat^{263}) and the 'deep' water (Wat^{338}) have moved 0.8 Å and 0.7 Å, respectively (Fig. 1). The hydrogen-bond system involving the zinc-bound water molecule, Oγ1 of Thr^{199} and Oε1 of Glu^{106} is maintained [1–3]. while the tetrahedral coordination geometry of the zinc ion is essentially maintained, the mutation has resulted in some changes of bond angles and zinc–ligand distances as shown in Table 1.

Although the overall structure of the protein remains very similar to that of wild-type enzyme, significant non-local perturbations of the structure were observed (Figs. 1 and 2). The Cα's of residues 125 through 136, forming an α helix and a turn region on the surface of the protein, have moved between 0.3 and 0.9 Å. In addition, the β-strands neighboring the zinc ion show a coordinated positional adjustment in response to the changes in metal coordination. For example, the side chains of

Table 1
Geometry of zinc coordination in His^{94}→Glu human carbonic anhydrase II

Atom	Distance (Å)	Angle (°)		
X	X–Zn	X–Zn-94	X–Zn-96	X–Zn-119
O 263	2.5 (2.1)	99.7 (111.1)	112.3 (113.6)	114.3 (112.9)
Oε1 (Nε2) 94	2.3 (2.1)		110.0 (103.9)	129.5 (115.3)
Nε2 96	2.4 (2.1)			90.9 (99.2)
Nδ1 119	2.3 (2.1)			

Values in parentheses refer to wild-type enzyme [3]. O 263 represents the oxygen atom of the zinc-bound water molecule.

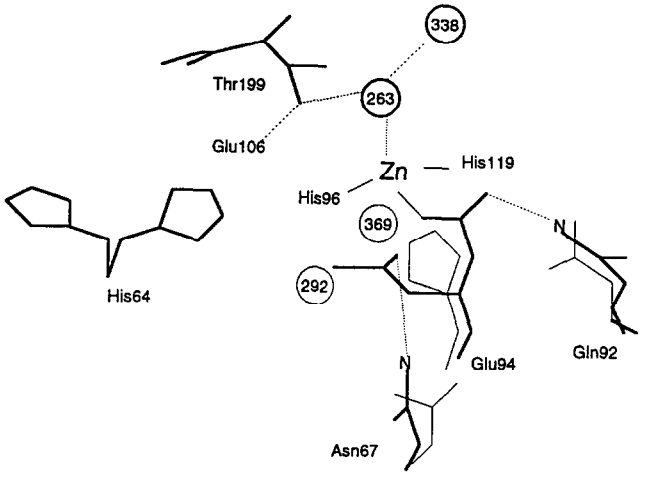

Fig. 1. Schematic diagram of part of the active site of human His^{94}→Glu carbonic anhydrase II. Both Glu^{94} and His^{64} are shown in two orientations. Circles indicate positions of water molecules. Positions of Asn^{67}, Gln^{92} and His^{94} in wild-type enzyme are drawn with thin lines.

Asn^{67}, Glu^{69} and Gln^{92} have undergone drastic rotations. The B factors of the side chain atoms of Gln^{92} are twice as high as for wild-type enzyme (23.4 and 11.7 $Å^2$, respectively). The Nε2 atom of Gln^{92} is hydrogen bonded to Oε2 of the metal-bound orientation of Glu^{94} and a water molecule. The Oε1 atom of Gln^{92}, hydrogen bonded to Nδ1 of His^{94} in wild-type enzyme, is linked to a water molecule in the mutant. In wild-type enzyme, Asn^{67} is within 4 Å of both His^{94} and Gln^{92} and linked to Asn^{62} via a water molecule. In the mutant structure, Asn^{67} is hydrogen-bonded directly to Asn^{62} as well as to the non-coordinated orientation of Glu^{94}. Two conformations with nearly equal occupancy (6:4) were observed for the side chain of His^{64} (Fig. 1).

4. Discussion

Three major observations have emerged from this study. First, the replacement of the strictly conserved zinc ligand, His^{94}, with Glu results in minor structural adjustments and the overall fold of the protein is intact. Second, the zinc ion interacts with Glu^{94}, but the affinity appears to be greatly reduced. Third, the modified enzyme has a residual catalytic activity.

Unmodified human carbonic anhydrase II binds zinc with a very high affinity. Lindskog and Nyman [19] estimated an affinity constant, K_a, of 1×10^{12} M^{-1} at pH 7 and Kiefer et al. [20] recently determined a value of 2.5×10^{11} M^{-1} at the same pH. While we have not estimated the affinity constant for zinc binding to the mutant with Glu^{94}, such data have been published for mutants with Asp^{94} ($K_a = 7 \times 10^7$ M^{-1}, pH 7) and Cys^{94} ($K_a = 3 \times 10^7$ M^{-1}, pH 7) [21,22]. The structure of the His^{94}→Glu mutant offers some explanations of the reduced zinc affinity. In wild-type enzyme, the three histidine ligands are rigidly posed to coordinate zinc in a suitable geometry. The positions of these ligands, as well as their hydrogen-bond contacts with other protein groups, are essentially unchanged in the apoenzyme, and a water molecule occupies the position left by the zinc ion [3]. Thus, the zinc ion can bind with a minimal structural change, which is an entropic advantage.

