Studies on the Function and Biogenesis of Cytochrome *b* in Mutants of *Saccharomyces cerevisiae* Resistant to 3-(3,4-Dichlorophenyl)-1,1-dimethylurea†

Liviu Clejan, Anita Sidhu, and Diana S. Beattie*

ABSTRACT: Mutants of the yeast *Saccharomyces cerevisiae* have been reported with resistance during growth to diuron, an inhibitor of electron transport between cytochromes *b* and c_1 in yeast mitochondria. Three of these mutants which map on mitochondrial DNA were chosen for biochemical characterization of possible changes in cytochrome *b*. All the mutant strains exhibited resistance to diuron when coenzyme QH_2–cytochrome *c* reductase activity was assayed in submitochondrial particles with the decyl analogue of coenzyme Q_1 as substrate. No *extrareduction* of cytochrome *b* was observed when either antimycin A or diuron was allowed to bind to oxidized submitochondrial particles prior to reduction by succinate. Similarly, the oxidant-induced reduction of cytochrome *b*, where succinate was added before the inhibitors, was negligible in the mutants compared to the wild type. Moreover, the spectral shift in the α band of cytochrome *b* induced by antimycin was qualitatively different in the mutants. Finally, reversed electron flow from cytochrome *b* to fumarate–succinate induced by ATP in cyanide- and antimycin-treated submitochondrial particles reduced with ascorbate–*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine was considerably lower in the mutants. Despite these changes in the response of cytochrome *b* to these inhibitors and the energy charge across the membrane, the activity of coenzyme QH_2–cytochrome *c* reductase was not impaired in the mutants. When the strains were grown on lactate instead of galactose, the mutant strains had a 50% or greater increase in the amount of cytochrome *b* per milligram of mitochondrial protein. The mitochondrial translation products appeared almost identical in the mutants and wild type. These results suggest that the mutations leading to a resistance to diuron during growth may lead to changes in the apoprotein of cytochrome *b* such that its role in energy coupling but not electron transport is affected.

Cytochrome *b*, a low-potential hemoprotein of the mitochondrial respiratory chain, catalyzes the transfer of electrons from coenzyme Q to the iron–sulfur protein and cytochrome c_1 of complex III (Erecinska et al., 1972). Several unusual features of cytochrome *b* in its response to either antimycin, the classical inhibitor of electron transfer from cytochrome *b*, or energization of the membrane have led to the formulation of cyclic mechanisms for electron flow in this part of the chain (Mitchell, 1975; Rieske, 1976). The oxidant-induced reduction of cytochrome *b* in the presence of antimycin and the concomitant red shift in the absorption maximum are best explained by the Q cycle (Mitchell, 1975), or one of its modifications (Trumpower, 1981), than by the classical electron transport chain.

Recently, the gene for cytochrome *b* has been localized on the mitochondrial DNA of both yeast (Nobrega & Tzagoloff, 1980a) and mammals (Anderson et al., 1981) and sequenced (Nobrega & Tzagoloff, 1980b; Lazowska et al., 1980). Mutants which are resistant to inhibitors of electron transport have been isolated and shown to be allelic with various regions of the cytochrome *b* gene (Colson & Wouters, 1980). We felt that such mutants might be useful for investigating the structural features of the cytochrome *b* protein which contribute to its functional behavior. The three mutants chosen for these initial characterizations were selected for their ability to grow on diuron (Diu),[1] a herbicide shown previously to inhibit electron transport between cytochromes *b* and c_1 in yeast mitochondria (Briquet & Goffeau, 1981). Genetic studies had indicated that the mutant Diu^r-742 mapped in the diuron-II locus which is allelic with box 4, or the α exon (Colson & Wouters, 1980), of the cytochrome *b* gene. The other two mutants, Diu^r-740 and Diu^r-752, were chosen because they mapped in a new locus, diuron-III, which appears to be allelic to box 3, the first intron of the mosaic cytochrome *b* gene (Lazowska et al., 1980).

The results obtained suggest that these mutations lead to changes in the cytochrome *b* protein which are manifest as subtle differences in its function. All the mutations confer resistance to diruon during growth on agar plates, but only partial resistance when the catalytic activity of complex III is assayed as coenzyme QH_2–cytochrome *c* reductase. The extrareduction with either diuron or antimycin and the oxidant-induced reduction along with reversed-electron flow in the presence of ATP are all affected differently in the various mutants.

