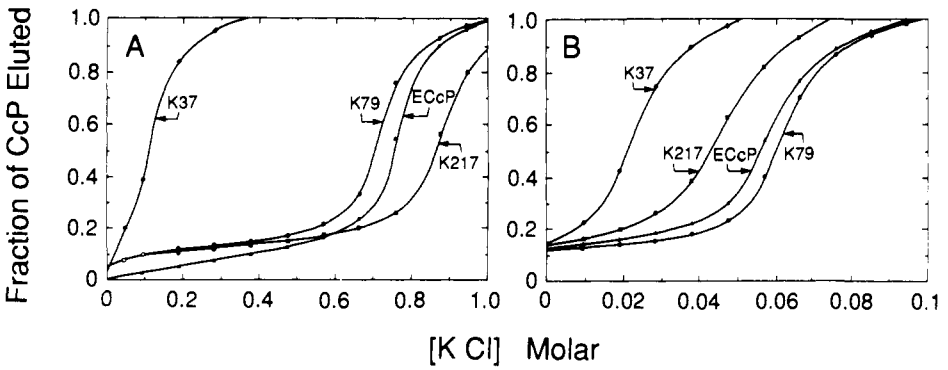

Figure 1. Panels A and B show binding curves to the cyt *c* affinity column as a function of potassium chloride concentration. Wild-type Ccp, obtained from yeast, is similar to the native recombinant Ccp (ECcp). The reduced column material was produced as previously described,[19a,b] the oxidized material was prepared according to the method of Azzi et al.,[20] and the oxidation state of the column material was determined spectroscopically.[19b] Several control experiments indicate that the derivatized cyt *c* binds Ccp and has binding and redox behavior identical to that of the cys 107 free cyt *c*.[19b,21] Elutions were conducted using the methods described in ref 19b. We obtained data in panel A using reduced cyt *c* column material and 25 mM sodium acetate pH 6.0 buffer. We obtained the data in panel B using oxidized cyt *c* column material and 5 mM sodium acetate pH 6.0 buffer. Note the difference in the horizontal axis [KCl] scale for the oxidized and reduced affinity materials. Experiments at pH 7 using TrisHCl equilibration buffer with either a TrisHCl or a KCl gradient for elution gave similar results.

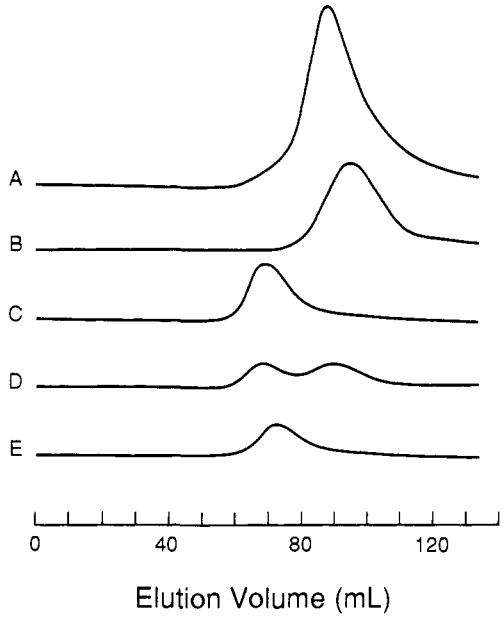

Figure 2. Gel permeation chromatograms of cyt *c*:Ccp elution profiles [using a Pharmacia FPLC apparatus with a 2.5 × 32.5 cm precision bore column, containing Sephadex G50 material, sodium acetate pH 6.0, 0.2 M ionic strength (KCl)]: (A) Reduced cyt *c* only, 78 mL; (B) oxidized cyt *c* only, 94 mL; (C) Ccp only, 69 mL; (D) Ccp plus oxidized cyt *c*, 70 and 87 mL, respectively; (E) Ccp plus reduced cyt *c*, 69.5 mL. The elution volumes listed correspond to the peak. Elution profiles were monitored at 405 nm. Note that, in chromatogram D, the peaks elute independently, showing *no* binding interaction, while in chromatogram E, only a single peak eluted, which contains both Ccp and cyt *c*, bound in a complex (as confirmed spectroscopically).

independently of Ccp (Figure 2).

The second observation is more surprising. The pattern of Ccp mutational effects at positions 79 and 217 observed in binding Fe(III) cyt *c* is *reversed* for binding to Fe(II) cyt *c*. For binding to Fe(III) cyt *c*, the observed pattern is D217K < Ccp < D79K, while for Fe(II) cyt *c*, the observed pattern is D79K < Ccp < D217K. At physiological ionic strengths, this reversal corresponds to a large change in relative affinity.

