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Effects of Cerulenin upon the Syntheses of Lipid and Protein and upon the Formation of Respiratory Enzymes in Adapting, Lipid-Limited *Saccharomyces cerevisiae*

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When bakers' yeast cells were grown anaerobically in a medium supplemented with Tween 80 and ergosterol, exposure during aeration to the fatty acid synthesis inhibitor, cerulenin, had little effect upon respiratory adaptation, the induction of enzymes of electron transport, or the in vivo incorporation of [^{14}C]leucine into mitochondrial membranes. These lipid-supplemented cells were apparently able to undergo normal respiratory adaptation utilizing endogenous lipids alone. The level of cerulenin used (2 μg/ml) inhibited the in vivo incorporation of [^{14}C]acetate into mitochondrial membrane lipids by 96%. If, however, the cells were deprived of exogenous lipid during anaerobic growth, subsequent exposure to cerulenin severely reduced their capacity to undergo respiratory adaptation, to form enzymes of electron transport, and to incorporate amino acid into both total cell and mitochondrial membrane proteins. This cerulenin-mediated inhibition of enzyme formation and of protein synthesis was nearly completely reversed by the addition of exogenous lipid during the aeration of the cells. In lipid-limited cells, chloramphenicol also had dramatic inhibitory effects, both alone (75%) and together with cerulenin (85%), upon total cell and mitochondrial membrane [^{14}C]leucine incorporation. This marked chloramphenicol-mediated inhibition was also largely reversed by exogenous lipid. It is concluded that, in lipid-limited cells, either cerulenin or chloramphenicol may prevent the emergence of a pattern of lipids required for normal levels of protein synthetic activity. The effect of cerulenin upon the formation of mitochondrial inner membrane enzymes thus appears to reflect a nonspecific effect of this antilipogenic antibiotic upon total cell protein synthesis.

During the aeration of bakers' yeast cells grown anaerobically on lipid-limited media, total fatty acids and sterols increase in parallel with increasing respiratory capacity (8). Observed changes in mitochondrial lipid composition which accompany respiratory adaptation include marked increases in the levels of long-chain unsaturated fatty acids, ergosterol, and ubiquinone (6, 12). These changes in lipid composition suggest an integral role for lipids in the adaptation process.

Cerulenin, an antibiotic isolated from culture filtrates of *Cephalosporum caerulens*, inhibits fatty acid synthetases from a variety of organisms, including bacteria, mycelial fungi, and yeasts (11). It inhibits fatty acid synthetases by binding in an equimolar ratio to beta-ketoacyl-acyl carrier protein synthetase (2), but does not inhibit either fatty acid elongation or desaturation in bakers' yeast cells (1). Moreover, cerulenin may not inhibit sterol synthesis in yeast (7).

As an inhibitor of fatty acid synthesis, cerulenin has been purported to be potentially useful as a tool for examining the role of lipid synthesis and availability in the biogenesis of cell membranes and organelles (1, 11, 15). For example, in bakers' yeast cells, the emergence of an aerobic pattern of membrane fatty acids and sterols during respiratory adaptation could represent a pivotal step in the assembly of functional mitochondrial inner membrane enzyme complexes. In the present study, data are presented which suggest that cerulenin does indeed prevent the formation of functional mitochondrial membrane enzymes, but that this effect is merely a consequence of a general inhibition of total cell protein synthesis in lipid-limited, adapting cells.

MATERIALS AND METHODS

Cell growth. The wild-type *Saccharomyces cerevisiae* D273-10B (α; ATCC 25657) was used. The cells were grown anaerobically in a 0.5% galactose-yeast

extract medium as described earlier (13) except that Tween 80 and ergosterol were absent from the medium for those cells designated "lipid-limited." The cells were harvested in early stationary phase.

Incubations. Upon harvesting, the cells were washed once in adaptation buffer containing 40 mM KPO_4 (pH 7.4), 0.3% glucose, and 2.0% ethanol; resuspended in the same at a cell concentration of 10 mg (wet weight) per ml; and shaken at 28°C in the dark. Where used, cerulenin was present at a final concentration of 2.0 μg/ml, chloramphenicol was present at 4.0 mg/ml, Tween 80 was present at 0.2% (vol/vol), and ergosterol was present at 0.001% (wt/vol). For all labeling experiments (Tables 1 and 3), the cells were preincubated for 30 min before the addition of either [^{14}C]acetate or [^{14}C]leucine. Labeling was then carried out for 60 min as described earlier (14).