Materials and Methods

Materials. Antimycin A, cycloheximide, ATP, cytochrome *c* (type VI, from horse heart), bovine serum albumin (fraction V), and $NaDodSO_4$ were Sigma products. Kodak NS.5T X-ray film was used for autoradiography. [^{35}S]Methionine (1000 Ci/mmol) was purchased from Amersham. DB[1] was a gift from Dr. B. Trumpower (Dartmouth), and diuron was a gift from Dr. A. Goffeau (University of Louvain). All other reagents were of the highest purity commercially available.

Growth of Cells. The strain KL14-4A and the three mutants Diu^r-740, Diu^r-742, and Diu^r-752 isolated from this strain (Colson et al., 1977) were cultured aerobically with either 3% galactose or 3% sodium DL-lactate as carbon source (Beattie

† From the Department of Biochemistry, Mount Sinai School of Medicine of the City University of New York, New York, New York 10029. *Received May 20, 1982.* This work was supported in part by Grant HD-04007 from the National Institutes of Health and Grant PCM 7824535 from the National Science Foundation. This work is in partial fulfillment of the requirements for the Ph.D. degree at the City University of New York (A.S.).

[1] Abbreviations: Diu, diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea]; Diu^r, diuron resistant; DBH_2, reduced 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone; BSA, bovine serum albumin; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; $NaDodSO_4$, sodium dodecyl sulfate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; SMP, submitochondrial particles; EDTA, ethylenediaminetetraacetic acid.

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et al., 1979; Pajot & Claisse, 1974). The levels of diuron resistance were determined by plating the yeast strains on YPG medium consisting of 1% yeast extract, 2% Bactopeptone, 3% glycerol, and diuron in varying concentrations up to 350 μM (Colson et al., 1977).

Preparation of Mitochondria and Enzyme Assays. Yeast cells were broken in a Bronwill shaker for preparation of mitochondria and submitochondrial particles as described previously (Kim & Beattie, 1973). Cytochrome *c* depleted submitochondrial particles were prepared according to Brown & Beattie (1978). Coenzyme QH_2–cytochrome *c* reductase activity was assayed with the decyl analogue of coenzyme Q (DBH_2)[1] as substrate (Brown & Beattie, 1977). Values of K_m were calculated from Eadie–Hofstee plots (Segel, 1975).

Spectral Determinations. A Perkin-Elmer double-beam spectrophotometer (Model 557) was used for spectral analysis at room temperature. A slit width of 2 nm was used, and spectra were scanned at 120 nm/min. In some experiments a slit width of 1 nm and a scan speed of 3 nm/min were used. Difference spectra were obtained by scanning the dithionite-reduced sample against the ferricyanide-oxidized reference.

The extrareduction of cytochrome *b* was studied in submitochondrial particles as described by Roberts et al. (1980). Submitochondrial particles were suspended in 0.1 M phosphate buffer, pH 7.5, at a concentration of 1.5–2 mg/mL, and the following spectra were recorded: (1) For succinate-reducible cytochrome *b* 15 min after the addition of 60 mM succinate the spectrum was recorded; (2) for succinate reducible cytochrome *b* in the presence of either diuron or antimycin, 2 min after the addition of the inhibitor, 60 mM succinate was added and the spectrum recorded after 15 min; (3) for total dithionite-reducible cytochrome *b*, the spectrum was recorded 2 min after the addition of a few grains of dithionite to the sample cuvette.

So that the oxidant-induced extrareduction of cytochrome *b* and the concomitant red shift could be studied, washed mitochondria were suspended at concentrations of 4–6 mg/mL in a buffer containing 0.65 M sorbitol, 20 mM KCl, 0.1 mM $MgCl_2$, 10 mM KH_2PO_4, 2 mM EDTA, pH 6.0, and 3 mg/mL BSA[1] (buffer S) (Meunier-Lemesle et al., 1980). Succinate (30 mM) was used as reductant in the sample cuvette. The absorption at wavelengths of 562–575, 564–575, and 553–539 nm was recorded 1, 2, 5, 10, and 15 min after the addition of succinate. In separate experiments antimycin (11.2 nmol/mg of protein) or diuron (500 μM, methanolic solution) was added to the sample cuvette followed by bubbling of 10 mL of air through the cuvette. An equal volume of methanol was added to the reference cuvette and the difference spectrum recorded after 2 min. The total cytochrome *b* content was obtained by reduction with a few grains of dithionite.

The ATP-induced oxidation of cytochrome *b*-562 was studied in submitochondrial particles from the wild-type and mutant strains, according to the method of Gopher & Gutman (1980). The submitochondrial particles, suspended at a concentration of 8–12 mg/mL in buffer S without BSA, were treated sequentially with 8 mM KCN, 133 μM TMPD, 8 mM ascorbate, 20 mM fumarate, and 2 mM succinate. Subsequently, 2 nmol of antimycin/mg of protein was introduced into the sample cuvette, followed by 2 mM ATP.

