Journal of Bioenergetics and Biomembranes, Vol. 12, Nos. 3/4, 1980

Inhibition of Electron Transfer in the Cytochrome b–c_1 Segment of the Mitochondrial Respiratory Chain by a Synthetic Analogue of Ubiquinone

Bernard L. Trumpower and John G. Haggerty

Department of Biochemistry
Dartmouth Medical School
Hanover, New Hampshire 03755

Received February 7, 1980

Abstract

A synthetic analogue of ubiquinone, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole, inhibits oxidation of succinate and NADH-linked substrates by rat liver mitochondria. Inhibition occurs both in the presence (state 3) and absence (state 4) of ADP. With isolated succinate–cytochrome *c* reductase complex from bovine heart mitochondria the quinone analogue inhibits succinate–cytochrome *c* reductase and ubiquinol–cytochrome *c* reductase activities but does not inhibit succinate–ubiquinone reductase activity. Inhibition of cytochrome *c* reductase activities is markedly dependent on pH in the range pH 7–8. At pH 7.0 inhibition occurs with an apparent $K_i \leq 1 \times 10^{-8}$ M, while at pH 8.0 the apparent K_i is more than an order of magnitude greater than this. Spectrophotometric titrations of 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole show a visibly detectable pK_a at pH 6.5 attributable to ionization of the 6-hydroxy group. These results indicate that this quinone derivative is a highly specific and potent inhibitor of electron transfer in the b-c_1 segment of the respiratory chain. Because of the structural analogy, it is likely that the mechanism of inhibition involves disruption of normal ubiquinone function. In addition, this inhibition depends on protonation of the ionizable hydroxy group of the inhibitory analogue or on protonation of a functional group in the b-c_1 segment.

Introduction

The mechanism of oxidation and reduction of ubiquinone in the mitochondrial respiratory chain is not understood. This problem is of special interest because ubiquinone possesses the intrinsic properties necessary to function as a redox-linked hydrogen carrier by a direct chemiosmotic mechanism [1]. The possible role of ubiquinone in proton translocation has been reemphasized by Mitchell's formulation of the proton-motive Q cycle mechanism, by

0145-479X/80/0800-0151$03.00/0

which ubiquinone might function as the hydrogen carrier for both the second and third coupling sites of oxidative phosphorylation [2].

Although there is extensive evidence to indicate that ubiquinone is an obligatory redox component of the respiratory chain (for a review see [3]), it is not established whether oxidation–reduction of ubiquinone is a transmembrane event as required to form a proton-translocating loop. It is thus important to identify which components of the respiratory chain are electron acceptors and donors for ubiquinone, ubisemiquinone, and ubiquinol and, eventually, to ascertain whether these are asymmetrically disposed across the mitochondrial membrane.

One approach to further understanding the mechanism of ubiquinone function is to obtain inhibitors which disrupt these processes. Folkers and co-workers have synthesized a variety of quinone compounds and examined their effects on growth and respiratory activity in yeast [4]. One such class of compounds is the alkyl-4,7-dioxobenzothiazoles, which were first synthesized as possible antimalarials [5].

One of the alkyl dioxobenzothiazoles, UHDBT,[1] has been shown to inhibit growth of yeast on ethanol, but not on glucose [4]. This quinone inhibited oxidation of succinate and NADH by yeast mitochondria, and a preliminary analysis of the redox poise of the cytochromes suggested this inhibitor acted in the b–c_1 segment in this organism. However, Halsey and Parson [6] showed that 5-pentadecyl-6-hydroxy-4,7-dioxobenzothiazole inhibited electron transfer between the primary and secondary acceptor quinones of the photochemical reaction center of *Chromatium vinosum* but did not report any effect in the cytochrome b region analogous to that observed in yeast.

Subsequently Bowyer and Crofts [7] showed that UHDBT inhibits rereduction of photooxidized cytochrome c_2 and reduction of cytochrome b_{50} in *Rhodopseudomonas capsulata* but found no inhibition of electron transfer between the reaction-center quinones in this organism or in *Rps. sphaeroides*.[2] Most recently it has been found that UHDBT prevents oxidation of the Rieske iron-sulfur center coincident with inhibition of the flash-induced cytochrome c_2 rereduction in *Rps. sphaeroides*, thus suggesting that UHDBT blocks a direct electron-transfer reaction between the iron-sulfur protein and cytochrome c_2 in this bacterium.[3]

[1]The abbreviations used are: UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; DBH, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; Tes, *N*-tris (hydroxymethyl)methyl-2-aminoethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; SCR, succinate–cytochrome *c* reductase complex; CCCP, carbonyl cyanide–*m*-chlorophenyl hydrazone.

