Modulated Growth of *Saccharomyces cerevisiae* by Altering the Driving Force of the Reactions of Cytochrome *c*: Marcus' Theory *in Vitro* and *in Vivo*

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ABSTRACT: According to Marcus' theory, rates of electron transfer reactions depend parabolically on the free energy of reaction. Amino acid replacements in the electron transport protein cytochrome *c* produced a series of proteins which changed the free energy of reaction for cytochrome *c* in oxidative phosphorylation. This study shows that Marcus' theory of electron transfer can be applied to the reactions of redox-altered cytochromes *c* with cytochrome c_1 both *in vitro* and *in vivo*. *In vitro*, isolation of physiologically relevant partners of cytochrome *c* suggests that a change in the free energy of reaction of cytochrome *c* changes the rate of electron transfer with the cytochrome bc_1 complex as would be predicted by Marcus' theory of electron transfer. Furthermore, the reactivity pattern observed *in vitro* is paralleled in *in vivo* studies. *In vivo* the rates of growth of *Saccharomyces cerevisiae*, in which these alterations have been incorporated, also are consistent with the change in free energy of the reactions of cytochrome *c* with the cytochrome bc_1 complex. This study suggests that Marcus' theory of electron transport can predict rates not only *in vitro*, in isolated protein−protein systems, but also *in vivo*, where the relative growth rates of yeast may be predicted from the *in vitro* results.

Ongoing work is helping to define the key parameters which control the rates of biological electron transfer reactions. One central insight has been Marcus' theory which predicts the parabolic dependence of reaction rate, k_{et}, on free energy, ΔG (or, equivalently, equilibrium constant, K): $k_{et} \propto (K)^{1/2}$ (Bolton et al., 1991; Marcus & Sutin, 1985). While such predictions are well established for isolated chemical electron transfer reactions, including protein reactants, there is no direct test of their relevance for respiratory electron transfer *in vivo*, where additional complexities might be expected.

Respiratory electron transport involves a series of metalloprotein reactions in the inner mitochondrial membrane of eukaryotic cells (Hatefi, 1985). In particular, the reactions of cytochrome *c*, the penultimate protein in the chain, have drawn much attention (Beratan et al., 1992; Siddarth & Marcus, 1993; Wuttke et al., 1992). With the routine applicability of site-directed alterations in proteins, it is now possible to directly modulate the reaction free energy of some metabolic steps in electron transport in a chemically rational fashion. Such ability is unusual. Since the change in free energy, ΔG, depends only on structure, ΔG can only be altered if the structure of the reactant or product is altered. Thus, while protein mutagenesis may alter a rate, it can only alter a metabolic free energy if the protein itself is a reactant metabolite, as in the case of electron transport studied here.

We previously reported that it was possible to tune the redox potential of a protein by producing a series of single and multiple amino acid replacements in the heme environment of iso-1-cytochrome *c* from the yeast *Saccharomyces cerevisiae* (Komar-Panicucci et al., 1992). The combination of replacements at amino acid residues 38, 52, and 82, all surrounding the heme, caused large shifts in the measured redox potential of cytochrome *c*. Most dramatic was the 123 mV shift in redox potential observed with the quadruply substituted protein, R38A N52I F82S C102A. With this alteration, the cytochrome bc_1 complex to cytochrome *c* step in aerobic respiration was shifted from a thermodynamically favored process (a release of 1.27 kcal·mol^{-1} of free energy) to one which consumes 1.57 kcal·mol^{-1} of free energy. An initial surprise was that, even when the reaction between the cytochrome bc_1 complex and cytochrome *c* became thermodynamically unfavorable, obligatory aerobic respiration could still be maintained at a functional level (Komar-Panicucci et al., 1992). However, no quantitative analysis of this growth was reported. We have reported a complete thermodynamic cycle of the free energy changes of unfolding each of the substituted proteins in both the Fe^{III} and Fe^{II} oxidation states (Komar-Panicucci et al., 1994). The structural consequences of each amino acid substitution have been discussed in terms of the observed redox potential, stability with respect to chemical denaturation, and the multiplicity of roles played by individual amino acid residues (Lo et al., 1995). This structural information has been obtained for each of the substituted proteins from high-resolution structural analysis (Lo et al., 1995).

We now report the comparative *in vitro* kinetics of these altered cytochromes *c* with their physiological protein partners. It is found that the reaction of cytochrome *c* with the cytochrome bc_1 complex shows a systematic dependence on cytochrome *c* redox potential as quantitatively predicted by using Marcus' theory. We also report the *in vivo* growth effects of these altered forms of cytochrome *c* by using a series of isogenic diploid yeast strains, containing altered cytochromes *c* which were grown on lactate and ethanol media. Differential growth on these nonfermentable carbon sources indicates that a redox-altered cytochrome *c* can in

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turn alter overall growth of *S. cerevisiae*. The changes in overall cell growth track the *in vitro* rates, consistent with the reaction between cytochrome *c* and the cytochrome bc_1 complex being partially the "rate-determining" step of electron transport in oxidative phosphorylation *in vivo*. Thus, the differential growth rates observed for these mutant strains track the predictions made by Marcus' theory of electron transfer.

EXPERIMENTAL PROCEDURES

Construction of Yeast Strains Containing Altered Iso-1-Cytochrome c. Altered cytochrome *c* alleles were constructed by site-directed mutagenesis, and a single copy of the altered allele was integrated into yeast chromosome of strain B-6748 (*MAT***a** *cyc1*-Δ::*lacZ cyc7*-Δ::*CYH2*$^+$ *ura3-52 his3*-Δ*1 leu 2*−*3 leu2-112 trp1-289 can1-100 cyh2*$^-$) as previously described (Komar-Panicucci et al., 1994).