For calculation of cytochrome concentration, the following molar coefficients were used: cyt *b* = 25.6 mM^{-1} cm^{-1} and cyt c_1 = 20.9 mM^{-1} cm^{-1} (Van Gelder, 1978).

The content of cytochrome *b*-565 was estimated aerobically, by recording the spectrum of mitochondria reduced by succinate plus antimycin vs. oxidized mitochondria. Since this spectrum represents both cytochromes *b*, the contribution of *b*-562 calculated from the spectrum of anaerobic mitochondria reduced with succinate is subtracted to yield the concentration of *b*-565 (Meunier-Lemesle et al., 1980).

Table I: Effect of Antimycin A and Diuron on DBH_2–Cytochrome *c* Reductase Activity in the Wild-Type and Diu^r Mutants[a]

	DBH_2-cyt *c* reductase				diuron resistance[d]	
strains	mito	SMP	V_{max}[b]	K_m[c]	mito	SMP
KL14-4A	231[b]	419[b]	400	60	8.28	5.92
740	185	555	700	44	11.2	7.72
742	244	465	480	50	12.0	21.2
750	218	636	780	59	7.94	20.6

[a] The specific activities were determined according to the procedure described under Materials and Methods. For the inhibition studies, mitochondria (mito) and submitochondrial particles (SMP) were incubated for 4 min with the antibiotic prior to addition of the substrate. [b] nmol min^{-1} (mg of protein)$^{-1}$. [c] μM DBH_2. [d] mM diuron (nmol of cyt c_1) giving 50% inhibition.

Labeling of Cells. Yeast cells were labeled in vivo with [^{35}S]methionine in the presence of cycloheximide. Electrophoresis of the labeled products were performed on sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gels as described previously (Beattie et al., 1979). Limited proteolytic digestion of the labeled bands with *Staphylococcus aureus* V-8 protease was performed according to Beattie & Clejan (1980).

Results

Resistance to Diuron. The diuron resistance of the three mutants used in this study was confirmed prior to further biochemical characterizations. The mutants were grown on glycerol medium supplemented with diuron ranging in concentration from 15 to 350 μM. Under these conditions, growth of the parental strain KL14-4A was completely blocked by 25 μM diuron. The mutant strains Diu^r-740 and Diu^r-752 grew on 130 μM diuron, while Diu^r-742 grew on 78 μM diuron, thus confirming their resistance to growth on diuron.

The resistance of the three mutants to diuron was also determined in vitro by examining cytochrome *c* reductase activity with the decyl analogue of coenzyme Q (DBH_2) as substrate (Brown & Beattie, 1977). Comparable rates of cytochrome *c* reduction were observed in mitochondria isolated from all four strains (Table I); however, a significantly higher reduction rate was obtained with submitochondrial particles isolated from two of the mutant strains. This higher enzymic rate was also reflected in a higher V_{max} when calculated by Eadie-Hofstee plots although all four strains had a similar apparent K_m for DBH_2.

Mitochondria obtained from all three mutant strains with the exception of Diu^r-752 were slightly resistant to diuron during the assay of DBH_2–cytochrome *c* reductase despite their greater resistance to comparable concentrations of diuron in assays of NADH oxidation in submitochondrial particles (Briquet & Goffeau, 1981). By contrast, significantly higher amounts of diuron were needed to attain 50% inhibition of cytochrome *c* reductase activity in submitochondrial particles from two of the mutants, Diu^r-742 and Diu^r-752, as compared to particles from the parental strain. Enzymic activity in submitochondrial particles from the other mutant studied, Diu^r-740, which is resistant to diuron during growth, was only slightly less sensitive to diuron than the wild type.

Functional Studies of Cytochrome b in Diu^r Mutants. Other possible functional changes in the behavior of cytochrome *b* which might have resulted as a consequence of the

Table II: Extrareduction of Cytochrome *b* in Submitochondrial Particles of KL14-4A and Diu^r Mutants[a]

	cytochrome *b* reduced (% of total)[b]			
	expt 1		expt 2	
strains	succinate	anti-mycin	succinate	diuron
KL14-4A	68	80	77	83
Diu^r-740	79	79	79	79
Diu^r-742	54	54	60	56
Diu^r-752	61	61	65	65

[a] Submitochondrial particles were suspended at 1.5–2 mg/mL in 0.1 M phosphate buffer, pH 7.5. Spectra were recorded 15 min after the addition of 60 mM succinate. For the extrareduction assays, antimycin A (5 μg/mg of protein) or diuron (500 mM) was incubated with submitochondrial particles 2 min before the addition of succinate (60 mM). Total cytochrome *b* was obtained by reduction with a few grains of dithionite. [b] In experiment 1, the total cytochrome *b* reduced by dithionite was 0.61, 0.29, 0.46, and 0.68 nmol (mg of protein)$^{-1}$ and in experiment 2, the total was 0.64, 0.29, 0.41, and 0.60 nmol (mg of protein)$^{-1}$ in strains KL14-4A, Diu^r-740, Diu^r-742, and Diu^r-752, respectively.