[2]J. Bowyer, personal communication.

[3]J. Bowyer, P. L. Dutton, R. Prince, and A. Crofts, personal communication.

The purpose of this paper is to show that UHDBT is a specific and highly potent inhibitor of electron transfer in the b–c_1 segment of the respiratory chain in mammalian mitochondria. In addition, results are presented which show that in these preparations inhibition is dependent on the protonation state of either the inhibitor or a functional component in the b-c_1 complex.

Experimental

Materials

Cytochrome *c* (Type III), succinic acid, malonic acid, glycylglycine, Tes, and DCPIP were obtained from Sigma. Reduced DBH, the analog of ubiquinol-2 which was used as substrate for ubiquinol–cytochrome *c* reductase assays, was prepared as described previously [8]. Lauroyl chloride and 2-amino-6-methoxybenzothiazole were obtained from Eastman and Aldrich Chemical Companies, respectively.

Preparation of Mitochondria and Isolated Succinate–Cytochrome c Reductase Complex

Liver mitochondria were prepared from 200–300 g Sprague Dawley rats. Livers were excised free of mesenteric tissue and placed in 0.25 M sucrose at 4°C. The lobes were minced into approximately 0.5-cm sections and rinsed with sucrose to remove blood and debris.

Approximately 8 g of liver mince was homogenized in 72 ml of 0.25 M sucrose with three to four short strokes of a loose-fitting Teflon–glass homogenizer (A. H. Thomas). The homogenate was centrifuged for 5 min at 600 *g* in a Sorvall SS-34 rotor to remove nuclei and debris, after which the heavy mitochondria were collected from the supernatant by centrifuging for 5 min at 1500 *g*. The mitochondria were washed by suspension and centrifugation from one-half the original volume of sucrose, and then suspended by hand homogenization to approximately 20 mg/ml in sucrose. Typically, the mitochondria showed acceptor control ratios of 8–10 (±ADP) with succinate as substrate.

Succinate–cytochrome *c* reductase complex was isolated from washed bovine heart mitochondria and used either immediately or following short-term storage at −70°C as described previously [9]. A detailed description of the cytochrome content and functional properties of the isolated reductase complex is available [3].

Synthesis of 5-n-Undecyl-6-hydroxy-4,7-dioxobenzothiazole

UHDBT was synthesized as described by Friedman and co-workers [5] with minor modifications. The 6-methoxybenzothiazole intermediate was synthesized by the described alternative route from 2-amino-6-methoxybenzothiazole. The UHDBT product was recovered as an ether solution from the concentrated product mixture as described, after which the ether was evaporated and the product dissolved in hexane and applied to a silica gel (Davison Chemicals, Baltimore) chromatography column. The column was first eluted with hexane to remove nonquinone reaction products derived from lauroyl peroxide, after which the bright yellow UHDBT was eluted from the column with ether-hexane (1:1). The solvent was removed under vacuum and UHDBT was dissolved in hexane, from which it readily crystallized on standing at 4°C.

The purity of UHDBT was confirmed by thin-layer chromatography on silica gel G (Analabs) in 60% ether–40% hexane (R_f = 0.6). The NMR spectrum of the purified UHDBT in $CDCl_3$ was identical to that reported [5]. Absorption spectra of UHDBT are reported below. UHDBT was stored as a dry powder at −10°C and stock solutions were prepared in ethanol. Based on absorption spectra and inhibitory potency, UHDBT appeared to be stable in ethanol at 4°C for at least several weeks.

Assays and Analytical Measurements

Succinate–cytochrome *c* reductase, ubiquinol–cytochrome *c* reductase, and succinate–ubiquinone reductase activities were measured as described previously [8, 9], except the buffers in these assay mixtures were adjusted to the pH values indicated below. Respiratory activity of mitochondria was measured at 30°C with a Clark type polarographic O_2 electrode in a mixture containing 20 mM glycylglycine, 120 mM KCl, 10 mM potassium phosphate, 5 mM $MgCl_2$, pH 7.0, and 1–2 mg/ml of mitochondria. The reaction was started by adding 20 mM succinate or 2 mM β-hydroxybutyrate.

Rates of cytochrome *c* reductase and succinate–ubiquinone reductase are expressed in units of 1 electron equivalent, a unit being defined as 1 μmol of cytochrome *c* or 1 μg equivalent of DCPIP reduced per minute. Rates of O_2 consumption are expressed as ng atom of oxygen ($\frac{1}{2}O_2$) per minute.

