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PURIFICATION AND CHARACTERIZATION OF HIGHLY PURIFIED CYTOCHROME *b* FROM COMPLEX III OF BAKER'S YEAST

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A simple, high-yield purification procedure for cytochrome *b* from yeast Complex III has been developed. This procedure involves solubilization using chemical modification of the lysine residues with 3,4,5,6-tetrahydrophthalic anhydride followed by hydroxyapatite column chromatography. This purified cytochrome *b* has a heme content of 37.0 nmol cytochrome *b*/mg and a molecular weight on SDS gels of 25000–26000. Amino acid analysis indicates high hydrophobicity and is very comparable to the composition deduced from the gene sequence (Nobrega, F.G. and Tzagoloff, A. (1980) J. Biol. Chem. 255, 9828–9837). The latter data indicate a molecular weight of 42000 for the polypeptide; our heme analyses thus imply the presence of two hemes per polypeptide chain. Optical and MCD spectra are typical of a low-spin *b*-type cytochrome. MCD-potentiometric titration indicates a one-electron carrier with a single midpoint potential of −44 mV at pH 7.4 and 25°C. The EPR spectrum of isolated cytochrome *b* has only one g_z signal at 3.70, indicating that the 'strained' heme structure (Carter, K., T'sai, A. and Palmer, G. (1981) FEBS Lett. 132, 243–246) is still maintained. No indication of antimycin binding was demonstrated either by the direct-fluorescence method or binding-precipitation method although stoichiometric binding to the parent Complex III was readily demonstrated.

Introduction

The cytochrome(s) *b* of the mitochondrial electron-transport chain have long been known for peculiar oxidation-reduction behavior which is assumed to have a close relationship with energy transduction [1,2]. This redox center appears heterogeneous when studied by spectroscopy, potentiometry and kinetic measurements on mitochondria and on isolated protein complexes. For example, two distinct species of cytochromes are observed in Complex III; cytochrome *b*-562 (b_k), has a higher midpoint potential and a lower *g* value by EPR measurement while cytochrome *b*-588/566 (b_t), has a lower midpoint potential and a higher *g* value. This heterogeneity has led to more confusion than clarification concerning the function of this component. The specific genetic origin of cytochrome *b* has recently become the focus of substantial investigation [3] and the sequence of the gene from both yeast [3] and mammalian [4] mitochondria has been established.

The isolation of this cytochrome is a necessary prerequisite for the detailed understanding of its chemical and physical properties and its biosynthesis. A number of purification methods for cytochrome *b* have been reported [3,5], including two procedures for the protein from yeast [6,7]; the procedure developed by Katan et al. [6] yields a product with significant contamination by other proteins while the protein isolated by Lin and Beattie [7] appears to have lost a substantial amount of heme during isolation.

Purification of this mitochondrial cytochrome has routinely relied on high concentrations of detergents to release the cytochrome *b* from its ex-

tremely hydrophobic membrane environment. Recently, Howlett and Wardrop [8] have used 3,4,5,6-tetra-hydrophthalic anhydride to dissociate reversibly and then reconstitute the protein components of the human erythrocyte membrane. This lysine-modifying reagent, which has a relatively effective membrane-penetrating capability, has been tested as a tool for the isolation of cytochrome *b*, and in this paper we introduce a simple, rapid and effective procedure for purifying cytochrome *b* from baker's yeast by using 3,4,5,6-tetrahydrophalic anhydride treatment to solubilize this cytochrome from the purified Complex III. The cytochrome *b* prepared by this method has a heme content of about 37 nmol/mg protein with the overall yield averaging 60%, implying that both heme centers are represented in the final product. EPR and MCD spectra of mitochondrial cytochrome *b* are presented for the first time.

Materials and Methods

Complex III was isolated from yeast (Red Star) according to the procedure of Siedow et al. [9] and had a purity of 9 nmol cytochrome *b*/mg protein. Cholic acid was obtained from Sigma and recrystalized from ethanol. 3,4,5,6-Tetrahydrophthalic anhydride was purchased from Aldrich and dissolved in a minimum volume of peroxide-free *p*-dioxane immediately prior to use. Hydroxyapatite was prepared using the procedure of Jenner [10]. The antimycin A (Sigma Chemical Co.) used for fluorescence-quenching experiments was prepared as 1 or 10 mM solutions in dimethyl sulfoxide. Acrylamide and bisacrylamide were BioRad products. All other chemicals were reagent grade.

Purification procedure

All steps were carried out at 4°C. Complex III in 0.1 M phosphate, pH 7.4, 0.5% cholate was centrifuged at 100000 × *g* for 2.5 h. The pellet was resuspended in 50 mM Tris-HCl buffer, pH 8.0, containing 0.5% cholate to 3 mg/ml and tetrahydrophthalic anhydride was added dropwise to a concentration of 2 mg/mg protein. The solution pH was maintained close to 8.0 by addition of 1 M KOH and the reaction was complete in approx. 50 min as judged by the absence of any further pH change. The clear red solution was then applied to a hydroxyapatite column (3 × 10 cm); the column was first washed with 80 ml of 50 mM Tris-HCl, pH 8.0, containing 0.5% cholate and subsequently eluted with a linear gradient constructed using 200-ml volumes of 50 mM potassium phosphate, pH 7.9, and 0.4 M potassium phosphate, pH 7.9; each buffer containing 0.5% cholate. Alternatively, following collection of the first orange band which eluted at about 150 mM potassium phosphate during the phosphate gradient step, the major cytochrome *b* fraction was stepwise eluted with 0.3 M potassium phosphate, pH 7.9, containing 0.5% cholate. Fractions with a constant ratio of A_{415}/A_{280} were pooled and, when necessary, concentrated using a Micro-ProDicon concentrator (Bio-molecular Dynamics, Beaverton, OR) with a vertical dialysis membrane.