Table III: Oxidant-Induced Extrareduction of Cytochrome *b*-562 in the Presence of Antimycin A or Diuron[a]

	cytochrome *b* reduced (% of total)[b] in the presence of		
strains	succinate	antimycin A	diuron
KL14-4A	72.8	92.0	84.7
740	60.0	60.0	62.5
742	77.4	77.4	77.4
752	70.7	73	73.0

[a] Mitochondria were suspended at concentrations of 4–6 mg/mL, as described under Materials and Methods. The spectrum was recorded 15 min after the addition of 30 mM succinate. Separate experiments were performed with antimycin or diuron followed by bubbling air, prior to recording the spectrum; total cytochrome *b* content was obtained as described in footnote *a* of Table II. [b] The total cytochrome *b* reduced by dithionite was 0.57, 0.43, 0.38, and 0.46 nmol (mg of protein)$^{-1}$ in strains KL14-4A, 740, 742, and 752, respectively.

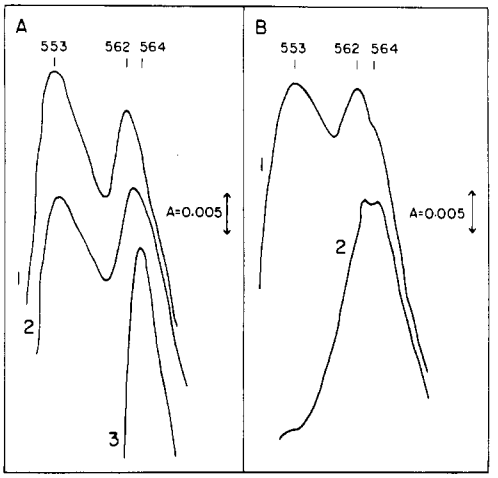

FIGURE 1: Red shift of cytochrome *b*-562 produced by antimycin A in the wild-type and Diu^r strains. Mitochondria were suspended at room temperature in the medium described by Meunier-Lemesle et al. (1980). (A) (Trace 1) Succinate (30 mM) was added to the sample cuvette, and 15 min later the difference spectrum was recorded; (trace 2) antimycin A (11 nmol/mg of protein) was introduced, and 2 min later the spectrum was recorded; (trace 3) 10 mL of air was bubbled through the sample, and the spectrum was recorded. The traces are representative of the wild type and the Diu^r-752 strain. (B) (Trace 1) Spectrum recorded 15 min after succinate (30 mM) addition; (trace 2) spectrum recorded after addition of antimycin A and air. The traces are representative of the Diu^r-740 and Diu^r-742 strains.

Table IV: ATP-Induced Oxidation of Cytochrome *b*-562 in Antimycin-Inhibited Submitochondrial Particles

strains	antimycin [nmol of *b* (mg of protein)$^{-1}$][a]	+ATP (% *b* reduced)	+CCCP (% *b* reduced)
KL14-4A	0.228 (100%)	54.1	81.1
740	0.066 (100%)	71.6	86.1
742	0.190 (100%)	74.5	69.2
752	0.185 (100%)	73.1	82.1

[a] Total cytochrome *b*-562 reduced after addition of antimycin A.

mutation-conferring resistance to diuron during growth were also studied. In the first approach, the *extrareduction* of cytochrome *b* was studied in submitochondrial particles, by comparing the amounts of the total cytochrome *b* reduced by succinate with that reduced in the presence of either diuron or antimycin plus succinate (Roberts et al., 1980). In these experiments, the antibiotic was preincubated with the particles prior to the addition of the substrate succinate. Table II indicates that no extrareduction of cytochrome *b* was observed in the presence of either antibiotic in particles from the mutants, although a significant extrareduction of cytochrome *b* was observed in the parental strain. It can be noted that considerably more of the cytochrome was reduced by succinate in mutant 740.

