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Effect of Ketoconazole on Isolated Mitochondria from *Candida albicans*

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Ketoconazole, an oral antimycotic imidazole drug, blocked the transport of electrons in the respiratory chain of *Candida albicans* under aerobic conditions with different substrates, such as NADH and succinate. This effect was a nonspecific inhibition of NADH oxidases and succinate oxidases. The addition of ketoconazole to *C. albicans* mitochondria without a substrate resulted in strong reduction of cytochrome a_3, as revealed by difference spectra (reduced versus oxidized). This indicated that there was a specific interaction between ketoconazole and cytochrome *c* oxidase. A spectrophotometric analysis confirmed that the cytochrome oxidases other than cytochome *c* oxidase were not inhibited because subsequent addition of any substrate caused an increased level of reduction of all of the other respiratory chain components compared with the control. Consequently, our data strongly suggested that the primary site of ketoconazole inhibition on isolated mitochondria from *C. albicans* is the most distal portion of the respiratory chain.

Ketoconazole (R41,400; Janssen Pharmaceutica) is the newest oral imidazole compound which has broad-spectrum antifungal activity, including activity against dimorphic fungi and pathogenic yeasts (2).

The suppression of experimental cryptococcosis, histoplasmosis, and coccidioidomycosis by ketoconazole has been described previously (6, 16). The efficacy of ketoconazole in the treatment of patients with paracoccidioidomycosis (11) and with chronic mucocutaneous candidiasis and other superficial mycoses (3) has also been reported previously.

Despite its relatively low activity against *Candida albicans* in vitro, we found that ketoconazole was very effective against systemic experimental candidiasis when it was administered intraperitoneally or orally compared with clotrimazole (13). The blood level obtained by oral administration was quite high and persistent, yet still far below the minimal inhibitory concentration for our test strain of *C. albicans* (≥50 μg/ml) in vitro. However, ketoconazole produced incomplete inhibition of this opportunistic yeast at concentrations ranging from 25 to 0.2 μg/ml (47 to 0.4 μM), as determined by the agar dilution method. This markedly broad concentration range for incomplete inhibition was observed only with *C. albicans*.

The experiments described below were based on findings which strongly suggested that the primary site of ketoconazole action against *C. albicans* was the respiratory chain of the organism (15). We found that at low drug concentrations, 2,3,5-triphenyl tetrazolium chloride, an electron acceptor, was reduced more rapidly by *Candida* cells exposed to ketoconazole than by control cells, indicating that ketoconazole acts on the electron transport system. Furthermore, we found that this effect was not reversed when *C. albicans* was grown in ergosterol-containing medium. On the other hand, inhibition of *C. albicans* respiration by ketoconazole was detected within minutes.

Very recently, Sud and Feingold (14) reported that the minimal inhibitory concentration of ketoconazole for *Saccharomyces cerevisiae* is much higher under anaerobic conditions than under aerobic conditions.

In this paper we describe the determination of the site of ketoconazole action at the mitochondrial level in *C. albicans*.

MATERIALS AND METHODS

Chemicals. Ketoconazole (KW1414) was kindly supplied by Kyowa Hakko Kogyo Co., Tokyo, Japan. Crystalline antimycin A was obtained from Boehringer Mannheim GmbH, Mannheim, Germany. Zymolyase 5000 (cell wall digestive enzyme) was obtained from Kirin Brewery Co., Ltd., Tokyo, Japan.

