X-ray Structure of β-Carbonic Anhydrase from the Red Alga, *Porphyridium purpureum*, Reveals a Novel Catalytic Site for CO₂ Hydration*

(Received for publication, October 4, 1999, and in revised form, November 22, 1999)

Satoshi Mitsuhashi‡‡, Tsunehiro Mizushima*, Eiki Yamashita†, Masaki Yamamoto, Takashi Kumasaka, Hideaki Moriyama**, Tatzuo Ueki**, Shigeto Miyachi‡, and Tomitake Tsukihara†††

From the ‡‡Marine Biotechnology Institute, Kamaishi Laboratories, Heita, Kamaishi, Iwate, 026-0001 Japan, the †Institute for Protein Research, Osaka University, Yamadaoka, Suita, Osaka, 565-0871 Japan, the ‡¶Harima Institute, RIKEN, Mikazuki, Sayo, Hyogo, 679-5143 Japan, and the **Japan Synchrotron Radiation Research Institute, Mikazuki, Sayo, Hyogo, 679-5198 Japan

The carbonic anhydrases (CAs) fall into three evolutionarily distinct families designated α-, β-, and γ-CAs based on their primary structure. β-CAs are present in higher plants, algae, and prokaryotes, and are involved in inorganic carbon utilization. Here, we describe the novel x-ray structure of β-CA from the red alga, *Porphyridium purpureum*, at 2.2-Å resolution using intrinsic wavelength anomalous diffraction. The CA monomer is composed of two internally repeating structures, being folded as a pair of fundamentally equivalent motifs of an α/β domain and three projecting α-helices. The motif is obviously distinct from that of either α- or γ-CAs. This homodimeric CA appears like a tetramer with a pseudo 222 symmetry. The active site zinc is coordinated by a Cys-Asp-His-Cys tetrad that is strictly conserved among the β-CAs. No water molecule is found in a zinc-liganding radius, indicating that the zinc-hydroxide mechanism in α-CAs, and possibly in γ-CAs, is not directly applicable to the case in β-CAs. Zinc coordination environments of the CAs provide an interesting example of the convergent evolution of distinct catalytic sites required for the same CO₂ hydration reaction.

---

*This work was supported in part by the New Energy and Industrial Technology Development Organization (NEDO). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ Member of the TARA project of University of Tsukuba and a senior visiting scientist of RIKEN.

††† Visiting scientist of RIKEN.

© 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.
carboxylase/oxygenase for CO₂ (12, 13). The CA monomer has a molecular mass of ~55 kDa and contains two atoms of zinc per monomer. We have isolated cDNA clones of the CA (14). The clones encode a 571-residue polypeptide in which two domains, each equivalent to that of other β-CAs (~25–30 kDa), are arranged in tandem and exhibit ~70% identity with each other. Thus, it is suggested that the CA gene of *P. purpureum* has been formed by duplication and fusion of a primordial β-CA gene. In this report, we describe the x-ray structure of *P. purpureum* CA. The study not only provides an understanding of the protein fold of the β-CA family but also reveals the novel architecture of the catalytic site, which is quite distinct from those of the α- and γ-CA enzymes.

**EXPERIMENTAL PROCEDURES**

**Crystallization**—Protein crystallization is described elsewhere. Brieﬂy, the hanging-drop, vapor diffusion method was used to crystallize *P. purpureum* CA, wherein 5 µl of the puriﬁed protein preparation (30 mg ml⁻¹ in 20 mM NaCl, 20 mM Tris-HCl, pH 8.5) was equilibrated against 5 µl of the reservoir solution (24% polyethylene glycol 4000, 300 mM ammonium sulfate, 50 mM sodium cacodylate, pH 6.75) at 20°C.

**Data Collection and Processing**—For data collection under cryogenic conditions, crystals were soaked in the reservoir solution for 1 h, supplemented with 5% (v/v) glycerol and flash-frozen in liquid nitrogen at 100 K. Diffraction data for the frozen crystal were collected at 100 K using RIKEN beamline I (BL44XU) at SPring-8, Japan (15). Multicrystal length anomalous diffraction (MAD) data for intrinsic zinc atoms were collected at remote (1.0000 Å), peak (1.2823 Å), and edge (1.2825 Å) wavelengths.

