Site-directed Mutagenesis Reveals the Essentiality of the Conserved Residues in the Putative Diiron Active Site of the Trypanosome Alternative Oxidase*

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Trypanosoma brucei possesses a non-cytochrome, salicylhydroxamic acid (SHAM)-sensitive ubiquinol:oxygen oxidoreductase known as trypanosome alternative oxidase (TAO). TAO and similar SHAM-sensitive alternative oxidases (AOXs) contain 2–3 conserved diiron-binding motifs (EXXH). Site-directed mutagenesis of residues H165A, E214A, E266A, and H269L within the conserved EXXH motif abolished the ability of TAO to complement the heme-deficient Escherichia coli strain GE1387. These mutations also reduced the growth of this E. coli auxotroph to about 5% of the control cells containing wild type TAO. In contrast, mutation of residues outside the EXXH motifs, e.g. V205A, L243A, C261A, and V271A, had little effect on complementation, and the reduction in the cell growth was about 5–10%. Mutations of the putative iron-binding residues within the EXXH motifs of TAO abolished the ability to confer SHAM-sensitive respiration to E. coli heme mutant, whereas mutations of the non-conserved/non-iron binding residues resulted in 20–30% reduction of SHAM-sensitive respiration of the E. coli auxotroph. Immunoblot analysis of the total cellular protein of transformed E. coli revealed that the expression level of mutated and wild type TAO (35 kDa) remained unaltered. Mutation at C261A produced a truncated but functional protein of 28 kDa. The addition of ortho-phenanthroline to the growth medium produces a non-functional TAO. The effect of ortho-phenanthroline on the activity of TAO was completely alleviated by the addition of iron in the medium, which suggests that iron is needed for the activity of TAO. This work demonstrates that His-165, Glu-214, and His-269 and the presence of iron are essential for the activity of TAO.

Trypanosoma brucei, the causative agent of sleeping sickness in humans and nagana in livestock, is an extracellular parasite transmitted by the tsetse fly Glossina. The parasite has a digenetic life cycle; it lives in the bloodstream and tissue fluid in the mammalian host as the bloodstream forms. In the insect alimentary gut it exists as the procyclic form. The blood-stream-form trypanosomes do not have cytochromes and use glycolysis as their sole source of energy (1). Reducing equivalents generated during glycolysis are re-oxidized by transferring electrons to a mitochondrial cyanide-resistant, salicylhydroxamic acid-sensitive terminal oxidase called trypanosome alternative oxidase (TAO)1 (2). TAO is a ubiquinol:oxygen oxidoreductase, is unique to the bloodstream form, and is not present in the mammalian host (3). Recent data showed that 40 μM SHAM could completely inhibit the growth of the in vitro cultured bloodstream form trypanosomes within 24 h (4). Therefore, TAO is a potential target for chemotherapy.

TAO has been cloned and characterized from T. brucei (5). Similar SHAM-sensitive, cytochrome-independent alternative oxidases have also been cloned from different fungi and plants (6–8). A considerable sequence homology has been observed among the deduced amino acid sequences of all AOX cDNAs. Analysis of the primary and the secondary structure of different alternative oxidases has revealed the presence of two hydrophobic domains at the center of the enzyme and putative diiron-binding motifs (EXXH) (9). The presence of the EXXH signature motifs places alternative oxidases as members of the class II type of diiron carboxylate proteins that include Escherichia coli ribonucleotide reductase R2 subunit (10, 11), methane monoxygenase (12), and stearoyl-acyl carrier protein (13, 14). The alternative oxidases consist of three EXXH motifs; the N-terminal and the C-terminal motifs are highly conserved, whereas the histidine residue in the middle EXXH motif varies among different alternative oxidases (AOXs). In TAO, this residue is isoleucine (Ile-218) (15).

Siedow et al. (9) first proposed an active site model of the alternative oxidase consisting of four putative α-helices, containing non-conserved and conserved EXXH motifs in helices 1 and 4, respectively. The two conserved hydrophobic regions of AOXs were previously postulated as the transmembrane domains. Recently, a revised model was proposed by Andersson and Nordlund (16). In this model alternative oxidase was proposed as an interfacial inner membrane protein. This model also consists of four α-helices with two EXXH motifs in helices 2 and 4, respectively (9).

