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Replacement of the Glu380 with Gln380 in subunit I of cytochrome *cbb*$_3$ oxidase from *Rhodobacter capsulatus* results in inactive enzyme

[*Rhodobacter capsulatus* ta bulunan sitokrom *cbb*$_3$ oksidazın I alt ünitesindeki Glu380'in Gln380 ile yer değiştirilmesi inaktif enzim ile sonuçlanmaktadır]

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ABSTRACT

Objective: To show the importance of conserved glutamate 380 residue located in the helix IX of catalytic subunit of the *cbb*$_3$-type oxidases from *Rhodobacter capsulatus* by site-directed mutagenesis.

Method: E380Q mutant was generated by site-directed mutagenesis using pMOZI vector as a template. After verification of the desired mutation and a few subcloning processes, pOX15/E380Q plasmid was transferred to GK32 strain of *R. capsulatus* by triparental mating. To determine the effect of the mutation on assembly of the *cbb*$_3$-type oxidase the chromatophore vesicles were separated by PAGE and than samples were analyzed with TMBZ staining and western blotting. The enzyme activity was determined by NADI staining of whole cells and spectroscopic measurement of the oxygen consumption rate of chromotophore vesicle.

Results: The sequencing results showed that E380Q mutation was created successfully during PCR reactions. Western blot analysis and TMBZ staining of the PAGE gel revealed that mutated *cbb*$_3$-type oxidase lacked all subunits of the enzyme. The E380Q mutant led to complete loss of activity of *cbb*$_3$-type oxidase which was observed by NADI test and oxygen consumption rate measurement (1%).

Conclusion: Our findings support the assumption that emphasizes the fact that the active site structure can be very different in the subfamilies of the heme-copper oxidases, and well conserved within individual subfamilies.

Key Words: Cytochrome *cbb*$_3$-type oxidase, D-proton pumping channel, site directed mutagenesis and *Rhodobacter capsulatus*

Conflict of interest: There is no conflict of interest among the authors.

ÖZET

Amaç: *Rhodobacter capsulatus*'a ait *cbb*$_3$-tipi oksidaz enziminin katalitik alt ünitesinin IX. sarmalında korunmuş olarak bulunan glutamat 380 aminoasidinin yönlendirilmiş mutagenez yöntemi ile öneminin gösterilmesi.

Metot: pMOZI vektörü kalıp olarak kullanılarak yönlendirilmiş mutagenez ile E380Q mutantı elde edilmiştir. İstenilen mutasyonun gerçekleştiği doğrulandıktan ve birkaç alt klonlamadan sonra pOX15/E380Q plazmidi *R. capsulatus*'un GK32 suşuna üçlü eşleşme ile aktarılmıştır. Mutasyonun *cbb*$_3$-tipi oksidazın alt ünitelerinin birleşmesi üzerine olan etkisini belirlemek için kromatofor zar proteinleri SDS-PAGE ile ayrıştırılmış, TMBZ boyama ve western blotlama ile analiz edilmiştir. Enzim aktivitesi, hücrelerin NADI boyanması ve kromatofor zar proteinlerinin spektrofotometrik ölçümle oksijen kullanım oranlarının hesaplanması ile belirlenmiştir.

Bulgular: Dizi analizi sonuçları E380Q mutasyonun PZR reaksiyonunda istenilen şekilde gerçekleştiğini göstermiştir. Western blot analizi ve SDS-PAGE jelinin TMBZ boyaması, mutant *cbb*$_3$-tipi oksidaz enziminin alt ünitelerinin eksik olduğunu göstermiştir. E380Q mutasyonun, NADI testi ve oksijen kullanım ölçümleri (%1) sonucunda enzim aktivitesinin tamamen kaybına neden olduğu belirlenmiştir.

Sonuç: Elde edilen bulgular, demir bakır oksidazların aktif bölge yapılarının alt gruplarında farklılık gösterebileceği ve her bir alt grup içerisindeki bireylerde korunmuş olabileceği varsayımını desteklemektedir.

Anahtar kelimeler: Sitokrom *cbb*$_3$-tipi oksidaz, D- proton pompalama kanalı, yönlendirilmiş mutagenez ve *Rhodobacter capsulatus*.

Çıkar çatışması: Yazarların çıkar çatışması bulunmamaktadır.