Diffraction data were recorded on a-RAXS IV image-plate detector with a crystal-to-detector distance of 250 mm. Two data sets at remote and edge wavelengths were simultaneously recorded in the same image plate. Diffraction data were processed with DENZO and intensity data were scaled together using SCALEPACK (16). Results of data collection are given in Table I.

**Structure Determination and Refinement**—The anomalous Patterson Patterson map showed very clear peaks for all vectors among the four zinc atoms in the asymmetric unit. The x-ray structure of CA was determined at 2.2-Å resolution using intrinsic zinc MAD phasing (17). Refinement of atomic parameters of zinc and MAD phase determination were performed at 2.2-Å resolution by program SHARP (18). The ﬁgure of merit was 0.48 for 50,294 reflections. The electron density map calculated with the MAD phases was reﬁned by the solvent flattening method and by the non-crystallographic symmetry averaging method using program SOLOMON (19). The R-factor, R = Σ(|Fo| - |Fc|)/Σ|Fo|, for the reﬁned electron density map was 0.246, where Fo and Fc are observed and calculated structure factors, respectively. An initial model was built using program O (20) and TURBO-FRODO. The model was reﬁned at 2.2-Å resolution with program X-PLOR (21) using non-crystallographic symmetry restraints for the two monomers in the asymmetric unit. The model were revised manually in omit maps or Fo -Fc maps after each cycle of X-PLOR reﬁnement. Twenty cycles of simulated annealing, positional, and temperature factor reﬁnement reduced the R-factor to 0.208 and the Rmerge to 0.274, where Rmerge was a R-factor estimated for 5% reflections excluded from the reﬁnement. In the ﬁnal model, interpretable electron density begins with residue Val84 and ends with Gly564 without interruption. The geometry of the ﬁnal model, as calculated using program PROCHECK (22), is satisfactory with 91.7% of the residues falling into the most favored regions, 8.3% into the additional allowed regions in a Ramachandran plot. The ﬁnal model also includes 4 zinc ions and 613 water molecules for the dimer. Results of MAD phasing and reﬁnement statistics are summarized in Table I.

**RESULTS AND DISCUSSION**

**Structural Duplication in a Monomer**—The monomeric structure of *P. purpureum* CA is shown in Fig. 1A. There are essentially two symmetrical structural motifs in one monomer, resulting from two homologous repeats (~70% sequence identity) in the CA polypeptide (Ref. 14, Fig. 2). The two motifs are related to each other by a pseudo 2-fold axis. Upon superposition of these two halves (N-terminal half, residues 86–309 and C-terminal half, residues 340–563), the root mean square deviations between the Ca atoms are 0.73 Å in monomer A and 0.74 Å in monomer B.

Each motif includes an α/β domain and three projecting α-helices. The α/β domain consists of α-βα units exhibiting Rossmann fold (23) and an anti-parallel β-strand (Fig. 1A). The motif is obviously distinct from that of either α- or γ-CAs. The three projecting α-helices run on the surface of the α/β domain formed by the opposite half of the same monomer. Six main chain hydrogen bonds are formed between the N- and C-terminal halves of a monomer. This means that the N- and C-terminal halves contact each other so intimately that they are unable to divide into two independent structural domains. Internal sequence repeats suggest that the structure of the monomer (~55 kDa) has evolved through gene duplication and fusion of an ancestral CA monomer. On the other hand, β-CA of pea has been reported to be an octameric enzyme with monomers of ~27 kDa (9). It is likely that two monomers of the unduplicated β-CA oligomers, such as pea CA, contact each other similarly as do the two halves of *P. purpureum* CA.