In the second approach the *oxidant-induced extrareduction* of cytochrome *b* with the concomitant red shift of the absorption maximum of the cytochrome in the presence of the antibiotics was studied (Convent & Briquet, 1978). In these experiments, mitochondria were reduced with succinate as substrate until anaerobiosis was obtained. Subsequently, either antimycin or diuron was added followed by bubbling air through the cuvette to oxidize those components on the oxygen side of the antimycin block. As seen in Table III, the amount of cytochrome *b* reduced after the oxidation of cytochrome c_1 was much less in the mutants than in the wild type despite the observation that succinate reduced cytochrome *b* to the same extent in mitochondria from the mutant and wild-type strains. The most unusual response of the mutants, however, was observed in an examination of the spectrum obtained. As seen in Figure 1A, the oxidation of cytochrome c_1 by air is absolutely necessary for the disappearance of the cytochrome *b* peak at 562 nm and the appearance of the new cytochrome *b* peak at 564 nm in either the mitochondria from the wild type or Diu^r-752 strains. By contrast, the other two mutants, Diu^r-740 and Diu^r-742, responded differently with the retention of the peak at 562 nm, concomitant with the appearance of the peak at 564 nm (Figure 1B) despite vigorous aeration of the cuvette for several minutes. Similar results were obtained when diuron was present as inhibitor (data not shown).

In the third experimental approach, the ATP-induced oxidation of cytochrome *b*-562 in the presence of antimycin was compared in submitochondrial particles from the parental strain and the three mutants. The submitochondrial particles were treated sequentially with KCN, ascorbate–TMPD, fumarate–succinate, and antimycin as described in the legend to Figure 2. The difference spectrum of the sample was recorded after each addition. Subsequently, ATP was added to energize the membrane, causing a reversed electron flow to occur resulting in the oxidation of nearly 50% of the cytochrome *b*-562 reduced by succinate in the presence of antimycin (Figure 2 and Table IV). This ATP-induced oxidation of cytochrome *b* was almost completely abolished by

Table V: Cytochrome Content of Cytochrome *c* Depleted Submitochondrial Particles[a]

strain	cytochrome *b* (nmol/mg of protein)					cytochrome c_1 (nmol/mg of protein)				
	n[b]	galactose grown	*n*	lactate grown		*n*	galactose grown	*n*	lactate grown	
KL14-4A	4	0.471 ± 0.115[c]	4	0.537 ± 0.1000	NS[d]	4	0.338 ± 0.117	4	0.341 ± 0.063	NS
D273-10B	3	0.538 ± 0.051	3	0.641 ± 0.194	NS	3	0.384 ± 0.126	4	0.439 ± 0.174	NS
740	3	0.356 ± 0.205	3	0.562 ± 0.072	$p < 0.02$	3	0.259 ± 0.187	3	0.420 ± 0.192	NS
742	3	0.357 ± 0.085	3	0.614 ± 0.044	$p < 0.005$	3	0.250 ± 0.090	3	0.473 ± 0.057	$p < 0.02$
752	3	0.386 ± 0.130	3	0.611 ± 0.060	$p < 0.05$	3	0.252 ± 0.046	3	0.523 ± 0.185	$p < 0.1$

[a] Yeast cells were grown to early stationary phase by using either 3% galactose or 3% sodium DL-lactate as energy source. Cytochrome *c* depleted submitochondrial particles were prepared, and cytochrome content was determined, as described under Materials and Methods. The level of significance between the two groups was obtained by using Student's *t* test. [b] *n* = number of experiments. [c] Values represent mean ± SD. [d] Not significant.

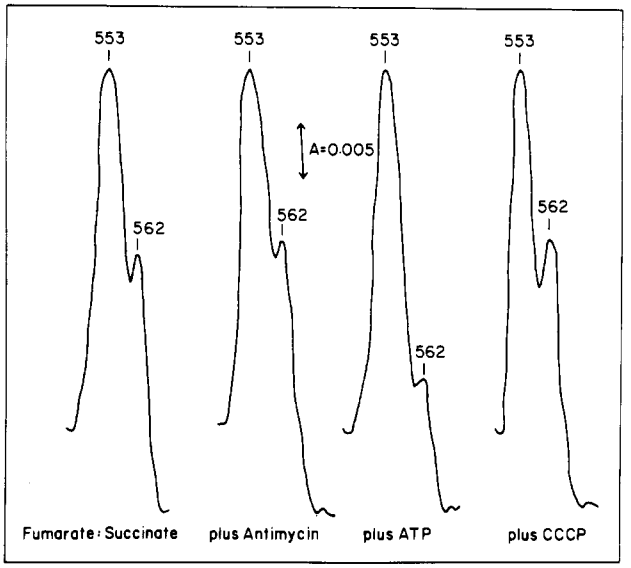

FIGURE 2: ATP-induced oxidation of cytochrome *b*-562 in antimycin-inhibited submitochondrial particles. Submitochondrial particles from strain KL14-4A were suspended at a concentration of 8.2 mg/mL in a buffer containing 0.65 M sorbitol, 20 mM KCl, 0.1 mM $MgCl_2$, 2 mM EDTA, and 10 mM potassium phosphate, pH 6.3. Spectra were recorded successively after each of the following additions to the sample cuvette: 8 mM KCN, 133 μM TMPD, 8 mM ascorbate, 20 mM fumarate, 2 mM succinate, 2.0 nmol of antimycin/mg of protein, 2 mM ATP, and 4 μM CCCP.

