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COMPETITION BETWEEN INHIBITORS OF THE TRYPANOSOME ALTERNATIVE OXIDASE (TAO) AND REDUCED COENZYME Q_9

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Abstract—The trypanosome alternative oxidase (TAO) is an attractive target for chemotherapy for the diseases caused by African trypanosomes because there is no equivalent enzyme in mammalian hosts. Many inhibitors of this enzyme have been described, but there have been no data on the mechanism of inhibition. In the present study, reduced 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (decyl-CoQ-H_2) was used as a substitute for the natural substrate CoQ_9-H_2 to allow direct measurements of the TAO in crude mitochondrial preparations from *Trypanosoma brucei brucei*. A K_m value of 3.8 μM was obtained for this substrate. The following five compounds that have alkyl side chains from 1 to 4 carbons and belong to three classes of inhibitors showed a competitive inhibition pattern with respect to decyl-CoQ-H_2: *p*-methoxybenzhydroxamic acid, *p*-ethoxybenzhydroxamic acid, *p*-*n*-butyloxybenzhydroxamic acid, methyl 3,4-dihydroxybenzoate and N-*n*-butyl-3,4-dihydroxybenzamide. The following four compounds belonging to the same chemical classes but having alkyl side chains from 10 to 12 carbons showed uncompetitive inhibition patterns: *p*-*n*-dodecyloxybenzhydroxamic acid, *n*-decyl 3,4-dihydroxybenzoate, *n*-dodecyl 3,4-dihydroxybenzoate, and N-*n*-decyl-3,4-dihydroxybenzamide. Clearly, the first group of inhibitors compete with CoQ-H_2 for the active site of the TAO. We propose that the uncompetitive patterns produced by the second group of inhibitors are due to the greater lipophilicity of these compounds and the resulting change in the interaction of the inhibitors and the membrane containing the TAO, thus affecting the local concentration of the inhibitors at the active site.

Key words: Trypanosoma; Trypanosoma brucei brucei; alternative oxidase; trypanosome alternative oxidase; coenzyme Q; coenzyme Q analogues; enzyme kinetics; enzyme inhibitors; drug mechanism

Pathogenic African trypanosomes, the agents of human African sleeping sickness and Nagana, a related veterinary disease, have no cytochromes in the life cycle stage infecting mammals (bloodstream form) but rely on a cyanide-insensitive mitochondrial terminal oxidase [1]. In *Trypanosoma brucei brucei*, this terminal oxidase has been shown to be similar to the alternative oxidases of plant mitochondria; thus, it has been called the TAO‖ [2, 3]. In the rapidly dividing long slender bloodstream forms, the TAO serves as the final step in a glycerol-3-phosphate shuttle transferring electrons from NADH (produced by glycolysis in the glycosomes) to O_2 in the mitochondria, thus maintaining the supply of NAD^+ needed for continued glycolysis. Long slender bloodstream forms have a high rate of TAO-mediated respiration, but this is not thought to be linked to ATP production in the mitochondria. Transitional short stumpy and intermediate bloodstream forms are partially developed towards the procyclic (vector) form in having some Krebs cycle enzymes, although they are still totally lacking cytochromes; in these forms, the TAO supports mitochondrial pathways that do produce ATP despite the lack of cytochromes [4].

Because the highly active, cyanide-insensitive respiratory system of these parasites is not shared by their hosts, this system is a rational target for the development of a selective chemotherapy for African trypanosomiasis. Over 20 years ago aromatic hydroxamates [5] were shown to be active against the enzyme we now refer to as the TAO. Rapid destruction of bloodstream trypanosomes *in vivo* was achieved by a combination of SHAM to inhibit respiration and glycerol to inhibit a glycerol-producing anaerobic glycolytic pathway [6]. Recently, one combination of glycerol with another respiratory inhibitor, N-*n*-butyl-3,4-dihydroxybenzamide, cured 17 of 19 mice infected with *T. brucei brucei* [3].