The simplest explanation for such a large change is that Fe(II) cyt *c* binds to a location centered about D37 and D79 on Ccp, which is different from the binding site for Fe(III) cyt *c*, which is centered around D37 and D217.[10] This difference might be viewed as analogous to the known differences in the binding of small anions to Fe(III) cyt *c* vs Fe(II) cyt *c*.[16] Indeed, the chromatographic technique works, in part, by the competition of Ccp and smaller anions for cyt *c*.[23] The present results show redox-dependent differences in binding, even with a large protein like Ccp, and roughly localizes the binding site for each oxidation state of cyt *c*. If the reactant, Fe(II) cyt *c*, and product, Fe(III) cyt *c*, binding sites indeed differ, as the present results suggest, then during oxidation or reduction, cyt *c* must move along the Ccp surface from the (reactant) Fe(II) cyt *c* site to the (product) Fe(III) cyt *c* site.

In summary, affinity chromatography has been used to study the binding to cyt *c* of single-site mutants of Ccp. The order of binding constants for the Ccp variants is different for Fe(II) cyt *c* and Fe(III) cyt *c*. This suggests that Ccp can sense the oxidation state of cyt *c*, so that the different redox states of cyt *c* may bind to different subdomains of the Ccp binding domain.

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Registry No. Ccp, 9029-53-2; cyt *c*, 9007-43-6; aspartic acid, 56-84-8.

Tuning the Redox Potential of Cytochrome *c* through Synergistic Site Replacements

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The free energy release along the electron transport chain of proteins in the inner mitochondrial membrane is sufficient to generate a proton-motive force that is used to drive the synthesis of the biological energy source, ATP. The penultimate reductant in this chain, cytochrome *c*, is positioned at an appropriate energy level between cytochrome reductase and cytochrome oxidase such that the electron transfer for each successive reaction is exergonic. Accordingly, eukaryotic cytochromes *c* have a high and essentially invariant redox potential (±20 mV). This invariance *presumably* reflects a strong coupling between the driving force for the reaction

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Table I. Reduction Potentials,[a] Thermodynamic Parameters,[b] and Midpoint of Gdn·HCl Unfolding[c] of Iso-1-cytochrome *c* Mutants

protein[d]	$E^{\circ\prime}$ (±2 mV vs SHE)	$\Delta E^{\circ\prime}$ (mV)	$\Delta G^{\circ\prime}$ (25 °C) (kcal·mol^{-1})	$\Delta H^{\circ\prime}$ (25 °C) (kcal·mol^{-1})	$\Delta S^{\circ\prime}$ (25 °C) (eu)	C_m [Gdn·HCl] (±0.1 M)
normal (R38 N52 F82)	285		−6.59	−11	−14	1.3
F82S	247	−38	−5.70	−11	−19	0.9
R38A	239	−46	−5.52	−8.9	−11	1.3
N52I	231	−54	−5.33	−5.4	0	2.1
R38A N52I	212	−73	−4.90	−4.9	0	2.1
R38A F82S	203	−82	−4.68	−4.7	0	0.7
N52I F82S	189	−96	−4.36	−4.4	0	1.8
R38A N52I F82S	162	−123	−3.74	−7.6	−13	1.7

[a] Determination of reduction potentials was carried out at 25 °C in 0.1 M potassium phosphate pH 7.0 relative to SCE reference.[12] Cyclic voltammograms were obtained with a Cypress Model 1087 or BAS Model 37 instrument. [b] Thermodynamic parameters were determined by temperature-dependent variation in the midpoint potentials of cyclic voltammograms between 3 and 38 °C. [c] The guanidine concentration necessary to half-denature the protein in guanidine hydrochloride induced equilibrium unfolding transitions. Equilibrium unfolding was monitored with fluorescence measurements using a Perkin-Elmer MPF-2A fluorimeter.[5b] [d] *Note*: All proteins have C102A; other substitutions are indicated by the single-letter convention as given.

and the biologically optimal rate of that reaction. However, it is not known to what extent the free energy for a metabolic step, such as cytochrome *c* dependent electron transport, can be altered while retaining biological function.

The present paper examines a key physical parameter of cytochrome *c*, the redox potential. Studies are directed at both physicochemical and physiological questions: (1) Do multiple substitutions interact synergistically, or are effects specifically additive? (2) Given the evolutionary invariance of the redox potential of cytochrome *c*, can in vivo function be retained if the redox potential changes markedly?

