Glutamate-286 mutants of cytochrome *bo*-type ubiquinol oxidase from *Escherichia coli*: influence of mutations on the binuclear center structure revealed by FT-IR and EPR spectroscopies

Motonari Tsubaki[a,*], Hiroshi Hori[b], Tatsushi Mogi[c]

[a] *Department of Life Science, Faculty of Science, Himeji Institute of Technology, Kamigoori-cho, Akou-gun, Hyogo 678-12, Japan*
[b] *Division of Biophysical Engineering, Graduate School of Engineering Science, Osaka University, Toyonaka, Osaka 560, Japan*
[c] *Department of Biological Sciences, Graduate School of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan*

Received 19 September 1997

Abstract Glutamate-286 mutants of cytochrome *bo*-type ubiquinol oxidase from *Escherichia coli* were examined by EPR and FT-IR spectroscopies. We confirmed a very low enzymatic activity for E286Q. However, E286D retained one-third of the wild-type activity, probably due to the presence of the carboxylic group on the side-chain. The effect of the mutations at position 286 on the binuclear site was observed clearly in the EPR spectral change for the air-oxidized state. The effect was more significantly manifested in the presence of cyanide or azide in the oxidized state. In contrast, the mutations only slightly perturbed the binuclear center of the CO-reduced enzymes. These results indicate the importance of a direct through-bond connectivity between Cu_B and Glu^{286} via Pro^{285} and His^{284}.

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Key words: Heme-copper terminal quinol oxidase; Cytochrome *bo*; Site-directed mutagenesis; Proton channel; EPR spectroscopy; FT-IR spectroscopy

1. Introduction

Cytochrome *bo*-type ubiquinol oxidase in the aerobic respiratory chain of *Escherichia coli* catalyzes the reduction of dioxygen to water with ubiquinol-8, and utilizes the redox reactions to drive vectorial translocation of protons across the cytoplasmic membrane [1]. The mechanism of redox-coupled proton pumping is not yet known; but recently much attention has been focused on the binuclear center [2,3] and the conserved charged amino acid residues in the putative proton channels [3,4]. Glu^{286} of subunit I is one of the best conserved amino acid residues among the heme-copper terminal oxidases [5], and present in the middle of transmembrane helix VI [3,4]. A side-chain of Glu^{286} is located at the end of the putative proton translocation pathway (the Asp^{135}-Glu^{286} channel) and is likely to make H-bonds with solvent molecules [3] whereas His^{284} and Tyr^{288} present at the opposite side of the same helix ligate to Cu_B and the hydroxyethylfarnesyl group of high-spin heme *o*, respectively [3,4]. These observations suggest that a carboxylic group of Glu^{286} can undergo protonation or H-bond changes upon alterations in the redox and/or ligation states of the binuclear metal center.

Earlier studies have shown that a mutation of Glu^{286} to alanine (E286A) results in an inactive enzyme [6,7]; whereas a substitution by glutamine (E286Q) leads to conflicting results. Kawasaki et al. demonstrated by a genetic complementation test that the E286Q mutant oxidase was unable to support the aerobic growth on non-fermentable carbon sources [1,8]. In contrast, Puustinen et al. [9], Thomas et al. [6] and Moody et al. [10] reported that E286Q has 95, 69 and 106% of the wild-type oxidase activity, respectively. Furthermore, about 70% of E286Q was found to be cytochrome oo_3 [9,11], not cytochrome *bo* [12]. However, the same group also reported that E286Q of aa_3-type cytochrome *c* oxidase from *Rhodobacter sphaeroides* showed a complete lack of the activity [13].

In the present study, we confirmed that the purified E286Q is indeed severely impaired, and examined further the effect of the substitutions of Glu^{286} on the heme-copper binuclear center employing Fourier-transform infrared (FT-IR) and electron paramagnetic resonance (EPR) spectroscopic techniques.

2. Materials and methods

The wild-type and mutant enzymes were isolated from the *E. coli* strains GO103 (*cyo*$^+$ Δ*cyd*)/pMFO2 (*cyo*$^+$) [12] and ST4676 (Δ*cyo cyd*$^+$)/pMFO9-E286Q or -E286D [8], respectively, which were grown in a jar fermentor in the presence of excess amount of copper and ferrous ions under highly aerated conditions.

The steady-state turnover rate was determined spectrophotometrically by measuring the oxidation of ubiquinol-1 in 50 mM Tris-HCl (pH 7.4), 0.1% sucrose monolaurate at 25°C [14]. Reactions were started by addition of ubiquinol-1 at a final concentration of 0.5 mM. Concentrations of the enzymes were 5 nM (wild-type), 100 nM (E286Q), 10 nM (E286D).