Besides the aromatic hydroxamates, other iron chelators are active against trypanosome respiration; these include benzotropolones, purpurogallins, alkyl esters of 3,4-dihydroxybenzoic acid and N-*n*-alkyl-3,4-dihydroxybenzamides [3, 7–10]. It was reasonable, therefore, to assume that inhibition was achieved by these inhibitors binding to an iron atom presumed to be associated with the terminal oxidase. However, since all these inhibitors also bear some structural resemblance to CoQ, this assumption had to be reconsidered in light of the knowledge that the terminal oxidase is a ubiquinol:oxygen oxidoreductase [11]. The alternative hypothesis would be that these compounds compete with CoQ-H_2. If these inhibitors operate by displacing CoQ-H_2, kinetic analysis should reveal a competitive inhibition pattern. Con-

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‖ Abbreviations: TAO, trypanosome alternative oxidase [EC number not assigned]; CoQ, coenzyme Q, ubiquinone; CoQ-H_2, reduced CoQ; decyl-CoQ, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; decyl-CoQ-H_2, reduced 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; and SHAM, salicylhydroxamic acid.

versely, if iron chelation were the mechanism of inhibition, kinetic analysis should not reveal a competitive pattern.

Decyl-CoQ-H_2 has been used successfully as a substitute for CoQ-H_2 in an assay of the TAO [11, 12], and we used this compound to examine the kinetic parameters of inhibitors of the TAO. The work reported here reveals that the inhibitors act by displacing CoQ-H_2 from the active site of the enzyme. The importance of defining such mechanisms of action is highlighted by a recent review on the potential of CoQ homologs as chemotherapeutic agents for the treatment of parasitic disease [13]. That review noted that although several antiparasitic agents have structural similarities to CoQ and produce biochemical consequences consistent with interference with CoQ function, the actual site of action is not known.

MATERIALS AND METHODS

Preparation of T. brucei brucei *cell homogenate*

Female Sprague-Dawley rats (100–125 g) were inoculated intraperitoneally with *T. brucei brucei* strain Lab 110 EATRO. Trypanosomes were harvested near the maximal parasitemia (~1 × 10^9 cells/mL blood) by intracardial exsanguination, separation from formed blood elements by DEAE-cellulose chromatography using a phosphate–saline–glucose buffer (PSG) [14] and removal of soluble blood components by washing three times in PSG. The final pellet was homogenized by grinding a cell paste with carborundum grit, and a crude mitochondrial preparation was made by differential centrifugation as previously described [8].

Preparation of decyl-CoQ and inhibitors

Although decyl-CoQ is now available from the Sigma Chemical Co. (St. Louis, MO), for this work we synthesized it by a modification of the method of Wan *et al.* [15], as described in Ref. 11. The complete reduction of decyl-CoQ was performed as described previously [11]. Care was taken to avoid exposing the decyl-CoQ-H_2 to oxygen until it was re-solubilized immediately prior to the assay. Auto-oxidation over the measurement period was shown not to interfere with the assay of the alternative oxidase. Syntheses of all inhibitors were as previously described [3, 8, 9]. Inhibitors were dissolved in 100% ethanol and added to the O_2 electrode chamber. The total volume of ethanol added to the chamber was always less than 100 μL, and this amount of ethanol was shown to have no effect on TAO activity.