Several investigators have been interested in modulating cytochrome *c* redox potential. These studies show that axial ligation,[1] surface charged residues,[2,3] internal residues,[4] and internal water molecules[5] are all determinants of cytochrome *c* redox potentials. The observed changes in redox potential from these investigations were all relatively small (<50 mV) except in the case of axial ligand replacements. Unfortunately, the axial mutations produced by the semisynthetic method of Gray and co-workers[1a,b] are substitutions which cannot readily be expressed in an in vivo system.[6]

Other amino acid replacements in the heme environment have been reported to shift the reduction potential of native cytochrome *c*. Cutler et al. have reported[7] that an Arg*38*Ala replacement results in a 50 mV shift in redox potential of cytochrome *c*; our group has reported[5a] a similar shift for an Asn*52*Ile replacement, and Rafferty et al. have reported[8] a 35 mV shift for a Phe*82*Ser substitution. Our first objective was to combine such single-site replacements to address the question of synergistic effects on the redox potential of cytochrome *c*.[9] Oligonucleotide directed mutagenesis was used to produce the desired amino acid replacements in iso-1-cytochrome *c* from the yeast *Saccharomyces cerevisiae*.[10] All of the iso-1-cytochromes *c* contained the Cys*102*Ala replacement to prevent dimerization in vitro.[11] The resultant proteins were isolated and shown to have a wide range of decreased measured formal redox potentials, listed in Table I.

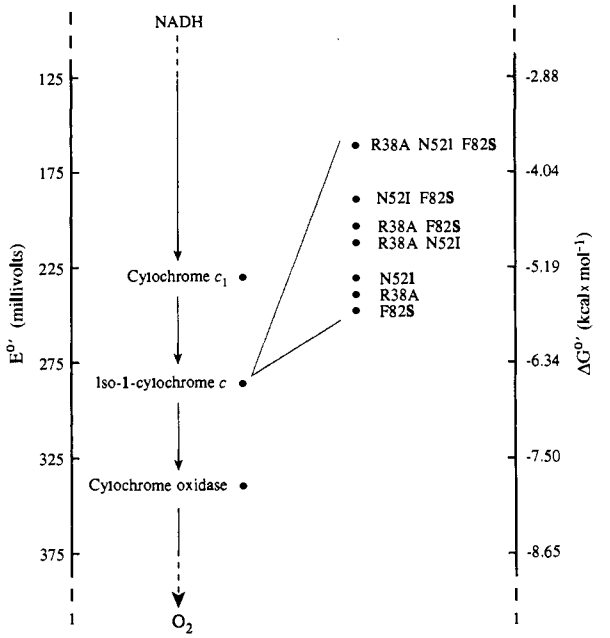

Figure 1. Electron transport energetics of iso-1-cytochromes *c* in oxidative phosphorylation. Reduction potentials for cytochrome c_1 and cytochrome *a* metal centers of cytochrome reductase and cytochrome oxidase, respectively, are estimates from refs 15a,b.

Analysis of the isolated proteins indicated that the heme environment was not spectroscopically altered by the substitutions. Soret values were all 410 ± 2 nm, and the conformationally sensitive charge-transfer band at 695 ± 5 nm was present in all proteins. Direct electrochemistry of the mutant cytochromes *c* was performed at 4,4′-dithiodipyridine modified gold disc electrodes.[12] A reversible electrochemical response was observed for

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(9) One previous example has suggested such synergistic effects.[4]

(10) Iso-1-cytochrome *c* is encoded by the nuclear gene CYC1. Altered alleles were constructed by site-directed mutagenesis as described in ref 5a. A single copy of the altered allele was integrated into the yeast chromosome of strain B-6748 (*MATa cyc1-Δ::lacZ cyc7Δ::CYH2$^+$ ura3-52 his3-Δ1 leu2-3, 112 trp1-289 can1-100 cyh2*), making all mutant strains isogenic.

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all mutants as indicated by linear plots of peak current vs (scan rate)$^{1/2}$. The peak-to-peak separations and equivalent anodic and cathodic peak currents also supported reversible behavior without kinetically coupled conformational effects on $E°'$. Thermodynamic parameters listed in Table I were determined by temperature-dependent variation in the midpoint potentials of cyclic voltammograms. The small observed changes in entropy suggested that the redox potential shifts were not a consequence of protein unfolding.

To further evaluate protein structure stability we compared the guanidine hydrochloride induced equilibrium unfolding transitions of the mutants.[5b] Changes in the midpoints (C_m) and the cooperativity of the guanidine hydrochloride induced transitions were observed. The C_m values given in Table I indicate that the mutant proteins are at least as stable as, if not more stable than, the native protein. Thus, multiple substitutions in the heme environment of iso-1-cytochrome *c* apparently retain stable, overall native-like structure.

