Structure of the Phenylhydrazine Adduct of the Quinohemoprotein Amine Dehydrogenase from *Paracoccus denitirifcans* at 1.7-Å Resolution

S. Datta,[1] T. Ikeda,[2] K. Kano,[2] F. S. Mathews[1]
[1]Washington University School of Medicine, St. Louis, MO, U.S.A.
[2]Kyoto University, Sakyo-ku, Kyoto, Japan, U.S.A.

Introduction

The bacterium *Paracoccus denitrificans* contains two amine dehydrogenases. One is the well-known tryptophan tryptophylquinone-containing methylamine dehydrogenase [1], and the other is a quinohemoprotein amine dehydrogenase (QHNDH), which is preferentially induced by growth on n-butylamine [2]. The natural electron acceptor for QHNDH is cytochrome c550 [3]. QHNDH is a heterotrimer of 109-kDa molecular mass. The largest subunit (α) is 60 kDa and contains two heme c per molecule. The second subunit (ß) is about 40 kDa. The smallest subunit (γ) is 9 kDa and stains positively for quinone-dependent redox cycling.

The crystal structure of QHNDH was determined at 2.05-Å resolution by multiple isomorphous replacement [4]. The γ-subunit was found to contain the novel quinone cofactor, cysteine tryptophylquinone (CTQ). In addition, it contains three chemical cross-links involving a cysteine side chain bridged to a carboxylic acid side chain, either aspartate or glutamate. The ß subunit is a seven-bladed ß-propeller. The γ-subunit is located above the ß-subunit, with the CTQ nearly coincident with the sevenfold axis of pseudosymmetry of the ß-propeller. The four domains of the α-subunit are wrapped around the γ-subunit, holding it in place in the center of the molecule.

The CTQ cofactor is located in an internal cavity ~160 $Å^3$ in volume that is the probable binding site for substrate. The cavity is lined by hydrophobic and aromatic groups, about half from the ß subunit and the remainders from the γ subunit. A large fragment of difference electron density within this cavity was modeled as t-butanol, and another strong difference peak was modeled as a sodium ion.

In QHNDH, amine oxidation is believed to follow a ping-pong mechanism in which the quinone cofactor is reduced to an aminoquinol (the reductive half reaction) and then reoxidized by an electron acceptor to regenerate the oxidized quinone form of the cofactor (the oxidative half reaction) [4]. The reductive half reaction proceeds through formation of a Schiff base intermediate, in which the imine nitrogen of the substrate replaces one of the quinone oxygens. O-6 of CTQ is likely to be the site of attack by amine substrates, since O-7 is also hydrogen bonded to a backbone amide group and since O-6 is closer to the substrate analogue, t-butanol, in the crystal structure. However, definitive proof of the identity of the site of attack by substrate has so far been lacking. In this report, we describe the structure determination of the phenylhydrazine inhibited form of QHNDH at 1.7-Å resolution. This structure clearly identifies O-6 of CTQ as the site of inhibitory attack by phenylhydrazine and also extends the resolution of the analysis from the initial 2.05 Å, thereby providing a more accurate structure of the enzyme.

Methods and Materials

The crystals were grown as described previously [4]. To prepare the complex with phenylhydrazine, the native crystals were soaked in solutions supplemented with 5-mM phenylhydrazine. The data were collected at 100K on the Bio Consortium for Advanced Radiation Sources (BioCARS) beamline 14-BM at APS with λ = 0.9 Å. The data were processed by using the HKL package [5]. The results of data collection and processing are given in Table 1.

The starting model for refinement was the structure of QHNDH previously determined at 2.05-Å resolution [4]. Ten percent of the reflections were set aside for cross-validation analysis by means of Rfree. Initial rigid-body refinement was carried out by using CNS [6] to an R factor of 26.3% in the 6.0- to 3.0-Å resolution range. The resolution was increased stepwise from 3.0 to 1.7 Å. After each step, 2Fo-Fc and Fo-Fc electron-density maps were calculated and the model was rebuilt as necessary by using TURBO-FRODO [7]. Water molecules were picked up from the difference map on the basis of the peak heights and distance criteria. A total of 1307 water molecules, three molecules of t-butyl alcohol, and one MES molecule were identified. A summary of the crystallographic refinement is given in Table 1.