The purity of cytochrome *b* was routinely verified by electrophoresis on a 12% gel or a 12.5–20% gradient gel. SDS-polyacrylamide slab gel electrophoresis was performed as described by Laemmli [12]. Heme concentration and extinction coefficients were determined via formation of the pyridine hemochrome [13] and protein concentration was determined by the biuret method [14] or according to Lowry et al. [15]. The enzyme assay for Complex III was the same as described by Siedow et al. [9], by measuring the reduction of 50 μM ferricytochrome *c* by 25 μM 2,3-dimethoxy-5-methyl-6-pentylbenzohydroquinol (PBQ) at 25°C in pH 7.4 buffer. A similar procedure was carried out with purified cytochrome *b*. The reduction of cytochrome *b* was followed at 561.5 nm; 50 μM reduced PBQ or 1 mM succinate was used to reduce 10 μM cytochrome *b* in the presence of 5–10 μg Complex II isolated from yeast in 0.1 M potassium phosphate, pH 7.4, containing 0.5% cholate. The amino acid composition of purified cytochrome *b* was determined by hydrolysis of 0.6–0.7-mg samples in 6 M HCl at 100°C for 48, 96, 120 h and quantitation on a Beckman 120-C amino acid analyzer. Tryptophan was released by hydrolysis in 4 M methanesulfonic acid plus 0.2% 3-(2-aminoethyl)indole and analyzed as above [11].

MCD spectra were obtained using a Jasco J-500C spectropolarimeter equipped with a Jasco MCD-1B electromagnet. Spectra were accumulated using a Jasco DP-500 data processor and transferred to the laboratory data system for stor-

age, manipulation and plotting. The temperature dependence of the MCD spectrum of oxidized cytochrome *b* was determined in a thin-walled cuvette with a 1.96 mm light path. A glass dewar with flat, unsilvered windows was fitted between the electromagnet pole-pieces and the sample in the dewar cooled by liquid N_2 boil-off gas. The temperature was monitored by a platinum resistance temperature sensor placed adjacent to the cuvette. Solvent contraction due to temperature change was corrected for by recording the Soret band absorbance at each temperature setting.

Potentiometric titrations monitored by MCD were performed as previously described [16].

EPR spectra were recorded with a Varian E-6 EPR spectrometer equipped with a liquid-helium transfer system.

The antimycin-binding experiments were carried out essentially as described by Berden and Slater [17] using an SLM-400 polarization fluorometer. The excitation wavelength was 355 nm and the emission wavelength 410 nm; 2-nm spectral bandwidths were used at both wavelengths. Antimycin binding was observed by two methods, direct fluorescence and a binding-precipitation method. The direct-fluorescence procedure consisted of titrating a known quantity of Complex III or cytochrome *b* with aliquots of antimycin and monitoring the antimycin fluorescence after each addition during the titration. Before and after each addition, samples of the reaction mixture were assayed for enzyme activity. This procedure was repeated with successive additions until the amount of antimycin in the reaction mixture substantially exceeded that needed for a complete loss of activity. The data obtained with Complex III were used as a control to ensure that inner filter effects were not obscuring the stoichiometric binding of antimycin to cytochrome *b*. The precipitation method involved incubating 7.5 nmol Complex III or 20 nmol cytochrome *b* with increasing quantities of antimycin at 4°C. After 10 min, the reaction mixture was centrifuged at 48000 × *g*, at 4°C, for 3 h and the supernatant used for fluorescence determination of free antimycin.

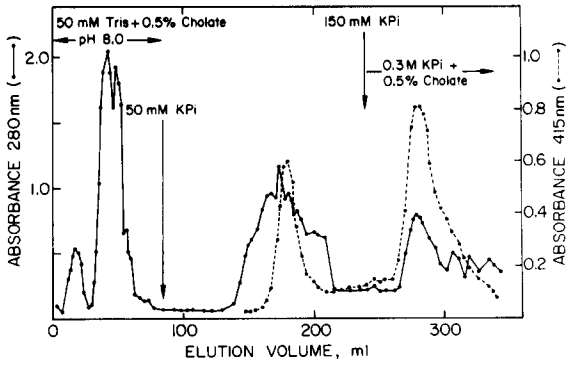

Fig. 1. Hydroxyapatite chromatography purification of cytochrome *b*. 46 mg of 3,4,5,6-tetrahydrophthalic anhydride-treated Complex III were applied to a hydroxyapatite column (3.5 × 10 cm) which was first washed with 30 ml of 50 mM Tris-HCl, pH 8.0, 0.5% cholate, and then developed by a linear phosphate gradient from 50 mM to 0.3 M phosphate (see Materials and Methods). The major cytochrome *b* fraction was eluted by 0.3 M phosphate after the first red band was completely eluted at about 150 mM phosphate. The flow rate was maintained at 0.4 ml/min. KPi, potassium phosphate.

Results

Fig. 1 illustrates the elution profile obtained after phthaloylated Complex III has been applied to a hydroxy apatite column; it shows three major peaks with absorbance at 280 nm. The first peak is predominantly excess unreacted anhydride, the second peak contains cytochrome c_1 and uncleaved Complex III and the third peak, which contained most of the 415 nm absorbing material, was the cytochrome *b* fraction. It takes approx. 14 h to obtain the purified cytochrome *b*.

Table I summarizes the purity and yield of a representative preparation of cytochrome *b* prepared by this procedure. The average yield for six preparations was 61%, varying from 46 to 79%. This high yield implies that this preparation of cytochrome *b* includes contributions from both species of cytochrome *b* present in Complex III assuming that the two cytochrome *b* species exist in a 1:1 ratio [9]. The purity of cytochrome *b* varied from 36.6 to 37.8 nmol cytochrome *b*/mg protein as determined by the pyridine hemochrome method. This value which is comparable to that obtained with the best preparations of the heart protein [18] is equivalent to a minimum molecular mass of 27000 Da. Attempts at further purification by gel filtration or density gradient centrifugation resulted in no significant improvement in purity.