Initially, to verify that the *in vivo* levels of iso-1-cytochrome *c* were not significantly altered by the site-directed alterations, whole cell levels of the cytochromes were determined by visual examination with a Hartree low-dispersion spectroscope (Sherman & Slonimski, 1964; Sherman et al., 1974). This was used as a qualitative screening technique where the relative intensity of the cytochrome *c* band in each yeast strain was analyzed. Low-temperature (−196 °C) spectrophotometric recordings of intact cells were quantitatively analyzed by using a modified Aviv Model 14DS spectrophotometer (Schweingruber et al., 1979; Sherman et al., 1974). Yeast cells were incubated for 3 days at 30 °C on YPS (yeast peptone sucrose) medium (Sherman, 1991). The cells were then scraped from the incubation plates and blotted on paper towels to absorb excess water. The dried cells were mounted into a 1 mm path-length cell holder, frozen in liquid nitrogen, and held in an unsilvered Dewar flask which kept the cells frozen and aligned in the spectrophotometer. The Aviv Model 14DS UV−vis spectrophotometer was modified with a Cary Model 1462 scattered transmission attachment and interfaced to an AT&T 6300 WGS computer. Spectra were scanned stepwise by 0.2 nm, a bandwidth of 0.4 nm, and an averaging time of 0.4 s. The Aviv-supplied program software extrapolates the baseline into the 620−535 nm region, subtracts that from the raw data, and smoothes the difference spectra to optimize the signal to noise. Data were processed with the SigmaPlot Scientific Graphing Software version 5.0 (Jandel Scientific, San Rafael, CA).

Protein Isolation and Purification. The isolation and *in vitro* structural characterization of cytochrome *c* have been reported (Komar-Panicucci et al., 1992, 1994; Lo et al., 1995).

The phospholipids of the membrane in which the cytochrome bc_1 complex is embedded have been shown to be essential to overall activity (Yu & Yu, 1993) and specifically to the activity of the cytochrome *b* containing subunits (Tsai & Palmer, 1986). Therefore, the method of Ljungdahl et al. (1987) was chosen for isolation of the cytochrome bc_1 complex. This method involves the use of the synthetic nonionic detergent dodecyl maltoside (DM). In the presence of DM both the cytochrome bc_1 complex and cytochrome oxidase were isolated as active, monomeric protein complexes.

All preparations were done at 4 °C in a cold room, and samples were kept on ice as much as possible. To inhibit protease activity, 1 mM phenylmethanesulfonyl fluoride [PMSF (Sigma)] was added to every buffer just prior to use. Submitochondrial particles (SMPs) were prepared, with slight modifications, following the procedure outlined by Graham and Trumpower (1991), which is a modification of another procedure (Needleman & Tzagoloff, 1975). Commercial yeast, Red Star brand, was purchased from Wegman's Bakery (Rochester, NY). Approximately 1300 g of yeast cells was crumbled into very small pieces and washed in water. The final cell slurry volume was 1500 mL, which was centrifuged at 4200*g* for 10 min. Washed cells were resuspended in 100 mL of 400 mM mannitol, 50 mM Tris, pH 7.6, and 2 mM EDTA buffer (MTE buffer). Cells were disrupted using a Biospec Products bead beater with 425−600 μm acid-washed glass beads (Sigma). Cells (250 ml) were added to the beating chamber together with approximately 120 g of rinsed glass beads. Cell disruption was done in 1 min intervals of the beater being off and on. This was repeated five times for each cell sample.

The disrupted cells were diluted with an equal volume of cold MTE buffer. The pooled cell extracts were centrifuged for 10 min at 1000*g*. The supernatant was centrifuged a second time to ensure removal of intact cells. SMPs were pelleted from the supernatant at 16000*g* for 20 min. The pellets were resuspended in 150 mM NaCl to a final volume of 600 mL and centrifuged at 14500*g* for 20 min. This was repeated twice to wash the membrane pellets. Then 6 mL of this sample was removed for Lowry analysis of SMPs (Lowry et al., 1951; Markwell et al., 1978). The membranes were not resuspended outlined (Graham & Trumpower, 1991) but were used immediately.

To deplete SMPs of cytochrome *c* and other water-soluble proteins (Jacobs & Sanadi, 1960), the pellet was resuspended in 600 mL of 15 mM NaCl, incubated on ice for 10 min, and centrifuged at 16000*g* for 20 min. The pellet was resuspended in 600 mL of 150 mM NaCl, incubated on ice for 10 min, and centrifuged at 16000*g* for 20 min. That pellet was resuspended in 400 mL of 150 mM NaCl, incubated on ice for 10 min, and centrifuged at 16000*g* for 20 min. The pellet was resuspended in 100 mL of buffer I. Buffer I is 50 mM Tris with the pH adjusted to 8.0 at 4 °C, 1 mM $MgSO_4$, and 1 mM PMSF. The sample was stored overnight at 4 °C.

Purification of the cytochrome bc_1 complex (Ljungdahl et al., 1987) involved diluting the depleted SMP solution to 30 mg of protein/mL of buffer I (100 mL total final volume). The pH of the suspension was adjusted to 8. The sample was centrifuged at 100000*g* for 90 min. The yellow-brown supernatant was disposed of, and the pellet was resuspended in buffer I to a final protein concentration of 10 mg/mL. The suspended membranes were solubilized by stirring for 30 min with 0.8 g of DM/g of yeast protein. The solubilized samples were centrifuged at 100000*g* for 90 min, and the pellet was discarded. The NaCl concentration of the pooled supernatants was adjusted to 100 mM, and the mixture was stirred for 60 min to facilitate separation of cytochrome oxidase from the cytochrome bc_1 complex.

A DEAE-Bio-Gel A (Bio-Rad) column (2.2 cm × 15 cm) was preequilibrated with 50 mM Tris-HCl, pH 8.0 at 4 °C, 1 mM $MgSO_4$, 100 mM NaCl, and 0.1 $mg \cdot mL^{-1}$ DM, referred to as buffer II. The samples were run at 75 $mL \cdot h^{-1}$. The adsorbed sample was washed with three column volumes of buffer II. A gradient was run with 150 mL of buffer II in the low salt buffer chamber of the gradient mixer and

150 mL of buffer III, 50 mM Tris-HCl, pH 8.0 at 4 °C, 1 mM $MgSO_4$, 300 mM NaCl, and 0.1 mg·mL^{-1} DM, in the high salt buffer chamber. Three sample aliquots were loaded. Fractions were collected in 5 mL sample volumes. The fractions were analyzed for absorption at 280 and 415 nm. Cytochrome oxidase eluted at ~225 mM NaCl and the cytochrome bc_1 complex eluted at ~275 mM NaCl. Pooled fractions were concentrated and exchanged into buffer II using an Amicon ultrafiltration cell equipped with an Diaflo PM30 membrane filter (W. R. Grace Co., Beverly, MA). The concentrated sample was diluted with an equal volume of glycerol and stored either at −170 °C (for long-term storage) or at −20 °C (for kinetic experiments). According to the literature protocol, glycerol dilution and storage at −20 °C for 2 weeks increases the activity of the cytochrome bc_1 complex (Ljungdahl et al., 1987). Therefore, the sample was not used for kinetic experiments until this period of time lapsed.

