Purification of active recombinant trypanosome alternative oxidase

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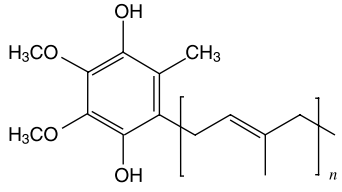
Abstract Trypanosome alternative oxidase (TAO) is the terminal oxidase of the respiratory chain in long slender bloodstream forms of African trypanosomes. TAO is a cytochrome-independent, cyanide-insensitive quinol oxidase. These characteristics are distinct from those of the bacterial quinol oxidases, proteins that belong to the heme-copper terminal oxidase superfamily. The inability to purify stable TAO has severely hampered biochemical studies of the alternative oxidase family. In the present study, we were able to purify recombinant TAO to homogeneity from *Escherichia coli* membranes using the detergent digitonin. Kinetic analysis of the purified TAO revealed that the specific inhibitor ascofuranone is a competitive inhibitor of ubiquinol oxidase activity.

Key words: *Trypanosoma brucei brucei*; Trypanosome alternative oxidase; Alternative oxidase; Parasite mitochondria; Ascofuranone; Quinol oxidase

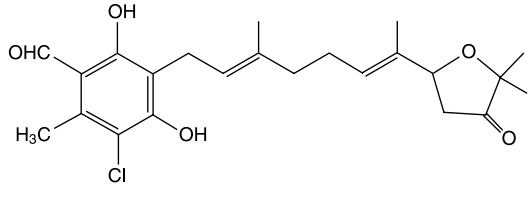
1. Introduction

African trypanosome is a parasite that causes African sleeping sickness in humans and Nagana disease in cattle. Because current drugs against trypanosomes have strong side effects and are not effective for chronic phase patients, new drugs are needed. We have found that the antibiotic ascofuranone (Fig. 1) strongly inhibits the glycerol-3-phosphate (G3P)-dependent mitochondrial oxygen consumption by the bloodstream form of *Trypanosoma brucei brucei*. The G3P oxidase system is composed of G3P dehydrogenase, ubiquinone and cyanide-insensitive ubiquinol oxidase, also known as the trypanosome alternative oxidase (TAO) (see [1] for review). This respiratory system is essential for survival of the bloodstream form of the parasite because it reoxidizes reducing equivalents produced during glycolysis in the glycosome. Ascofuranone, isolated from the phytopathogenic fungus *Ascochyta visiae*, specifically inhibits the quinol oxidase activity of TAO in the mitochondria [2,3]. Since TAO does not occur in the mammalian host, it has been targeted for anti-trypanosomal chemotherapy [1,4].

TAO is a member of the cyanide-insensitive alternative oxidases (AOXs), which have been found in higher plants, algae, yeast, slime molds and free-living amoebae [5–7]. The ubiquitous presence of these enzymes suggests that AOXs have a general physiological role, including maintenance of the cellular redox balance and protection against oxidative stress (see [8] for a review). Like AOXs, the heme-containing terminal oxidases, such as cytochrome *c* oxidase, catalyze the four-electron reduction of oxygen to form water. However, AOXs do not translocate protons, allowing electron transfer to proceed without coupling to ATP synthesis. Spectroscopic studies of the partially purified enzyme indicate that AOXs are also distinct because they do not contain a heme or an iron–sulfur center [8]. Despite this lack of a heme or an iron–sulfur center, studies in the fungus *Pichia anomala* (formerly *Hancenula anomana*)[9] and *T. brucei brucei* [10] show that the enzyme activity requires iron. This iron binding may be contributed by the conserved sequence motif of AOXs, -E (D)- and -EXXH-, which is similar to the binuclear iron binding sequences of the hydroxylase enzymes ribonucleotide reductase R2, methane monooxygenase and Δ9-desaturase [11]. Finally,



Ubiquinol



Ascofuranone

Fig. 1. Structures of ubiquinol and ascofuranone.