Heme and copper contents were analyzed as described previously [12,15]. Heme content was calculated as a sum of hemes B and O using a molar extinction coefficient for heme B [12].

EPR spectra were measured at 5K as described previously [12]. Infrared spectra were recorded at 10°C with a Perkin-Elmer (model 1850) FT-IR spectrophotometer [12].

3. Results

Steady-state turnover rate of the mutant enzymes was estimated from the ubiquinol-1 oxidase activity, and E286D and E286Q exhibited 31 and 4%, respectively, of the wild-type activity (1036 electrons/s). Heme to copper ratios of E286D

*Corresponding author. Fax: +81 (7915) 8 0189.
E-mail: tsubaki@sci.himeji-tech.ac.jp.

Abbreviations: EPR, electron paramagnetic resonance; FT-IR, Fourier-transform infrared; H-bond, hydrogen bond

This work was supported in part by Grants-in-aid for Scientific Research on Priority Areas (08249106 and 09257213), for Scientific Research (B) (08458202), and for Exploratory Research (08878097) from the Ministry of Education, Science, Sports and Culture, Japan. This is paper XXX in the series 'Structure-function studies on the *E. coli* cytochrome *bo*'.

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PII S0014-5793(97)01218-0

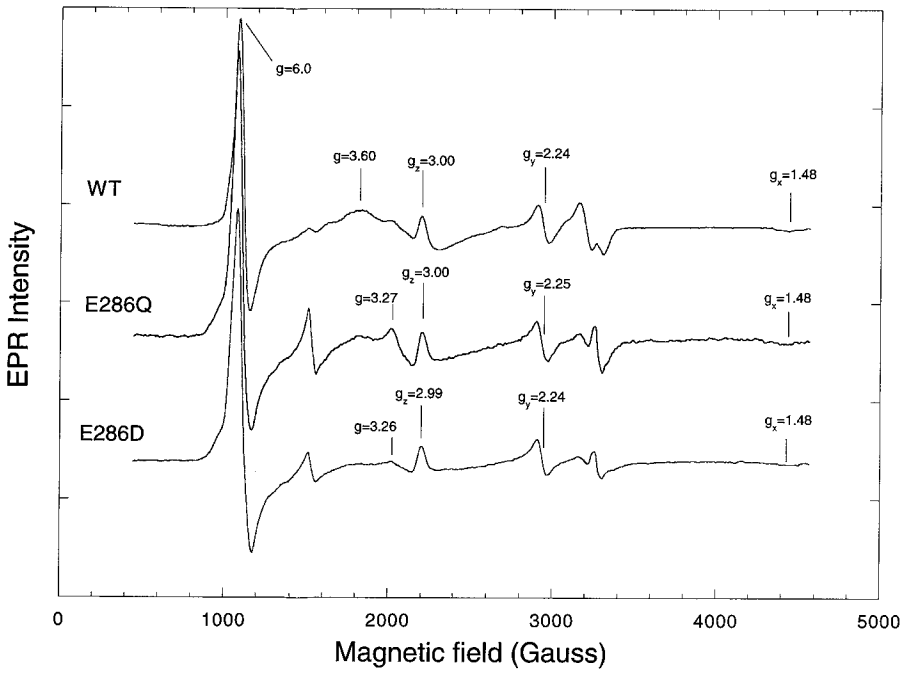

Fig. 1. EPR spectra of wild-type, E286Q, and E286D in the air-oxidized state at 5K. Conditions: microwave frequency (X-band), 9223 MHz; 100 kHz modulation with 5 Gauss width; incident microwave power, 5 mW; enzyme concentrations, 405 μM (wild-type), 247 μM (E286Q), and 544 μM (E286D).

and E286Q were 2:1.6 and 2:1.5, respectively, and were similar to the wild-type value, 2:1.1. These data indicate that a copper ion (Cu_B) stoichiometrically binds to the binuclear site of the mutant enzymes.

The EPR spectra of the wild-type and mutant enzymes in the air-oxidized state are shown in Fig. 1. All spectra contain typical low-spin heme *b* signals ($g_z = 3.00$, $g_y = 2.24$, $g_x = 1.48$) [12]. The signals at *g*6 and *g*4.3 are due to a minor population of high-spin heme *o* and adventitiously bound Fe^{3+} ions, respectively. The broad signal around $g = 3.6$, which is derived from the integer spin system comprised of high-spin heme ($S = 5/2$) and cupric Cu_B ($S = 1/2$), of the wild-type enzyme becomes weaker, and the $g = 3.27$ signal (another type of the integer spin system) gains intensity in E286Q (Fig. 1, center). A similar change was observed for E286D (Fig. 1, bottom). Our data for E286Q are critically different from the wild-type phenotypes of Moody et al. [10].