Midpoint potentials of UHDBT were measured in collaboration with R. Prince and P. L. Dutton at the University of Pennsylvania. Measurements were made on 40 μM UHDBT in 20 mM arginine, 100 mM KCl, and 1 mM $MgCl_2$ containing 1% ethanol. The pH was adjusted as indicated in Fig. 2 by addition of HCl, Mes, or KOH. A glassy carbon electrode was used over the potential range +300 to −250 mV and a dropping mercury electrode was

used over the range −100 to −400 mV. Where the midpoint potential could be measured by either electrode, there was excellent agreement in the experimentally determined E_m values obtained by the two methods as shown above.

Results

Properties of 5-n-Undecyl-6-hydroxy-4,7-dioxobenzothiazole

UHDBT is a structural analogue of ubiquinone in which the ring methyl group of ubiquinone is replaced by a hydroxy group and the two methoxy groups are replaced by a fused thiazole ring. Both ubiquinone and UHDBT contain hydrocarbon side chains, which account for their hydrophobic properties, although the isoprenoid side chain which is a distinguishing feature and may be important to the biological function of ubiquinone [10] is replaced by a saturated side chain in UHDBT.

Hydroxybenzoquinones consist of a tautomeric mixture of *ortho-* and *para*-quinone. The tautomers of UHDBT are shown in Fig. 1. On addition of

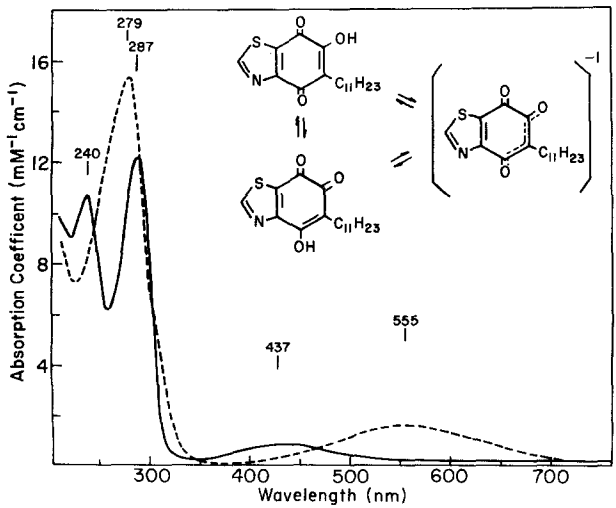

Fig. 1. Absorption spectra and structures of 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole showing the effect of ionization of the 6-hydroxy group. The solid line shows the spectrum of UHDBT obtained under acidic conditions in ethanol containing 0.1 mM acetic acid. The dashed line shows the spectrum of UHDBT obtained under alkaline conditions in ethanol containing 0.1 mM NH_4OH. The two structures to the left are of the *ortho-* and *para*-quinone tautomers of UHDBT under conditions where the 6-hydroxy group is un-ionized. The structure to the right approximates the resonance hybrid of UHDBT under conditions where the 6-hydroxy group is ionized.

alkali to an ethanolic solution of UHDBT the yellow quinone changes color to a rose-violet, and on subsequent addition of acid the solution instantly returns to yellow. This pH-dependent change is due to ionization of the hydroxy group of UHDBT and is common to hydroxybenzoquinones [11]. Absorption spectra of the hydroxyquinone and its anion are shown in Fig. 1. The un-ionized UHDBT has absorption maxima at 241, 287, and 445 nm (ϵ_{mM} = 10.6, 12.2, and 0.77, respectively). Ionization causes a shift of the UV absorbance to 279 nm and an increase in extinction coefficient (ϵ_{mM} = 15.6). The visible spectrum of the anion has a peak at 555 nm (ϵ_{mM} = 1.60) which was monitored in spectrophotometric titrations. The structure of the hydroxyquinone anion is most closely approximated by the resonance hybrid shown in brackets.