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Abbreviations: AOX, alternative oxidase; rTAO, recombinant trypanosome alternative oxidase; TAO, trypanosome alternative oxidase

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the structure and membrane-inserted portions of AOX has been predicted from amino acid sequences of the enzyme [12,13], but biochemical validation has not been available because of the lack of pure protein.

The cDNA for TAO has been cloned and characterized, and TAO has been verified as a member of the AOX family [14,15]. To study the molecular properties of TAO and the mechanisms of the trypanocide ascofuranone, large-scale purification of active enzyme is essential. However, similar to other AOXs, purification of TAO from *T. brucei brucei* is difficult because the enzyme is unstable when solubilized from the mitochondrial membrane [16]. To overcome this problem, the recombinant enzyme must be produced by bacterial overexpression. Such a system has not yet been established, although recombinant TAO (rTAO) has been expressed in *Escherichia coli* [14,15]. As pointed out by Affourtit and coworkers, the lack of a stable fully purified enzyme has severely hampered biochemical studies of AOX family [8]. In addition, the ubiquinol oxidase activity of *E. coli* terminal oxidases (cytochrome *bo* and *bd* complexes) interferes with the kinetic analysis of rTAO [15,17].

In the present study, we expressed rTAO in the *E. coli* Δ*hemA* mutant FN102/pTAO. The rTAO was purified to homogeneity from the *E. coli* membrane using the detergent digitonin. Kinetic analysis of the purified enzyme revealed that ascofuranone is a competitive inhibitor vs. the substrate ubiquinol.

2. Materials and methods

2.1. Establishment of overproduction system for rTAO in E. coli

To obtain the heme-deficient host strain for the expression of rTAO, the Δ*hemA*::Km^R mutation [18] was introduced into the strain BL21(DE3) by P1 transduction. The FN102 heme-deficient strain was then transformed using expression vector pTAO, which carries the cDNA for TAO isolated from *T. brucei brucei* TC221 [15,17]. Ampicillin (100 μg/ml) and kanamycin (50 μg/ml) were used to select rTAO-expressing cells.

2.2. Membrane preparation

The strain FN102/pTAO was grown aerobically at 30°C in 10 l of medium containing 104 g K_2HPO_4, 30 g KH_2PO_4, 7.5 g Na-citrate $2H_2O$, 25 g $(NH_4)_2SO_4$, 100 g tryptone peptone, 50 g yeast extract, 50 g casamino acids, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.25 g $FeSO_4{\cdot}7H_2O$, 2 g glucose and 100 mg ampicillin. Expression of rTAO was induced by the addition of IPTG (100 μM) when the bacterial culture reached 0.1 absorbance units at OD_{550}. The cells were collected after 3 h of culture and resuspended in 60 mM Tris–HCl (pH 7.5), broken by French Pressure Cell (Ohtake, Tokyo) and centrifuged at 27 000 × *g* for 15 min (TOMY MRX-150). The cytosolic supernatant was separated into cytoplasm and a membrane pellet by further centrifugation at 200 000 × *g* for 1 h (Hitachi CS120). The membrane pellet was resuspended in 60 mM Tris–HCl (pH 7.5) and used as the membrane sample.

2.3. Solubilization and purification of rTAO

To find a detergent which solubilizes active rTAO, membrane containing rTAO was incubated with various detergents (1 mg/ml of protein in 60 mM Tris–HCl, pH 7.5) for 1 h at 4°C and centrifuged at 200 000 × *g* for 1 h (HITACHI CS120). The quinol oxidase activities of the samples before centrifugation, as well as of supernatant and pellet were determined.

For the purification of rTAO, membrane was treated with 3% (w/v) digitonin (1 mg/ml of protein in 60 mM Tris–HCl, pH 7.5) at 4°C for 1 h. After centrifugation at 200 000 × *g* for 1 h, the supernatant was applied to a nickel column (2 ml). The column was washed with water, treated with 50 mM $NiSO_4$, then equilibrated with binding buffer containing digitonin (5 mM imidazole, 0.5 M NaCl, 20 mM NaCl, 20 mM Tris–HCl, pH 7.5, 1% (w/v) digitonin). His-tagged rTAO was diluted by 8× binding buffer to a final concentration of 1× and applied to the column, then was eluted with a gradient of 5–250 mM imidazole (1.3 mM imidazole/min; flow rate 0.3 ml/min) in 20 mM Tris–HCl (pH 7.5) and 1% (w/v) digitonin.