Thus, all alternative oxidases possess six conserved amino acid residues proposed as ligands to the iron center. These consist of two bridging bidentate glutamates (Glu-162 and Glu-266), two terminal glutamates (Glu-123 and Glu-214), and two monodentate histidines (His-165 and His-269) (Fig. 1). Histidine residues as well as the bridging glutamates form part of

* This work was supported in part by National Institutes of Health Grant RO1AI21159 and Minority Biomedical Research Support Grant F606GM08037 (to G. C. H.). Meharry Medical College is the recipient of RCMI Grant G12RR03032 from the National Institutes of Health. In addition, support was provided from NRSF Training Grant 2732A07281. 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.
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1 The abbreviations used are: TAO, trypanosome alternative oxidase; AOX, alternative oxidase; SHAM, salicylhydroxamic acid; IPTG, isopropylthiogalactoside; OP, ortho-phenanthroline.
the EXXH motif that holds the putative metal-containing active center. The amino acid residue number indicated in parentheses are from the TAO sequence (Fig. 1). Andersson's model has been further modified by changing one of the proposed glutamate terminal ligand in helix 3 based on the sequence data of the recently cloned Arabidopsis thaliana IMMUTANS (Im) gene, a distant relative of the AOXs (17).

Earlier, we have demonstrated that TAO was able to rescue the heme mutant of *E. coli* strain GE1387 by conferring on them the SHAM-sensitive alternative pathway of respiration (5). In *Hansenula anomala*, iron has been suggested to be required for the activity of the alternative oxidase (18). Previously, site-directed mutagenesis of a conserved glutamic acid residue of the Sauroratium guttatum alternative oxidase (19) as well as mutation of the corresponding residue (Glu-215) in the TAO (15) revealed that this residue is essential for the activity of the enzyme.

Given the information about the proposed models, we hypothesize that mutations of the conserved residues in the EXXH motifs will alter the activity of the TAO. To address this idea, we performed site-directed mutagenesis of the conserved amino acid residues His-165, Glu-214, Glu-266, and His-269. The experiments reported here present for the first time with any alternative oxidase a systematic analysis of key residues in the EXXH motifs and establish conserved amino acid residues His-165, Glu-214, Glu-266, and His-269 as essential for the alternative oxidase activity. These experiments provide biochemical confirmation of the proposed active site model for the alternative oxidases.

### EXPERIMENTAL PROCEDURES

**E. coli Strains and Growth Medium—** *E. coli* heme mutant strain GE1387 (P-145 lys5 trp R55 pro 48 tsc b4 hema A204 rpsL80 r-9) was a gift from Dr. D. Soll, Yale University, and was grown on M9 minimal medium containing glycerol (0.2%) as the carbon source, supplemented with 5-aminolevulinic acid (50 μg/ml). Methionine, histidine, tryptophan, and proline (50 μg/ml) were also added when needed. The strain was also grown on LB agar plates supplemented with 5-aminolevulinic acid and in liquid medium containing ampicillin (100 μg/ml). *E. coli* strain DH5α (Invitrogen) and M15SpE65C (ChattWest, CA) were used for cloning, mutagenesis studies, and the expression of recombinant protein. TAO was expressed after the addition of IPTG (1.0 mM) in the medium, and the cells were grown for 2–4 h. The *E. coli* heme mutant transfectants were grown in LB liquid medium containing ampicillin (100 μg/ml) without 5-aminolevulinic acid supplement. The cultures were grown for 48 h at 37 °C in a shaker incubator. Bacterial growth was measured using a spectrophotometer at 600 nm.

**Growth was measured using cultures were grown for 48 h at 37 °C in a shaker incubator.**

**RESULTS**

**TABLE I**

| Residues within the EXXH motifs | Results, growth of colonies |
|--------------------------------|-----------------------------|
| H165A                          | No growth                   |
| H269L                          | No growth                   |
| E266A                          | No growth                   |
| N1-conserved residues outside the EXXH | Growth                  |
| V205A                          | Growth                      |
| L243A                          | Growth                      |
| C261A                          | Growth                      |
| V205A                          | Growth                      |
| L243A                          | Growth                      |
| C261A                          | Growth                      |
| Conserved residue outside the EXXH motifs | No growth               |
| E214A                          | No growth                   |