addition of the uncoupler CCCP, resulting in the appearance of more reduced cytochrome *b*. In the mutant strains, the addition of ATP induced the oxidation of only 25–28% of the cytochrome *b* reduced in the presence of antimycin A (Table II). A less dramatic change in the amount of reduced cytochrome *b* was observed upon addition of CCCP to the three mutants.

Growth and Biogenesis. The results described above suggest that the mutations causing a resistance to diuron during growth in glycerol cause subtle changes in the function of cytochrome *b*. It was thus of some interest to study the effects of these mutations on both the growth of these yeast strains and the biogenesis of cytochrome *b*. The wild type and the three mutants had identical growth curves in galactose. When cells were precultured in 3% galactose until stationary phase and then transferred into growth medium containing 3% galactose, a variable lag period from 6 to 8 h was observed prior to doubling; however, the same cell density was achieved at stationary phase 22 h after the initial transfer. The characteristics of the growth curves obtained in lactate with both mutants and the wild type varied with the preculture conditions. When KL14-4A and either Diu^r-740 or Diu^r-742 were precultured in 3% galactose and then transferred into growth medium containing 3% sodium lactate, a 4-h lag period was observed prior to cell division. The wild type and Diu^r-740 reached approximately the same cell density at stationary phase after 40 h. By contrast, Diu^r-742 stopped growing after 25 h and attained a much lower cell density. When cells were precultured in 3% lactate, prior to transfer to the growth medium containing lactate, the mutants and the parental strain responded identically, each having a 4-h lag before growth began and reaching stationary phase after 70 h.

Since the strain Diu^r-742 grew at the same rate as the wild type in lactate after being precultured in lactate, it was interesting to determine if the use of this energy source affected the concentrations of components of the respiratory chain. The total cytochrome *b* and cytochrome c_1 content was determined in cytochrome *c* depleted submitochondrial particles obtained from cells grown in lactate and galactose (Table V). All three mutant strains contained higher levels of cytochrome *b* when grown in lactate instead of galactose, although the difference obtained with strain 740 is not as statistically significant. Diu^r-742 also had a higher cytochrome c_1 content when grown in lactate. No change in either cytochrome *b* or cytochrome c_1 content was observed when the wild-type strains, KL14-4A or D273-10B, included for comparison, were grown in lactate.

All of the mutant strains with the exception of Diu^r-752 had amounts of cytochrome *b*-565 comparable to those of the wild type. The *b*-565/*b*-562 ratio in mitochondria isolated from KL14-4A was 0.20, while the ratio in strains Diu^r-740, Diu^r-742, and Diu^r-752 was 0.25, 0.16, and 0.05, respectively. In a similar study, the ratio of *b*-565/*b*-562 was 0.25 in mitochondria from strain 777-3A grown at 28 °C (Meunier-Lemesle et al., 1980).

The mitochondrial translation products were compared in the three mutant strains and the wild type by pulse labeling the cells in the presence of sufficient cycloheximide to block cytoplasmic protein synthesis. The autoradiogram indicates an almost identical pattern of labeled bands in mitochondria from all four strains (Figure 3). The three subunits of cytochrome oxidase are present as indicated except in strain 742 which lacks subunit II. In addition, an apparent double band is observed in the region corresponding to cytochrome *b* in the gel pattern from all four strains. These two labeled bands which differ in calculated molecular weight by 1000 were excised from the gel of the mitochondria from the wild-type cell and subjected to limited proteolytic digestion. As seen in Figure 4, the peptide fragments observed appear the same in the two proteins, suggesting that they are two forms of cytochrome *b* differing slightly in their apparent molecular weight. It can also be noted that the lower band corresponding to cytochrome *b* appears to be more heavily labeled in the two mutants 740 and 742 but equally labeled in the wild type and mutant 752.