2.4. Assay of ubiquinol oxidase

Ubiquinol oxidase activity was determined by measuring the absorbance change of ubiquinol-1 at 278 nm (Shimadzu spectrophotometer UV-3000) [19]. The reaction was initiated with the addition of a membrane sample to a mixture containing 60 mM Tris–HCl (pH 7.5), 0.01% (w/v) digitonin and 150 μM of ubiquinol-1 (millimolar extinction coefficient of 15 for ubiquinone-1) at 25°C.

2.5. Chemicals

All detergents were purchased from Dojin Chemicals (Tokyo, Japan). Digitonin used in this study was biochemistry grade with highest purity. Ubiquinone-1 was purchased from Sigma.

3. Results and discussion

3.1. Overproduction of rTAO

To remove the quinol oxidase activity of heme-containing cytochrome *bo* and *bd* complexes from the host strain, the Δ*hemA*::Km^R mutation [18] was introduced into strain BL21(DE3) by P1 transduction. Because the *hemA*-deficient mutant lacks glutamyl-tRNA reductase needed to catalyze the first step of heme synthesis, the mutant does not grow aerobically in the absence of 5-aminolevulinic acid (ALA), a precursor for protoheme IX synthesis. However, the FN102 heme-deficient strain containing the pTAO plasmid, which contains the cDNA for TAO from *T. brucei brucei* TC221 [15,17], showed similar aerobic growth to wild-type even in the absence of ALA. This indicates that rTAO functions as a terminal oxidase in the respiratory chain of *E. coli*. Finally, to simplify purification of rTAO, we introduced a histidine-tag to the amino terminus of rTAO [15].

When the cells were grown at 37°C, rTAO was produced in inclusion bodies and had no ubiquinol oxidase activity. For this reason, screening of culture conditions was necessary to

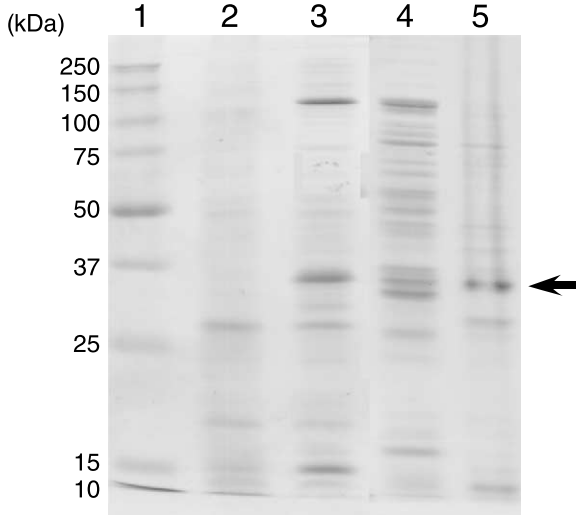

Fig. 2. SDS–PAGE of rTAO. The *E. coli hemA*-mutant was grown as described in Section 2 in the presence of 100 μM IPTG. 5 μg of protein was loaded onto each lane of a 12.5% (w/v) SDS–PAGE. Lane 1, marker; lane 2, membrane from FN102/pET; lane 3, membrane from FN102/pTAO; lane 4, supernatant solubilized from the membrane by digitonin; lane 5, precipitate after solubilization by digitonin.

establish a system for expressing large quantities active rTAO. After extensive testing, we identified optimal conditions for large-scale culture in volumes of more than 10 l (see Section 2). Under these conditions, rTAO accounted for more than 80% of the total membrane protein (Fig. 2, lane 3). The specific activity of ubiquinol oxidase (approximately 5 μmol/min/mg) in the membrane from FN102/pTAO was similar to that of the membrane from the wild-type strain (*hemA*$^+$), which contains cytochrome *bo* and *bd* complexes [19,20]. As expected, there was no ubiquinol oxidase activity in the membrane from the FN102 heme-deficient strain.