**pTAO 25 (T. brucei alternative oxidase)**

**pAOX (A. thaliana alternative oxidase)**

**pQE (vector alone)**

### Materials and Methods

**Primers, Plasmid Constructs, and Site-directed Mutagenesis—** A 416-bp fragment (amino acid residues 179–319) from TAO open reading frame was subcloned into pGEM vector (Promega, Madison, WI) at NdeI and MluI sites and designated pGEM416. This 416-bp TAO cDNA fragment, containing residues from the proposed EXXH signature sequence of diiron active site, was used for site-directed mutagenesis studies using the QuikChange mutagenesis protocol (Stratagene, La Jolla, CA). Each mutated clone was designated according to the amino acid residue mutated, the amino acid residue number, and respective substitution (e.g., E214A, where the codon (GAA) for glutamic acid was changed to the codon (GCC) for alanine). Other changes were in the EXXH motifs (H165A, E266A, and H269L) and outside the EXXH motifs (V205A, L243A, C261A, and V271A). Mutation H165A was performed using full-length TAO cDNA subcloned in the pQE vector.

### Mutations of Conserved Residues within EXXH Motifs in TAO Did Not Complement *E. coli* Heme Mutant GE1387—The *E. coli* auxotroph GE1387 is an excellent system to test the effect of mutations on TAO function because it is defective in the synthesis of heme and produces no cytochromes if not supplemented by 5-aminolevulinic acid. This strain of *E. coli* does not grow aerobically in medium without 5-aminolevulinic acid, and wild type TAO is able to complement this defect and provide the SHAM-sensitive alternative pathway of respiration to the heme mutant (5). The complementation ability of TAO mutants was analyzed in comparison to non-mutated TAO (Table I). The results showed that mutation of residues Glu-214, the bridging bidendate glutamate, His-165, His-269 motoneutrate histidines, and Glu-214, the terminal ligand in the bridging bidentate histidines. The experiments reported here present for the first time with any alternative oxidase a systematic analysis of key residues in the EXXH motifs and establish conserved amino acid residues His-165, Glu-214, Glu-266, and His-269 as essential for the alternative oxidase activity. These experiments provide biochemical confirmation of the proposed active site model for the alternative oxidases.

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### Mutations of Conserved Residues within EXXH Motifs in TAO Did Not Complement *E. coli* Heme Mutant GE1387—The *E. coli* auxotroph GE1387 is an excellent system to test the effect of mutations on TAO function because it is defective in the synthesis of heme and produces no cytochromes if not supplemented by 5-aminolevulinic acid. This strain of *E. coli* does not grow aerobically in medium without 5-aminolevulinic acid, and wild type TAO is able to complement this defect and provide the SHAM-sensitive alternative pathway of respiration to the heme mutant (5). The complementation ability of TAO mutants was analyzed in comparison to non-mutated TAO (Table I). The results showed that mutation of residues Glu-214, the bridging bidendate glutamate, His-165, His-269 motoneutrate histidines, and Glu-214, the terminal ligand in the bridging bidentate histidines. The experiments reported here present for the first time with any alternative oxidase a systematic analysis of key residues in the EXXH motifs and establish conserved amino acid residues His-165, Glu-214, Glu-266, and His-269 as essential for the alternative oxidase activity. These experiments provide biochemical confirmation of the proposed active site model for the alternative oxidases.
Val-271 (Fig. 1) showed little difference from the complementation ability of the wild type TAO (Table I). These results demonstrate that His-165, Glu-214, Glu-266, and His-269 are essential for TAO function.

To evaluate these mutations further, growth of the transformants in liquid medium was measured at A_{600}. As shown in Fig. 2, mutations at H165A, E214A, E266A, and H269L in TAO reduce the growth of the E. coli heme mutant to the level of the growth of the same E. coli strain transformed or with vector containing no insert. However, cells transformed with mutants V205A, L243A, C261A, and V271A supported to 70–80% the growth of the E. coli heme mutant compared with the cells transformed with wild type TAO.