Growth of *Candida* cells. *C. albicans* 7N, which was isolated from a patient with pulmonary candidiasis and was maintained in our laboratory, was grown aerobically with shaking in yeast-peptone-glucose broth containing the following (per liter): 3.5 g of yeast extract, 3.5 g of peptone, 2.0 g of KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, 1.0 g of $MgSO_4$, and 10 g of glucose. The cells were grown for 16 h at 36°C and then harvested by centrifugation at 700 × *g* and washed twice with distilled water

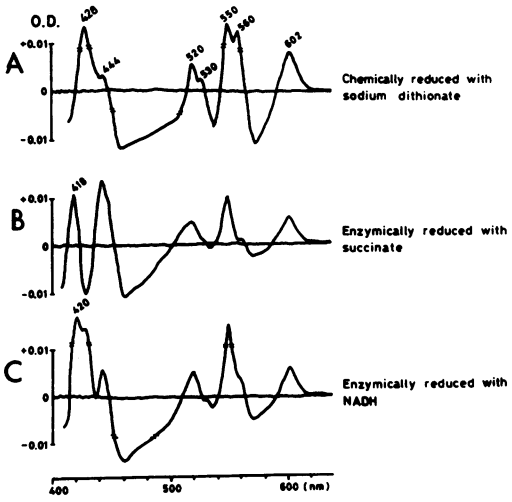

FIG. 1. Difference spectra of *C. albicans* 7N mitochondria. Spectra were recorded at room temperature immediately after the addition of a trace of sodium dithionite (A), 1 mM succinate (B), or 0.2 mM NADH (C) without any inhibitor. The protein concentration was 4 mg/ml, and the final volume was 3 ml. O.D., Optical density.

and three times with a solution containing 1.3 M sorbitol, 0.1 mM EDTA, and 10 mM Tris buffer (pH 7.4) (SET).

Formation of protoplasts and isolation of mitochondria. Mitochondrial preparations were obtained by combined enzymatic and mechanical procedures, as follows. Washed cells (10 g) were suspended in a solution containing 20 ml of SET, 0.4 ml of 0.1 M EDTA, and 0.3 ml of beta-mercaptoethanol and incubated for 1 h at 36°C in order to facilitate the digestion of *C. albicans* cell walls.

After centrifugation, the cells were suspended in 20 ml of SET containing 150 mg of zymolyase and incubated for 2 h at 36°C. The formation of protoplasts was followed roughly by diluting a sample of the incubation mixture 10-fold with water and checking the number of remaining cells. After the incubation period, the cells and protoplasts were centrifuged at 2,000 × *g* for 15 min at 4°C. The pellet was washed with 20 ml of SET and centrifuged again. The protoplasts were suspended in SET and sonicated for 10 min at 10 kHz and 0°C. The cell debris and undisrupted cells were removed by centrifuging the homogenate three times at 2,500 × *g* for 5 min. The resulting supernatants were pooled and centrifuged in a Hitachi type 30 rotor at 106,000 × *g* and 0°C for 1 h. The pellet was suspended in SET with the aid of a Teflon homogenizer, and the centrifuged mitochondria were stored at −80°C and used within 3 days.

For studies with NADH as a substrate, sub-mitochondrial particles were prepared by sonicating mitochondria in the presence of sodium pyrophosphate (pH 7.4).

Determination of protein content. Protein contents were determined by the method of Lowry et al. (7), using crystalline bovine serum albumin as a standard.

TABLE 1. Concentrations of the respiratory chain components of *C. albicans* 7N mitochondria

Component	Concn (nmol/mg of protein) with:[a]		
	Chemically reducible sodium dithionite	Enzymically reducible NADH[b]	Enzymically reducible succinate[c]
Flavoprotein	1.415	0.518	0.226
Cytochrome *b*	0.425	0.100	0.027
Cytochrome c_1+c	0.447	0.263	0.152
Cytochrome $a+a_3$	0.227	0.089	0.075

[a] Concentrations were determined immediately after the chemical was added. The protein concentration was 4 mg/ml, and the final volume was 3 ml. The chemically reducible concentrations of flavoprotein and cytochromes refer to the total concentrations reduced by the addition of a trace of sodium dithionite in the absence of substrate.

[b] NADH concentration, 0.2 mM.

[c] Succinate concentration, 1 mM.