**Dimeric Structure with a Pseudo 222 Symmetry**—An asymmetric unit cell of a crystal contains a dimer of two identical subunits, each with a molecular mass of 55 kDa (Fig. 1B). This CA also exists as a dimer in solution based on gel filtration. The dimer has approximate dimensions of 90 Å × 70 Å × 60 Å. The two monomers are related by a 2-fold axis perpendicular to the pseudo 2-fold axis in the monomer. Consequently, the dimer has a pseudo 222 symmetry. Each monomer makes close contacts with its symmetry counterpart with a contact surface area of 4,000 Å² of each monomer (19% of a monomer’s surface area). A long turn segment (residues 310–339) connecting the

---

**Table I**

| Data collection, MAD phasing, and refinement statistics | Remote | Peak | Edge |
|--------------------------------------------------------|-------|------|------|
| Wavelength (Å)                                         | 1.0000| 1.2823| 1.2825|
| Resolution (Å)                                         | 60.0–2.2 | 60.0–2.2 | 60.0–2.2 |
| Reflections                                            | 49,836| 49,836| 49,836|
| Completeness (%)                                       | 99.8 (99.8)| 97.2 (77.9)| 96.1 (72.9)|
| Rmerge (%)                                             | 7.1 (22.7)| 7.7 (25.0)| 12.8 (38.6)|
| Multiplicity                                           | 6.5 (5.4)| 6.1 (3.6)| 5.4 (3.0)|
| Mean (|Fo|−|Fc|)                                      | 16.4 (4.5)| 12.5 (2.1)| 6.8 (1.2)|

---

2 S. Mitsuhashi, T. Mizushima, E. Yamashita, S. Miyachi, and T. Tsukihara, manuscript in preparation.

3 S. Mitsuhashi, N. Kurano, S. Harayama, and S. Miyachi, unpublished results.
N- and C-terminal halves sticks out from one monomer toward the surface of the counter monomer (Fig. 1B).

Zinc Binding Environment—The two zinc-binding sites reside in two clefts (Fig. 3A) on both sides of a monomer. They are located at the C-terminal ends of the parallel $\beta$-sheets, as often found in $\alpha/\beta$ enzymes with Rossmann folds. One of the catalytic zinc atoms is coordinated in a tetrahedral manner with the S-$\gamma$ atom of Cys149, the O-$\psi$ of Asp151, the N-$\varepsilon$ of His205, and the S-$\gamma$ of Cys208 in the N-terminal half and the other is with equivalent atoms of Cys463, Asp465, His459, and Cys462 in the C-terminal half (Fig. 3B). These residues are strictly conserved among all $\beta$-CAs sequenced to date (Fig. 2). Here, zinc coordinated by the former tetrad is designated Zn-N and that by the latter Zn-C.

The electron density of a water molecule is not found within coordination radius to the zinc atoms, not only in the initial MAD map but also in the $F_o - F_c$ map calculated after the final refinement cycle, indicating that no water or hydroxide participates in zinc coordination in the present x-ray structure. This is the most remarkable difference from the structures of $\alpha$- and $\gamma$-CAs in which a hydroxide or water molecule occupies the fourth liganding site and the fourth ligand has been suggested to be a nucleophile of the CO$_2$ hydration reaction. Thus, we propose that the zinc-hydroxide mechanism is not directly applicable to $\beta$-CAs. Previously, two research groups proposed a Cys-His-Cys-H$_2$O ligand scheme to bind zinc at the active site in higher plant $\beta$-CAs based on extended x-ray absorption fine structure (10, 11) presuming that a zinc-hydroxide catalytic mechanism similar to that of $\alpha$-CAs exists in $\beta$-CAs.

The three zinc-liganding residues other than the Asp suggested in these earlier studies are in agreement with those found in the structure of $P. purpureum$ CA. Kinetic studies of $\beta$-CAs of higher plants have revealed that a basic form of the enzyme is the active species and the $pK_a$ for the activity linked group is approximately 8.5 (10). The present x-ray structure has been determined at pH 6.75. Considering that the side chain carboxyl groups of Asp151 and Asp465 would be more deprotonated at higher pH values, these residues should firmly bind the zinc under more alkaline conditions. In other words, zinc liganding by these Asp residues at neutral pH may not be a crystallization artifact and can also be observed in the basic forms of the enzyme. Based on the structure of $P. purpureum$...
CA, the extended x-ray absorption fine structure data indicate that one of the nitrogen/oxygen atoms involved in the zinc coordination is not that of a water molecule, but of the Asp residue. It is also of interest to note the results of the site-directed mutagenesis analysis of potential zinc ligands in the \( \beta \)-CA of spinach (15), in which the Asn residue was substituted for Asp152 (corresponding to Asp151 and Asp405 in \( P. \) purpureum CA). The mutant enzyme retained little CO2 hydration activity, although the mutant enzyme could bind 80% of the zinc compared with the wild type CA. This result indicates that only one of the two carboxyl oxygen atoms of Asp152 is necessary for zinc binding, and the other carboxyl oxygen or its negative charge is essential for catalytic activity.