TAO measurement

Respiration was measured with a Clark-type oxygen electrode (YSI, Yellow Springs, OH) in a manner similar to that described previously [8]. The assay was begun by adding either 50 or 100 μL of crude mitochondria (depending on the protein concentration which ranged from 7 to 13 mg/mL) to 3.0 mL of a buffer composed of 100 mM sodium phosphate, 73 mM sodium chloride, 5 mM EGTA and 1% defatted bovine serum albumin (Cat. No. A-7030, Sigma Chemical Co.), pH 7.5. Measurements were not considered valid unless the oxygen consumption rate indicated by the electrode (no cells and no substrate) was zero, and addition of decyl-CoQ-H_2 did not increase the rate unless mitochondria were also present. To be certain that there was no oxygen consumption due to any other enzyme, at the end of each assay 30 μL of an ethanolic solution of SHAM (Aldrich Chemical Co., Milwaukee, WI) was added to a final concentration of 2 mM, and the rate of oxygen consumption was observed; the assay was rejected if this rate did not return to the zero rate baseline. For these studies it was necessary to measure oxygen uptake at many different substrate concentrations. Initially this was done in two ways: (1) by performing multiple assays on multiple aliquots of mitochondrial suspensions each with a different initial substrate concentration, and (2) by performing a single assay on a single aliquot of mitochondria but steadily increasing the concentration of the substrate and observing the response in oxygen consumption rate. Kinetic plots indicated that both approaches were valid; therefore, we used only the single aliquot of mitochondria with increasing concentrations of substrate. This had the advantage of being a faster assay system and one that allowed an entire set of kinetic data to be collected on a single mitochondrial aliquot, thus enhancing the precision of the work. However, at least two independent sets of data were collected for each inhibitor, and they always produced the same kinetic pattern. The plots presented represent data from single experiments and were prepared with the aid of SigmaPlot (Jandel Corp., San Rafael, CA) using the linear regression feature of this program without any preset anchor.

RESULTS

CoQ_9 found in *T. brucei brucei* [11, 16] and other natural CoQs are virtually water insoluble and, therefore, not suitable substrates for kinetic analyses. Decyl-CoQ-H_2 is a slightly water-soluble analogue of CoQ_9 in which the 45 carbon isoprenoid chain is substituted by a saturated, straight 10-carbon chain. Decyl-CoQ-H_2 thus serves as a substitute for CoQ for which the concentration can be controlled, and this compound has been used for measurement of the TAO [11, 12]. The measured K_m value of TAO relative to decyl-CoQ-H_2 was 3.8 μM. Specific activities of mitochondrial preparations ranged from 5 to 20 nmol $O_2 \cdot min^{-1} \cdot (mg\ protein)^{-1}$.

Kinetic data for two sets of inhibitors were collected, the difference between the two sets being in the length of the alkyl chain attached to the aromatic ring and the resulting difference in lipophilicity. The first group had alkyl chain lengths from 1 to 4 carbons and the second from 10 to 12 carbons. Data for the first set is presented in Fig. 1 as a panel of Lineweaver-Burk plots. These showed an overall pattern of competitive inhibition by five compounds belonging to three classes of inhibitors: *p-n*-alkyloxybenzhydroxamic acids (*p*-methoxybenzhydroxamic acid, *p*-ethoxybenzhydroxamic acid and *p-n*-butyloxybenzhydroxamic acid), esters of 3,4-dihydroxybenzoic acid (methyl 3,4-dihydroxybenzoate), and an N-n-alkyl-3,4-dihydroxybenzamide (N-*n*-butyl-3,4-dihydroxybenzamide). Figure 2 presents the kinetic data for the other set of inhibitors, which suggest an overall pattern of uncompetitive kinetics. The second set of inhibitors belonged to the same chemical classes: a *p-n*-alkyoxybenzhydroxamic acid (*p-n*-dodecyloxybenzhydroxamic acid), esters of 3,4-dihydroxybenzoic acid (*n*-decyl 3,4-dihydroxybenzoate and *n*-dodecyl 3,4-dihydroxybenzoate) and an N-*n*-alkyl-3,4-dihydroxybenzamide (*N-n*-decyl-3,4-dihydroxybenzamide). Lineweaver-Burk plots were used due to the familiarity of this data transformation and the simplicity of interpretation