TABLE I

YIELD AND PURITY OF THE PREPARATION PROCEDURES

Step or fraction	Protein (mg)	Heme *b* (nmol)	Heme *b*/protein (nmol/mg)	Yield (%)
Cytochrome *b*-c_1 complex	46.2	415	9.0	100
Anhydride treatment and hydroxyapatite column chromatography	6.9	253	36.7	61

After 42 h of dialysis against 0.1 M Mes, pH 6.0, containing 2.0% taurocholate to remove the chemically bound 3,4,5,6,-tetrahydrophthalic anhydride [8], the purified cytochrome *b* precipitated from solution and the resultant highly aggregated product was extremely difficult to redissolve, even when the solvent contained high concentrations of ionic or nonionic detergents.

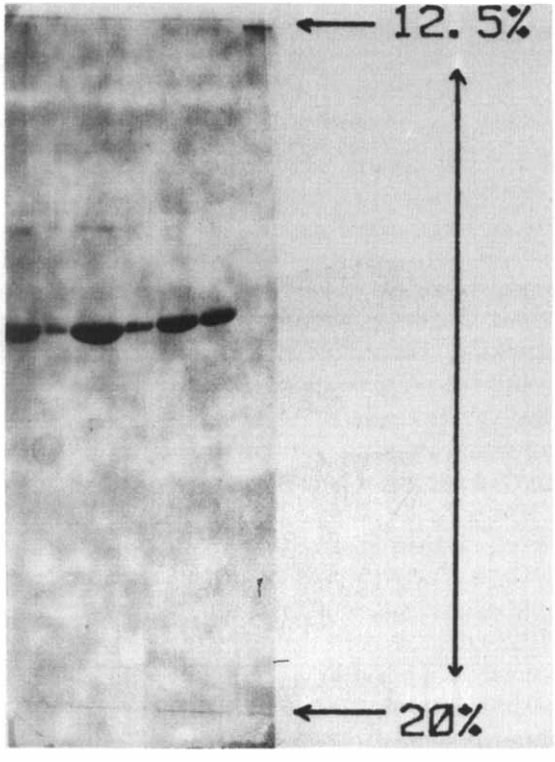

Fig. 2. SDS-polyacrylamide gel electrophoresis of purified cytochrome *b*. A 12.5–20% gradient acrylamide gel with 5% stacking gel was used. 10 μg cytochrome *b* treated at 100°C for 5 min was applied on the rightmost column. Samples applied on the other columns were, from right to left: 5, 10, 2, 25, 2, 15 μg. These samples were previously treated with 80 mM Tris-HCl, pH 6.8, containing 2% SDS, 0.1 M dithiothreitol, 10% glycerol. 0.2% bromothymol blue at 25°C overnight.

A typical polyacrylamide slab gel pattern for purified cytochrome *b* is shown in Fig. 2. A band on the top of the separating gel in the rightmost column is the superaggregate formed by treating the cytochrome *b* sample at 100°C for 5 min. The other gels correspond to varying amounts of cytochrome *b* treated at 25°C overnight before applying to the gel. A dark protein band in each column can be clearly seen with a second visible band of higher molecular weight which can be observed in the fifth gel to which 25 μg protein were applied. These two bands are also conspicuous when Complex III is treated and electrophoresed by the same procedure. Due to different conditions for sample treatment, this result is not the same as that previously reported [9]. An estimate of the molecular weight was obtained by electrophoresis of the cytochrome *b* sample together with four protein markers on a 12% acrylamide analytical slab gel. The molecular mass of cytochrome *b* as judged by this method is approx. 25000–26000 Da, in good agreement with the heme content. Isolated cytochrome *b* apparently exists in an aggregated form, since it is eluted in the void volume on gel filtration through Biogel P-60. A small and variable amount of a species of molecular weight 50000–52000 is often observed. Although the possibility cannot be ruled out that this is a true contaminant, this value suggests that it might be a dimer of cytochrome *b*. This high molecular weight species does not correspond to any component observed in Complex III when run in the same electrophoresis system. We found no evidence for two species

TABLE II

AMINO ACID COMPOSITION OF PURIFIED CYTOCHROME *b* FROM YEAST

Amino acid	Mole percentage		
	Data from gene sequence [14]	This work	From Katan et al. [6]
Aspartic acid (Asx)	7.3–7.5	7.9	8.6
Threonine [a]	4.1	2.8	5.3
Serine [a]	7.3	5.3	5.7
Glutamic acid (Glx)	2.8–3.1	4.0	6.6
Proline	4.9	5.0	4.9
Glycine	6.5	7.9	6.5
Alanine	5.7	6.5	7.6
Cysteine	1.0	0	–
Valine [b]	9.3	9.6	9.4
Methionine	4.1	3.9	2.6
Isoleucine [b]	10.6	9.4	8.1
Leucine [b]	11.2	11.1	11.5
Tyrosine	5.4	5.4	3.4
Phenylalanine	9.3	8.8	8.4
Tryptophan	1.8	1.8	–
Lysine	1.8	3.1	4.4
Histidine	3.1–3.4	3.4	2.9
Arginine	2.8	4.0	3.9
Polarity	29.2–30.00	30.5	37.4

[a] Corrected for destruction by zero-time extrapolation.
[b] From 120-h hydrolysis.

of closely related molecular weight in the main band even when examined in highly resolved gradient gels; thus, the two forms of cytochrome *b* recently reported by Chen and Beattie [19] are apparently not present in our preparation.