The zinc-binding geometries of the N- and C-terminal halves of the \( P. \) purpureum CA monomer are shown superimposed on each other in Fig. 3C. Using the zinc atoms and all atoms of the four ligands as references, a root mean square deviation of 0.73 Å is obtained. Coordination geometries of the two zinc atoms are very similar to each other. However, side chain conformations of the two equivalent Asps are different from each other. These residues have higher temperature factors than the other three zinc ligands and may have lower occupancy and flexibility resulting in conformational variations. The similarity in the coordination geometries is consistent with the results obtained with CA mutants of \( P. \) purpureum CA. When each of the zinc-binding residues in the N- and C-terminal halves was mutated, each of the mutants retained nearly half of the wild-type CA activity. However, results with these mutants differ from previous observations, in which the C-terminal half-polypeptide lost activity but the N-terminal half one retained activity (14). Since the C-terminal half-polypeptide with the long turn segment was expressed, this segment may have seriously impaired folding around the active site.

Active Site Clefts—Out of 23 residues which are strictly conserved in the \( \beta \)-CAs in Fig. 2, including that from higher plants, algae, and prokaryotes, 14 residues are clustered on concave surfaces of the clefts (Fig. 3A). For purpose of clarity, only the cleft surrounding Zn-N is discussed here, but that surrounding Zn-C is basically equivalent. It should be noted that Zn-C is not exclusively surrounded by residues from C-terminal half of the protein. The conserved residues in the cleft are classified into two groups: Group I residues (in the boxes with blue background) are located around Zn-N and Zn-C and reside in the N- and C-terminal halves of the protein, respectively. Zinc ligands are marked by open circles. Group II residues (in the boxes with red background) from the C- and N-terminal halves of the protein are oriented toward Zn-N and Zn-C, respectively. The location of secondary structure elements of the \( P. \) purpureum CA is indicated over the aligned sequences. Wedges represent cleavage sites to generate enzyme subunits of higher plants. Abbreviations used are PorN and PorC for the N- and C-terminal halves of \( P. \) purpureum CA (D86050), Spi for spinach CA (P16016), Coc for CA from the green alga Coccomyxa sp. (U49976), Syn for icfA product from Synechococcus sp. PCC7942 (M77095), and Eco for cynT product from Escherichia coli (M23129). Numbers in parentheses are GenBank accession numbers.

CA
FIG. 3. Zinc coordinating environment of P. purpureum CA. A, stereo diagram of concave surface of active site cleft in which the zinc-binding site is located. The arrangement of the amino acid residues around Zn-C is depicted here, but that around Zn-N is fundamentally equivalent. Conserved residues in the cleft are shown. Group I and group II residues (see Fig. 2) are colored blue and red, respectively. Pro142, Phe168, and Leu195 form a hydrophobic environment adjacent to the zinc ligand, Asp405. B, stereo diagram of the zinc-binding ligands of P. purpureum CA. Experimental electron density derived by MAD phasing obtained after solvent flattening and 2-fold non-crystallography symmetry averaging shows zinc liganding participants (orange, zinc; green, sulfur; red, oxygen; and blue, nitrogen atoms). Cages of electron density were drawn at 1.9σ in blue. In addition, the (Fo - Fc) difference map calculated before assignment of the water molecule is drawn at 3.0σ in green. A water molecule is hydrogen-bonded to O-e1 of the Asp405. Only Zn-C binding site is shown here, but the nature of the site around Zn-N is fundamentally equivalent (see C). C, stereo diagram showing superposition of the zinc ligands of the N- on the C-terminal halves, colored blue and green, respectively. Using the zinc atom and all atoms of the four ligands as references, the root mean square deviations obtained are 0.73 Å. Coordination geometries of Zn-N and Zn-C are very similar. However, side chain conformations of two equivalent Asp residues differ from each other. These residues with higher temperature factors may have flexibility affecting conformational variations. Coordination bond distances (Å) of Cys149, Asp151, His205, and Cys208 for Zn-N are 2.34, 1.90, 1.98 and 2.34, respectively and that of Cys403, Asp405, His459, and Cys462 for Zn-C are 2.31, 1.85, 1.96, and 2.32, respectively. The length were calculated as the average value between the two copies in the crystallographic asymmetric unit. D, hydrogen bond formations of zinc ligands with residues in the immediate vicinity of Zn-C. Four zinc ligands, the Asn and two Tyr residues are conserved in all β-CAAs sequenced to date. Hydrogen bonds are depicted by dotted lines. Note that Tyr190 derives from the N-terminal half of the same monomer. The region around Zn-N is basically equivalent.
Proposed CO₂ hydration mechanism based on the x-ray structure of P. purpureum CA. See text for the explanation of each step.