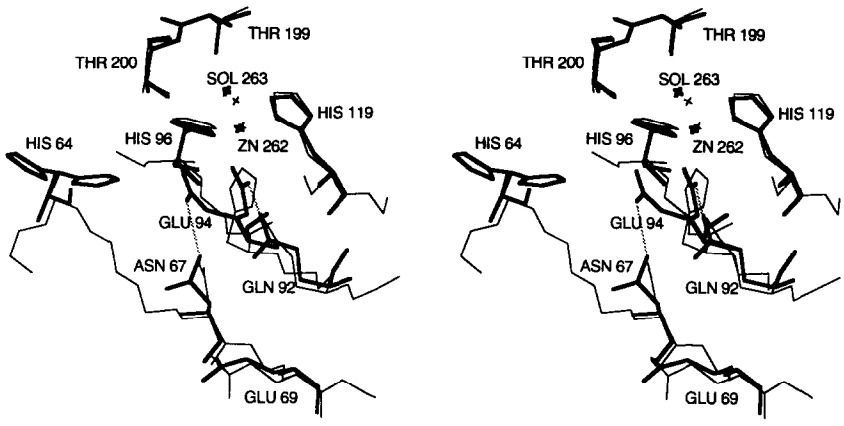

Fig. 2. Stereo diagram of part of the active site of human $His^{94} \rightarrow Glu$ carbonic anhydrase II. The zinc ion and the zinc-bound water molecule are represented by crosses. Thin lines and crosses represent wild-type enzyme. Dotted lines represent hydrogen bonds. All hydrogen bonds are not shown.

In the Glu^{94} mutant, our data suggest that this ligand changes its position as the zinc ion is removed, breaks its hydrogen bond with Gln^{92} and establishes a new hydrogen bond with Asn^{67} (Fig. 1). In addition, the adjustment of the structure to accommodate Glu^{94} as a zinc ligand has resulted in significant increases of all zinc- ligand bond lengths from 2.1 Å in the wild-type enzyme to 2.3–2.5 Å in the mutant (Table 1).

The interaction of carboxylate groups with metal ions in protein structures has been surveyed by Chakrabarti [23]. Both bidentate and monodentate binding of carboxylate groups have been observed and both *syn* and *anti* geometries relative to the carboxylate group have been found. Carboxylate–zinc interactions normally show distinct *syn* geometry [24]. The geometry of the Glu^{94}-zinc interaction in our mutant is rather different from what has been found in zinc protein structures currently available in the Brookhaven Protein data Bank. The binding is monodentate with the projection of the Zn-Oε1 bond on the carboxylate plane being close along the Oε1–Cδ direction (5.4°) and with a deviation of 62° from the carboxylate plane. In this $His^{94} \rightarrow Asp$ mutant, a monodentate carboxylate coordination is also observed, but the interaction is close to the plane of the carboxylate group in a *syn* geometry [21].

Carbonic anhydrase is the only known zinc protein in which all three protein ligands are from tightly lined β strands. When protein carboxylate groups are involved in metal binding, they usually come from helices, turns or regions with no regular secondary structure [23], all of which normally form more flexible binding sites than β structures. While the rigidity of the β-structure scaffold presumably contributes to the strong binding of zinc to wild-type enzyme, it might prevent any alternative protein ligand from forming optimal interactions with the metal ion.

The substitution of Glu^{94} for His^{94} results in a decrease of the maximal rate of catalyzed CO_2 hydration by a factor of 2000. Wild-type carbonic anhydrase II has an exceptionally high turnover rate, which has been partly attributed to a high nucleophilicity of a hydroxide ion bound to zinc in a complex of low coordination number with otherwise neutral ligands [25]. Increased bond lengths and the introduction of a negatively charged carboxylate group in the coordination sphere are factors that might lead to a decreased reactivity of a coordinated OH^- in the mutant. In addition, Glu^{94} probably interferes with the formation of the appropriate transition state of the CO_2–HCO_3^- interconversion step. It has been proposed that the structure of this transition state is similar to that of the HCO_3^- complex with $Thr^{200} \rightarrow His$ carbonic anhydrase II [1]. In this mutant, the protonated oxygen of the HCO_3^- ion replaces the zinc-bound OH^- ion, while a negatively charged oxygen atom is located 3.1 Å from Cε1 of His^{94} [26]. Superimposing this HCO_3^- orientation on the structure of the Glu^{94} mutant, we find that the distance between this negatively charged HCO_3^- oxygen and a carboxylate oxygen of Glu^{94} is only 2.9 Å.

Acknowledgements: We thank Ms. K. Wallgren for help with mutagenesis, Dr. A.L. Svensson and Dr. J. Vidgren for help with data collection and Dr. K. Håkansson for allowing access to his program for calculation of bond angles. This work was financially supported by grants from the Swedish Natural Science Research Council to S.L. and to A.L. and by a grant from NUTEK to B.H.J. and A.L.

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