The second chromatographic purification step presented in the literature (Ljungdahl et al., 1987) was assessed to be unnecessary for the purposes of this study because only a small increase in activity, and a large decrease in yield, is reported. Following a suggestion by Professor Bernard Trumpower (Dartmouth Medical School, personal communication), any cytochrome oxidase impurity present in the cytochrome bc_1 complex sample can be adequately inhibited with 20 μM KCN.

The concentrations of cytochrome b and cytochrome c_1 were determined by using a dithionite-reduced minus ferricyanide-oxidized absorption-difference spectrum of the pooled cytochrome bc_1 complex samples (Vanneste, 1966). The dithionite-reduced minus the oxidized (untreated) absorption-difference spectrum of the pooled cytochrome oxidase samples was used to calculate the concentration of cytochromes aa_3 in cytochrome oxidase (VanGelder, 1966).

A 10−15% gradient SDS−polyacrylamide gel was run with SDS buffer strips using a PhastSystem electrophoresis apparatus (Pharmacia LKB Biotechnology, Uppsala, Sweden) to examine protein subunit composition.

In Vitro Steady-State Kinetic Activity. To assess the functional role of cytochrome c oxidase with respect to the redox-altered cytochromes c, we studied initial steady-state kinetics. Concentrated (usually 0.5−1.0 mM) samples of iso-1-cytochrome c were reduced by adding a few grains of solid sodium dithionite ($Na_2S_2O_4$). The reduced sample was run down a small (1 mL) DE-52 column equilibrated in 100 mM potassium phosphate, pH 7.0, buffer to remove excess reductant. The most concentrated fraction of cytochrome c was collected and sealed under a stream of N_2. This was necessary because some samples would air oxidize in the time that elapsed from sample preparation to kinetic experiments.

Cytochrome c final concentrations ranged from 1 to 100 μM in each reaction mixture. Cytochrome c (0.3 mL in 100 mM potassium phosphate, pH 7.0) was added to 1.7 mL of stock reaction buffer containing 25 mM potassium phosphate, pH 7.0, with 0.1% DM. The reaction was initiated by adding 1 μL of cytochrome c oxidase (2 pmol) which was diluted from enzyme stock 1:8 with 50 mM Tris-HCl pH 8.0 at 4 °C, 1 mM $MgSO_4$, 100 mM NaCl, and 0.1 mg·mL^{-1} DM. DM was present to ensure the cytochrome oxidase sample remained solubilized in a monomeric conformation. The change in absorbance at 550 nm was monitored as a function of time using a Hewlett-Packard 8452A diode array spectrophotometer with HP 89531A MS-DOS−UV/VIS operating software (1989) in the kinetic mode.

The initial rate of oxidation, v_i, of cytochrome c was calculated from a zero-order fit of the initial change in absorbance with time. The amount of time chosen for the zero-order fit was dependent on the concentration of cytochrome c. For example, approximately 10 s were fit typically for samples less than 20 μM cytochrome c, and up to 20 s were fit for samples around 40 μM. The criterion was to minimize the relative standard deviation reported from the HP operating software. Initial rates are reported as moles of cytochrome c oxidized per mole of enzyme per second. The ΔAbs·s^{-1} values were calculated from a zero-order fit using the available software. The difference in molar absorptivity between oxidized and reduced cytochrome c at 550 nm is equal to 18.5×10^3 M^{-1}, and the concentration of enzyme used in the reaction mixture was 1.0×10^{-9} M.

The cytochrome c concentration dependence of the initial reaction rate was fit to the Michaelis−Menten equation using the nonlinear least-squares fitting program from SigmaPlot Scientific Graphing Software (Jandel Scientific, San Rafael, CA). The data were also fit to the Michaelis−Menten equation using a nonlinear least-squares regression program, Enzfitter (Elsevier BIOSOFT, Cambridge, U.K.), which reports the error involved in the data analysis.

The procedure used for the enzymatic reduction of cytochrome c by the isolated cytochrome bc_1 complex follows the basic procedure outlined by Kubota et al. (1992). Iso-1-cytochrome c was oxidized by addition of a few grains of $K_3Fe(CN)_6$ to the protein sample, and excess oxidant was then removed by eluting the protein on a small (1 mL) DE-52 column equilibrated in 100 mM potassium phosphate, pH 7.0, buffer. Cytochrome c (300−400 μM final concentration) was added to a 2 mL reaction mixture which contained 29 mM potassium phosphate, 50 mM EDTA, 20 μM KCN, and 0.1 mg/mL dodecyl maltoside (DM). The KCN was added prior to each kinetic run to inhibit cytochrome oxidase activity as discussed above.

Decylubiquinol (DBH) was prepared following a procedure for the reduction of duroquinone (Nelson & Gellerfers, 1978) and was stored in 100 μL sample aliquots at −20 °C. DBH (2 μL) was added to the reaction cuvettes (20 μM final concentration). The increase in absorbance at 550 nm was monitored for 40 s in the absence of the cytochrome bc_1 complex to evaluate the nonenzymatic reduction of ferricytochrome c.

The enzymatic reduction of an equivalent cytochrome c solution was initiated by addition of 1 μL of freshly diluted enzyme. The final enzyme concentration was 2.25×10^{-10} M. The stock enzyme solution, 26 μM, was diluted 1:50 with buffer comprising 50 mM Tris (pH 8.0 at 4 °C), 1.0 mM $MgSO_4$, 100 mM NaCl, and 0.1 mg/mL DM and kept on ice prior to use. The increase in absorbance at 550 nm was monitored as a function of time (reaction time = 6 min) using a Hewlett-Packard diode array spectrophotometer, as described above.

At the end of each enzymatic run a few grains of $Na_2S_2O_4$ were added to the reaction mixture to determine A_∞ at 550 nm. The enzymatic data were fit to first-order kinetics using this value. The rate of electron transfer, k_{12}, from DBH to the redox-altered cytochrome c, catalyzed by the cytochrome bc_1 complex, was corrected for the nonenzymatic reduction of ferricytochrome c by decylubiquinol. At least two replicate determinations were used to obtain values for k_{12}.