The amino acid composition of cytochrome *b* is given in Table II, together with the composition reported by Katan et al. [6] for their preparation of cytochrome *b* isolated from yeast mitochondria and that deduced by Nobrega and Tzagoloff [20] from the gene sequence of this protein. Except for a few polar amino acids, i.e., arginine, lysine, threonine and glumatic acid, the amino acid compositions obtained directly in this work and indirectly by Nobrega and Tzagoloff [20] are very similar, especially with respect to the content of nonionic amino acids. (The phthaloyl group attached to lysine residues does not affect the amino acid analysis, since the amide bond formed between phthalic anhydride and the free amino group of lysine is completely hydrolyzed by the 6 M HCl.) This highly hydrophobic amino acid composition is consistent with location of cytochromes *b* as an integral protein in the mitochondrial inner membrane. The amino acid composition of the cytochrome *b* preparation of Katan et al [6] indicates a greater polarity for this preparation which may well be due to the impurities which can be observed in the gel pattern reported.

Enzyme activity

Enzyme activity was tested by following the absorbance changes at 561.5 nm after mixing a 5- or 10-fold excess of substrate with oxidized cytochrome *b* at pH 7.4 in 0.1 M potassium phosphate and room temperature. This preparation of cytochrome *b* is not reduced by succinate in the presence of a catalytic amount of Complex II, nor does it catalyze electron transfer from the reduced coenzyme Q analog, 2,3-dimethoxy-5-methyl-6-pentylbenzohydroquinol (PBQ), to oxidized cytochrome *c*. Preincubation of the purified cytochrome *b* with excess CoQ_{10} extracted from the yeast Complex III and asolectin suspension did

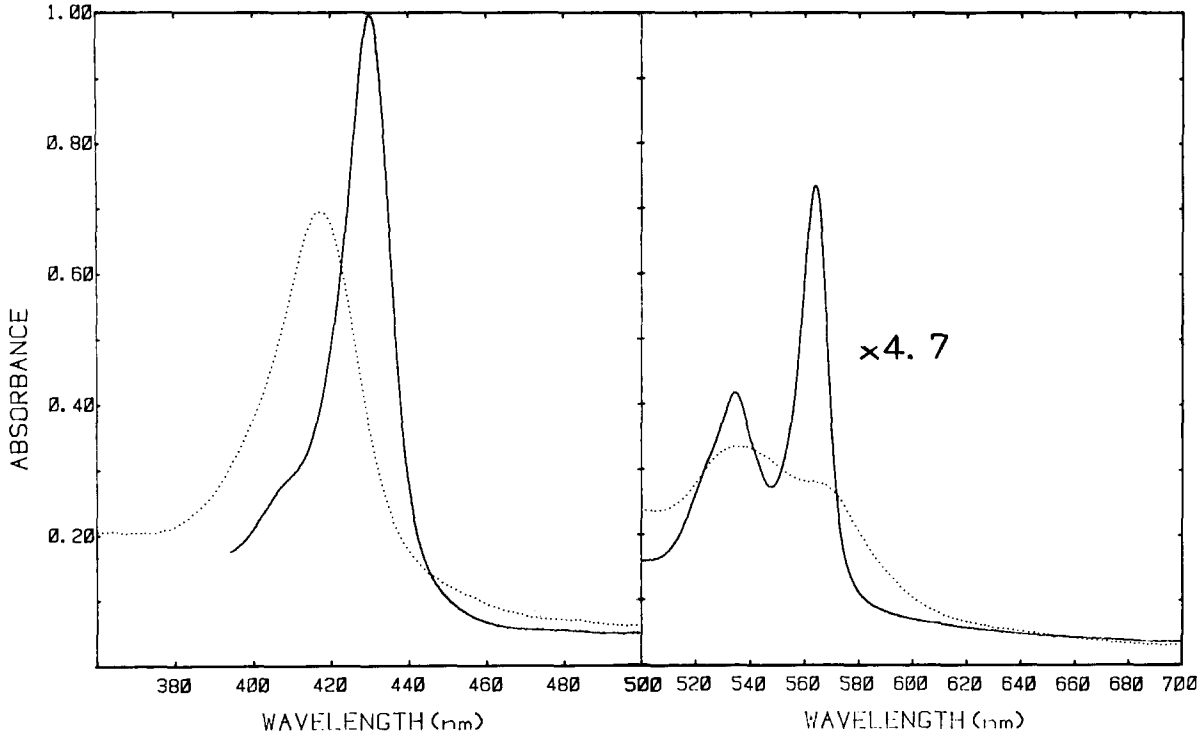

Fig. 3. Optical spectra of purified cytochrome *b*. The spectrum in the α region was taken with 23.0 μM cytochrome *b*; 4.88 μM cytochrome *b* was used for the Soret region. A meaningful spectrum in the ultraviolet region could not be obtained due to the absorption of phthaloyl substituents. Samples were dissolved in 0.1 M potassium phosphate, pH 7.4, containing 0.5% cholate. The temperature was 24°C.

not restore activity. Furthermore, at the completion of the derivatization reaction the Complex III had no measurable activity in this assay.

Optical spectra

The optical spectra of oxidized and reduced cytochrome *b* in the α and Soret regions are presented in Fig. 3; selected parameters are summarized in Table III. The absorption maxima of oxidized cytochrome *b* occur at 567, 530 and 415 nm, and those for the reduced protein are at 561.5, 532 and 428 nm. These spectra have exactly the same extrema as those found for the cytochrome *b* component(s) in the intact Complex III as judged from the difference spectra between dithionite-reduced and ascorbate-reduced Complex III [9].

Carbon monoxide reactivity was investigated by incubating reduced cytochrome *b* under an atmosphere of CO in a stoppered cuvette with frequent mixing. There was an approx. 60% decrease in the absorbance at 561.5 nm during a 40 min period. Addition of 1% sodium lauryl sarcosinate to the incubation mixture led to a further large decrease in the peak at 561.5 nm, suggesting that some of the cytochrome *b* was originally in a form unavailable for reaction with CO.