Binding of the inhibitory ligands to the binuclear site was investigated by FT-IR spectroscopy. Even at 5 mM, cyanide ions were unable to form a stable bridging conformation at the binuclear site of E286Q, in contrast to the sharp 2146 cm^{-1} band for the wild-type enzyme (Fig. 2, upper) [12]. There seems to be an equilibrium between the 2146 and 2126 cm^{-1} bands (Fig. 2, center). Similarly, the 2146, 2132, and 2118 cm^{-1} bands of E286D appeared in an equilibrium (Fig. 2, bottom). For E286D, the 2170 cm^{-1} band, the Cu_B-cyano complex released from the auto-reduced enzyme [15], was observed much faster and more significantly than for the wild-type enzyme (Fig. 2, bottom).

Binding of azide to the binuclear site in the air-oxidized state also very affected in the mutant oxidases. E286Q showed a very broad band of free azide ions centered at 2048 cm^{-1} (Fig. 3, center), indicating the absence of the binding to either metal center or both. For E286D, there is an indication of the azide binding on the basis of spectral changes (Fig. 3, bottom), but the configuration may be very different from the wild-type enzyme where a sharp 2040.5 cm^{-1} band with a

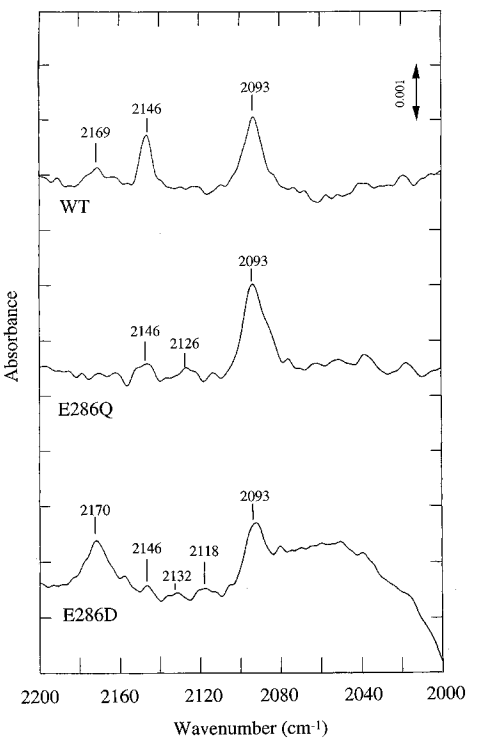

Fig. 2. FT-IR spectra of wild-type (top), E286Q (center), and E286D (bottom) in the cyanide-inhibited air-oxidized state. Conditions; temperature, 10°C; cyanide concentration, 5 mM; 200 cycles (~40 min) accumulation; spectral resolution, 4.0 cm^{-1}. The concentrations of the enzymes are the same as in Fig. 1.

clear shoulder at 2061 cm^{-1} can be easily seen (Fig. 3, upper) [12].

In the reduced state, carbon monoxide binds to the ferrous heme *o* of the mutant enzymes in a very similar manner with the wild-type oxidase [12], although the C-O stretching frequency shifted slightly to 1958.9 and 1959.1 cm^{-1} for E286Q and E286D, respectively, without any significant change in their half bandwidths (~4.0 cm^{-1}) (Fig. 4).

4. Discussion

The present study has confirmed a very low enzymatic activity for E286Q, as found for E286Q of cytochrome *c* oxidase from *R. sphaeroides* [13] and E286A of the *E. coli* cytochrome *bo* [6,7]. This result is also consistent with the previous genetic complementation test [1,8] and X-ray crystallographic studies on cytochrome *c* oxidases [3,4]. On the other hand, E286D retained one-third of the wild-type activity, probably due to the presence of the carboxylic group on the side-chain which is one methylene group shorter than that of glutamate. These results indicate that protonation changes at the carboxylic group of Glu^{286} are essential for the catalytic activity. However, it does not seem to participate significantly in the fundamental molecular architecture at the binuclear site, since there is no obvious H-bond between Glu^{286} and nearby amino acid residues in the X-ray structures of the oxidized cytochrome *c* oxidase at 2.8 Å resolution [3,4].

The effect of the Glu^{286} mutations on the binuclear site was observed clearly in the EPR spectral change for the air-oxidized state (Fig. 1). The effect was more significantly manifested in the presence of exogenous ligands (cyanide and azide) in the oxidized state (Figs. 2 and 3). E286Q was unable to bind azide ions whereas E286D did bind these ligands but with very different configuration (not clear whether in a bridging or in a terminal fashion) (Fig. 3). In contrast, these mutations only slightly perturbed the binuclear center of the CO-reduced enzymes (Fig. 4).