Discussion

The results of this study suggest that the mutations which alter the sensitivity of KL14-4A to growth on diuron also cause

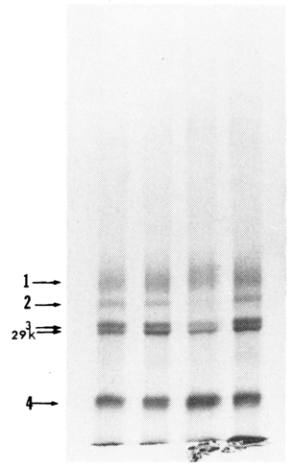

FIGURE 3: Autoradiogram of mitochondrial translation products. Cells were labeled for 15 min at 28 °C with [^{35}S]methionine, 1 mCi/g wet weight cells, in the presence of cycloheximide (1 mg/mL) in a medium containing 2% glucose and sulfate-free inorganic salts (Beattie et al., 1979). The mitochondrial translation products were separated on NaDodSO$_4$–10% polyacrylamide gels and autoradiographed on NS–5T X-ray film. From left to right: (lane 1) KL14–4A; (lane 2) 740; (lane 3) 742; (lane 4) 752. The arrows show (1) cyt oxidase subunit I, (2) cyt oxidase subunit II, (3) cyt *b*, and (4) cyt oxidase subunit III; 29k, 29 000-dalton polypeptide.

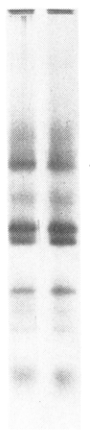

FIGURE 4: Fingerprint pattern after limited proteolytic digestion of apocytochrome *b* and the polypeptide of apparent molecular weight of 29 000. The [^{35}S]methionine-labeled bands of apocytochrome *b* and of the polypeptide of 29 000 daltons were excised from a 10% NaDodSO$_4$–polyacrylamide gel and subjected to limited proteolytic digestion with *Staphylococcus aureus* V-8 protease as described by Beattie & Clejan (1980). The proteolytic products were separated on a 15% NaDodSO$_4$–polyacrylamide gel: apocytochrome *b* (left lane) and the 29 000-dalton polypeptide (right lane) from strain KL14–4A.

subtle changes in the biochemical functions of cytochrome *b*. While these modifications are not sufficient to alter the gross properties of cytochrome *b* such as its apparent molecular weight or spectrum, the changes in biochemical function suggest that these mutations occur in regions which are related to the activity of the protein. The functional changes in cytochrome *b* included resistance to diuron at the level of the respiratory chain, measured as QH_2–cytochrome *c* reductase, no *extrareduction* of cytochrome *b* when either antimycin or diuron was allowed to bind to submitochondrial particles in the oxidized state prior to addition of succinate, a greatly lowered *oxidant-induced extrareduction* when succinate was added before the inhibitors, and a diminished reversed electron flow induced by ATP in KCN and antimycin-treated submitochondrial particles reduced with fumarate–succinate. Generally these changes in cytochrome *b* behavior were observed in all three mutant strains; however, the observed differences in the three diuron-resistant mutants confirm the genetic studies, indicating that each mutation maps in a different locus on the mitochondrial DNA. Furthermore, the results suggest a nonfunctional equivalence of these loci. For example, Diu^r-742 mutant, which maps in the first exon of the cytochrome *b* gene, exhibited more pronounced changes in general in the parameters studied than were observed in the mutants Diu^r-740 and Diu^r-752, which appear to be allelic to box 3, or the first intron locus of the cytochrome *b* gene in this strain (Lazowska et al., 1980; Colson & Wouters, 1980). The functional changes in cytochrome *b* observed in the antibiotic-resistant mutants confirm the previous suggestions that cytochrome *b* is the protein of complex III to which diuron and antimycin bind (Roberts et al., 1980; Briquet & Goffeau, 1981). Although spectral studies have suggested that antimycin and diuron bind to different sites on cytochrome *b*, similar biochemical effects have been observed in mutations of yeast conferring resistance to both antibiotics.

In the current study, it was noted that the oxidant-induced reduction of cytochrome *b* in the presence of both antibiotics did not result in a complete shift in the maximum absorption of cytochrome *b* from 562 to 564 nm as observed in the wild type. This result provides further evidence that the binding of the antibiotics is insufficient to cause a shift in the potential such that cytochrome *b*-564 is fully reduced. The lack of extrareduction when the antibiotics were bound to the submitochondrial particles prior to succinate can also be explained by a change in the binding of the inhibitors to the mutated cytochrome *b*.