Introduction

Heme-copper oxidases (HCOs) are the terminal respiratory enzyme, catalyzing the reduction of a molecule of dioxygen to two molecules of water, consuming four protons and four electrons. Recent modeling [1-2] and experimental studies [3] have provided that K and D channels containing hydrophilic amino acids facilitate the proton translocation from the bacterial cytoplasm or mitochondrial matrix into the active site. The superfamilies of HCOs comprise the aa_3-, ba_3- and cbb_3-type oxidases [4-5] along with other possible subfamilies [6]. Cytochrome cbb_3 is an interesting and distinct member of the heme-copper oxidase superfamily. The extremely low degree of conservation between the different protein subfamilies presents a possibility to identify shared structural features that are from a different part of the over all structure but serve the same function of coupling electron transfer and proton transport.

It was recently found that glutamate, a key amino acid in transferring protons to be pumped a cross the membrane and to the site of oxygen reduction, fulfills the proton-conducting D-channel in aa_3-type oxidases [7]. Glu-278 (*Pseudomonas denitrificans* numbering) is one of the best conserved residues among the heme-copper oxidases, and mutations at this locus have been shown to block the oxygen reaction and the uptake of protons, as well as proton translocation [8-9] suggesting that this residue plays an important role in the catalytic mechanism and that it is a key residue for proton translocation in aa_3-type oxidases [10-11]. However, almost nothing is known about proton channels in the cbb_3 oxidases. According to sequence alignments glutamate in helix VII is completely missing from the cbb_3-type oxidases despite the presumed catalytic importance of the histidine-glutamate ligand [12, 5]. Our amino acid sequence alignment from cbb_3-type oxidase from fifty different organisms has suggested that another residue, glutamate 380 (the numbering refers to subunit CcoN of *Rhodobacter capsulatus* cytochrome cbb_3), might structurally replace the missing glutamate in the active site and thereby possibly fulfill the mechanistic requirement also in the cbb_3-type of the heme-copper oxidase family.

In this work, sequence alignments are used to identify residues which might form putative proton channels or histidine ligand in the cbb_3-family of oxygen reductases, although sequence alignments shows that E380 is not completely conserved within the cbb_3-type oxidases. In this study, to show the functional importance of the identified residue, glutamate 380 in helix IX, it was substituted for glutamine by site-directed mutagenesis and then mutant was analyzed in respect to activity and assembly of the cbb_3-type respiratory oxidases from *R. capsulatus*.

Material and Method

Bacterial strains, plasmids and growth conditions:

The bacterial strains and plasmids are listed in Table 1. *Escherichia coli* strains were grown aerobically at 37 °C in Luria Bertani (LB) broth supplemented with appropriate antibiotics (tetracycline, kanamycin, spectinomycin, and ampicillin at final concentrations of 12.5, 50, 10, and 100 μg/ml, respectively) [13-14]. *Rhodobacter capsulatus* strains were grown chemoheterotrophically (Res growth) at 35 °C on enriched (MPYE) or minimal (MedA) media [15] supplemented with appropriate antibiotics (tetracycline, kanamycin, and spectinomycin at final concentrations of 2.5, 10, and 10 μg/ml, respectively) [13].

Molecular Genetic Techniques:

Standard molecular techniques were performed according to Sambrook et al., 2001 [16]. pMOZI vector containing 2.8 kb *XhoI* fragment from the structural operon *ccoNOQP* of *R. capsulatus* which encoding the subunit CcoN [17] was used as template for generating the point mutation by site-directed mutagenesis. Site- directed mutagenesis was performed by using oligonucleotides: E380Q F 5'-GGCATGTCGACCTTTCAGGGC-CCGATGATGTCG-3', E380QR5'- CGACAT-CATCGGG CCCTGAAAGGTCGACATGCC-3'. Sequence verification of the mutagenesis reaction was performed at the DNA Sequencing facility, Iyontek corp. and University of Pennsylvania. The 2.8 kb *Xho*I fragments of pMOZIIE380Q was replaced with pOX15 and transferred to HB101 [18] competent cells of *E. coli*. The orientation check of the colonies was performed by using *Hind*III restriction enzyme. Finally pOX15/E380Q was transferred to GK32 strain of *R. capsulatus* [19] by triparental mating in the presence of HB101/pRK2013 [20].