3.2. Solubilization and purification of rTAO

We first examined the ability of 20 detergents to extract active TAO. Only digitonin, a non-ionic detergent, solubilized a functional TAO from the membranes of FN102/pTAO cells. Other detergents that have been reported to solubilize AOXs from plant mitochondria, including deoxycholate, BIGCHAP, deoxyBIGCHAP and taurocholate [21–25], were much less effective than digitonin. Some detergents, such as sarcosyl, were able to solubilize TAO but were unable to maintain its quinol oxidase activity. Therefore, we used digitonin for further purification of rTAO.

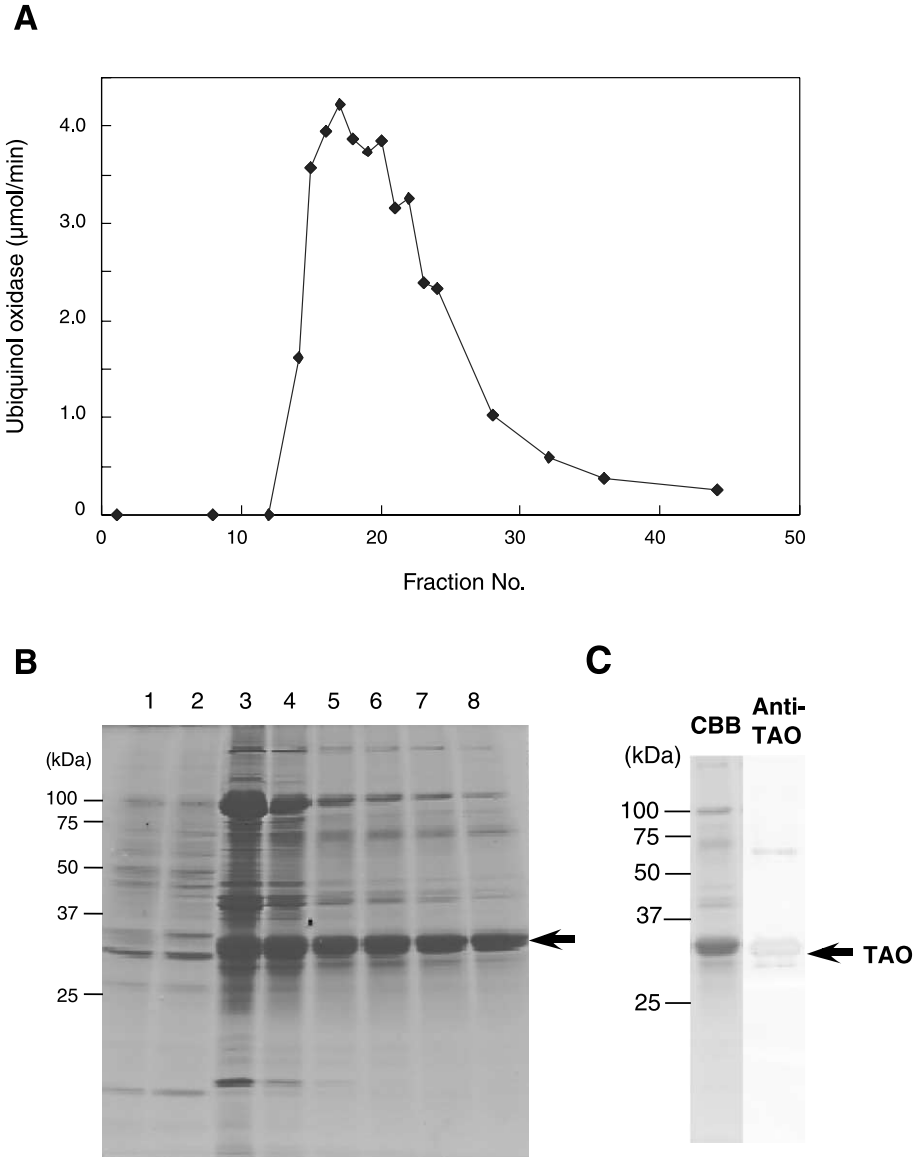

Fig. 3. Purification of rTAO. A: Elution pattern of rTAO from nickel column. The supernatant, solubilized from the cytoplasmic membrane fraction of *E. coli* with 3% (w/v) digitonin, was applied to a nickel column. His-tagged rTAO bound to the nickel column was eluted with a gradient of 5–250 mM imidazole (1.3 mM imidazole/min; flow rate 0.3 ml/min) in 20 mM Tris–HCl (pH 7.5) and 1% (w/v) digitonin. B: 12.5% SDS–PAGE of eluted fractions from nickel column chromatography. Each 850 μl of digitonin-extract and flow through fraction (lanes 1 and 2) were concentrated by trichloroacetate precipitation prior to loading on the gel. Similarly, each 160 μl of eluted fractions 15–20 (lane 3–8) was concentrated by trichloroacetate precipitation prior to loading on the gel. After SDS–PAGE, the gel was stained with Coomassie brilliant blue. The arrow indicates rTAO with an apparent molecular mass of 34 kDa. C: SDS–PAGE and Western blot of purified rTAO obtained by rechromatography of nickel column. Antibody against highly purified rTAO obtained by nickel column in the presence of guanidine was used. Epitope recognized by this antibody was C-terminal domain of the enzyme. Same result was observed when antibody against plant AOX [23] was used (data not shown).

Table 1
Purification of TAO by nickel ion chromatography

Fractions	Protein mg	Total activity μmol/min	Specific activity μmol/min/mg protein	Recovery %
Membrane	94.0	506	5.38	100
Digitonin extract	21.8	86.3	3.96	17.1