Results and Discussion

The crystal structure of the phenylhydrazine derivative of QHNDH (p-QHNDH), determined at 1.7-Å resolution, is consistent with the native QHNDH structure determined earlier at 2.05-Å resolution [4]. p-QHNDH is composed of three subunits. The 489-residue α-subunit, carrying two heme c groups, is folded into four domains.

Table 1. Data collection and refinement statistics.

Data collection statistics	Value
Wavelength (Å)	0.9
Resolution (Å)	30–1.7
Space group	$P4_12_12$
Unit cell	
a (Å)	99.24
c (Å)	213.06
No. of unique reflections	110131
R_{symm} (outer shell)[a,b]	6.0 (48.1)
Completeness (outer shell) (%)	94.2 (75.4)
Redundancy	6.7
I/σ(I) (outer shell)	27.7 (2.4)
Refinement statistics	
R/R_{free} (%)[c]	19.12/22.49
Average B-factors (Å)	22.9
r.m.s deviations	
Bonds (Å)	0.006
Angles (Å)	1.4

[a]The values in parentheses are for the highest-resolution shell.

[b]$R_{symm} = \sum \left| I_i - \langle I \rangle \right| / \sum I_i$, where I_i is the intensity of the i^{th} observation and $\langle I \rangle$ is the mean intensity of the reflections.

[c]$R = \sum || F_o | - | F_c || / \sum | F_o |$, where F_o and F_c are the observed and calculated structure factor amplitudes. R_{free} is calculated by using 10% of reflection omitted from the refinement for both the native data sets.

The ß subunit, a seven-bladed ß-propeller, contains 337 amino acid residues. The γ-subunit contains 82 residues.

The first domain of the α-subunit is a diheme cytochrome consisting of two subdomains, each having three α-helices and a heme group. The other three domains are antiparallel ß-barrel structures, one having eight ß-strands and the other two having seven ß-strands. The 82-residue γ-subunit has little secondary structure. The concave top surface of the ß-subunit provides a base upon which the α and γ subunits rest.

The overall γ-subunit construct is similar to that of the native QHNDH. It is a highly cross-linked globular structure containing four cysteine residues. All four cysteines are involved in highly unusual covalent cross-links to other amino acid side chains. Two are to aspartates, one is to glutamate, and one is to tryptophan, all as carbon-sulfur thioether linkages. The most important cross-link is between Cys37γ and Trp43γ and makes up the part of the CTQ redox cofactor in native QHNDH [4].

In p-QHNDH, the Fourier map clearly indicated positive electron density near the C-6 position of the CTQ, which could be modeled nicely by the hydrazone of CTQ with phenylhydrazine bound to the C-6 position (Fig. 1). The phenyl ring accommodated in the hydrophobic cavity near the active site displaces the sodium ion and the t-butanol found in native QHNDH (Fig. 2). This high-resolution structure of p-QHNDH clearly identifies the catalytic site of CTQ as C-6. Thus we propose that in the catalytic process, the substrate amine reacts with the C-6 carbonyl group CTQ, forming a Schiff base intermediate similar to the proposed reaction mechanism of the TTQ-dependent methylamine dehydrogenase [1].

Acknowledgments

This work was supported by U.S. Public Health Service (USPHS) Grant No. GM31611 and National Science Foundation (NSF) Grant No. MCB-0091084 (to F. S. Mathews). Use of the APS was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. W-31-109-ENG-38. We would also like to thank the staff at BioCARS for their assistance and the use of their equipment.