Variations in daily enzyme activity were observed. Therefore, the cytochrome bc_1 complex reduction of the C102A altered protein was repeated daily. This enzyme activity was used to normalized the enzyme activity with the other, multiply substituted, cytochrome *c* data. Best reproducibility was obtained under saturation conditions (>200 μM cytochrome *c* and K_m <30 μM). These saturating concentrations of cytochrome *c* should appropriately reflect physiological conditions where the mitochondrial concentration of cytochrome *c* is much higher, being estimated as ≥2 mM.

In Vivo Growth of Yeast Strains Containing Redox-Altered Iso-1-Cytochrome c. Diploid strains were constructed by crossing each of the haploid strains containing the altered forms of iso-1-cytochrome *c* to a haploid strain B-7682 (*MAT*α *cyc1-31 cyc7-67 trp2-1 his1*) that completely lacked both iso-1-cytochrome *c* (*cyc1-31*) and iso-2-cytochrome *c* (*cyc7-67*). Thus, each diploid strain contained only one *CYC1* gene encoding the desired altered proteins. All diploids were constructed in duplicate. Strains containing R38A F82S C102A and R38A N52I F82S C102A altered proteins were constructed from two independent *MAT***a** haploid strains. Diploid strains containing *CYC1*$^+$ and *cyc1-363* genes were used as controls with 100% and 0% relative specific activity.

Liquid lactate media for growth curves were prepared by combining 25 mL of solution I with 225 mL of solution II 12 h before the growth curves were started. The lactate media were sterile filtered prior to distributing 10 mL aliquots to 125 mL sterile Erlenmeyer flasks. Solution I is an autoclaved solution of 10% DL-lactic acid with the pH adjusted to 5.1 using KOH. Solution II is an autoclaved solution of 0.75% Difco-yeast nitrogen base (without amino acids) and 0.056% Difco-yeast extract.

Liquid ethanol media were prepared by combining 980 mL of solution III with 20 mL of 95% ethanol. Solution III is 12.3% Difco-yeast extract and 22.8% Difco-peptone. The media were prepared immediately prior to use and sterile filtered by gravity to avoid evaporation of the ethanol. For growth curve samples, the media was distributed in 10 mL aliquots to 125 mL sterile Erlenmeyer flasks.

YPD [yeast−peptone−dextrose (Sherman, 1964)] cultures were inoculated with diploid yeast strains and grown overnight to saturation. Cells were left dormant at 4 °C for 24 h. Each sample was diluted by adding 100 μL of saturated YPD sample into 900 μL of media. A 40 μL aliquot of that sample was counted in 20 mL of Isotone II, electrolyte solution provided for use with a Coulter counter Model ZM system (Coulter Electronics Ltd., Hialeah, FL). Typically, a 500 μL sample was counted on the Coulter counter, and an appropriate dilution was made to start the growth curves in the Erlenmeyer flasks, described above, with 4.0×10^5 cells/mL.

Cells were grown in 10 mL of the nonfermentable liquid medium and were shaken vigorously at 30 °C. Samples (100 μl) were removed from the growing cells every 1.5−3.0 h to monitor growth. The time the sample was removed was noted as the growth time. Samples were transported to the Coulter counter on ice. Samples (40 μL) were diluted into 20 mL and counted as described above. The volume of cells counted was reduced appropriately for cell samples which were very concentrated. The samples were also monitored closely for signs of flocculating yeast (clumping of cells), and data reported do not include samples which had begun to flocculate.

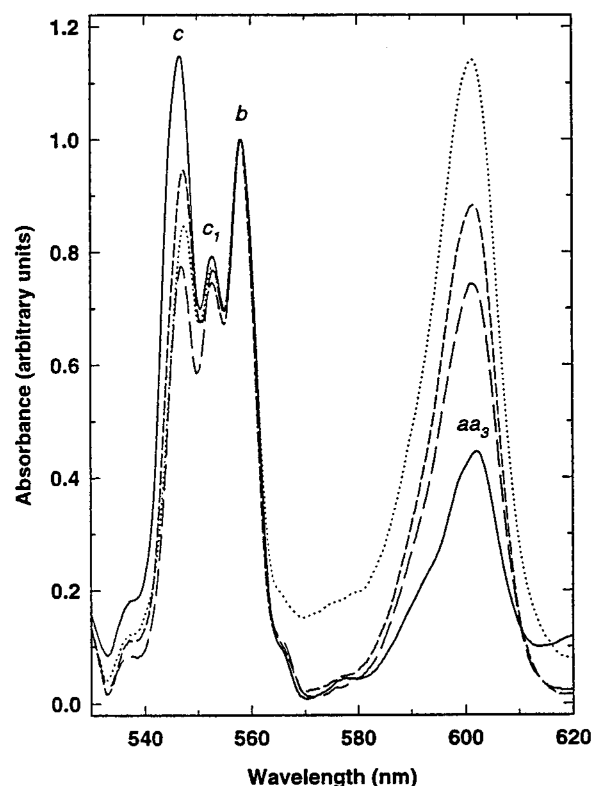


FIGURE 1: Low-temperature spectra of cytochromes in intact yeast cells with increasing ratio of cytochrome aa_3 to cytochrome *b*: solid line, C102A; long-dashed line, R38A F82S C102A; short-dashed line, N52I F82S C102A; dotted line, R38A N52I F82S C102A.

A second set of Erlenmeyer flasks was started after 12 h of growth of the first set. Thus, cells did not have to be counted overnight and the second set served as the 12−24 h samples. The second set was counted the second day while the first set was being counted as the 24−36 h samples. Growth curves were repeated three times in liquid lactate media.

RESULTS AND DISCUSSION

The resultant haploid strains grow on media having nonfermentable substrates, lactate or glycerol, as the sole carbon and energy source. This is indicative of an intact and functional assembly of electron transport proteins in the inner mitochondrial membrane (Komar-Panicucci et al., 1994). As reported elsewhere, the substitutions studied here did not significantly alter the overall structural integrity of the isolated cytochrome *c* variants (Lo et al., 1995).

Low-temperature (−196 °C) spectrophotometric recordings of intact cells confirmed that levels of cytochrome *c* were normal for most of the mutant haploid strains. An example is given by the solid line in Figure 1. The average ratio of cytochrome *c* to cytochrome *b* for all the substituted proteins is 1.1 ± 0.2 as given in Table 1. Cytochrome *c* levels are slightly lower for the R38A F82S C102A substituted protein and slightly higher for the N52I C102A substituted protein. The change in concentration of cytochrome oxidase as indicated by the cytochrome aa_3 absorbance is discussed below.