Difference spectrophotometry. Difference spectra (reduced versus oxidized) of the respiratory chain of *C. albicans* were recorded at room temperature with a Hitachi model 557 double-beam, double-wavelength spectrophotometer. The sample and reference cuvettes contained *C. albicans* mitochondria which were suspended in a reaction mixture containing 0.65 M mannitol, 10 mM phosphate, 10 mM Tris-maleate buffer (pH 6.6), 10 mM KCl, and 0.1 mM EDTA. The different states of mitochondria were defined as described by Onishi et al. (9).

Concentrations of respiratory chain components. The concentrations of cytochrome $a+a_3$, cytochrome c_1+c, cytochrome *b*, and flavoprotein were estimated by using extinction coefficients of 16.5, 19.0, 20.0, and 10.6 $mM^{-1}\ cm^{-1}$, respectively. Enzymically reducible amounts were determined immediately from the reduction obtained after the addition of 0.2 mM NADH or 1 mM succinate without any inhibitor.

RESULTS

The difference spectra of *C. albicans* mitochondria chemically reduced with sodium dithionite showed different absorption peaks (reduced) typical of the alpha bands of cytochrome $a+a_3$ at 602 nm and cytochrome $c+c_1$ at 550 nm and peaks typical of the beta bands of cytochrome *b* at 530 nm and cytochrome $c+c_1$ at 520 nm, as well as the typical absorption band from 460 to 510 nm, which corresponded to the flavoprotein region (Fig. 1A). Absorption peaks at 444 and 428 nm were also observed in the Soret region, corresponding to cytochrome a_3 and cytochrome *b* (which is specific to yeasts), respectively.

Figure 1B shows the difference spectrum for *C. albicans* mitochondria enzymically reduced with 1 mM succinate under aerobic conditions (state 4). Absorption peaks occurred at 602, 560, 550, 530, 520, and 444 nm with different intensi-

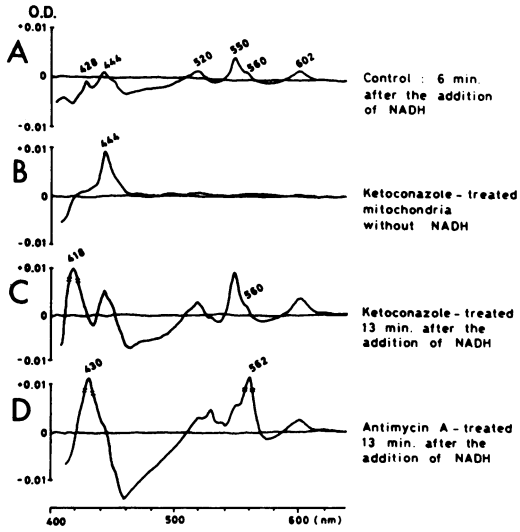

FIG. 2. Effect of ketoconazole on the difference spectra of *C. albicans* 7N mitochondria. The ketoconazole and antimycin A concentrations used were 100 μM. The protein concentration was 4 mg/ml, and the final volume was 3 ml. (A) Control. (B) Ketoconazole-treated mitochondria. (C) Ketoconazole-treated mitochondria with NADH. (D) Antimycin A-treated mitochondria. O.D., Optical density.

ties, as in Fig. 1A. However, in the Soret region the absorption peak at 428 nm was not present, but another peak at 418 nm (cytochrome c_1+c) was observed.

Figure 1C shows the difference spectrum of mitochondria that were enzymically reduced with 0.2 mM NADH as a substrate. In the Soret region an absorption peak at 420 nm corresponding to cytochrome c_1+c was observed. The difference spectra shown in Fig. 1 were recorded immediately after the reductant was added.

Table 1 shows data from a spectrophotometric analysis of chemically reducible respiratory chain components (total amounts of cytochromes and flavoprotein present in the sample), as well as enzymically reducible amounts of *C. albicans* mitochondria. As this table shows, sodium dithionite strongly reduced all of the components of the respiratory chain of *C. albicans*. In contrast, the enzymically reducible amounts depended on the substrate used.