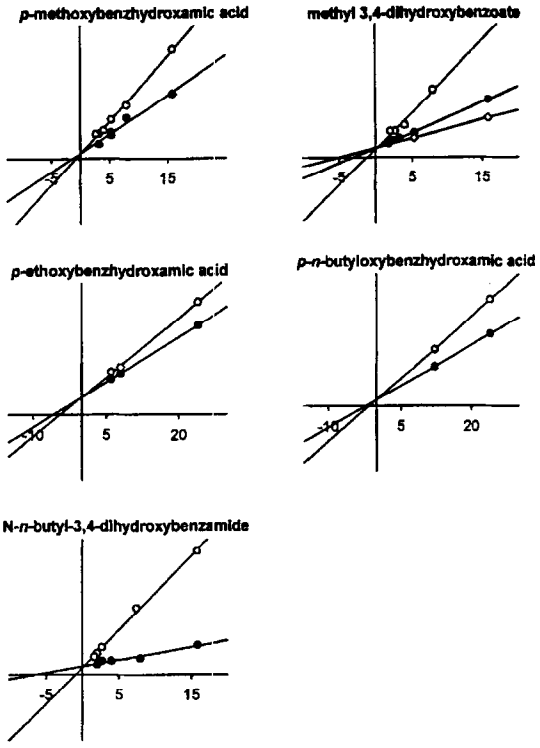

Fig. 1. Lineweaver-Burk plots with inhibitors that displayed competitive kinetics. 1/S (x-axis) was derived from micromolar substrate values. 1/V (y-axis) was derived from slopes from an oxygen electrode recording. Other conditions were as described in Materials and Methods. The concentrations of inhibitors used were as follows: *p*-methoxybenzhydroxamic acid, 240 (○) and 72 (●) μM; *p*-ethoxybenzhydroxamic acid, 75 (○) and 25 (●) μM; N-*n*-butyl-3,4-dihydroxybenzamide, 16 (○) and 1.6 (●) μM; methyl 3,4-dihydroxybenzoate, 48 (○), 28 (●) and 0 (◇) μM; and *p*-*n*-butyloxybenzhydroxamic acid, 17 (○) and 0 (●) μM.

of the graphic representation. The significance of these data lies in the overall patterns of the less hydrophobic inhibitors as compared with the overall pattern of the more hydrophobic inhibitors.

DISCUSSION

When used as a substitute for the natural substrate CoQ_9-H_2, decyl-CoQ-H_2 was observed to have a K_m value of 3.8 μM, which is similar to the 2.1 μM K_m value reported for O_2 [17], the other natural substrate. Based on molecular structures, we had proposed previously that TAO inhibitors operate as competitive inhibitors blocking the transfer of electrons from CoQ-H_2 to O_2 [9–11]. Here we have demonstrated that the overall pattern displayed by several of these inhibitors is one of competitive inhibition with respect to the decyl-CoQ-H_2 substrate, which substitutes for CoQ_9-H_2 (Fig. 1). We conclude that these and related compounds do indeed compete with CoQ_9-H_2 for the active site of the TAO. This conclusion is not refuted by the fact that related compounds failed to show competitive kinetics (Fig. 2). We propose that for these compounds the interaction with the active site of the TAO is exactly the same as for those compounds that show competitive kinetics but that the kinetic pattern is altered by an additional factor affecting the local concentration of the inhibitor in the region of the active site, i.e. increased lipophilicity. This conclusion is supported by the following argument. The TAO is likely an inner mitochondrial membrane protein and its natural substrate, CoQ_9-H_2, is a component of the membrane bilayer. Therefore, any partition of an inhibitor into the membrane will be kinetically significant because this will control the effective concentration of the inhibitor at the active site of the enzyme and may control the orientation of the active moiety of the inhibitor *vis-à-vis* the active site. Since the inhibitor-membrane interaction is necessarily upstream from the interaction of the inhibitor and the active site, the uncompetitive pattern we observed would be expected.