Protein Purification. The method employed for isolating the cytochrome bc_1 complex involves separation of this protein complex from cytochrome oxidase in a single chromatographic step following the procedure outlined above. As confirmed by an independent isolation and

Table 1: Ratio of Cytochromes As Determined by Low Temperature Spectrophotometric Recordings

cytochrome *c* replacements	cyt *c*/cyt *b* ratio	yt aa_3/cyt *b* ratio
normal R38 N52 F82 C102	1.3	0.48
no cytochrome *c*		
C102A	1.2	0.45
F82S C102A	0.96	0.38
R38A C102A	1.3	0.31
N52I C102A	1.4	0.40
R38A N52I C102A	1.3	0.37
R38A F82S C102A	0.77	0.74
N52I F82S C102A	0.95	0.88
R38A N52I F82S C102A	0.84	1.15

characterization of cytochrome oxidase (Taanman & Capaldi, 1992), the best separation of the cytochrome bc_1 complex from cytochrome oxidase was in the presence of 0.1 mg/mL DM solubilizing detergent. Previous methods which used Triton X-100 for the isolation of cytochrome oxidase identified only 9 subunits in yeast cytochrome oxidase whereas 12 subunits are isolated in the presence of DM. Thus, the cytochrome oxidase isolated by the procedure used in the current work, although not extensively purified nor characterized (see Experimental Procedures), should resemble the protein as found under physiological conditions.

The procedure as outlined yielded 6.4 g of total protein in the SMP preparation and 5.9 g of total protein in the depleted SMP preparation.

The reduced minus the oxidized absorption-difference spectrum of the cytochrome oxidase sample had λ_{max} at 604 nm, which is as expected from the literature. The final volume of concentrated cytochrome oxidase was 5.6 mL with a cytochrome aa_3 concentration of 8.2 μM. This sample was further concentrated and filtered through a 0.2 μm filter to a final stock volume of 2.5 mL.

Dithionite-reduced minus ferricyanide-oxidized absorption-difference spectra of the cytochrome bc_1 complex appeared, as given in the literature, with λ_{max} at 561 nm and an absorption shoulder at 555 nm. The procedure yielded 26 μM cytochrome c_1 and 59 μM cytochrome *b* in a 50% glycerol, 3.3 mL stock sample.

The SDS−polyacrylamide gel electrophoresis (SDS−PAGE) of the purified cytochrome bc_1 complex indicated that all subunits were present in this preparation and could be compared to the literature isolation (Ljungdahl et al., 1987).

In Vitro Kinetic Studies. Isolation of the redox-altered proteins provided an independent opportunity to assess *in vitro* the role of the driving force in the reaction of cytochrome *c* with its physiologically relevant partners. The cytochrome bc_1 complex donates electrons to cytochrome *c* while cytochrome oxidase, the terminal oxygen reducing enzyme, accepts electrons from cytochrome *c*. Both of these membrane-bound, multisubunit complexes were isolated from yeast. Similarly, other assays of binding and activity with physiological partners cytochrome b_2 (lactate dehydrogenase) and cytochrome *c* peroxidase have been studied. Differences, which do exist in the latter two systems, while interesting, do not correlate with functional growth in aerobic respiration. Thus, details of these effects will be discussed elsewhere.

Cytochrome oxidase has a complex mechanism, involving multiple electron transfer steps, not only with cytochrome *c* but also between the various redox cofactors within cytochrome oxidase. A simple dependence of k_{cat} on ΔE will be observed only if the initial oxidation of cytochrome *c* is rate determining. Therefore, initially overall trends in binding and/or reactivity with the cytochrome *c* mutants were sought. As shown in Table 2, there are no systematic trends in the reaction of cytochrome oxidase with the change in cytochrome *c* redox potential. This suggests, perhaps not surprisingly, that under these conditions the cytochrome *c* to cytochrome oxidase electron transfer is *not* the slow step in overall oxidase turnover. The parametric constants determined by curve fitting with the SigmaPlot program were within the reported error of the Enzfitter data given in Table 2. Data marked with a superscript *a* have been normalized to account for variations in enzymatic activity over different weeks of the experiments.

Table 2: Kinetic Parameters from Numerical Analysis of Cytochrome Oxidase Reactions with Redox-Altered Cytochromes *c*

iso-1-cytochrome *c*	$E°' \pm 2$ mV vs SHE	k_{cat} (mol of cyt *c*/mol of cyt aa_3) (s)	K_m (M)
C102A	285	1230 ± 82	15 ± 2
F82S C102A	247	740 ± 41	10 ± 1
R38A C102A	239	918 ± 145[a]	8 ± 2
N52I C102A	231	1053 ± 61	10 ± 2
R38A N52I C102A	212	949 ± 55[a]	9 ± 2
R38A F82S C102A	203	1117 ± 34	24 ± 2
N52I F82S C102A	189	605 ± 10	10 ± 1
R38A N52I F82S C102A	162	833 ± 31	14 ± 1

[a] Data are corrected for variation in enzyme activity as described in the text.

Table 3: Kinetic Parameters from Numerical Analysis of the Cytochrome bc_1 Complex Reactions with Redox-Altered Cytochromes *c*

iso-1-cytochrome *c*	$k_{12}^{measured}$ (mol of cyt *c*/mol of cyt c_1)	$k_{12}^{normalized}$ (mol of cyt *c*/mol of cyt c_1)	K_{12}[a]
C102A	84 ± 9	84	8.54
F82S C102A	53 ± 1	44	1.93
R38A C102A	20 ± 3	30	1.43
N52I C102A	59 ± 6	34	1.03
R38A N52I C102A	19 ± 2	23	0.49
R38A F82S C102A	18 ± 2	16	0.35
N52I F82S C102A	7 ± 5	11	0.20
R38A N52I F82S C102A	5 ± 5	6.6	0.07

[a] The change in free energy of reaction, ΔG, for cytochrome *c* and the cytochrome bc_1 complex is $\Delta G = nF\delta E°'$, where, for example, $\Delta E°' = 60$ mV for the difference between C102A cytochrome *c* (285 mV) and the cytochrome bc_1 complex (225 mV) (Hatefi, 1985). The equilibrium constants, K_{12}, were calculated from $K_{12} = \exp(-\Delta G/RT)$ for each cytochrome *c*−cytochrome bc_1 complex reaction pair.