As shown below, spectrophotometric titrations showed that ionization of the hydroxy group of the quinone occurs with pK = 6.5. Absorption spectra recorded at various pH values during spectrophotometric titrations showed isobestic points both in the visible and UV range (see Fig. 1), thus indicating

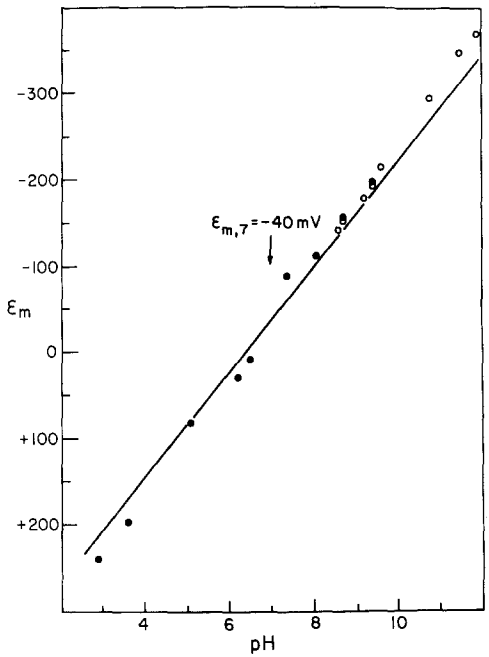

Fig. 2. Effect of pH on the midpoint potential of 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole. The solid circles are experimentally determined points obtained with a glassy carbon electrode. The open circles are experimentally determined points obtained with a dropping mercury electrode. The solid line shows a theoretical slope of 60 mV per pH unit.

that the pH-dependent spectral change is due to a simple two-component system.

In order to relate the possible oxidation–reduction reactions of UHDBT to its inhibitory properties, measurements were made of its midpoint potential at various pH values as shown in Fig. 2. Over the pH range 3–12 the experimentally determined values of E_m closely fit a theoretical slope of 60 mV/pH unit, shown by the solid line. A linear regression analysis of the data indicated a best fit of 68 mV/pH unit. Thus the midpoint potential of UHDBT shows a pH dependence typical of quinones, described by a Nernst relationship of $2H^+/2e^-$ with $E_{m,7} = -40$ mV. There appeared to be little, if any, change in the slope of the E_m versus pH curve in the pH range 6–7, corresponding to the spectrophotometrically measured pK_a of the quinone hydroxy group. This result indicates that the pK_a of the ionizable hydroxy group is nearly identical in the quinone and hydroquinone forms of UHDBT.

*Effect of 5-*n*-Udecyl-6-hydroxy-4,7-dioxobenzothiazole on Respiratory Activity of Rat Liver Mitochondria*

UHDBT inhibits succinate oxidase activity of rat liver mitochondria, and inhibition occurs both in the presence and absence of ADP, as shown in Fig. 3. Similar results were obtained when β-hydroxybutyrate was used as substrate and likewise when the mitochondria were uncoupled by addition of CCCP (results not shown). These findings agree with those of Roberts and co-workers [4], who found that UHDBT inhibited oxidation of succinate and NADH by yeast mitochondria.

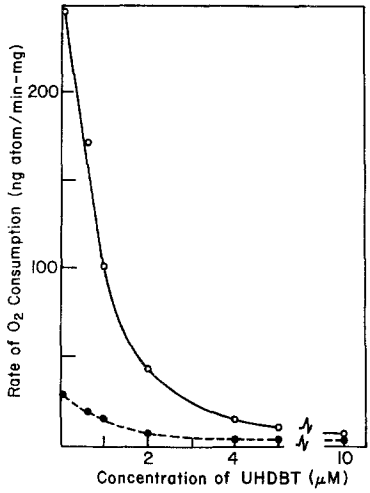

Fig. 3. Effect of 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole on succinate oxidase activity of rat liver mitochondria. The open circles show the effect of UHDBT on succinate oxidation under state 3 conditions, in the presence of 0.62 mM ADP. The solid circles show the corresponding effect of UHDBT on succinate oxidation under state 4 conditions, in the absence of ADP.

It is interesting to note that, at concentrations which fully inhibit respiration, UHDBT appears not to act as an uncoupler as evidenced by the lack of any increase in the state 4 respiration rate on addition of inhibitor. In contrast, if CCCP was added to an assay in which the state 4 respiratory rate was partially inhibited by UHDBT, there was an increase in the respiratory rate to a level comparable to that obtained by addition of UHDBT to mitochondria respiring in state 3 (results not shown). A possible explanation for lack of uncoupling by this hydrophobic weak acid is discussed below.

In the experiments shown in Fig. 3 half maximal inhibition was obtained with $\simeq 1$ μM UHDBT. However, as noted below, the apparent K_i is dependent on protein concentration in the assay due to the nearly stoichiometric binding of UHDBT to the b–c_1 complex.