Processing of labeled samples for counting. For the labeling experiments presented in Table 3, the termination of labeling, cell breakage, membrane isolation, and the washing and extraction of the trichloroacetic acid precipitates were carried out as described earlier (14) except that the samples were collected and processed on glass fiber filters (Whatman GF/A). Those samples designated "total cell" consisted of portions of the low-speed (2,000 × *g*) supernatant of the French press cell homogenate. For the labeling experiments presented in Table 1, each washed membrane pellet, prepared as described earlier (14), was suspended in 2.0 ml of distilled water, and a portion was removed for protein estimation. A 1-ml amount of each sample was then extracted with a 2:1 (vol/vol) mixture of chloroform and methanol by the procedure of Folch et al. (3). Each extract was evaporated to near dryness under vacuum, and 10 ml of a toluene counting mixture containing 5 g of PPO (2,5-diphenyloxazole) and 300 mg of POPOP {1,4-bis[2-(5-phenyloxazolyl)]benzene} per liter was added directly.

Assays, analytical procedures, and reagents. Aliquots of cells taken at the times indicated in Fig. 1 were assayed for QO_2 with a Gilson Medical Electronics model K-1C oxygraph equipped with a Clark-type electrode. QO_2 values were corrected for cyanide-insensitive oxygen uptake and are expressed as microliters of O_2 consumed per hour per milligram (dry weight). Coenzyme QH_2–cytochrome *c* reductase and cytochrome *c* oxidase activities, expressed as micromoles of cytochrome *c* reduced or oxidized per minute per milligram of protein, were determined as described earlier (13) on crude, washed membranes obtained from cells that had been aerated for 6 h (Table 2). Protein concentrations used in specific activity and in specific radioactivity calculations were estimated by the method of Lowry et al. (9). The [1,2-^{14}C]acetate and [*U*-^{14}C]leucine used were obtained from New England Nuclear Corp. The coenzyme Q_2 was a generous gift supplied by F. Hoffmann-LaRoche & Co., Basel, Switzerland. The cytochrome *c* was Sigma Chemical Co. type III horse heart material. The cerulenin used in initial experiments (not reported here) was a generous gift supplied by Satoshi Omura of Kitasato University, Tokyo, Japan. The cerulenin used for all later experiments was obtained from Makor Chemicals, Ltd., Jerusalem, Israel. All other chemicals used were of reagent grade.

RESULTS

The experiment reported in Table 1 shows that cerulenin at a final concentration of 2.0 μg/ml inhibited the in vivo incorporation of [^{14}C]acetate into total mitochondrial membrane lipids by 96%. A maximal inhibition of the inductions of coenzyme QH_2–cytochrome *c* reductase and cytochrome *c* oxidase activities occurred at even lower cerulenin levels than employed here, i.e., at approximately 1.0 μg/ml (15).

The experiment depicted in Fig. 1B shows that when cells were grown in a galactose–yeast extract medium containing Tween 80 and ergosterol as described previously (13), the addition of cerulenin to the adaptation medium had virtually no effect upon respiratory adaptation. These lipid-supplemented cells were apparently able to undergo normal respiratory adaptation using endogenous lipids alone. However, when lipid-limited cells were used (Fig. 1A), the addition of cerulenin blocked adaptation by about 80%. The adaptation which did occur in lipid-limited cells in the presence of cerulenin is thought to be due to the rapid formation of low levels of "aerobic" lipids in the few minutes which elapsed during cell harvesting before the addition of cerulenin to the cells. Additionally, there presumably was some desaturation of preexisting membrane fatty acids occurring upon aeration of the cells even after the addition of cerulenin.

The results of studies on the effects of cerulenin on the formation of two inner membrane respiratory enzyme complexes in both lipid-limited and lipid-supplemented cells are shown in Table 2. The addition of the inhibitor to lipid-limited cells prevented the appearance of coenzyme QH_2–cytochrome *c* reductase activity by about 50% after 6 h of aeration. That of cytochrome *c* oxidase was reduced to roughly 25% of controls. The addition of exogenous lipid to the adaptation medium completely reversed the effects of cerulenin upon the induction of coen-

TABLE 1. *In vivo [^{14}C]acetate incorporation into the total lipid of a washed, crude mitochondrial membrane fraction*[a]

Addition	cpm/mg of protein	% of control
None	6,657	100.0
Cerulenin	273	4.1

[a] The cells used in this experiment were grown under lipid-limited conditions. They were aerated in 50-ml batches in the dark. A 10-μCi amount of [^{14}C]-acetate was added to each flask 30 min after the beginning of the incubation, and labeling was carried out for 60 min. Where used, cerulenin was present at a final concentration of 2.0 μg/ml.