MCD spectra

The MCD spectra of ferro- and ferricytochrome *b* in the Soret region are given in Fig. 4. The spectrum of the reduced sample has extrema at 431 and 418 nm with a crossover point at 427 nm, close to the maximum (428 nm) in the corresponding optical spectrum. The oxidized cytochrome *b* has more conspicuous extrema at 407 and 420 nm; the crossover is at 413 nm, close to the absorbance maximum. These spectra closely resemble corresponding spectra of cytochrome *c* and cytochrome b_5 [21] and are typical of low-spin ferric hemes. The intensity of the Soret signal (Fig. 5, inset)

TABLE III

PARAMETERS OF OPTICAL ABSORPTION AND MCD OF THE PURIFIED CYTOCHROME *b*

Oxidation-reduction state	Absorption		MCD	
	λ (nm)	ε $(cm \cdot mM)^{-1}$	λ (nm)	$\Delta\epsilon/H$ $(cm \cdot M \cdot T)^{-1}$
Oxidized	567	11.3	551	7.0
	530	14.6	558	0
			569	−12.0
	415	142.6	407	78
			413	0
			420	−97
Dithionite reduced	561.5	32.0	555	160
	532	18.1	558	0
			561	−190
	428	205	431	7.8
			427	0
			418	−14.2

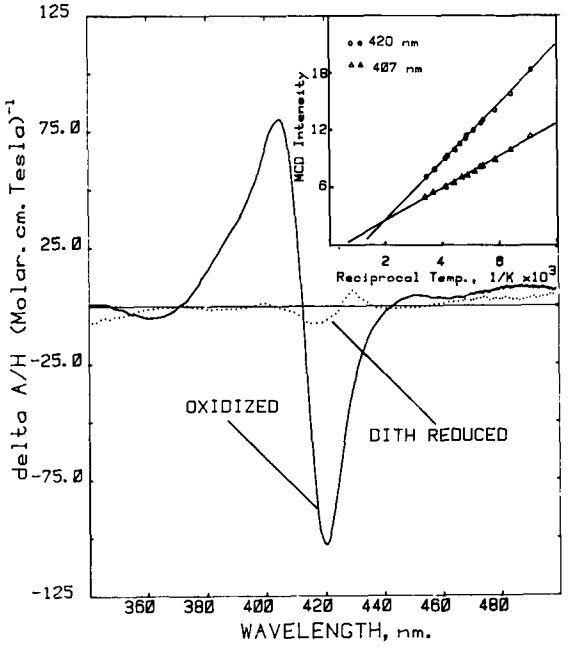

Fig. 4. MCD in the Soret region of purified cytochrome *b* and the temperature dependence of the signal at 420 and 407 nm (inset). The sample for the temperature study was prepared by mixing 3 parts 127 μM cytochrome *b* solution in 0.1 M potassium phosphate, 0.5% cholate, pH 7.4, with 7 parts glycerol. The temperature was cycled between 292 and 141 K. the open and solid symbols represent data obtained with decreasing and increasing temperature, respectively. DITH, dithiothreitol.

exhibits a linear dependence on the reciprocal of the temperature at both 420 and 407 nm. This linear dependence demonstrates that this derivative-shaped feature is composed primarily of MCD C-terms, similar to other low-spin ferric heme proteins [21,22].

Fig. 5 presents the MCD spectra for both oxidized and reduced cytochrome *b* in the α region. The spectrum of the oxidized sample has extrema at 551, and 569 nm and a crossover point at 558 nm, while the reduced sample displays a spectrum with a peak at 555 nm, a trough at 561 nm and a crossover at 558 nm. The very large intensity in the α region for reduced cytochrome *b* (Fig. 5a), 350 $m^{-1} \cdot cm^{-1} \cdot T^{-1}$ (peak-to-trough amplitude), is due to an MCD A-term which arises through the removal of the double degeneracy of the excited state for the π-π^* electronic transition by the external magnetic field [23]; this A-term is similar to that observed for reduced cytochrome b_5 and myoglobin-imidazole [21] and is typical of D_{4h} hemes. The visible MCD of the oxidized protein (Fig. 5b) shows great similarity with other bisimidazole heme proteins [21] and the bis(1-methylimidazole) protoheme model compound [24]. There is no trace of the characteristic charge-transfer transition (Fig. 5b) which is usually observed at about 620–640 nm in high-spin protoheme proteins [22].