Effect of 5-n-*Undecyl-6-hydroxy-4,7-dioxobenzothiazole on Electron Transfer Activities of Isolated Succinate–Cytochrome c Reductase Complex*

In view of the above results and previous evidence that UHDBT acts on the b–c_1 segment of the respiratory chain [4], we undertook a more detailed analysis of the effects of UHDBT on the electron-transfer activities of isolated succinate–cytochrome c reductase complex. With the isolated reductase complex UHDBT inhibits electron transfer from succinate to cytochrome c, as illustrated in Fig. 4a. However, under comparable conditions there was no inhibition of succinate–ubiquinone reductase activity (Fig. 4c).

The tracings in Fig. 4b demonstrate that UHDBT inhibits ubiquinol–cytochrome c reductase activity, thus localizing the site of inhibition in the b–c_1 segment. The tracing on the left shows the control activity, in which the reduced quinone substrate (DBH) was added first to show the nonenzymic rate, and after approximately 5 sec the enzymic reaction was initiated by addition of reductase complex. As shown in the tracing on the right, if UHDBT is added to the reductase complex there is extensive inhibition of cytochrome c reduction, which becomes maximal approximately 5 sec after the reaction is started by adding DBH.

The differential effect of UHDBT on the electron-transfer activities of isolated reductase complex is shown quantitatively in Fig. 5. Both succinate–cytochrome c reductase and ubiquinol–cytochrome c reductase activities are inhibited by UHDBT, and half maximal inhibition occurs at ≤ 25 nM under the conditions of these assays. Under the same conditions of pH and enzyme concentration only approximately 5% of the succinate–ubiquinone reductase activity is inhibited by 25 nM UHDBT. Even at concentrations of UHDBT as high as 25 μM it was not possible to inhibit more than 40% of the succinate–ubiquinone reductase activity (results not shown), and at higher

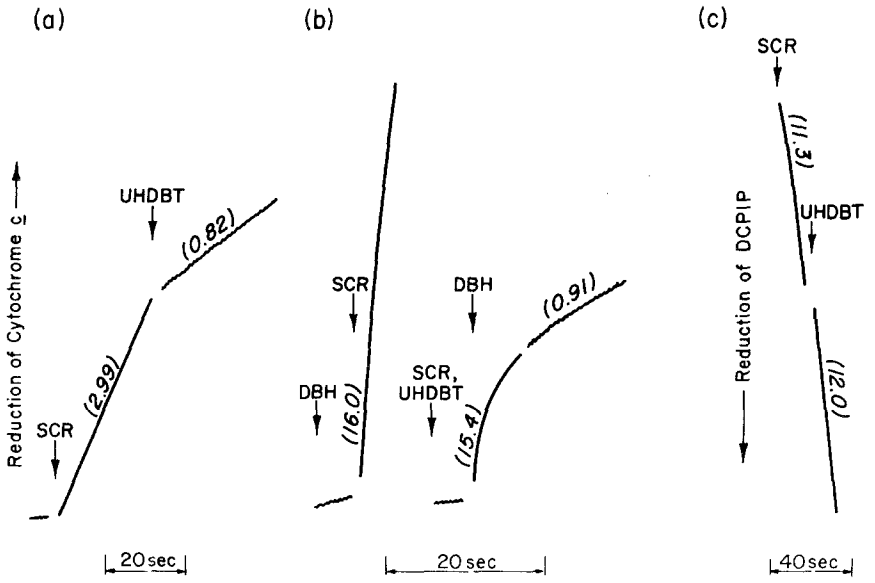

Fig. 4. Effect of 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole on electron-transfer activities of isolated succinate–cytochrome *c* reductase complex. The tracing in (a) is of succinate–cytochrome *c* reductase activity. The reaction was started by adding 1.8 nM reductase complex (SCR), and 40 nM UHDBT was added as indicated. The tracing on the left in (b) is of ubiquinol–cytochrome *c* reductase activity in the absence of inhibitor. The nonenzymic rate of cytochrome *c* reduction was obtained by adding 60 μM DBH, after which the reaction was started by adding 1.8 nM reductase complex. The tracing on the right in (b) is of ubiquinol–cytochrome *c* reductase activity in the presence of UHDBT. After adding 1.8 nM reductase complex (SCR) and 40 nM UHDBT, the reaction was started by adding 60 μM DBH. The tracing in (c) is of succinate–ubiquinone reductase activity. The reaction was started by adding 1.8 nM reductase complex (SCR), after which 50 nM UHDBT was added as shown. All of the activities were measured at pH 7.0, and the numbers in parentheses are rates of electron transfer in units/mg.

concentrations the UHDBT was not soluble. The assay conditions in the experiments shown in Fig. 5 actually minimize somewhat the differential effect of UHDBT on these activities since, as shown below, the efficacy of inhibition of cytochrome *c* reductase activity increases as the pH is lowered, and this pH effect was not observed with the slight inhibition which could be obtained in the succinate–ubiquinone reductase reaction.