By contrast, the reaction between the cytochrome bc_1 complex and cytochrome *c* directly tracks the change in cytochrome *c* redox potential, in a manner quantitatively predicted by Marcus' theory. The enzymatic reduction of cytochrome *c* by the cytochrome bc_1 complex is summarized in Table 3. Data are corrected for the nonenzymatic reduction of ferricytochrome *c* by decylubiquinol as described in Experimental Procedures. Data are also normalized for variations in the enzymatic activity of the cytochrome bc_1 complex between experiments.

As shown in Table 3 and illustrated in Figure 2 reactivity *is* correlated with driving force for the reaction of the cytochrome bc_1 complex and cytochrome *c*. The solid line drawn in Figure 2 is a predicted line derived from the Marcus correlation equation. For a reaction in which the cytochrome *c* heme to cytochrome bc_1 heme electron transfer is rate determining, the Marcus cross relation (eq 1) predicts that

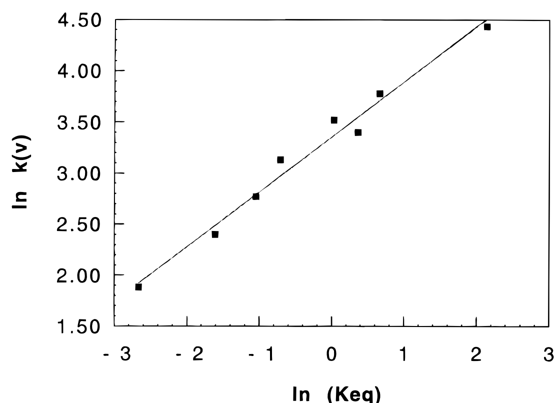


FIGURE 2: *In vitro* electron transfer rates, k_{12}, of redox-altered cytochromes *c* catalyzed by the cytochrome bc_1 complex ln (k_v) vs reaction free energy ln K_{eq} (equilibrium constant = K_{eq}).

the observed rate, k_v, is proportional to $K_{eq}^{1/2}$.

$$k_{12} = (k_{11}k_{22}K_{eq}f_{12})^{1/2} \quad (1)$$

Here K_{eq} is the equilibrium constant for the reaction between cytochrome *c* and the cytochrome bc_1 complex. Parameter f_{12} depends on K_{eq} and the collision frequency, and f_{12} is derived from the cross term in the Marcus quadratic equation. When $K_{eq} \approx 1, f_{12} \approx 1$. The parameters k_{11} and k_{22} are the self-exchange rate constants for cytochrome *c* and the cytochrome bc_1 complex, respectively. While the precise value of k_{22} is not known, it will be constant across all experiments. We assume that k_{11} is constant for the various mutants studied here. This assumption is supported by both electrochemical and NMR self-exchange studies. Thus a plot of ln k_v vs ln K_{eq} should be linear, with a slope of 0.5 as shown in Figure 2.

In earlier work, mechanisms for the cytochrome c_1 to cytochrome *c* reaction were proposed which involved specific conformational changes (Kim & King, 1989). For a reaction in which a conformational change is rate determining, no dependence on ΔG is expected or observed (Hoffman & Ratner, 1987). The microscopic parameters K_m and k_{cat} measured in this work are composites of individual rate processes and are not themselves elementary rate constants. In some cases, however, k_{cat} can be equated with a single rate process and associated rate constant. For the present data, the clear dependence of k_{cat} on ΔG suggests that the overall steady state k_{cat}, as measured, corresponds to a simple electron transfer reaction between cytochrome c_1 and cytochrome *c* in which the rate is governed by a single, structurally determined, reorganization energy. This assumes that the cytochrome *c* reorganization is constant for all the mutants. As already noted, this indeed appears to be the case based on both small molecules cross reactivities and electron self-exchange as maintained by NMR and by cyclic voltammetry.

There has been a previous report (Wallace & Proudfoot, 1987) of a linear relationship between the logarithm of electron transfer rates from cytochrome *c* reductase and the redox potential of semisynthetic analogs of cytochrome *c* in depleted mitochondria. In the current work, the structures of the redox-altered cytochromes *c* have been carefully characterized, and the reactivities of the proteins have been well defined in an isolated *in vitro* system. The rate of electron transfer from the cytochrome bc_1 complex is directly related to the redox potential of the altered cytochromes *c*.

Table 4: Relevant *MATa* Genotypes and Yeast Strain B-Numbers from Constructing Diploids Used for Growth Curves *in Vivo*[a]

cytochrome *c*	relevant *MATa* genotype	haploid strain	diploid strain
normal R38 N52 F82 C102	*CYC1*$^+$	B-7553	B-8479
no cytochrome *c*	*cyc1-363*	B-6442	B-8482
C102A	*CYC1-820*	B-7706	B-8483
F82S C102A	*CYC1*-1042	B-8181	B-8487
R38A C102A	*CYC1-1016*	B-8134	B-8485
N52I C102A	*CYC1-899*	B-7907	B-8484
R38A N52I C102A	*CYC1-1017*	B-8135	B-8486
R38A F82S C102A	*CYC1-1058*	B-8219	B-8489
	(CYC1-1059)	(B-8431)	(B-8491)
N52I F82S C102A	*CYC1-1045*	B-8198	B-8488
R38A N52I F82S C102A	*CYC1-1057*	B-8217	B-8490
	(CYC1-1060)	(B-8433)	(B-8492)

[a] In parentheses are listed genes that were constructed in duplicate to assess reproducibility and to verify that molecular manipulations had not introduced an external, deleterious mutation which affected growth.

Furthermore, the reactivity of cytochrome *c* with the cytochrome bc_1 complex can apparently be quantitatively rationalized *in vitro* using Marcus' fundamental theory of electron transfer. Therefore, the current work substantiates that electron transfer from the cytochrome bc_1 complex to cytochrome *c* is under thermodynamic control. The *in vitro* studies reported here suggest an approach to understanding the *in vivo* growth rates of yeast containing redox-altered cytochromes *c* as discussed below.