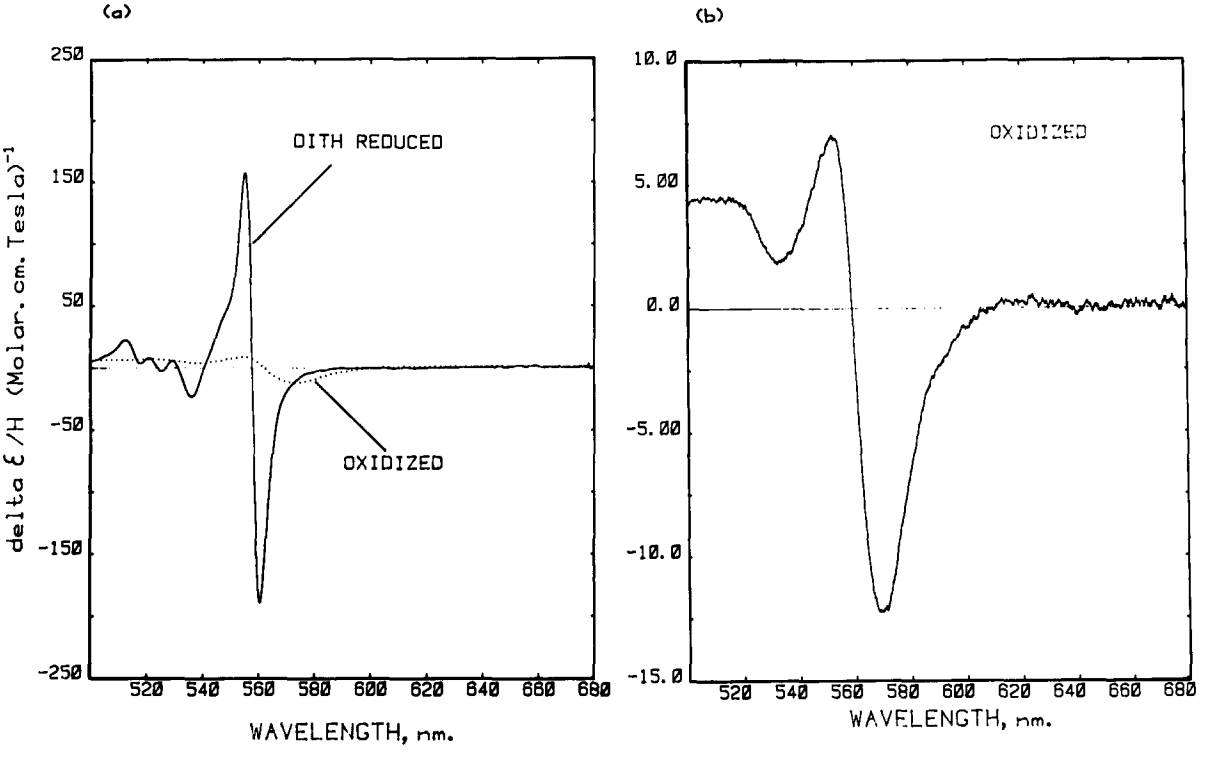

Fig. 5. MCD spectra of purified cytochrome *b*. (a) Oxidized and dithionite-reduced cytochrome *b* spectra. (b) Enlarged MCD spectrum of oxidized cytochrome *b*. Condition for these spectra were as in Fig. 3.

EPR spectra of purified cytochrome b

Two g_z signals are observed for cytochrome *b* in yeast Complex III, with values of 3.60 and 3.76 [9]; slightly different values are found with Complex III from beef heart mitochondria [25,26]. These different *g* values correspond to cytochrome *b* species having different environments and, apparently, midpoint potentials [9]. The *g* 3.7 resonance has an unusual line shape as was previously noted for the mammalian counterpart [26]; no explanation has been advanced for this line shape. The purified cytochrome *b*, however, exhibits a single EPR signal at *g* 3.70 (Fig. 6) midway between the two values found in Complex III. This change in EPR, which is already complete at the end of the derivatization reaction, is indicative of a slight modification in the immediate coordination environment of the metal iron in both heme centers. Nevertheless, this *g* value is much larger than that found with cytochrome b_5 or bisimidazole protoheme. Thus, it appears that this preparation of purified cytochrome *b* still maintains the proposed 'strained' coordination structure [24]. Treatment of the protein with a strong denaturant such as SDS (greater than 1%) causes the *g* 3.70 signal to be shifted upfield to *g* 2.90, the normal g_z position for bisimidazole heme [27]. Sonication also produces this transition. Cytochrome *b* purified from aged or less active Complex III shows both *g* 3.70 and 2.90 signals.

Midpoint potential

The midpoint potential of purified cytochrome *b* was determined by an MCD-potentiometric titration in a manner similar to that described by Wilson and Dutton [28]. The Nernst log plot (Fig. 7) strongly resembles that of a simple one-electron carrier and yields a value of −44 mV for the midpoint potential. The same titration procedure performed on Complex III treated with 3,4,5,6-tetrahydrophthalic anhydride resulted in the same midpoint value. However, two potentio-

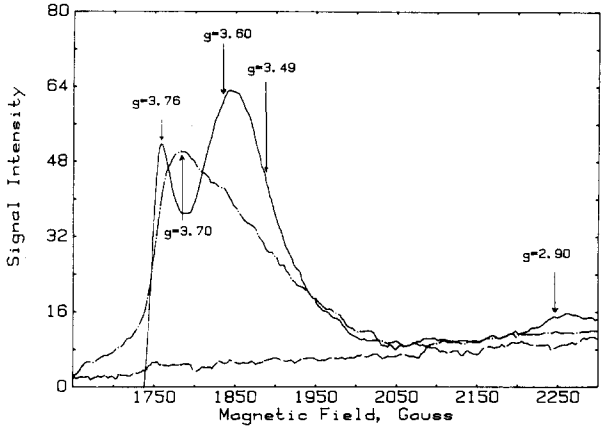

Fig. 6. EPR spectra of oxidized Complex III (172 μM cytochrome *b*) (———) and oxidized cytochrome *b* (110 μM) (– · –). The spectrum of reduced cytochrome *b* is also shown (– –). The EPR spectra were recorded under the following conditions modulation amplitude, 20 G; microwave power, 40 mW; microwave frequency, 9.238 GHz; temperature, 8.7 K; scan rate, 250 G/min; time constant, 1 s.

metrically different cytochrome *b* species ($E_{m,7.4}$ = +62 and −20 mV, respectively) are found in intact yeast Complex III (Tsai, A.-L. and Palmer,

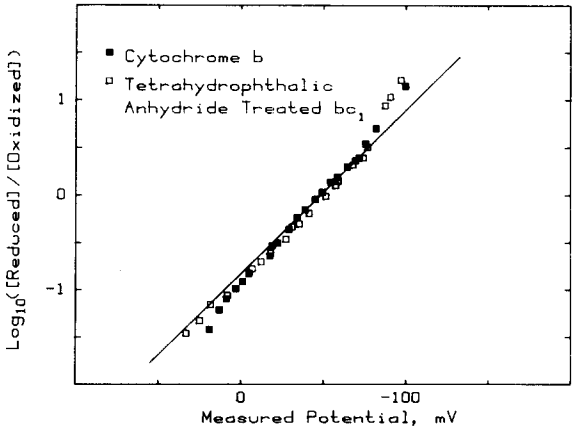

Fig. 7. MCD-potentiometric titration of purified cytochrome *b* and anhydride-treated Complex III. Titrations were conducted at 25°C, in 0.1 M potassium phosphate, pH 7.4, containing 0.1% deoxycholate and 0.1% Triton QS-30, with 9.5 μM cytochrome *b* and 4.0 μM anhydride-treated Complex III. 20 μM each of the following dyes was introduced: *N,N,N′,N′*,-tetramethyl-*p*-phenylenediamine dibydrochlororide, 2,6-dichlorophenolindophenol, 1,2-naphthoquinone, phenazine methosulfate, methylene blue, indigo tetrasulfonate (sodium salt) and 2-hydroxy-1,4-naphtoquinone. Dithionite and ferricyanide solution were used to adjust the potential.