As illustrated by the experiment in Fig. 4b, there is a lag in the inhibition of ubiquinol–cytochrome *c* reductase activity by UHDBT. Although this lag is not evident in the succinate–cytochrome *c* reductase assay shown in Fig. 4a, this is probably due to the lower turnover number of this activity, since with higher amounts of reductase complex there was a delay in inhibition in this assay comparable to that illustrated with the ubiquinol–cytochrome *c* reduc-

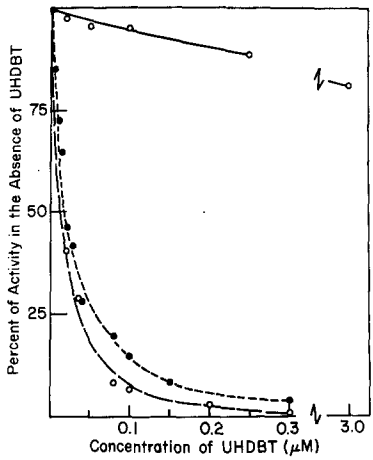

Fig. 5. Effect of various concentrations of 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole on electron transfer activities of isolated succinate–cytochrome *c* reductase complex. In the absence of UHDBT succinate–ubiquinone reductase activity (—O—O—) was 12.4 units/mg, succinate–cytochrome *c* reductase activity (--●--●--) was 3.0 units/mg, and ubiquinol–cytochrome *c* reductase activity (--O--O--) was 15.4 units/mg. All of the activities were measured at pH 7.0.

tase assay. This delayed inhibition is observed if the inhibitor is added to a reaction which is initiated by addition of reductase complex (results not shown), and the duration of the lag is not diminished by adding the inhibitor to the reductase complex prior to starting the reaction with reduced quinone substrate. Likewise the delay was not eliminated by allowing the reductase complex to mix in the cytochrome *c* containing reaction mixture prior to addition of UHDBT and substrate. These results suggest the lag in inhibition cannot be ascribed to a simple time-dependent binding phenomenon nor to a requirement that the redox components of the reductase complex be converted to the oxidized state.

Inhibition of cytochrome *c* reductase activities by UHDBT is dependent on the pH of the reaction mixture. The pH dependence of inhibition of succinate–cytochrome *c* reductase activity is illustrated in Fig. 6. At pH 7.5 half maximal inhibition requires approximately 150 nM UHDBT. As the pH is lowered the efficacy of inhibition is increased, such that at pH 6.8 half maximal inhibition occurs at $\simeq$10 nM UHDBT. This pH dependence of inhibitor efficacy cannot be attributed to the pH dependence of the succinate–cytochrome *c* reductase rate. Although there is a substantial change in the control rate of cytochrome *c* reduction as the pH is lowered, the most extensive change in efficacy of inhibition by UHDBT occurs between pH 7.5 and 7.2, in which range there is only a slight pH dependence of the succinate–cytochrome *c* reductase activity (Fig. 6). This same pH dependence of inhibition was observed in the ubiquinol–cytochrome *c* reductase assay (results not shown).

A comparison of the effect of pH on the efficacy of inhibition by

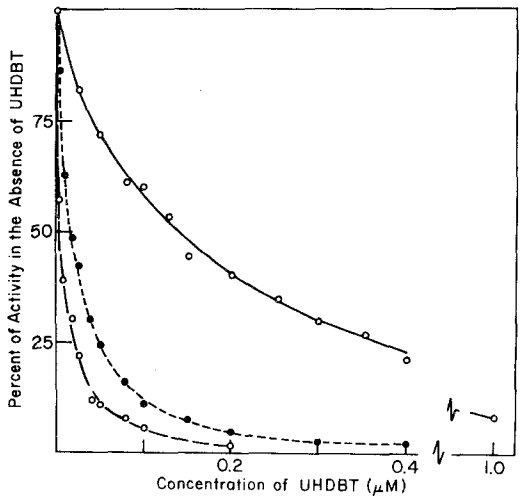

Fig. 6. Effect of pH on inhibition of succinate–cytochrome *c* reductase activity by 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole. The titration curves were obtained at pH 7.5 (—O—O—), pH 7.2 (--●--●--), and pH 6.8 (--O--O--). In the absence of UHDBT the succinate–cytochrome *c* reductase activities were 5.6 (pH 7.5), 5.3 (pH 7.2), and 3.7 (pH 6.8) units/mg.