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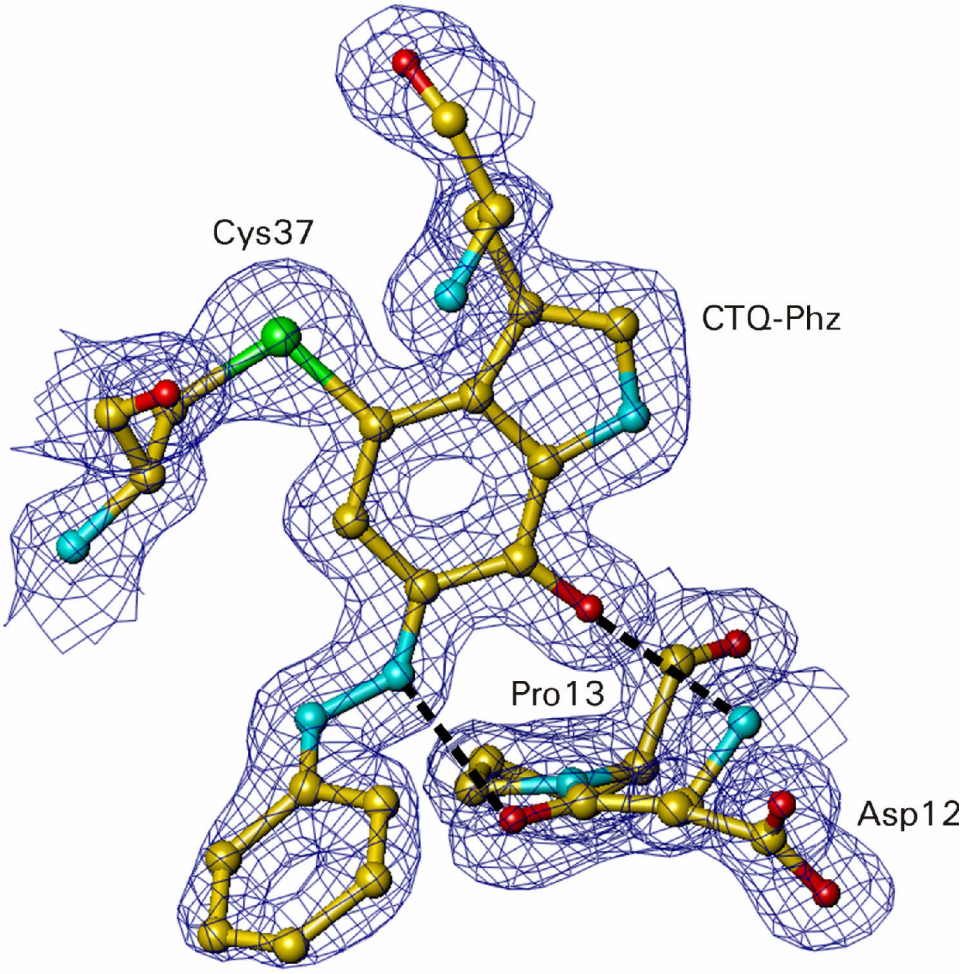

FIG. 1. Electron density of the phenylhydrazine adduct of CTQ in QHNDH bound to the C-6 position as the hydrazone. The covalent link of Trp 43 to Cys37 is also shown as are the backbone amide and carbonyl hydrogen bonds of Pro13 to N-6 and O-7 of CTQ, respectively.

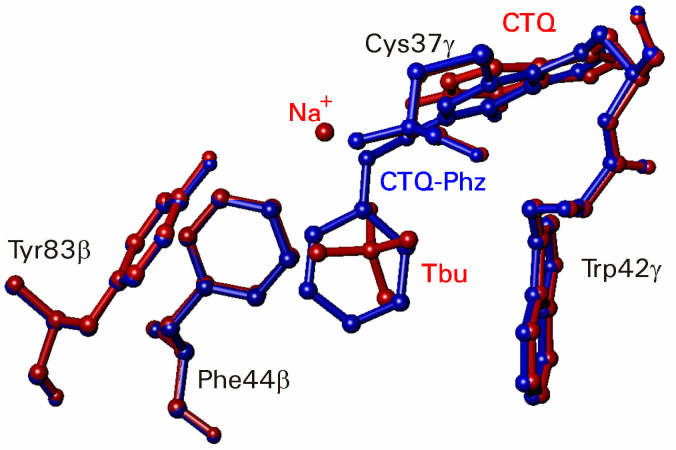

FIG. 2. Comparison of the structures of the active sites of the native (red) and the phenylhydrazine adduct (blue) of QHNDH. The bound phenylhydrazine displaces the molecule of t-butanol and the sodium ion found in the active site of the native enzyme. There is also a tilting of the indole ring of CTQ by about 10º-15º upon binding phenylhydrazine.