In Vivo Growth Results and Discussion. The *in vitro* kinetic studies described above suggest an approach to understanding the growth rates of yeast containing altered cytochrome *c*. As the overall reaction free energy decreases, the rate of the cytochrome bc_1 complex to cytochrome *c* reaction correspondingly decreases as shown in Figure 2. At some point, the rate of electron transfer *in vivo* between the cytochrome bc_1 complex to cytochrome *c* may become sufficiently slow as to become (at least partially) the rate-determining step for overall electron transport and, thus, overall growth. If so, the rate of growth of yeast containing altered cytochrome *c* genes can be predicted.

For small changes in redox potential, the rate of reaction between the cytochrome bc_1 complex and cytochrome *c* may remain relatively fast, and no change in growth is predicted. When the reaction between the cytochrome bc_1 complex and cytochrome *c* becomes at least partially rate determining for overall electron transport, then the rate of cell growth can begin to track the rate of reaction between the cytochrome bc_1 complex and cytochrome *c* as measured *in vitro*.

In order to compare the approximate relative specific activities of altered iso-1-cytochromes *c*, an isogenic series of diploid strains was constructed as outlined in Table 4. All the altered *CYC1* genes were constructed by using *in vitro* site-directed mutagenesis into the same parent haploid yeast strain B-6748. Yeast strain B-7553 (from Professor Fred Sherman's laboratory stock, Department of Biochemistry, University of Rochester) was also constructed from the same parent strain. This strain contains an unaltered *CYC1*$^+$ gene and was used as a 100% control. Therefore, with the exception of the 0% control, from B-6442 (*MAT***a** *cyc1-363 cyc7-*Δ::*CYH2*$^+$ *ura3-52 his3-*Δ*7 leu2-3 leu2-112 trp1-289 can1-100 cyh2*$^-$) which is only slightly different, all the halploid strains with different iso-1-cytochromes *c* are isogenic. Diploid constructs eliminated any recessive lesions in the parent strain that may affect the growth rate.

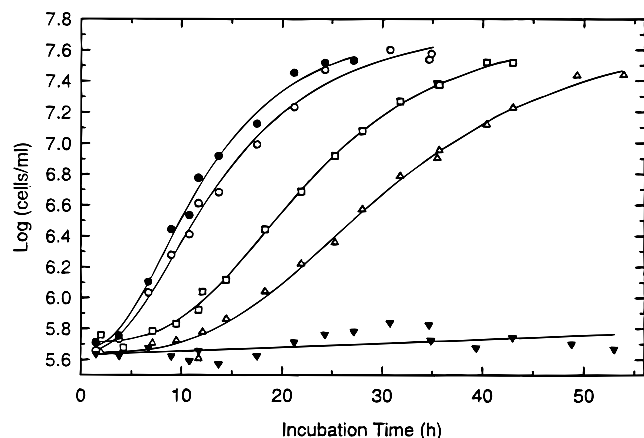


FIGURE 3: Differential growth of isogenic diploid yeast strains containing redox-altered cytochromes *c* in lactate medium: ● = wt; ○ = N52I; □ = N52I F82S; △ = N52I R38A F82S; ▼ = *CYC1⁻* (control).

Table 5: *In Vivo* Rate of Growth of Diploid Yeast Cells in Lactate Media[a]

iso-1-cytochrome *c*	K_{12}	$t_d + 0.5$ (h)	$k_{vivo} \pm 0.01$ (h^{-1})
C102A	8.54	2.5	0.28
F82S C102A	1.93	2.5	0.28
R38A C102A	1.43	2.5	0.28
N52I C102A	1.03	2.5	0.28
R38A N52I C102A	0.49	3.6	0.19
R38A F82S C102A	0.35	6.0	0.11
N52I F82S C102A	0.20	9.7	0.07
R38A N52I F82S C102A	0.07	14.7	0.05

[a] Doubling time, t_d, data were obtained from the average of three growth experiments in lactate medium. $k_{vivo} = 0.693/t_d$ and K_{12} is calculated as described in Table 3.

Constructing diploid strains also avoided complications from interfering recessive mutations that may have arisen during genetic manipulations.

Diploid yeast strains were grown in nonfermentable liquid medium, and cell growth was monitored as a function of time. Under these conditions of obligatory respiration, the *in vivo* function of redox-altered iso-1-cytochromes *c* can be compared to the normal function of unaltered iso-1-cytochrome *c*. Thus, this study is an investigation of the *in vivo* relationship between the driving force in the reactions of cytochrome *c* and the related effects of oxidative phosphorylation on cellular function.

Figure 3 illustrates the observed differential growth of isogenic diploid yeast strains containing redox-altered cytochromes *c* in lactate medium. Filled circles were derived from growth of the *CYC1⁺/cyc1-31* diploid, the 100% control, containing one copy of an unaltered iso-1-cytochrome *c*. The standard sigmoidal fit of these data is similar for all the following strains studied which contained altered cytochromes *c*: C102A, F82S C102A, R38A C102A, and N52I C102A. Differential growth is shown for diploid strains containing R38A N52I C102A (open circles), N52I F82S C102A (open squares), and R38A N52I F82S C102A (open triangles) substituted iso-1-cytochrome *c*. Filled triangles denote "no growth" of the *cyc1-363/cyc1-31* diploid, the 0% control. The fact that cytochrome *c* is rigorously required for growth rules out a mechanism(s) involving alternate pathways which bypass cytochrome *c*. It is known that ethanol medium is less sensitive to small differences in growth than is lactate medium. Nonetheless, the same basic differential growth trends were recapitulated on ethanol (data not shown for R38A N52I F82S < N52I F82S < N52I ≈ unsubstituted cytochrome *c*).

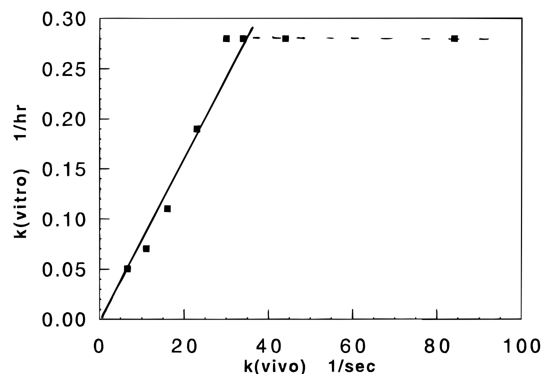
The overall growth rates are consistent with the predictions discussed above. Initial small shifts in redox potential (<50 mV) produce no changes in observed growth rates. As the cytochrome *c* potential further decreases, however, the observed growth rates change substantially, as qualitatively indicated by the time required to reach saturation.