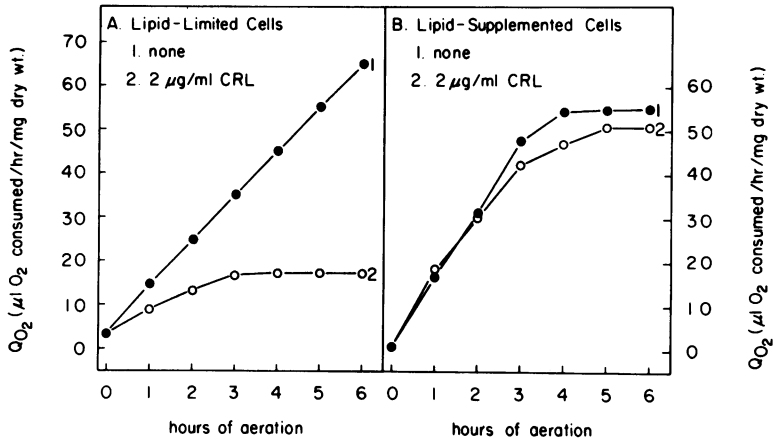

FIG. 1. *Adaptation of (A) lipid-limited and (B) lipid-supplemented cells in the absence and presence of cerulenin (CRL) at a final concentration of 2.0 μg/ml. The cells were grown anaerobically and harvested as described in the text. They were suspended at 10 mg (wet weight) per ml in adaptation buffer containing 40 mM KPO_4 buffer (pH 7.4), 0.3% (wt/vol) glucose, 2.0% (vol/vol) ethanol minus and plus CRL and shaken at 28°C in the dark. Portions were removed at the times indicated and resuspended in fresh adaptation buffer, and cyanide-sensitive rates of oxygen uptake were determined.*

TABLE 2. *Specific activities of reduced coenzyme Q cytochrome c reductase and of cytochrome c oxidase*[a]

Addition(s)	Lipid-limited cells				Lipid-supplemented cells			
	QCR sp act[b]	% of control	CO sp act[b]	% of control	QCR sp act[b]	% of control	CO sp act[b]	% of control
None	0.354	100.0	0.893	100.0	0.225	100.0	0.817	100.0
CRL[c]	0.174	49.2	0.210	23.6	0.198	88.3	0.714	87.3
CRL + Tween 80 + ergosterol	0.364	102.9	0.777	87.0	0.229	102.0	0.722	88.4

[a] Tween 80 and ergosterol were present in the adaptation buffer at final concentrations of 0.2% (vol/vol) and 0.001% (wt/vol), respectively.

[b] QCR, Reduced coenzyme Q cytochrome *c* reductase; CO, cytochrome *c* oxidase. Specific activity is expressed as micromoles of cytochrome *c* reduced or oxidized per minute per milligram of protein after 6 h of aeration.

[c] CRL, Cerulenin.

zyme QH_2–cytochrome *c* reductase and nearly completely restored cytochrome *c* oxidase activity to a normal level. Consistent with its effects upon the induction of whole-cell respiratory function, cerulenin prevented the formation of enzymes of electron transport in lipid-limited cells, but had only a slight effect upon cells grown anaerobically in a lipid-supplemented medium.

Studies on the effects of cerulenin both by itself and in combination with chloramphenicol on the in vivo incorporation of [^{14}C]leucine into total cell and mitochondrial membrane proteins during the adaptation of both lipid-limited and lipid-supplemented cells are presented in Table 3. As may be seen, cerulenin inhibited total cell and membrane protein synthesis by about 50% in lipid-limited cells, but had no effect on lipid-supplemented cells. In agreement with many earlier reports, chloramphenicol inhibited mitochondrial membrane protein synthesis by approximately 15% in lipid-supplemented cells. In contrast, its effect on lipid-limited cells was much more dramatic; here a 75% inhibition was observed. Moreover, when cerulenin was added together with chloramphenicol to lipid-limited cells, the two agents caused a 85% inhibition of total cell and membrane protein synthesis. This combined inhibitory effect was nearly completely reversed by the addition of exogenous lipid to the adapting cells, as was the inhibition of amino acid incorporation by cerulenin alone.

DISCUSSION

In 1970 Vary et al. (16) reported that chloramphenicol inhibited the oxygen inductions of malate and succinate dehydrogenases and of fumarase in anaerobically grown, lipid-depleted yeast cells, but had no effect upon the formation of these same (cytoplasmically translated) enzymes in anaerobically grown, lipid-supplemented cells. Shortly thereafter, Gordon et al.