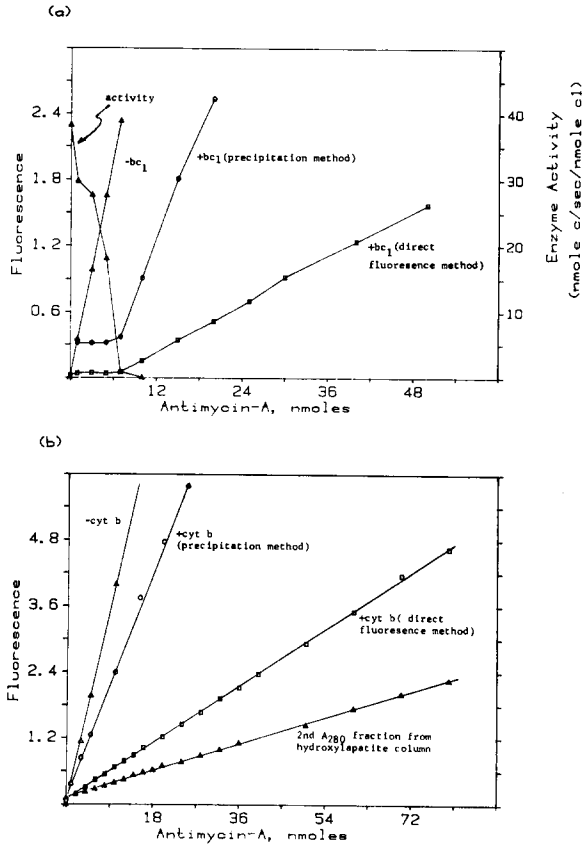

Fig. 8. Antimycin A-binding measurement performed on (a) Complex III and (b) purified cytochrome *b*. The direct fluorescence method (□ ——— □) was implemented with 7.5 nmol cytochrome bc_1 or 20 nmol purified cytochrome *b* in 0.1 M potassium phosphate, pH 7.4, containing 0.5% cholate. The same amounts of cytochtromes bc_1 and *b* were used for the precipitation method (○ ——— ○). Centrifugation was done at 4°C and 48 000 × *g* for 3 h. The enzyme activity (△ ——— △) was measured as described in 9 using a reduced CoQ analog and oxidized cytochrome *c* as substrates at 25°C. △ ——— △ in b represents the direct-fluorescence measurement on the pooled second fraction from the hydroxyapatite column. This sample contained 8 μM cytopchrome c_1. cyt, cytochrome.

G., unpublished results). This result combined with the above EPR studies, and with the demonstration of a single species by both optical (Fig. 3) and MCD (Fig. 5) spectroscopy, demonstrates that 3,4,5,6-tetrahydrophthalic anhydride treatment removes the apparent environmental difference between the two *b* hemes in Complex III and results in a single species.

Antimycin A binding

Antimycin A binding was studied by the fluorescence-quenching method described by Berden and Slater [17,29]. The results of direct fluorometric measurement and determination by the precipitation method of antimycin A binding to Complex III are presented in Fig. 8. Slope changes are readily observable in data obtained for Complex III (Fig. 8a) by both methods; however, no breakpoints can be found in comparable data for cytochrome *b* (Fig. 8b). The fluorescence end point and the elimination of enzyme activity observed with Complex III correspond to a 1:1 binding stoichiometry of antimycin A. These results clearly show that antimycin A binds to yeast Complex III stoichiometrically and totally inhibits electron transfer as was previously reported by Slater [29] for the beef heart mitochondrial complex. However, the purified cytochrome *b* did not show any binding with antimycin A by either the fluorescence or precipitation method (Fig. 8b). Binding of antimycin A also could not be demonstrated with the second fraction (Fig. 1) eluted from a hydroxyapatite column (Fig. 8b) nor with Complex III at the completion of the reaction with phthaloyl anhydride.

Discussion

A convenient high-yield procedure for purified cytochrome *b* from yeast has been developed. This cytochrome preparation is homogeneous and has a high heme content which has facilitated the study of the heme coordination environment by spectroscopic methods. [24]. The major step in the procedure is the dissociation of the cytochrome *b* subunit from Complex III by chemically modifying the ε-amino group of lysine residues. The hydrophobic substituted benzene ring of tetrahydrophthalic anhydride appears to be necessary for effective penetration of the hydrophobic environment around cytochrome *b*, since other acid anhydrides, such as 2,3-dimethylmaleic anhydride, in a 3:1 (w/w) ratio to protein were not sufficient to dissociate cytochrome *b* from its native complex using the same procedure. Although 3,4,5,6,-tetrahydrophthalic anhydride is a potent dissociating reagent, the immediate heme environment remained relatively unperturbed. Thus, the optical and MCD spectra of this purified cytochrome *b* closely resemble those difference spectra of complex III which isolate the spectral contributions of the *b* cytochromes while the EPR spectrum showed only a slight change in the low-field *g* value compared to Complex III.