Several points should be noted. First, it can be seen that the growth rates diminished in diploid strains having cytochromes *c* with redox potentials altered by greater than 70 mV. In the case of the R38A N52I C102A substituted cytochrome *c* this corresponds to a process which is endothermic by approximately 0.42 kcal·mol^{-1}. Second, the growth yield (the total availability of useful energy for growth) has not changed. Thus, the saturation level is the same for all strains [except the *CYC1-1058* diploid which contains a relatively unstable R38A F82S C102A substituted cytochrome *c* (Komar-Panicucci et al., 1994; Lo et al., 1995)]. The most obvious differences in growth among the various yeast strains are observed in the first doubling time, t_d, as listed in Table 5.

In principle, one might as well or better measure doubling time within the exponential growth phase. However, unless chemostat conditions are used, this is a more difficult and less reproducible measurement than is doubling within the log phase. By using t_d, or an equivalent rate constant k_{vivo} (where $k_{vivo} = 0.693/t_d$), as a sensitive parametric measure of growth, it is clear that this parameter indeed tracks the *in vitro* electron transfer rates in the manner predicted above. For $\Delta E° < 50$ mV, t_d is unaffected by $E°$. As the cytochrome *c* reduction potential decreases (and the rate constant for the cytochrome bc_1 to cytochrome *c* reaction correspondingly decreases), the observed doubling time systematically tracks the *in vitro* measured rate constant for the reaction between the cytochrome bc_1 complex and cytochrome *c* (Figure 4).

Obviously, the net growth of a eukaryotic cell is quite complex. Nonetheless, the systematic relationship observed between cell doubling times and the underlying *in vitro* rates (which obey fundamental Marcus' theory) is striking, as illustrated in Figure 4. The solid line in Figure 4 shows a precise correlation between the measured *in vitro* rate of the $bc_1 \rightarrow$ (variant) reaction and the doubling of a cell containing that *cc* variant. As discussed above, when k_{et} exceeds ca. 30 s^{-1} in the *in vitro* cytochrome *c*—cytochrome bc_1 kinetic system, the dependence of growth rate on this reaction rate ceases to hold, as another process becomes limiting for growth. Such data suggest that a molecular understanding at the level of fundamental (Marcus) theory may ultimately be possible for at least some *in vivo* electron transport reactions.

A final interesting observation *in vivo* is that the levels of cytochrome aa_3 increase for the cytochromes *c* with the greatest change in reduction potential. Spectra for strains *CYC1-1058*, *CYC1-1045*, and *CYC1-1057* all have increased levels of cytochrome aa_3 as illustrated in Figure 1. These are also the strains for which we have noted a change in rate of growth. Such an increase helps assure that any reduced cytochrome *c* produced in the reaction with the cytochrome bc_1 complex is followed by a rapid, effectively irreversible reaction with cytochrome oxidase. As already noted, this rate depends only on overall cytochrome oxidase



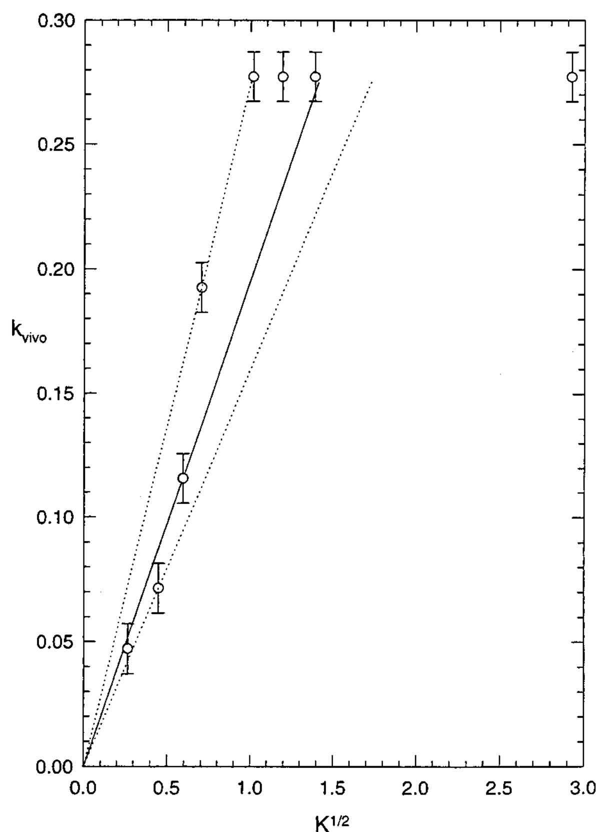


FIGURE 4: (A, top) *In vivo* reactivity of diploid yeast strains containing redox-altered cytochrome *c* measured as the rate constant for growth on lactate media, $k_{vivo} = (1/t$ doubling), vs the *in vitro* rate constant for $C_1 \rightarrow c$ reduction, k_{vitro}. (B, bottom) Equilibrium constants for the $C_1 \rightarrow c$ reduction vs k_{vivo} in the form of the relative Marcus expression ($K^{1/2}$ vs k_{vivo}).

concentration and not on cytochrome *c* redox potential. Thus, a maximal electron flux from cytochrome *c* and cytochrome oxidase is maintained in the redox-altered derivatives and the thermodynamically favorable back-transfer from cytochrome *c* to the cytochrome bc_1 complex avoided. While the value of this adaptation is clear, the detailed mechanism for linkage between the cytochrome *c* redox potential and aa_3 levels remains unclear.

CONCLUSIONS

Three primary conclusions are drawn from these studies: (1) Altered proteins can be used to modulate, in a functionally significant manner, the free energy of a key metabolic step in respiration. This ability to change ΔG for a metabolic reaction is unusual, since ΔG only depends on structures of reactants and products. In this study, ΔG could be altered because the protein *is* the product of an electron transfer reaction and not simply a catalyst. (2) The results of this modulation can apparently be quantitatively rationalized for the *in vitro* reaction of the cytochrome bc_1 complex with cytochrome *c* by using Marcus' fundamental theory of electron transfer. (3) Furthermore, these molecular kinetic parameters measured for altered cytochrome *c in vitro* predict the relative growth rates *in vivo* of yeast strains containing these altered cytochromes.

SUPPORTING INFORMATION AVAILABLE

One figure showing reduction of C102A cytochrome *c* by bc_1 (a) monitored at 550 nm and (b) fit to first-order kinetics (1 page). Ordering information is given on any current masthead page.

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