Figure 2A shows the difference spectrum of *C. albicans* mitochondria in the NADH pathway 6 min after the substrate was added. A remarkable feature of this mitochondrial system was that added NADH was oxidized at a high rate. This feature is evident compared with Fig. 1C.

A direct, specific interaction between ketoconazole and cytochrome *c* oxidase was observed when 100 μM ketoconazole was added under aerobic conditions in the absence of any substrate; this immediately resulted in a strong reduction of cytochrome a_3, as shown by the absorption peak at 444 nm (Fig. 2B). This effect was also detected at lower concentrations of ketoconazole. On the other hand, ketoconazole per se did not show any absorption peak in this range. Subsequent addition of NADH to the reaction mixture resulted in stronger reduction of all the other components of the respiratory chain, as shown by the intensities of the absorption peaks (Fig. 2C). This was due to a specific blockage at the cytochrome *c* oxidase level. Consequently, this effect gave rise to the anaerobic state of mitochondria (exhaustion of oxygen or state 5).

Cytochrome *b* was reduced and its peaks (560 and 428 nm) were shifted 2 nm to longer wavelengths (red shift) when mitochondria from *C. albicans* were treated with antimycin A (Fig. 2D). This spectral profile confirmed the mitochondrial integrity of *C. albicans* prepared by enzymatic and mechanical means.

Table 2 shows the normally reduced concentrations of the components of the respiratory chain throughout the NADH pathway as a function of time. The activities of the NADH oxidases (e.g., NADH dehydrogenase, cytochrome *c* oxidase, etc.) were markedly high, as shown by the quick reduction of the components of the respiratory system. Furthermore, almost no detectable reduced amounts remained after 9 min due to the high oxidative rate.

As expected, Table 3 shows the striking difference in the reduced amounts of flavoprotein, cytochrome c_1+c, and cytochrome $a+a_3$ in the presence of ketoconazole. This effect was clearly detectable as early as 6 min after the addition of NADH and the drug. This effect was dose dependent up to a concentration of 0.4 μM. However, to show a clear effect of ketoconazole action, Table 3 shows only representative results obtained with 100 μM ketoconazole.

Figure 3A shows the difference spectrum of *C. albicans* mitochondria in the succinate pathway

TABLE 2. Concentrations of respiratory chain components reduced with NADH as a function of time[a]

Component	Concn (nmol/mg of protein) after:		
	3 min	6 min	9 min
Flavoprotein	0.377	0.070	0.070
Cytochrome *b*	0.100	0.018	0.015
Cytochrome c_1+c	0.210	0.065	0.028
Cytochrome $a+a_3$	0.075	0.037	0.030

[a] The concentrations of respiratory chain components that could be reduced by 0.2 mM NADH were determined in the absence of any inhibitor.

TABLE 3. Concentrations of respiratory chain components reduced with NADH in the presence of ketoconazole[a]

Component	Concn (nmol/mg of protein) after:	
	6 min	10 min
Flavoprotein	0.183	0.207
Cytochrome *b*	0.013	0.017
Cytochrome c_1+c	0.155	0.150
Cytochrome $a+a_3$	0.048	0.042

[a] Ketoconazole (100 μM) was added 1 min before the substrate was added. The protein concentration was 4 mg/ml, and the final volume was 3 ml.

TABLE 4. Concentrations of respiratory chain components reduced with succinate as a function of time[a]

Component	Concn (nmol/mg of protein) after:		
	10 min	13 min	35 min
Flavoprotein	0.155	0.122	0.073
Cytochrome *b*	0.027	0.012	0.006
Cytochrome c_1+c	0.152	0.086	0.059
Cytochrome $a+a_3$	0.075	0.060	0.045

[a] The concentrations of respiratory chain components that could be reduced by 1 mM succinate were determined in the absence of any inhibitor.