Nickel column (fractions 17–20)	0.378	15.7	41.5	3.10

Although the specific activity of rTAO was decreased and a significant amount of rTAO remained in the membrane during solubilization (Fig. 2, lane 5), about 17% of the membrane ubiquinol oxidase activity was solubilized by 3% (w/v) digitonin. Proteins with smaller size than rTAO in the solubilized fraction (Fig. 2, lane 4) may not be proteolytic product of rTAO because only one band was observed in Western blot of this fraction by using monoclonal antibody against C-terminal fragment of the enzyme, although a possibility that this band is N-terminal part of rTAO can not be ruled out. After digitonin-solubilization from the membrane of FN102/pTAO, histidine-nickel affinity chromatography was used to purify rTAO. Fig. 3A shows a typical elution profile of ubiquinol oxidase activity during the purification of rTAO. The isolated rTAO has a molecular mass of 34 kDa and possessed ubiquinol oxidase activity (Fig. 3B). Highly purified enzyme obtained by repurification with nickel chromatography was estimated to be 90% pure by SDS–PAGE (Fig. 3C). We identified a protein band with a molecular mass of 70 kDa in the Western blot, even though SDS–PAGE was run under reducing conditions (Fig. 3C). This may be a dimer of rTAO because this band was recognized by monoclonal antibody against TAO. Dimeric form of TAO in the Western blot was also observed previously (Fig. 3 in [16]).

The specific activity of the purified rTAO was quite high compared to that of the enzymes partially purified from trypanosome [16] and *Sauromatum guttatum* [23] mitochondria, and always exceeded 40 μmol/min/mg protein when quinol oxidase was determined using 150 μM of ubiquinol-1 as substrate. This value is about twice that of partially purified enzyme from *Arum maculatum* reported by Zhang et al. [25]. Table 1 summarizes the purification. More than seven-fold purification was attained, and 3.1% of the activity was recovered from the membrane of FN102/pTAO cells. These results also show that digitonin is essential for keeping the enzyme in an active form during the purification.