Mutations of the Conserved Amino Acid Residues within EXXH Motifs Did Not Confer SHAM-sensitive Respiration on E. coli Transformants—The ability of the wild type TAO to complement the E. coli heme mutants corroborates the presence of SHAM-sensitive respiration conferred by TAO in these auxotrophs that normally do not exhibit any SHAM-sensitive respiration. Thus, to study the effect of different mutations on TAO function, we compared the SHAM-sensitive respiration of the E. coli cells transformed with the two groups of mutations. E. coli auxotroph GE1387 transformed with TAO mutants V205A, L243A, C261A, and V271A grown in LB/ampicillin broth without supplementation of the 5-aminolevulinic acid. The respiration of these transformants grown in this ampicillin broth was about 70–80% SHAM-sensitive. Cells transformed with the wild type TAO were 100% sensitive to SHAM (Table II). Mutations of glutamate and histidines residues H165A, E214A, E266A, and H269L (EXXH motif mutations) did not rescue the E. coli heme mutants in media deficient in 5-aminolevulinic acid.

To determine whether these TAO mutants also confer SHAM-sensitive respiration to bacteria, we transformed the normal E. coli cells that have the standard cytochrome system and cyanide-sensitive respiration with mutated or wild type TAO. Respiration of these transformants grown with or without IPTG was assessed in the presence or absence of cyanide, SHAM, or in the presence of both respiratory inhibitors. In E. coli transformed with wild type TAO but not induced with IPTG, 90% of the observed respiration was KCN-sensitive. However, when grown in the presence of IPTG, 45% of the respiration of these transformants was KCN-sensitive, and the residual KCN-resistant respiration of these cells was inhibited by SHAM (Table III). These observations demonstrate that detection of the SHAM-sensitive, KCN-resistant respiration of the cells transformed with TAO mutants or wild type TAO is correlated with the ability of the TAO polypeptide to confer SHAM-sensitive respiration. The kinetics of the respiration of the E. coli transformants containing mutations within the putative EXXH motifs (H165A, E214A, E266A, and H269L) was similar to those of the wild type E. coli cells without plasmid or E. coli cells containing wild type TAO but without IPTG. These mutations completely abolished the ability of TAO to promote SHAM-sensitive respiration in the heme mutant. On the other hand, the respiration of the E. coli transformants containing V205A, L243A, C261A, and V271A located outside of the proposed active site was inhibited 65% by KCN and about 90% by the combination of KCN and SHAM. Therefore, E. coli cells expressing wild type TAO showed about 55% KCN-resistant/SHAM-sensitive respiration. This respiration was reduced to 35% by mutations at V205A, L243A, C261A, and V271A (non-EXXH motif) and was absent in mutations H165A, E214A, E266A, and H269L (EXXH motifs).

Expression of the Mutated and Non-mutated TAO in E. coli Transformants—To analyze whether mutations of TAO have any effect on expression or stability of TAO protein, we measured the steady state level of TAO protein in all E. coli transformants by using immunoblot analysis. Fig. 3 shows that the 35-kDa recombinant TAO protein was expressed in equal amounts in all E. coli cells transformed with wild type TAO and TAO mutants. Coomassie Blue staining of the duplicate gel showed equal loading of the protein. This suggests that mutation of these residues probably has no effect on the level of expression of TAO protein. However, as detected by monoclonal antibody, the size of the protein in E. coli transformed with a TAO mutant C261A was 28 kDa, probably due to truncation of one end of the protein (Fig. 3).

Essentiality of Iron (Fe^{2+}) in the Medium for the Activity of TAO Protein—To demonstrate that iron is needed for the functional activity of TAO, E. coli cells containing wild type TAO were grown in the presence and absence of iron, and KCN-resistant/SHAM-sensitive respiration was measured to assess the activity of TAO. The level of the expressed protein was measured by immunoblots. When OP was added to chelate the metal ions at the time of induction of TAO expression by IPTG,
no SHAM-sensitive/KCN-resistant respiration was observed (Fig. 4A). However, the simultaneous addition of excess iron with OP restored the SHAM-sensitive/KCN-resistant respiration of the *E. coli* as conferred by the wild type TAO to 90% (Fig. 4A). Moreover, we have found that the activity of the expressed TAO in *E. coli* was not restored when equal amounts of Cu²⁺ (Fig. 4A) or other metal ions (e.g. Zn²⁺ and Mn²⁺) (not shown) were added in the medium with OP. The level of the expressed protein remained unaltered in all cases as revealed by immunoblot analysis using monoclonal antibody against TAO as the probe (Fig. 4B). Therefore, iron is essential for the activity of this enzyme.