TABLE 3. *In vivo [^{14}C]leucine incorporation into total cell homogenate and into a washed, crude mitochondrial membrane fraction*[a]

Addition(s)	Lipid-limited[b]				Lipid-supplemented[c] (mitochondria)	
	Total cell		Mitochondria			
	Protein (cpm/mg)	% of control	Protein (cpm/mg)	% of control	Protein (cpm/mg)	% of Control
None	387,851	100.0	244,632	100.0	325,010	100.0
CRL[d]	197,370	52.0	127,575	52.1	325,719	100.2
CRL + T/E[d]	379,729	100.2	244,876	100.1	326,052	100.3
CAP[d]	92,837	24.5	64,338	26.3	275,431	84.7
CAP + CRL	54,148	14.2	36,450	14.9	271,310	83.4
CAP + CRL + T/E	282,943	74.6	182,250	74.5	278,133	85.5

[a] The cells were aerated in 50-ml batches in 250-ml flasks at 28°C in the dark. A 10-μCi amount of [^{14}C]-leucine was added to each flask 30 min after the beginning of the incubation, and labeling was carried out for 60 min. All values presented in this table are from a single experiment with one culture of lipid-limited cells and one of lipid-supplemented cells grown concomitantly. The experiment was run several times, and the relative values reported here are typical.

[b] Tween 80 and ergosterol were absent from the growth medium.

[c] Tween 80 and ergosterol were present in the growth medium at final concentrations of 0.2% (vol/vol) and 0.001% (wt/vol), respectively.

[d] Cerulenin (CRL) was present in the labeling medium at a concentration of 2.0 μg/ml; chloramphenicol (CAP) was present at 4.0 mg/ml; and Tween 80 and ergosterol (T/E) were present at 0.2% (vol/vol) and 0.001% (wt/vol), respectively.

(4) demonstrated that chloramphenicol inhibited the oxygen-induced synthesis of lipids in yeast. Using the unsaturated fatty acid auxotroph KD115, they further showed that lipid availability affected the synthesis of both membrane-bound and soluble enzymes of both mitochondrial and extra-mitochondrial origin (5). These findings suggested that a restriction in unsaturated fatty acid availability either in the fatty acid mutant or in wild-type cells exposed to chloramphenicol caused a general inhibition of total cell protein synthesis (5). One explanation proposed by these authors for the effect of a restricted unsaturated fatty acid content upon total cell protein synthesis was that membrane-bound ribosomes might not function in protein synthesis unless bound to a membrane matrix with a minimal unsaturated fatty acid content. Marzuki et al. (10) later showed that the activity of the mitochondrial protein synthesizing system in yeast was critically dependent upon the unsaturated fatty acid content of the cell. Below a 28% unsaturated fatty acid content, no mitochondrial ribosomal subunits could be detected (10).

In the present study, chloramphenicol was again shown to inhibit total cell protein synthesis in lipid-limited cells. That this effect is a consequence of its action as a fatty acid synthesis inhibitor is strongly suggested by the substantial reversal of this inhibition by exogenous lipid. Moreover, the effect of chloramphenicol upon the incorporation of amino acid into mitochondrial membranes mirrored almost exactly its effect upon total cell protein synthesis (Table 3), suggesting that the inhibitory effects of either chloramphenicol or the unsaturated fatty acid restriction prevailing in these cells were not limited to any particular class of proteins in the cell. Rather, the data in Table 3 suggest a general slowing of the synthesis of all cell proteins.

The relatively new antilipogenic antibiotic, cerulenin, was initially viewed by me as a promising tool for the investigation of the coupling of lipid availability to the assembly of mitochondrial inner membrane enzyme complexes during respiratory adaptation (15). Thus, I had begun to investigate the possibility that certain membrane polypeptides, perhaps the more hydrophobic ones, might exhibit a more stringent dependency upon lipid availability for either their synthesis or for their subsequent processing and integration into the membrane.

The data reported in Table 3, however, strongly suggest that cerulenin, like chloramphenicol, only caused a general slowing of the synthesis of all cell proteins in lipid-limited cells. The inhibition of mitochondrial membrane protein synthesis by cerulenin, as in the case of chloramphenicol, reflected exactly that inhibition seen for total cell protein synthesis.

When the cells were grown in a lipid-supplemented medium, cerulenin had no effect on mitochondrial membrane protein synthesis; chlor-

amphenicol had only the relatively small effect which has been reported previously by many workers.

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