UHDBT and on the ionization of the 6-hydroxy group of the inhibitor is shown in Fig. 7. Spectrophotometric titrations of UHDBT showed that ionization of the hydroxy group occurs with $pK_a = 6.5$, and spectra obtained at different pH values showed excellent agreement between the pK_a calculated from the absorbance increments in the visible and UV regions of the spectra. There appeared to be no significant change in the spectrophotometrically determined pK_a if the concentration of ethanol in the buffer mixture was increased to 5%.

As shown in Fig. 7, there is an extensive change in efficacy of inhibition of cytochrome *c* reductase activity by UHDBT which extended from pH 6.5 to pH 8.0. Although these results show quite clearly that there is a pronounced pH dependence of inhibition by UHDBT, it is not possible to calculate an apparent pK_a for this effect since the pH-dependent decline of the cytochrome *c* reductase activity precludes extending this analysis to lower pH.

As noted above, the concentration of UHDBT required to inhibit respiratory activity of mitochondria and cytochrome *c* reductase activities of isolated reductase complex is dependent on the concentration of protein in the assay mixture due to nearly stoichiometric binding of UHDBT to the b–c_1 complex. For inhibitors with high affinities the relationship between fraction

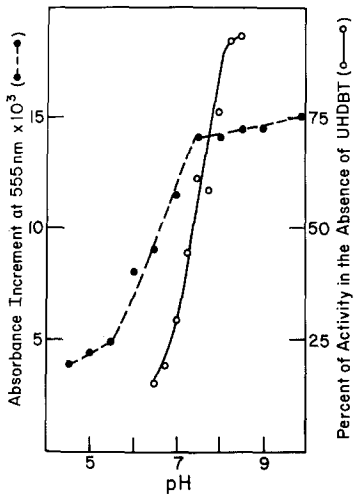

Fig. 7. Effect of pH on ionization of the 6-hydroxy group of 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole and on efficacy of inhibition of succinate–cytochrome *c* reductase activity of isolated reductase complex. The dashed line shows the spectrophotometric titration of UHDBT, in which the ionization of the 6-hydroxy group was measured from the absorbance increment at 555 nm (see Fig. 1). The spectrophotometric titration was performed on 10 μM UHDBT in 20 mM sodium phosphate containing 1% ethanol. The solid line shows the extent of inhibition of succinate–cytochrome *c* reductase activity by 50 nM UHDBT, which was calculated as percent of the control activity in the absence of UHDBT, at the indicated pH values.

of activity remaining in the presence of inhibitor (α), total amount of inhibitor added (i_t), molarity of enzyme (e), and K_i is described by the equation $i_t\,[\alpha/(1-\alpha)] = K_i + \alpha e$ [12]. Applying this analysis to the results shown in Fig. 6 indicated that at pH 6.8 and 7.2 inhibition occurs with K_i = 5–10 nM, and at pH 7.5 K_i increases to approximately 75 nM. In these assays the concentration of b–c_1 complex was $\simeq$1.5 nM. These results demonstrate that UHDBT binds with high affinity; however, it is not possible to extend this analysis to calculate the number of binding sites due to the pH dependence of the cytochrome *c* reductase reaction. The high affinity of UHDBT binding probably explains why this hydrophobic weak acid does not uncouple state 4 respiration as noted above. At concentrations which inhibit respiration it is likely that most of the UHDBT added is bound to the b–c_1 segment and not freely mobile in the membrane to otherwise act as a proton ionophore.

Discussion

The results presented here show that the synthetic analog of ubiquinone, UHDBT, is a highly potent and specific inhibitor of respiration in the cytochrome b–c_1 segment of the respiratory chain of bovine heart and rat liver mitochondria. These findings agree with those of Roberts and co-workers [4], who reported that UHDBT inhibited respiration in yeast mitochondria. Thus, although there appear to be species-specific differences in the effects of

UHDBT in photosynthetic bacteria (see Introduction), it is likely that this inhibitor is generally applicable to investigating the mechanism of ubiquinone involvement in the respiratory chain of eukaryotic mitochondria.