**DISCUSSION**

TAO is the only terminal oxidase of the bloodstream form of *T. brucei*, an obligate aerobic parasitic protozoa (4). Thus, TAO is probably essential for the parasite survival and an important chemotherapeutic target for African trypanosomiasis. From the similarities of the predicted primary and the secondary structure of TAO and other similar SHAM-sensitive, KCN-insensitive plant and fungi AOXs with known diiron proteins, it has been speculated that the active site structure of this class of oxidases consists of a bimolecular iron center (9, 16). However, neither the native nor the recombinant alternative oxidase (pAOX) and vector alone (pQE30).

**TABLE II**

Respiration assay of the heme-deficient *E. coli* GE1387

| Strains                        | Inhibition by SHAM |
|-------------------------------|--------------------|
| *E. coli* GE1387 containing pTAO | 100                |
| *E. coli* GE1387 without plasmid | 0                   |
| Mutations of the non-conserved residues |             |
| *E. coli* GE1387 containing pTAO V205A | 79±1               |
| *E. coli* GE1387 containing pTAO I218H | 70±3               |
| *E. coli* GE1387 containing pTAO L243A | 74±2               |
| *E. coli* GE1387 containing pTAO C261A | 78±3               |
| *E. coli* GE1387 containing pTAO V271A | 74±1               |

**TABLE III**

Respiration assay of the transformed M15 *E. coli*

Transformed *E. coli* cells were grown in appropriate medium. Cells were harvested, and the oxygen consumption of the whole cells was measured by Instech O₂ electrode as described under "Experimental Procedures.” KCN (1.0 mM) and SHAM (2.0 mM) were used to inhibit the respiration via cytochrome and alternative oxidase, respectively. Results are the mean of three independent experiments, and the standard deviations are represented.

| M15 with or without plasmid | Inhibition of respiration by KCN | Inhibition of respiration by KCN + SHAM |
|-----------------------------|---------------------------------|---------------------------------------|
| No plasmid                  | 100                             | 100                                   |
| pTAO (uninduced)            | 82 ± 4                          | 100                                   |
| pTAO (induced)              | 45 ± 5                          | 100                                   |
| M15 containing TAO mutations |                                 |                                       |
| V205A                       | 55 ± 6                          | 100                                   |
| I218H                       | 68 ± 3                          | 100                                   |
| L243A                       | 55 ± 5                          | 100                                   |
| C261A                       | 65 ± 1                          | 100                                   |
| V271A                       | 63 ± 3                          | 100                                   |
| H165A                       | 84 ± 4                          | 100                                   |
| E214A                       | 90 ± 3                          | 100                                   |
| E266A                       | 83 ± 3                          | 100                                   |
| H269L                       | 84 ± 3                          | 100                                   |

TAO in *E. coli* was not restored when equal amounts of Cu²⁺ (Fig. 4A) or other metal ions (e.g. Zn²⁺ and Mn²⁺) (not shown) were added in the medium with OP. The level of the expressed protein remained unaltered in all cases as revealed by immunoblot analysis using monoclonal antibody against TAO as the probe (Fig. 4B). Therefore, iron is essential for the activity of this enzyme.
dase protein from any system has yet been purified; similarly, no characteristic spectral evidence has been identified. Thus, to understand the active site structure of this enzyme, we have investigated the effect of mutations of different amino acid residues within the proposed active site on the structural and functional properties of this enzyme. We have previously shown that Glu-215 in TAO is essential for its functional activity (15). Here we extend our site-directed mutagenesis studies for several other conserved and non-conserved residues within the proposed active site of TAO. Results revealed that the proposed iron ligands His-165, Glu-214, Glu-266, and His-269 are essential for TAO function. Moreover, it has been found that the presence of iron, $\text{Fe}^{2+}$, in the medium is required for the functional activity of the recombinant TAO. These results provide evidence that TAO is possibly a diiron protein.