Biochemical Techniques:

Isolation of recombinant chromatophore membranes and characterization of mutant

The chromatophore membranes (intracytoplasmic membrane vesicles -ICM) of *R. capsulatus* were isolated in 50mM MOPS (3- (N-morpholino) propanesulfonic acid) (pH 7.0) containing 100 mM KCl by using a French Pressure Cell as described in [21]. 1mM PMSF was added to minimize proteolysis during cell disruption. Protein concentrations were determined using the bicinchoninic acid method [22] with bovine serum albumin as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (either on 11%or 16.5%) was performed as described in [23] and [24]. Samples were solubilized in 5%*β*- mercaptoethanol and incubated at 37 °C for 10 minutes before loading on gels. Immunoblot analyses were conducted as described in [19] using polyclonal antibodies raised against *R. capsulatus* CcoN or CcoP. Ctys *c* were visualized by endogeno-

us peroxidase activity of covalently attached heme with TMBZ staining as described in [25].

Oxidase Activity Measurement

The cytochrome *c* oxidase activity of whole cells on plates was determined by using the NADI test [26]. Mutant was tested for its cyt *c* oxidase activity by the treatment with 1:1 (v/v) mixture of 35 mM α-naphtol in ethanol and 30 mM *N,N*-deimethy-*p*-phenylenediamine in water. The cytochrome *c* oxidase activity was also carried spectrophotometrically by monitoring the oxidation of reduced horse heart cyt *c* (Sigma, St. Louis, MO) at 550 nm and 25°C using a U3210 UV-visible spectrophotometer described in [27].

Results

Mutation of Glutamate 380 and Enzyme Activity Test

The sequencing results showed that mutation was created successfully and no other unwanted mutations were generated during PCR reactions. The replacement of glutamate 380 to glutamine led to loss of activity which first observed by the utilization of NADI test that allows visual detection of the oxidase activity. The NADI staining of the GK32/E380Q mutant exhibited NADI negative phenotype similar to negative control strain, GK32 (Fig. 1 a-c).

Measurement of Oxygen Consumption Rate

The oxygen consumption rates of chromatophore membranes of MT1131 (wild type), GK32/pOX15 (positive control), GK32 (negative control) and GK32/E380Q mutant were measured spectrophotometrically by monitoring the oxidation of reduced horse heart cyt *c*. The mutant showed less than 1%activity of positive control, confirming its cyt *c* oxidase phenotype (Table 2). NADI test and oxygen measurement results strongly support that glutamate residue at the position of 380 has critical role in the enzyme activity or assembly.

Effects of E380Q Mutation on Protein Assembly

By using the SDS/PAGE-TMBZ staining technique, membrane-bound *c*-type cyts with molecular masses of 32, 31, 29 and 28 kDa of *R. capsulatus* can be visualized by their peroxidase activity. The 32 kDa protein is the cyt c_1 subunit of the cyt bc_1 complex [21, 14, 28], and the 29-kDa protein is the membrane-associated electron carrier cyt c_y [13]. The two remaining cyts of 32 and 28 kDa (cyts c_p and c_o, respectively) correspond to the heme *c*-containing subunits of the cyt cbb_3 oxidase. In comparison with wild type, MT1131, and positive control strain, GK32/pOX15, mutant GK32E380Q had no CcoP and CcoO subunits. In respect to heme *c*-containing subunit composition, E380Q mutant is similar to negative control strain, GK32 (Fig. 2). Western blot analyses revealed that CcoN was absent in GK32/E380Q establishing that it lacked all subunits of the cyt cbb_3 oxidase (Fig. 3).

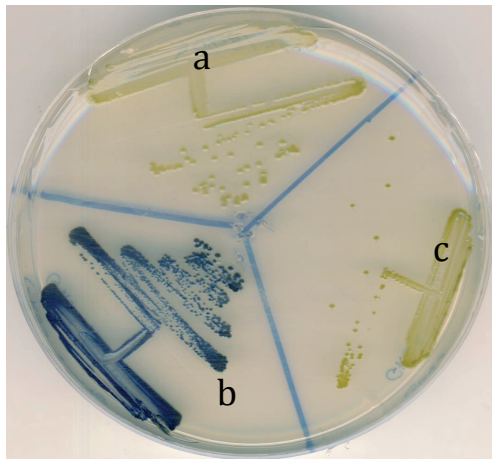

Figure 1. NADI staining of target mutant (E380Q) with negative (GK32) and positive (GK32/pOX15) controls. (a) GK32, NADI negative as expected, (b) GK32/pOX15, NADI positive as expected. (c) GK32/pOX15E380Q, NADI negative.