Replacements at heme residues 38, 52, and 82 combine to cause large shifts in the measured redox potential of cytochrome *c*, illustrated in Figure 1. Wells has suggested that the free energy change that results when multiple replacements are made is often equal to the sum of the independent free energy changes: $\Delta\Delta G_{X,Y} = \Delta\Delta G_X + \Delta\Delta G_Y + \Delta G_I$.[13] Exceptions to simple additivity are reflected in the ΔG_I term, which reflects electrostatic or structural interactions between the independent sites. The results shown in Figure 1 and Table I clearly demonstrate that multiple replacements can result in synergistic shifts in potential. Furthermore, ΔG_I does not equal 0, but may increase or decrease $\Delta\Delta G_{X,Y}$. Since charge–charge and charge–dipole effects operate over relatively long distances, the observed nonadditive effects, reported in this communication, may be attributable to these phenomena in the heme environment.

The second question which we are addressing in the current study is the following: Given such changes, do the mutant proteins retain function in vivo? All mutant yeast strains contained an integrated *CYC1* gene in an isogenic background.[10] The resultant yeast strains appeared to have normal amounts of iso-1-cytochrome *c* as determined by low-temperature (−196 °C) spectroscopic examination of intact cells.[14]

All yeast strains grew on a nonfermentable carbon source. This observed obligatory aerobic respiration in vivo established that the electron transport assembly of proteins in the inner mitochondrial membrane was intact and functional. However, we have not yet thoroughly analyzed the growth rates, which may reveal quantitative functional differences. Functional behavior was not necessarily expected for the mutants. For example, the 123-mV shift in redox potential observed with the triple Arg*38*Ala, Asn*52*Ile, Phe*82*Ser mutant corresponds to a thermodynamically uphill shift of 2.8 kcal·mol^{-1} in the free energy position of cytochrome *c* (Figure 1). Equivalently, the shift in redox potential corresponds to a 100-fold shift in the equilibrium constant for the cytochrome reductase–cytochrome *c* couple.

In this light, detailed examination of rates in vitro and further investigation of in vivo function may reveal the significance of the thermodynamic driving force of electron transfer in oxidative phosphorylation.

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A Total Synthesis of (±)-Bilobalide

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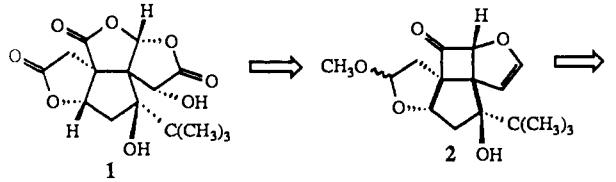
Ginkgo biloba is an ancient plant species whose extracts have been used as medicinal agents for approximately 5000 years.[1] The bitter principles responsible for the healing powers of the ginkgo extracts were first isolated by Furakawa.[2] The structures of three of the key components were later independently determined by two groups.[3] These compounds were C20 hexacyclic trilactones, which were given the names ginkgolides A, B, and C. Subsequently, a C15 tetracyclic trilactone, bilobalide, was isolated from the ginkgo extracts by Nakanishi and shown to have the structure **1**.[4] The unusual structural features of bilobalide include the presence of a *tert*-butyl group, known only to the ginkgolide class of terpenoids, and three contiguous five-membered-ring lactones. The only synthesis of bilobalide to date was reported by E. J. Corey in 1987[5] with a subsequent report by the Corey group on the enantioselective synthesis of bilobalide.[6] Herein we report a total synthesis of bilobalide employing an intramolecular [2 + 2] photocycloaddition as the key step.[7]



The approach outlined here relies on a regioselective Baeyer–Villiger oxidation of cyclobutanone **2** and an intramolecular [2 + 2] photochemical cycloaddition of α-acyloxy cyclopentenone **3**, which was stereoselectively prepared by the addition of the lithium enolate **5** to hydroxy aldehyde **4**.

Aldehyde **4** was prepared in four steps from commercially available 3-furaldehyde as illustrated in Scheme I. Addition of 3-furaldehyde to the reagent[8] prepared by the addition of *tert*-butyllithium to dry cerium trichloride in THF at −78 °C produced the secondary alcohol, which was oxidized under Swern[9] conditions to produce the *tert*-butyl furyl ketone. This ketone was then condensed with lithioacetonitrile to generate the β-hydroxy nitrile **6** in 82% overall yield.[10,11] Reduction of the nitrile with diiso-

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