13 min after the addition of the substrate. Compared with Fig. 1B, the activities of the succinate oxidases (e.g., succinate dehydrogenase, etc.) were somewhat lower than those of the NADH oxidases under the conditions used.

Treatment of mitochondria with 100 μM ketoconazole also blocked the normal flow of electrons in the succinate pathway by inhibiting cytochrome *c* oxidase, as shown in Fig. 3B. No inhibition of other oxidases by the drug brought the mitochondrial suspension to a reduced state (anaerobic state or state 5).

The difference spectrum of mitochondria 1 min after antimycin A was added is shown in Fig. 3C. Flavoprotein and cytochrome *b* were strongly reduced, and the absorption peak at 560 nm was shifted 2 nm to a longer wavelength. This spectral pattern confirmed the integrity of this mitochondrial preparation.

Table 4 shows the normally reduced concentrations of the components of the respiratory chain in the succinate pathway as a function of time. As this table shows, reduced amounts of the respiratory components were still detectable 35 min after the addition of the substrate due to the low rate of electron flow.

Despite this fact, greater amounts of all of the components of the respiratory chain were reduced in the presence of ketoconazole (Table 5) than in the control system (Table 4). This effect was evident 35 min after the addition of succinate and the drug.

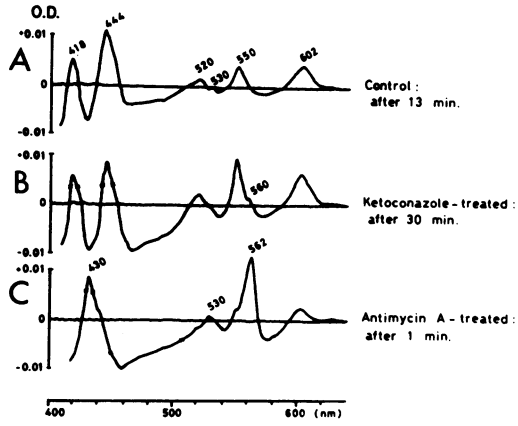

FIG. 3. Effect of ketoconazole on the difference spectra of succinate-reduced *C. albicans* 7N mitochondria. The succinate concentration was 1 mM. The other conditions were as described in the legend to Fig. 2. O.D., Optical density.

DISCUSSION

C. albicans mitochondria isolated from zymolyase protoplasts and purified by differential centrifugation gave a reproducible and well-resolved spectral profile. Other experiments (15), in which the oxygen uptake rates were determined polarographically, also gave reproducible values when measurements were made within 3 days after isolation. Thereafter, the cytochrome oxidase activity was reduced gradually by storage.

A prominent feature of our electron transport system from *C. albicans* mitochondria (as revealed by the spectral profile) was that in the Soret region the absorption peaks (reduced) appeared to be located at different wavelengths depending on the substrate used. This might be due to electron flow through different kinds of

TABLE 5. Concentrations of respiratory chain components reduced with succinate in the presence of ketoconazole[a]

Component	Concn (nmol/mg of protein) after:		
	6 min	10 min	35 min
Flavoprotein	0.273	0.198	0.169
Cytochrome *b*	0.025	0.020	0.012
Cytochrome c_1+c	0.207	0.176	0.147
Cytochrome $a+a_3$	0.092	0.089	0.757

[a] Ketoconazole (100 μM) was added 1 min before the substrate was added. The protein concentration was 4 mg/ml, and the final volume was 3 ml.

cytochrome *b*. As shown in Fig. 1B, the absorption peak at 428 nm (cytochrome *b* in yeasts) was not expressed (not reduced), probably because it did not participate in active electron transport throughout the succinate pathway.