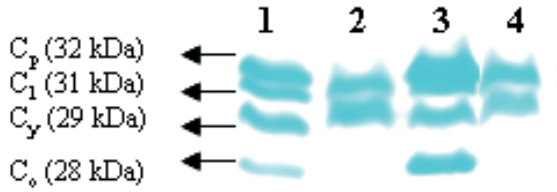

Figure 2. Cytochrome *c* profile of target mutant (GK32/pOX15E380Q) with wild-type (MT1131), negative control (GK32), positive control (GK32/pOX15). Approximately 50 mg of protein was loaded in each lane of a 16.5%SDS-polyacrylamide gel. *c*-type cytochromes were detected by TMBZ staining. Lane 1, wild-type MT1131; lane 2, GK32; lane 3, GK32/pOX15 and line 4, GK32/pOX15E380Q. Cyt c_p and c_o are the subunits II and III of cyt cbb_3 oxidase, and cyts c_1 and c_y correspond to the cyt c_1 subunit of the bc_1 complex and the membrane-attached electron carrier c_y, respectively.

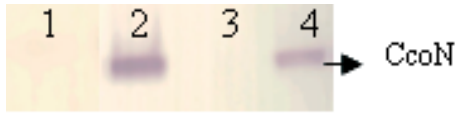

Figure 3: Western blot analysis of CcoN subunits with anti-CcoN antibodies. CcoN was detected with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G with $NiCl_2$-enhanced 3,3′-diaminobenzidine as the substrate. Lane 1, GK32/pOX15E380Q; lane 2, GK32/pOX15; lane 3, GK32 and line 4, wild-type MT1131. 10 µg of membrane protein was loaded on SDS-PAGE.

Discussion

The aa_3-type of oxygen reductases is by far the largest and most studied group of enzymes in the heme-copper superfamily. Studies on aa_3-type cytochrome oxidase showed that it has histidine-glutamate/aspartate structural motif. This motif has been shown to modulate substrate binding, catalytic activity, redox potentials as well as the metal coordination [29-30]. Glutamate, was sug-

Table 1. Bacterial strains and plasmids used in this study.

Strain	Genotype	Phenotype	Reference
E. coli			
XL1-Blue	rec A end A 1 gyr A986 thi-1hsdr17supE44 rel A1 lac		Stratagene
XL1-Blue/pMOZIE380Q		Amp^r	This work
HB101	F-proA2 hsdS20 (Rb-, mB-) recA13 ara14 lac Y1		[l8]
HB101/pACYC177	F-ara-14leu fhuA2 Δ (gpt-proA) 62 lac Y1glnV44 galK2 rspL20 xyl-5 mtl-1 Δ (mcrC-mrr) $_{HB101}$		New England Biolabs
HB101/pMOZI		Amp^r	[17]
HB101/pMOZII		Amp^r	[17]
HB101/pMOZIIE380Q		Amp^r	This work
HB101/pOX15	15.3 kb with pOX15 DNA	Tet^r	[34]
R. capsulatus			
MT1131	*crtD121 Rifr*	Wild type	[14]
GK32	Δ (cco*NO*::kan)	Kan^r	[19]
GK32/ pOX15E380Q		Tet^r	This work
Plasmid	**Phenotype**	**Phenotype**	**Reference**
pMOZI	pBluescript SKII with 2.8 kb *NO*' *Xho*I insert	Amp^r	[17]
PMOZI/E380Q	Substitution of glutamate to glutamine in pMOZI	Amp^r	This work
pMOZII	pACYC177 with 2.8 kb *NO*' *Xho*I insert	Amp^r, Kan^r	[17]
pMOZII /E380Q	pMOZII replaced 0.6 kb *Ppu*I fragment of pMOZI/E380Q	Amp^r	This work
pOX15	pRK404 with 4.7 kb *ccoNOQP* operon	Tet^r	[19]
pOX15/E380Q	pOX15 replaced 2.8 kb *XhoI* fragement of pMOZII/E380Q	Tet^r	This work
pOX12	pOX 15 lacks 2.8 kb *NO*' fragment (12.5 kb)	Tet^r	[17]
pRK2013	self-transmissible plasmid *tra*$^+$ (RK2)	Kan^r, helper	[20]