Proposed CO₂ Hydration Mechanism—Site-directed mutagenesis of zinc ligands of higher plant β-CAs (11, 24) and of P. purpureum CA³ have demonstrated that zinc is essential for catalysis. Although there is no water bound directly to zinc in the x-ray structure, a water molecule exists near each of the sites of zinc in an Fₐ – F₁ electron density map (Fig. 3B). The water molecule is hydrogen-bonded to O-ε1 of the zinc-liganding Asp¹⁵¹ and Asp⁴⁰⁵, but the water does not exist in the direct zinc coordination radius. The CO₂ hydration reaction should require a catalytic water molecule which acts as a nucleophile and which must be at least transiently bound to the zinc. In addition, averaged temperature factors of main chain atoms for tripeptides starting from zinc-liganding Asp are 37 Å² and 39 Å² for Asp¹⁵¹-Ser¹⁵²-Arg¹⁵³ and Asp⁴⁰⁵-Ser⁴⁰⁶-Arg⁴⁰⁷ of monomer A, respectively, and 37 Å² and 40 Å² for those of monomer B, respectively, while those for all residues of monomer A and B are 21 Å² and 21 Å², respectively. The tripeptide sequence is strictly conserved in β-CAs. The ligands other than the Asps have temperature factors of main chain atoms as low as around 12 Å². Higher temperature factors for the tripeptide segments, including the Asp ligands, suggest that the segments are mobile.

We propose a possible mechanism of CO₂ hydration cycle as shown in Fig. 4. Hydrophobic pockets formed by Pro¹⁴², Phe¹⁶⁸, and Leu¹⁹⁵ beside Zn-C and Pro³⁹⁶, Phe⁴²², and Leu⁴⁴⁹ beside Zn-N are candidates for the site of CO₂ association. Presumably, the CO₂ association triggers the subsequent catalytic steps. The zinc-bound aspartate functions as a base to accept a proton from its hydrogen-bonded water and yields a nucleophilic hydroxide (Fig. 4, step 1). As a consequence, the protonated aspartate will be released from the zinc and the resulting nucleophilic hydroxide moves toward and binds the zinc (Fig. 4, step 2). In the next step, the hydroxide attacks the CO₂ molecule to generate zinc-bound HCO₃⁻. The proton is transferred from the protonated aspartate to the bulk solvent or buffer, possibly through one of the hydrogen-bonded pathways immediately surrounding the zinc ligands (Fig. 3D and Fig. 4, step 3). Then the zinc-bound HCO₃⁻ is replaced with a deprotonated Asp, releasing the HCO₃⁻ and leaving a zinc-bound Asp (Fig. 4, step 4). Finally, a water molecule binds O-ε1 of the zinc-bound Asp to regenerate the initial stage (Fig. 4, step 5).

The present results show that β-CA differs from α- and γ-CAs, not only in overall protein folding, but also in the nature of the architecture coordinating and surrounding the catalytic zinc. The absence of a water molecule in the zinc coordination sphere suggests that the zinc-hydroxide mechanism in α-CAs, and possibly in γ-CAs, is not directly applicable in the case of β-CAs. Knowledge of the x-ray structure of P. purpureum CA should provide a more detailed description of the catalytic mechanism of β-CAs.