The high NADH oxidation rate shown in Fig. 2A (control system) without the addition of any inhibitor indicated the high NADH oxidase activity in our mitochondrial preparation. NADH oxidases were very sensitive to ketoconazole, as clearly shown by spectrophotometric analysis. Furthermore, the difference spectrum recorded immediately after the addition of ketoconazole in the absence of any substrate revealed that the action was specific at the cytochrome *c* oxidase level. This enzyme is required in the initial phase of growth, when the cells need large quantities of oxygen to provide sufficient energy for growth and division (1). The other NADH oxidases seemed to be not inhibited since subsequent addition of this substrate led to an accumulation of electrons throughout the respiratory chain. In other words, the difference spectrum of the control system became flat (oxidized) within 13 min, whereas that of the ketoconazole-treated system expressed more intense peaks (reduced) in the spectral profile.

Despite the lower activities of succinate oxidases than NADH oxidases, the effect of ketoconazole was clearly demonstrated 30 min after the addition of succinate and the drug.

It is well known that antimycin blocks the electron flow between cytochromes *b* and *c*, resulting in reduction of the former and oxidation of the latter. Therefore, cytochromes c_1+c and $a+a_3$ were expected to be oxidized as in other mitochondrial preparations, as reported with *Saccharomyces carlsbergensis* by Onishi et al. (9). However, in our *C. albicans* mitochondrial preparation the complete disappearance of the absorption peak (indicating oxidation) corresponding to cytochrome $a+a_3$ was not observed with NADH even after 13 min, in contrast to the difference spectrum with succinate.

Spectrophotometric analysis revealed that the concentration of the flavoprotein present in the respiratory chain of *C. albicans* mitochondria was much higher than the concentration in mammalian mitochondria reported by Norling et al. (8). On the other hand, the concentrations of cytochrome *b*, c_1+c, and $a+a_3$ were much lower than the concentrations in the mammalian system. This pronounced difference might explain the fact that ketoconazole was a markedly less potent inhibitor of respiration in mammalian mitochondria (15).

With respect to the enzymically reducible amounts of the components (without inhibitor) in our mitochondrial preparation, we found that NADH oxidase reduced greater amounts of all the components than succinate oxidase did. Therefore, the electron flow was more dynamic by the NADH pathway than by the succinate pathway in *C. albicans* mitochondria. Consequently, the interaction of ketoconazole with cytochrome *c* oxidase was demonstrated more rapidly with the former than with the latter. Although much greater amounts of flavoprotein and cytochromes $c_1 + c$ and $a + a_3$ were reduced in the presence of ketoconazole (due to a block in the electron flow) than in the controls when NADH was used as a substrate, only the concentration of cytochrome *b* remained almost unchanged. This might have been due to the high energy potential of cytochrome *b* or to the existence of an alternate pathway in *C. albicans* (as described previously [5, 12]), which bifurcated at the point between coenzyme Q and cytochrome *b* and allowed this cytochrome to be oxidized easily. The presence of such an alternate pathway was predicted when NADH was used as a substrate since immediate reduction of cytochrome *b* followed by rapid oxidation was observed in our mitochondrial preparation. This might be explained by fast electron transfer to both normal and alternate pathways.

In light of our results, we concluded that the primary site of ketoconazole action on *C. albicans* mitochondria might be at the most distal portion of the respiratory chain. De Nollin et al. (1) described cytochemical and biochemical studies of yeasts after in vitro exposure to miconazole. These authors observed a similar effect, but they assumed that miconazole acted on the synthesis of cytochrome *c* oxidase. Our data for ketoconazole suggested a direct interaction with cytochrome *c* oxidase since the effect was detected within a short time after the addition of the drug. The activities of the other enzymes in the respiratory chain appeared to be intact. This was reflected by the accumulation of electrons in the respiratory chain. Data providing further evidence for these conclusions were obtained from experiments in which no ketoconazole effect was observed on other NADH oxidases (e.g., NADH dehydrogenase) or on other succinate oxidases (e.g., succinate dehydrogenase) with artificial electron acceptors, such as potassium ferricyanide (4), and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT method) (10).

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