The effect of mutations on TAO functional activity was analyzed using the heme mutant of *E. coli*, which is devoid of a functional cytochrome system and a 5-aminolevulinic acid auxotroph. Kumar and Soll (23) first demonstrated that complementation of the *E. coli* heme mutants with *A. thaliana* alternative oxidase permits aerobic growth. TAO was demonstrated to complement the heme mutant of *E. coli* and confer the unique SHAM-sensitive respiration to bacteria (5). This strain of *E. coli* has also been used for the selection of AOX mutants of *A. thaliana* AOX that are resistant to SHAM (24). Therefore, the ability of these heme mutants to express a functional alternative oxidase and to sustain growth and respiration in the absence of 5-aminolevulinic acid was utilized to measure the effect of mutations of different amino acid residues in TAO.

In our studies, mutation of the amino acid residues His-165, Glu-214, and Glu-266 to alanine and that of His-269 to leucine drastically reduces the complementation ability of TAO to *E. coli* heme mutants. These residues are highly conserved among all AOXs characterized to date. Glu-266 and His-269 are in the second EXXH motif in the active site models of AOXs recently proposed by Andersson and Nordlund (16) as well as the model proposed previously by Seidow et al. (9). According to the Nordlund model, Glu-266 residue is the proposed bidentate ligand of iron in helix 4 of the four-helical bundle of the diiron structure (16). His-165 is located in the first EXXH motif, and it is also one of the proposed iron ligands of this model (16). Glu-214 is in the conserved LEEEA region of AOXs and is centrally located in the protein. Results of complementation for these four TAO mutants suggest that these residues are essential for TAO function.

For further characterization of the effect of the mutations, these mutated forms of TAO were expressed in *E. coli* that possesses the normal cytochrome system. The SHAM-sensitive respiration of the transfectants conferred by TAO was measured after inhibition of the cytochrome-mediated oxygen consumption by KCN (1.0 mM). This TAO-dependent SHAM-sensitive respiration can be distinguished from any cytochrome bo activity in *E. coli* that is SHAM- and KCN-sensitive by the amount of inhibitor used in the inhibition studies (24, 25). Thus, sensitivity to SHAM coupled with resistance to cyanide
has been considered to be the functional definition of AOXs (5, 7, 23). E. coli cells expressing wild type TAO showed 45–55% of the respiration cyanide-resistant and SHAM-sensitive. In contrast, the mutated versions of TAO did not produce any SHAM-sensitive respiration, and the cells acted similarly to the wild type E. coli cells and respired by cyanide-sensitive pathway. These results provide strong evidence that His-165, Glu-214, Glu-266, and His-269 are essential for TAO activity.

The LEEEA region has also been found to be highly conserved in all AOXs including TAO. From the primary sequence alignment of the A. thaliana IMMUTANS (Im), which is a plastid enzyme distantly related to AOXs, Berthold et al. (17) postulated that the first Glu of the LEEEA region (Glu-213 in TAO) is possibly involved in the binding of iron. Currently, it is not possible to conclude which of the three Glus is the true ligand of iron. Previously, we have mutated the last Glu (Glu-215 in TAO) to Ala, Leu, Asn, Gln, Asp, and His and assessed the effect of these mutations on TAO function (15). Our results demonstrate that mutation of Glu-215 to Ala, Leu, Asn, and Gln results in the loss of the complementation ability of TAO to heme mutants of E. coli. The conservative substitution of Glu-215 to Asp and His reduced the growth of the E. coli auxotrophs by ~80%. Thus, Glu-215 is essential for the activity of TAO.

This residue has also been mutated to asparagine in S. guttatum AOX, and the protein was expressed in Schizosaccharomyces pombe, which does not have alternative oxidase activity (19). The results demonstrate that the mutation produces a non-functional but properly targeted protein to the mitochondria, which also verifies the essentiality of this glutamate for the functional activity of AOX (19). In the studies reported here, we have further mutated E214A, the second glutamate of the LEEEA region and have found that this mutation drastically reduces the functional complementation ability as well as the ability to confer SHAM-sensitive respiration to the bacteria by TAO. Thus, this provides additional evidence that Glu-214 is also essential for the functional property of TAO. At the present time, we cannot tell from our experiments whether Glu-214 or Glu-215 could be the residue needed for iron binding. However, it is most likely that the conservation of the amino acid residues of this region is essential for maintenance of the structural integrity of the active site of TAO. Berthold (25) demonstrated that the effect of mutation of Phe-215 and Met-218 in A. thaliana alternative oxidase, which are at 7 and 3 amino acids apart from the LEEEA region, respectively, showed partial resistance to SHAM. From this observation Berthold (25) speculated that these residues are possibly involved in SHAM binding as well as for quinone binding because SHAM is thought to be the competitive inhibitor of AOXs. Therefore, it could be anticipated that any alteration of this LEEEA region could possibly hinder the substrate binding or substrate accessibility to the active site.