The interpretation above is compatible with the following observations. First, the inhibitors that display uncompetitive kinetics have longer, more hydrophobic side chains, which is consistent with insertion into, and concentration within, the inner mitochondrial membrane. Second, one would expect that if an inhibitor were inserted into the inner mitochondrial membrane, this would enhance activity, and previous observations support this expectation. We have made measurements of apparent K_i values, as well as I_{50} and I_{90} values, under conditions where glycerol-3-phosphate was supplied as an electron source for the reduction of endogenous CoQ_9 via glycerol-3-phosphate dehydrogenase, thus supplying CoQ_9-H_2 to the TAO [3, 8, 9]. These apparent K_i values were obtained from Dixon plots using data collected at one glycerol-3-phosphate concentration; they are not true kinetic constants and are only useful for comparing the effectiveness of different compounds in blocking the TAO *in situ*. Table 1 presents the values obtained from this earlier work. In every case, the compounds with alkyl side chains of 1–4 carbons were at least 10-fold less active than the corresponding compounds with side chains of 10–12 carbons. Third, the active moieties of

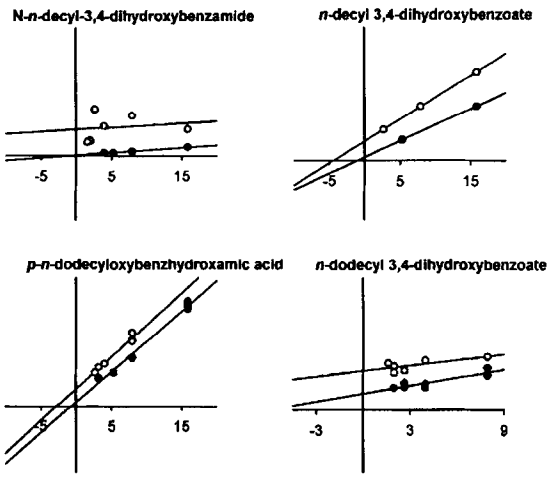

Fig. 2. Lineweaver-Burk plots with inhibitors that did not display competitive kinetics. Conditions were as for Fig. 1. These plots suggest uncompetitive inhibition. The concentrations of inhibitors used were as follows: N-*n*-decyl-3,4-dihydroxybenzamide, 1.9 (○) and 0 (●) μM; *p*-*n*-dodecyloxybenzhydroxamic acid, 1.4 (○) and 0.7 (●) μM; *n*-decyl 3,4-dihydroxybenzoate, 1.75 (○) and 0.7 (●) μM; and *n*-dodecyl 3,4-dihydroxybenzoate, 0.36 (○) and 0 (●) μM.

Table 1. Comparative activities of inhibitors of the TAO

Compound	Apparent K_i (μM)	I_{90} (μM)
Competitive inhibitors		
p-Methoxybenzhydroxamic acid*	18.6	191
p-n-Ethoxybenzhydroxamic acid*	13.9	120
p-n-Butyloxybenzhydroxamic acid*	3.9	136
Methyl 3,4-dihydroxybenzoate†	68	238
N-*n*-Butyl-3,4-dihydroxybenzamide‡	4.0	9.4
Uncompetitive inhibitors		
p-n-Dodecyloxybenzhydroxamic acid*	1.1	10
n-Decyl 3,4-dihydroxybenzoate†	0.4	3.3
n-Dodecyl 3,4-dihydroxybenzoate†	§	0.9
N-*n*-Decyl-3,4-dihydroxybenzamide‡	0.3‖	0.6

* Values are taken from Ref. 8.
† Values are taken from Ref. 9.
‡ Values are taken from Ref. 3.
§ No K_i or I_{50} value was available.
‖ This is an I_{50} value because a K_i was not available.

the inhibitors are the same despite varying lengths of the *n*-alkyl substituents; therefore, the interaction with the active site is probably the same. Accordingly, we conclude that since the compounds presented in Fig. 1 clearly compete with decyl-CoQ-H_2, it is likely that the compounds shown in Fig. 2 do so also.

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