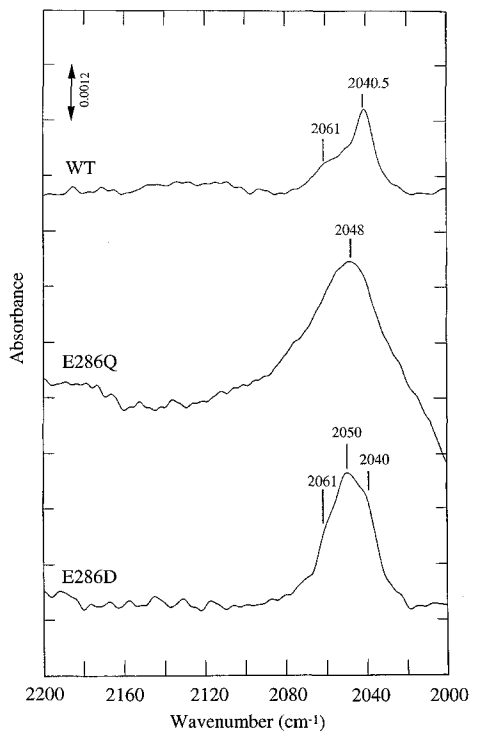

Fig. 3. FT-IR spectra of wild-type (top), E286Q (center), and E286D (bottom) in the azide-inhibited air-oxidized state. To minimize the interference from the free azide band around 2048 cm^{-1}, the concentration of azide was adjusted to 0.3 mM (wild-type), 0.25 mM (E286Q), and 0.5 mM (E286D) based on the concentration of the oxidases. Other conditions are the same as in Fig. 2.

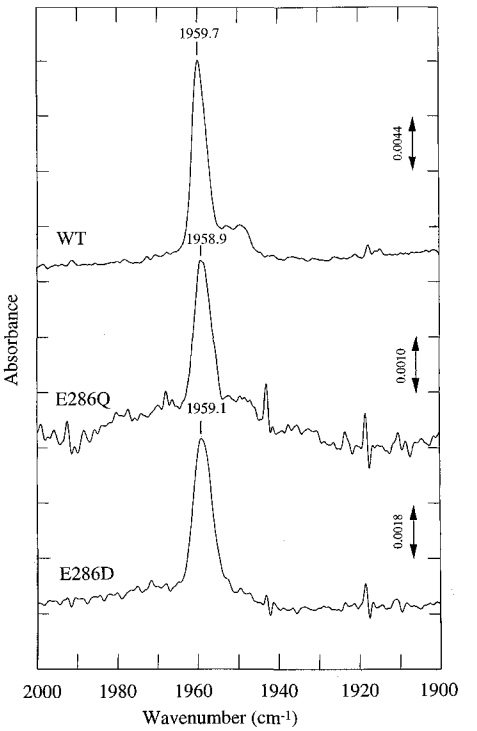

Fig. 4. FT-IR spectra of wild-type (top), E286Q (center), and E286D (bottom) in the CO-inhibited reduced state. Spectral resolution was chosen at 1.0 cm^{-1}. Other conditions are the same as in Fig. 2.

There is a direct through-bond connectivity between Cu_B and Glu^{286} via Pro^{285} and His^{284}, a Cu_B ligand [3,4]. The effects of the mutation at position 286 could be directly transmitted to the Cu_B center through this connection. Alternatively, perturbations of the H-bond network in the Asp^{135}-Glu^{286} channel by the Glu^{286} mutations could also indirectly influence the structure of the binuclear center. In the air-oxidized state, relative orientation and distance between high-spin heme and cupric Cu_B must be finely tuned to accommodate a bridging ligand(s). On the other hand, in the CO-reduced state, there is no direct connectivity between the heme-bound CO and the cuprous Cu_B center, and therefore, the effect of the mutation on the heme-bound CO could be marginal. It is also possible that the H-bond network including water molecule(s), Glu^{286} and possibly one of the Cu_B ligands (most likely His^{334}, the closest residue to Glu^{286}) may have some roles in binding of the exogenous ligands to the binuclear center of the oxidized enzyme.

Recently, Svensson-Ek et al. postulated that Glu^{286} is involved in proton uptake after the formation of the peroxy intermediate at the binuclear site [7]. It is not clear whether this proton(s) is directly participated in the dioxygen reduction or proton pumping [7]. Present observations clearly indicate that the Glu^{286} mutations affect the conformation at the binuclear site. Reciprocally, such a conformational change

during the dioxygen reduction might affect the protonation state of the carboxylic group of Glu^{286}. This connection may be very important for the proton translocation by the heme-copper terminal oxidases.

Acknowledgements: We wish to thank R.B. Gennis of University of Illinois for strain GO103, S. Ohsono of Eisai Co. Ltd. for ubiquinone-1, and E. Yoshimura of University of Tokyo for metal analysis.

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