In the first proposed model of the active site of AOX by Siedow et al. (9), one of the two iron-binding motifs (EXXH) is located in this LEEEA region. In TAO, this region involves residues 215–218. His in this position is not conserved in all AOXs. In TAO and soybean AOX3, this residue is Ile; in H. anomala and Orzya sativa this is Ser, and in Chlamydomonas species this residue is Lys (16). To investigate whether His in this position has any effect on enzyme function, we have mutated Ile-218 in TAO to His. Our results demonstrated that I218H mutation did not show any marked difference in the functional complementation to E. coli heme mutant. However, the ability of this mutated version of TAO to confer cyanide-resistant, SHAM-sensitive respiration to E. coli was reduced about 20% in comparison to the wild type TAO.

Because all the conserved amino acid residues within the proposed active site of TAO we tested showed dramatic reduction in the functional complementation ability, we wanted to test the effect of mutation of some non-conserved residues within this region on TAO function. These included amino acid residues Val-205, located in the interconnecting region between helices 2 and 3, Leu-243 present in the interconnecting region between helices 3 and 4, and Cys-261 and Val-271 in helix 4 in the four-helix model proposed by Nordlund et al. (16), which were all mutated individually to Ala. Mutation of these four residues individually to Ala restored the functional complementation ability of TAO to E. coli heme mutants. The ability to confer SHAM-sensitive respiration to bacteria was reduced about 15–20% in comparison to wild type TAO. Mutation of V205A showed the least effect. All these results suggest that these regions, which consist of mostly conserved residues, are important for AOX function. Mutational analysis of many other conserved and non-conserved amino acid residues in this region and their effect on TAO function is needed for elucidation of the TAO active site structure.

The level of expression of all mutated versions and of wild type TAO in E. coli was analyzed by immunoblot analysis using TAO monoclonal antibody as the probe. Results showed that none of the mutations have any effect on protein expression or the stability of the expressed protein in this system. The only variation has been found for mutation C261A, from which a truncated version of TAO protein (28 kDa) was detected on the immunoblot. The remarkable feature of the diiron protein like ribonucleotide reductase subunit 2 is the stability of its three-dimensional fold (11). The crystal structure analysis of several mutant forms of R2 protein revealed that metal contributes very little to the protein fold or the protein stability (10, 26). This could possibly explain our observation that mutation of several proposed iron ligands in TAO causes total loss of functional activity, whereas the amount of the expressed proteins remained unaltered. C261A mutation in TAO could have possibly altered the protein fold, and as a result, the protein was truncated from one end. However, this cleavage did not affect the functional property of TAO.

Finally, to understand whether iron is needed for TAO functional activity, we expressed the recombinant TAO in E. coli from an inducible expression vector. ortho-Phenanthroline, a metal chelator, was added at the time of induction for the expression of the recombinant protein. Results showed that the amount of protein expression remained almost unaffected in comparison to the cells where OP was not added to the medium. However, the ability of the TAO to confer SHAM-sensitive respiration was reduced about 90–95%. Moreover, the effect of ortho-phenanthroline could be completely negated by addition of excess ferrous iron but not by any other metal ions such as CuSO4. These findings strongly suggest the necessity of iron for the activity of TAO.

These results suggest that TAO is a diiron protein. The structure-function analysis of several other conserved and unique amino acid residues in TAO, purification of the recombinant protein, Mossbauer spectroscopic analysis, and crystal structure determination of this important enzyme will certainly be helpful to confirm the active site structure and hopefully to design successful drugs for African trypanosomiasis.

Acknowledgments—We thank Dr. D. Soll, Yale University, for the generous gift of the E. coli heme mutants. We appreciate the fruitful discussions with Dr. Jim Siedow at Duke University and Dr. Gautam Chaudhuri at Meharry Medical College.

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J. Biol. Chem. 2002, 277:8187-8193.
doi: 10.1074/jbc.M111477200 originally published online December 18, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M111477200

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