3.3. Properties of purified rTAO and the mechanism of inhibition by ascofuranone

Once we had purified rTAO, we analyzed its enzymatic properties. We found that the ubiquinol oxidase activity of rTAO was cyanide-insensitive. Addition of 10 mM of cyanide did not have any effect on the ubiquinol oxidase activity of rTAO. Table 2 summarizes the kinetic properties of TAO in the mitochondria of *T. brucei brucei*, rTAO in the *E. coli* membrane and purified rTAO. The K_m values for ubiquinone obtained from Lineweaver–Burk plot were almost identical between mitochondrial enzyme and rTAO (Fig. 4A and Table 2). These findings suggest that purified rTAO retains the properties of native mitochondrial TAO.

We further examined the mechanism of inhibition by the specific TAO inhibitor ascofuranone. Using either *T. brucei brucei* mitochondria or purified rTAO, ascofuranone was a competitive inhibitor vs. ubiquinol shown by Lineweaver–Burk plot (Fig. 4A). Then, result was analyzed by Dixon plot, because only lower range of substrate concentrations than K_m could be assayed due to the low solubility of ubiquinone as discussed later (Fig. 4B). The analysis showed clearly a competitive inhibition of quinol oxidase activity by ascofuranone, indicating that the site of inhibition of ascofuranone is the quinol binding domain of TAO. The K_i values obtained from Dixon plot were almost identical between mitochondrial and purified enzymes (Fig. 4B and Table 2). The competitive inhibition of quinol oxidase activity by ascofuranone is not surprising given that it and ubiquinol have similar ring structures (Fig. 1). The idea that the ring structure plays an important role in the inhibitory mechanism is further supported by the fact that the inhibitory effect of ascofuranone is dramatically reduced when its 4-OH group is changed to 4-*O*-methoxycarbonylmethyl or 4-*O*-carboxymethyl [2].

Accurate analysis of kinetic property of the enzyme was hampered by the high K_m value for ubiquinone and its low solubility. Because of hydrophobic isoprenoid chain of ubiquinol-1, maximum concentration, which we were able to prepare in the assay mixture, was 150–200 μM (about 20% of the K_m values). Therefore, apparent K_m obtained here may be higher than real K_m values, although V_{max} values obtained from Lineweaver–Burk plot and Dixon plot were almost identical (Fig. 4A,B).

This finding of competitive inhibition contrasts with our previous report that ascofuranone inhibits ubiquinol-dependent oxygen uptake in a non-competitive manner [2]. One possible reason may be low K_i value of ascofuranone. Inhibitor with K_i value of nM order tends to show non-competitive kinetics even when it is competitive with respect to a given substrate [26]. Another possible reason is that this apparent contradiction may be due to the different assay systems used. Specifically, in the previous report, ubiquinol-dependent oxy-

Table 2
Kinetic parameters of TAO in *E. coli* FN102/pTAO cells and *T. brucei brucei* mitochondria

Sample	K_m for ubiquinol-1[a] μM	V_{max}[a] μmol/min/mg protein	K_i[b] nM
T. brucei brucei mitochondria	625	0.77	1.40
E. coli FN102/pTAO membrane	714	10.5	1.29
Purified rTAO	662	240	1.69

[a]Apparent values obtained from Lineweaver–Burk plots.
[b]Values obtained from Dixon plots.