A wide range of estimates for the molecular weight of yeast cytochrome *b* has been reported. Katan et al. [30] obtained a value of about 32000 both with purified cytochrome *b* and radioactivity labeled Complex III while Lin and Beattie [7] obtained 28000 for the purified protein. Tzagoloff and Nobrega [31] deduced a value of 44000 from the gene sequence, but could only isolate a cytochrome *b* with a molecular weight of 20000. We find a molecular weight of 25000–26000 for cytochrome *b* isolated by our procedure. This is the third instance in which the size of a hydrophobic polypeptide determined by SDS gel techniques is found to be substantially smaller than that anticipated from the polypetide sequence inferred from the gene sequence. Thus, polypeptides I and III of mammalian cytochrome oxidase exhibit M_r values on SDS gels of 36000 and 21000, respectively [32], while the respective genes specify polypeptides of size 57000 and 30000 [4]. The value of 57000 for subunit I is supported by results from chemical labelling of the single cysteine residue present in the polypeptide [33]. Clearly, size estimates obtained by SDS gel electrophoresis must be viewed with considerable caution although this ambiquity will not be completely resolved until a complete amino acid sequence on the posttranslational purified cytochrome *b* species is obtained.

Assuming that the results from the gene sequence are in fact correct, than the heme content of 38 nmol per mg (equivalent to a minimum molecular weight of 26000) implies that there are two heme centers per polypeptide; our preparation would thus contain about 1.5–1.6 mol of heme per mol protein. It is then plausible to equate one of these with cytochrome *b*-562 and the other with *b*-566 as we have no persuasive evidence from either absorbance or MCD measurements of the presence of more than two heme centers in our preparation of yeast Complex III.

The MCD and EPR characteristics of the isolated protein are characteristic of a low-spin hemoprotein in both redox states. The visible MCD of

the ferric protein is quite similar to authentic bisimidazole systems, suggesting that this coordination also exists in mitochondrial cytochrome *b*. The value of g_z in the EPR spectrum is dramatically larger than that found in bisimidazole protoheme and cytochrome b_5 but values approaching this have recently been demonstrated in low-spin protoheme derivactives containing imidazole analogs which are sterically hindered by virtue of methylation at C_2 [24]. It thus seems plausible that the unusual *g* values of the mitochondrial cytochromes *b* arise by virtue of structural tension at the iron. Such a structural instability provides a simple explanation for the facile reaction of the isolated protein with CO; the strained structure appears to be substantially maintained in this preparation (Fig. 6).

The precise number of cytochrome *b* species associated with mammalian Complex III is unclear. For example, Berden et al. [34] have obtained optically derived potentiometric data from beef heart mitochondria which demonstrate that all species of cytochrome *b* are heterogeneous in their response to the addition of ATP. They correlated the behavior of the 585 nm spectrophotometric feature with the low-potential cytochrome *b* species. In a different approach, De Vries et al. [26] have analyzed the lineshapes of the low-field EPR of the *b* cytochromes and concluded that the peak at *g* 3.76 was compared of separate broad and narrow resonances, each contributing 0.5 equivalents of a *b* heme which correlated with the 558 and 566 nm optical species although specific assignments could not be made. However, the response of inhibited rat liver mitochondria to succinate led Higuti et al. [35] to conclude that the optical absorption at 558 nm is unrelated to cytochrome to *b*-566 and may in fact be due to a species unrelated to the mitochrondrial respiratory chain. Insofar as we have not obtained any evidence for more than two species of cytochrome *b* in the yeast system, the simplest interpretation of these facts assumes that cytochrome *b*-566 has a highly malleable structure and the exact chemical and physical characteristics of this species depend sensitively on the history of the enzyme preparation.

Potentiometrically, the purified cytochrome *b* behaves like a single one-electron carrier as do most other purified cytochromes *b*. However, data from potentiometric titrations of yeast Complex III obtained under the same conditions exhibit the traditional biphasic character [36], implying the presence of two different redox-active cytochrome *b* species; apparently, this benchmark of multiple cytochromes *b* has been removed by the derivative-formation procedure. Notably, Von Jagow et al. [37] have purified a cytochrome *b* from beef heart which retained the two-cytochrome *b* character demonstrated by potentiometric titration, although the midpoint potentials of this purified cytochrome *b* preparation are quite different from those values obtained with intact Complex III. This multiplicity in potentiometric properties was removed by urea treatment. EPR characterization of this beef heart cytochrome *b* would provide valuable confirmation of the presence of distinct cytochrome species.

Recently, Roberts et al. [38] tested the antimycin A-binding capabilities of several cytochrome *b*-deficient yeast mutants and concluded that cytochrome *b* is the antimycin A-binding component of Complex III. However, this conclusion has yet to be substantiated using purified cytochrome *b* (Refs. 17, and 37, and this work). In our hands, stoichiometric binding of antimycin A to Complex III was readily demonstrated whereas we could obtain no evidence for antimycin A binding to the purified cytochrome under identical experimental conditions. If cytochrome *b* is indeed required for antimycin A binding, then either the binding site for antimycin A was modified by the derivitive-formation procedure or the binding of antimycin A results from the cooperative action of this protein and an additional component of Complex III. The latter possibility is supported by the observation by Berden and Slater [17] that partially purified cytochrome *b* [39] still maintained the capacity to bind antimycin A, although the stoichiometry is much higher than 2:1; this was attributed to partial denaturation. However, the absence of antimycin A binding in derivatized Complex III could be taken as evidence in support of the former alternative.

Antimycin A is known to destabilize the coenzyme Q radical signal [40] in Complex III and also competes with the same binding site for HOQNO (2-heptyl-4-hydroxyquinoline *N*-oxide), a coen-

zyme Q analog and a cytochrome bc_1 inhibitor [41]. in contrast, the Q-binding protein, QP_s, is found to stabilize semiquinone radicals in succinate:cytochrome *c* reductase [42]. A second Q-binding protein reportedly exists in Complex III, Qp_c [43], playing a role similar to QP_s. This protein may be the antimycin A-binding protein reported by DasGupta and Rieske [44].

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