Acknowledgments—We are very grateful to Y. Hara (Osaka University) for screening crystallization conditions. We thank S. Harayama and N. Kurano (Marine Biotechnology Institute) for helpful comments.

REFERENCES
1. Tashian, R. E. (1990) Bioscience 10, 186–192
2. Badger, M. R. & Price, G. D. (1994) Annu. Rev. Plant Physiol. Plant Mol. Biol. 45, 369–392
3. Hewett-Emmitt, D. & Tashian, R. E. (1996) Mol. Phylogenet. Evol. 5, 50–77
4. Kisker, C., Schindelin, H., Alber, B. E., Ferry, J. G. & Rees, D. C. (1996) EMBO J. 15, 2323–30
5. Liljas, A., Kannan, K. K., Bergstén, P. C., Waara, I., Fridborg, K., Strandberg, B., Carlsson, U., Jarup, L, Levgren, S. & Petef, M. (1972) Nature New Biol. 235, 131–137
6. Håkansson, K., Carlsson, M., Svensson, L. A. & Liljas, A. (1992) J. Biol. Chem. 267, 1192–1204
7. Coleman, J. E. (1967) J. Biol. Chem. 242, 5212–5219
8. Steiner, H., Jonsson, B. H. & Lindskog, S. (1975) Eur. J. Biochem. 59, 253–259
9. Johansson, I. M. & Forsman, C. (1993) Eur. J. Biochem. 218, 439–446
10. Bowdler, R. S., Chance, M. R., Wirt, M. D., Sideling, D. E., Royal, J. R., Woodroofe, M., Wang, Y. F., Saha, R. P. & Lam, M. G. (1994) Biochim. Biophys. Acta 1253–1262
11. Bracey, M. H., Christiansen, J., Tovar, P., Cramer, S. P. & Barlett, S. G. (1996) Biochemistry 35, 2323–30
12. Aizawa, K. & Miyachi, S. (1986) FEBS Letters 215, 223–233
13. Yagawa, Y., Muto, S. & Miyachi, S. (1987) Plant Cell Physiol. 28, 1253–1262
14. Mitsuhashi, S. & Miyachi, S. (1986) J. Biol. Chem. 271, 28703–28709
15. Yamamoto, M., Kumasaka, T., Fujisawa, T. & Ueki, T. (1998) J. Synchrotron Rad. 5, 222–225
16. Otwinowski, Z. & Minor, W. (1997) Methods Enzymol. 276, 307–326
17. Hendrickson, W. A. (1991) Science 254, 51–58
18. de a Fortelle, E. & Bricogne, G. (1997) Methods Enzymol. 276, 472–494
19. Abramhs, J. P. & Leslie, A. G. W. (1996) Acta Crystallogr. Sect. D 52, 30–42
20. Jones, T. A., Zou, J. Y., Cowan, S. W. & Kjeldgaard, M. (1991) Acta Crystallogr. Sect. A 47, 110–118
21. Brünger, A. T. (1992) X-PLOR: A System for X-ray Crystallography and NMR, Version 3.1, Yale University Press, New Haven, CT
22. Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291
23. Rossmann, M. G., Moras, D. & Olsen, K. W. (1974) Nature 250, 194–199
24. Provat, N. J., Majeau, N. & Coleman, J. R. (1993) Plant Mol. Biol. 22, 937–943
X-ray Structure of β-Carbonic Anhydrase from the Red Alga, *Porphyridium purpureum*, Reveals a Novel Catalytic Site for CO₂ Hydration

Satoshi Mitsuhashi, Tsunehiro Mizushima, Eiki Yamashita, Masaki Yamamoto, Takashi Kumasaka, Hideaki Moriyama, Tatzuo Ueki, Shigetoh Miyachi and Tomitake Tsukihara

*J. Biol. Chem.* 2000, 275:5521-5526.
doi: 10.1074/jbc.275.8.5521

Access the most updated version of this article at http://www.jbc.org/content/275/8/5521

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 23 references, 3 of which can be accessed free at http://www.jbc.org/content/275/8/5521.full.html#ref-list-1