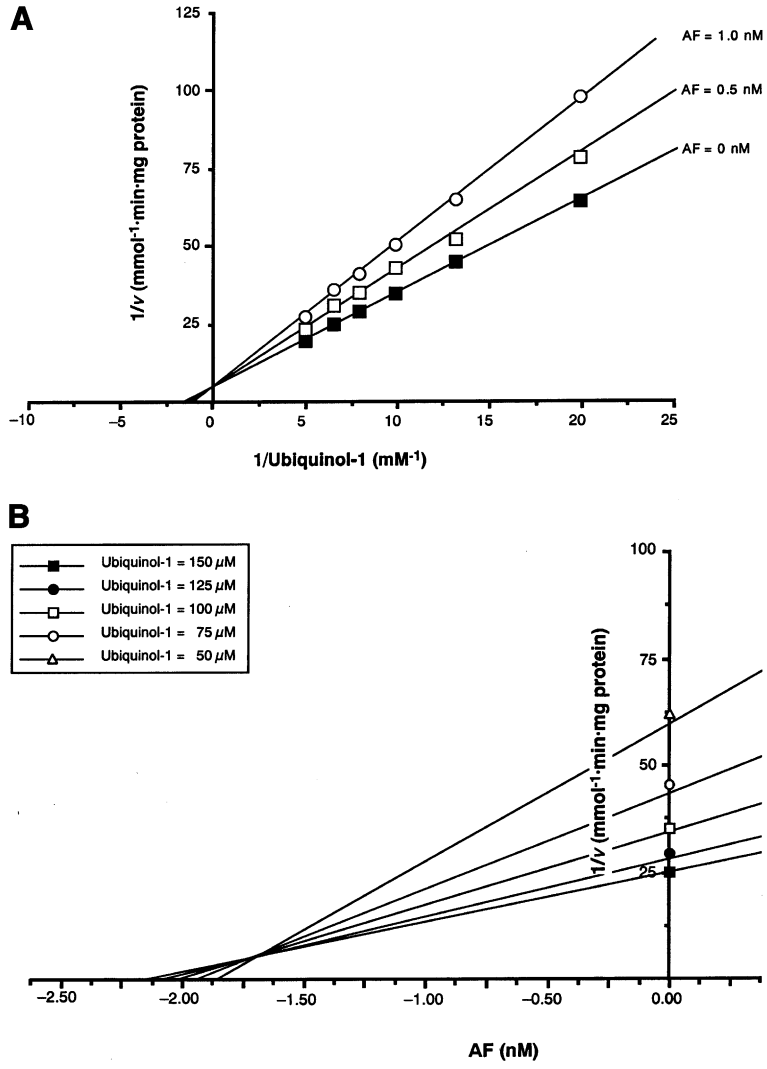

Fig. 4. Kinetic analysis of ascofuranone inhibition of rTAO. The assays were performed in 60 mM Tris–HCl (pH 7.5), ubiquinol-1 (50–200 μM) and 5 μg purified rTAO at 25°C. The reaction was initiated by the addition of rTAO to the reaction mixture. The absorbance at 278 nm (millimolar extinction coefficient of 15 for ubiquinone-1) was used to determine ubiquinol oxidase activity. A: Lineweaver–Burk plot of ascofuranone inhibition of ubiquinol oxidase activity. B: Dixon plot for K_i value determination. Data were taken from Fig. 4A except the points with 200 μM ubiquinol-1.

gen uptake was determined polarographically with an oxygen electrode, while the present study utilized a spectrophotometric assay. The present result showing competitive inhibition is much more reliable because ubiquinol concentration is difficult to control in the polarographic assay due to adsorption to the electrode and the assay chamber made by plastics. For quinol oxidase measurement, spectrophotometric assay is recommendable rather than polarographic assay when the reaction mixture contains hydrophobic substrates with low solubility such as ubiquinol-1. Competitive inhibition of TAO in mitochondria by ascofuranone was also observed when activity was measured by specrophotometric assay (Table 2). Similar K_i values for inhibition by ascofuranone between mitochondrial enzyme and rTAO also suggest that purified rTAO retains the properties of native mitochondrial TAO.

4. Conclusions

Although several groups have reported expression of AOX, including TAO in *E. coli*, information on the specific activity and kinetics of the quinol oxidase activity has been unavailable because of the inability to produce a purified, active enzyme [10,15,27,28]. The present study is the first report in which AOX with high purity and quinol oxidase activity was isolated. Three factors were critical for the purification of rTAO: (i) overproduction of rTAO by the *hemA*-deficient mutant; (ii) solubilization of active enzyme by digitonin; and (iii) addition of an amino-terminal poly-histidine tag, enabling one-step purification of the protein by nickel chromatography. Isolation of pure enzyme enabled analysis of its kinetic properties and revealed the competitive inhibition by ascofuranone. Furthermore, this purified rTAO will provide important information about the cyanide-insensitive quinol oxidases because biochemical analysis of the AOXs, including prosthetic groups, enzyme kinetic mechanisms and three-dimensional structures has not been determined.

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