One of the most interesting effects observed in the experiments reported here is the lack of response of cytochrome *b* in the mutants to an energy charge across the membrane during reversed electron transport (Table IV). The addition of ATP to submitochondrial particles reduced with fumarate–succinate in the presence of antimycin resulted in considerable oxidation of cytochrome *b*. In a previous study, Gopher & Gutman (1980) have concluded that it is the $\Delta\Psi$ rather than the ΔpH component which provides the energy for the observed reversed electron transfer. In all three diuron-resistant mutants the oxidation of cytochrome *b* after addition of ATP is practically negligible, suggesting that the mutated cytochrome *b* can no longer respond to an externally imposed energy charge. If this is indeed the case, it also suggests the converse that cytochrome *b* in the mutants may be inefficient in energy transduction at site II of the respiratory chain. Two observations of the present study support this suggestion. First, strain Diu^r-742, the most severely affected of the three mutants, only attains 50% of the cell density at stationary phase during growth in lactate, suggesting that the total energy available may become limiting in this strain. Second, all three diuron-resistant strains have a significantly higher content (60–89%) of both cytochromes *b* and c_1 per milligram of protein during growth on lactate. It is also of some interest that the mutants with the exception of Diu^r-752 all contain cytochrome *b*-565 which has been shown not to be involved in oxidation phosphorylation in yeast (Chevillotte-Brivet & Meunier-Lemesle, 1980). Perhaps, if the energy-transducing capability of cytochrome *b* is reduced as a consequence of the mutation-conferring diuron resistance, the cell compensates by synthesizing a higher quantity of the functionally impaired protein. To test this possibility we are currently studying the rate of proton translocation using purified complex III reconstituted into liposomes. A comparison

of the capacity of complex III isolated from the mutants and the wild type to energize the membrane can thus be made directly. Despite the postulated decrease in the energy coupling capacity in the diuron-resistant mutants, the rate of electron transport through this segment of the chain, measured as DBH_2–cytochrome *c* reductase was greater in the mutants than in the wild type. This result indicates that a functional respiratory chain is not impaired in the mutants.

Interestingly, less resistance to diuron was observed in mitochondria when cytochrome *c* reductase activity was assayed with DBH_2 as substrate. Likewise, the resistance to diuron in submitochondrial particles was less pronounced when DBH_2–cytochrome *c* reductase was assayed than in previously reported studies in similar diuron-resistant mutants in which NADH and succinate oxidation were measured with an oxygen electrode (Briquet & Goffeau, 1981). Perhaps, this lack of sensitivity to diuron during cytochrome *c* reduction with the coenzyme Q analogue may reflect the pattern of reduction of this compound in the respiratory chain. Previously we had reported that DBH_2 when added to either mitochondria mitoplasts or submitochondrial particles achieved a very different pattern of cytochrome *b* and c_1 reduction during anaerobiosis as compared to succinate (Beattie et al., 1981). For example, addition of DBH_2 and succinate to mitoplasts resulted in the reduction of 29 and 72% of the total cytochrome *b*, while their addition to submitochondrial particles resulted in the reduction of 60 and 70%, respectively. One explanation for this observation might be that the decyl analogue of coenzyme Q, which is less hydrophobic than the natural coenzyme Q_6, may not equilibrate completely with the coenzyme Q pool but may directly reduce the cytochrome or other component of the chain.

Finally, the mitochondrial translation products in the diuron-resistant mutants appear very similar to those in the parental strain. Of some interest is the presence of two bands in the region of the gel corresponding to cytochrome *b* under these labeling conditions. We would like to suggest that these represent the two forms of cytochrome *b* recently isolated in our laboratory (Chen & Beattie, 1981). In that study, the two forms of cytochrome *b* had molecular weights of 31 000 and 29 000, a considerably larger difference in molecular weight than that present in the two forms of cytochrome *b* after pulse labeling in vivo. The well-characterized anomalous migration behavior of cytochrome *b* during polyacrylamide gel electrophoresis may have led to these observed differences (Beattie et al., 1979). Of some interest is the indication that the diuron-resistant mutants may have relatively more of the lower molecular weight form of cytochrome *b* than the wild-type strain. The 31 000-dalton form of cytochrome *b* appears to be the initial translation product which may be processed in a second step to the lower molecular weight form. Perhaps in the diuron-resistant strains, the mutation in the cytochrome *b* protein may result in a changed regulation of protein processing.

In conclusion, the diuron-resistant mutants described in this study and previous ones should be useful for studies of both the bioenergetics and the biogenesis of the cytochrome b–c_1 region of the respiratory chain. These mutants may be especially useful in elucidating the role of cytochromes *b* in energy transduction either by the Q cycle or by the proton pumping models.

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Registry No. ATP, 56-65-5; diuron, 330-54-1; coenzyme QH_2–cytochrome *c* reductase, 9027-03-6; cytochrome *b*, 9035-37-4; cytochrome *b*-562, 9064-79-3; succinic acid, 110-15-6; antimycin A, 1397-94-0; galactose, 59-23-4; sodium lactate, 312-85-6; cytochrome *b*-565, 9083-17-4; cytochrome oxidase, 9001-16-5; cytochrome c_1, 9035-42-1.

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