Table 2. Quantitative analysis of cytochrome *c* oxidase activity in *R. capsulatus* membranes with spectrophotometer

Strain	**%of wt activity**
GK32/pOX15	100
GK32	0
MT1131	47,3
s	<1

*For cytochrome *c* oxidation, 100%activity corresponds to 1602 nmol of cyt *c*/mg of protein per min. (semiaerobic condition). Values shown are means of three independent measurements.

gested to ile as close as 11 Å from the proximal histidine ligand of the high spin heme of aa_3-type oxidases [2]. Some glutamate mutations within the D-channel can eliminate proton pumping [31, 32, 33] consequence of structural changes that regulate the proton-transfer rates to the catalytic site and acceptor site of pumped protons. In addition to this, Kaila et al [7, 34] suggested that a conserved glutamate at the end of the D channel in aa_3 type oxidase worked as valve in minimizing leakage of the pumped proton back to the D-channel. However, It has been noted that the oxygen reductases in the cbb_3-type oxidases do not appear to have glutamate equivalent to those corresponding to the D-channel of the aa_3-type of oxygen reductases [9, 10, 2].

An alternative proton input channel has been suggested between D364 (*R. sphaeroides* cbb_3 oxidase numbering) on the cytoplasmic side and E383 (*R. sphaeroides*) in helix IX near the high-spin heme [5]. However, sequence analysis shows that neither D364 nor E383 are completely conserved within the cbb_3-type oxidase. In many sequences the residue equivalent to E383 is a glutamine [10]. On the other hand, E383Q mutation in cbb_3-type oxidase of *R. spheroides* results in complete loss of activity [35]. Recently, Rauhamäki et al. [35] found that the proximal histidine (C: H405) of the active site heme b_3 most probably forms a hydrogen bond to a conserved glutamate (E383, only in cbb_3 oxidases) in helix IX of subunit CcoN. It was further shown by Sharma et al. [36] that the catalytic mechanism involves redox-coupled proton transfer between the proximal histidine ligand of heme b_3 and a conserved glutamate. It has also been proposed that by Buschmann et al [37] proton transfer in cbb_3- type oxidases may occur via Thr-378, Ser-377 and Glu-380 (*Rhodobacter capsulatus numbering*). However there is no protein assembly and pumping data have been published yet which are done by mutated glutamate residue. Our findings about protein assembly and enzyme activity partially support these results and proposals because E380Q mutation results in inactive and disassembled cbb_3-type oxidase in *R. capsulatus*. It has been supposed that deeply buried two heme *b* and the Cu cofactor might be critical for proper folding and stabilization of cbb_3 oxidase enzyme. Thus any residue which has an interaction with any of these might also has role in the folding and stabilization of the enzyme. Our findings about protein assembly support that E383Q mutation in cbb_3-type oxidase of *R. capsulatus* caused disassembled enzyme.

It has been shown that E380 residue is not completely conserved within the C- family oxygen reductases or other families. It has been also shown by sequence alignments, the residue equivalent to E380 is glutamine (Hemp et al., 2007), but this concerns few very distinct members of the cbb_3 subfamily of enzymes. In this study, we thought that substitution of glutamate residue to glutamine may explain why in some sequences this residue is conserved as glutamate rather than glutamine. In site-directed mutagenesis studies it is recommended to change the target aminoacid to other amino acids which have different characteristics than the target one to see the effect of the mutation. Sharma et al., 2006 was tested the possible functional role of E380 residue by mutation to aspartate in *R. sphaeroides.* It has been shown that E to D mutation retained approximately one- third of the wild type activity with normal H^+/e^- ratio of proton translocation. This also shows that substitutions to similar residues restore the function and the assembly of the enzyme.

Although nucleotide alignments and structural predictions indicate that glutamate is not conserved within subfamilies of the cbb_3-type oxidase, analysis of glutamic acid substitutions in helix IX of the subunit I of cbb_3-type oxidase indicates that histidine-glutamate/aspartate structural motif is found in individual subfamilies of the cbb_3-type oxidases. Our findings support the assumption [35] that emphasizes the fact that the active site structure can be very different in the subfamilies of the heme-copper oxidases, and well conserved within individual subfamilies, even though the overall function is shared.

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