With isolated succinate–cytochrome *c* reductase complex it has been possible to show conclusively that the site of inhibition by UHDBT is in the b–c_1 segment, and, in addition, these experiments have revealed several noteworthy aspects of this inhibition. One potentially important finding is that inhibition by UHDBT is pH dependent. This pH dependence was either absent or not noted in yeast mitochondria [4] and likewise has not been observed in photosynthetic bacteria [7]. It will be interesting to learn the basis for this apparent difference.

The pH dependence may be due to ionization of the weakly acidic hydroxy group of the inhibitor. Alternatively, inhibition by UHDBT may depend on protonation of a functional group in the b–c_1 segment. Previous work with pigeon mitochondria and reductase complex therefrom has shown that the iron–sulfur protein of the b–c_1 segment has a pK_a at pH 8.0 in the oxidized form [13], and cytochrome b-566 exhibits a pH-dependent midpoint potential above pH 7.0 [14]. Further experimentation is required to establish whether the redox components in our preparations of reductase complex exhibit pH-dependent behavior and, if so, to devise methods for testing whether the efficacy of UHDBT inhibition manifests a pK_a which can be correlated either with that of one of the redox components or with the pK_a for ionization of the UHDBT hydroxy group under conditions where the inhibitor is bound to the b–c_1 segment.

We have also found that there is a time-dependent lag in the inhibition of cytochrome *c* reductase activities by UHDBT. This lag could not be attributed to a slow binding of inhibitor, and it could not be eliminated by converting the redox components of the reductase complex to the fully oxidized form. We considered the possibility that the lag may result from a slow prerequisite reduction of UHDBT. However, in experiments not described above we tested and found that UHDBT was not reduced by reductase complex under conditions comparable to those in the cytochrome *c* reductase assays, which is consistent with the finding that the midpoint potential of this quinone ($E_{m,7} = -40$ mV) is substantially lower than that of the succinate–fumarate couple.

It is possible that inhibition of UHDBT occurs only under conditions which generate an intermediate redox poise in the b–c_1 segment. In this regard it should be noted that we have recently detected a novel stable form of ubisemiquinone in the b–c_1 segment of isolated reductase complex [15], and it is conceivable that the inhibitory analog may alter the stability of such a

semiquinone or its oxidation–reduction reactions with other redox components in the b–c_1 segment.

Acknowledgments

This investigation was supported by an NIH Research Grant, GM 20379. B.L.T. is an Established Investigator of the American Heart Association.

References

1. P. Mitchell. *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation,* Glynn Research Ltd., Bodmin, Cornwall, England (1966).
2. P. Mitchell. *J. Theor. Biol.,* **62** (1976) 327–367.
3. B. L. Trumpower and A. Katki, in *Membrane Proteins in Energy Transduction,* R. A. Capaldi, ed., Marcel Dekker, New York (1979), pp. 89–200.
4. H. Roberts, W. M. Choo, S. C. Smith, S. Marzuki, A. W. Linnane, T. H. Porter, and K. Folkers. *Arch. Biochem. Biophys.,* **191** (1978), 306–315.
5. M. D. Friedman, P. L. Stotter, T. H. Porter, and K. Folkers, *J. Med. Chem.,* **16** (1973) 1314–1316.
6. Y. A. Halsey and W. W. Parsons, *Biochim. Biophys. Acta,* **347** (1974) 404–416.
7. J. R. Bowyer and A. R. Crofts, in *Frontiers of Biological Energetics,* P. L. Dutton, J. S. Leigh, and A. Scarpa, eds., Vol. 1, Academic Press, New York (1978), pp. 326–333.
8. B. L. Trumpower and C. A. Edwards, *J. Biol. Chem.,* **254** (1979) 8697–8706.
9. B. L. Trumpower and Z. Simmons, *J. Biol. Chem.,* **254** (1979) 4608–4616.
10. B. L. Trumpower and C. E. Landeen, in *The Ealing Review,* pp. 4–7, Sept./Oct (1977).
11. R. A. Morton, in *Biochemistry of Quinones,* R. A. Morton, ed., Academic Press, New York (1965) pp. 23–65.
12. M. Dixon and E. C. Webb, *Enzymes,* 2nd edn., Academic Press, New York (1964).
13. R. Prince and P. L. Dutton, *FEBS Letts.,* **65** (1976) 117–118.
14. J. S. Leigh and M. Erecinska, *Biochim. Biophys. Acta,* **387** (1975) 95–106.
15. T. Ohnishi and B. L. Trumpower, *J. Biol. Chem.* **255** (1980) 3278–3284.