Application of Exciton Coupling Theory to the Structure of Mitochondrial Cytochrome b[†]

Graham Palmer*,[‡] and Mauro Degli Esposti[§]

Department of Biochemistry & Cell Biology, Rice University, P.O. Box 1892, Houston, Texas 77251-1892, and Department of Biology, University of Bologna, Via Irnerio 42, 40126 Bologna, Italy

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ABSTRACT: The circular dichroism of the reduced bis-heme mitochondrial cytochrome b in the Soret region has been analyzed using exciton theory. The theory was applied to a geometric model that reflects the current consensus of the structure of this membrane protein [Degli Esposti, M., De Vries, S., Crimi, M., Ghelli, A., Patarnello, T., & Meyer, A. (1993) *Biochim. Biophys. Acta 1143*, 243–271]. The analysis suggests that the iron–iron distance is about 19.5 Å, with a possible range of 17–23 Å; the two hemes appear to be canted at 45° with respect to one another. It appears that the two hemes undergo a relative angular displacement upon reduction, suggesting some flexibility in the protein structure. Some applications of this analysis to other bis-heme proteins are considered.

Certain of the redox enzymes present in the energy-conserving membranes of living organisms possess two hemes which serve to facilitate electron-transfer reactions coupled to the formation of a membrane potential. These enzymes include those that use (i) cytochrome c as electron donor and dioxygen as acceptor (the cytochrome c oxidase), (ii) a quinol as electron donor and dioxygen as electron acceptor (the quinol oxidases), and (iii) a quinol as electron donor and either a cytochrome (the bc_1 complex of mitochondria) or a copper protein (the b_6f complex of green plant photosynthesis) as electron acceptor. In each case, part of the catalytic apparatus appears to be a single polypeptide containing two hemes: subunit I in the oxidase family and cytochrome b in the bc_1 and b_6f complexes.

The possibility that electron transfer within these membrane-embedded bis-heme complexes may result from heme–heme interactions has intrigued biochemists and biophysicists. A technique that is sensitive to the through-space electronic coupling between two chomophores is circular dichroism (CD)[1] spectroscopy (Woody, 1985; Myer, 1985). Redox-linked conformational changes suggested by changes in heme–heme interactions have been deduced from the CD spectra of cytochrome oxidase (Urry *et al.*, 1967; Storey & Lee, 1973), a bacterial *cd* oxidase (Orii *et al.*, 1977), and the mitochondrial bc_1 complex (Reed *et al.*, 1979; Degli Esposti *et al.*, 1987, 1989a). However, the early CD data implying heme–heme interaction between cytochromes a and a_3 in cytochrome oxidase (Urry *et al.*, 1967; Storey & Lee, 1973) have been subsequently criticized, since the shapes of the CD spectra were found to be very sensitive to the detergents used in the preparation of the enzyme (Myer, 1985). The usefulness of CD spectroscopy in this context has also been demonstrated by Woody (1985), who showed that a feature of the CD spectra of hemoglobin in the Soret region resulted from heme–heme interactions. This finding is particularly interesting when one considers the large distances (>24 Å) which exist between the several hemes present in this protein (Bolton & Perutz, 1970).

Recent measurements of the CD spectra of the mitochondrial bc_1 complex clearly showed that cytochrome b displays an intense, bisignate Cotton effect in the Soret region consistent with exciton coupling between its two hemes (b_H and b_L), particularly when they are both reduced (Degli Esposti *et al.*, 1987, 1989a; Degli Esposti & Palmer, 1988).

The theory of exciton coupling is well understood and constitutes a field of optical activity which has been of considerable utility in stereochemistry (Mason, 1982; Harada & Nakanishi, 1983), and exciton coupling calculations are routinely utilized in organic chemistry for deducing the absolute conformation of aromatic oligomers (Harada & Nakanishi, 1983; Mason *et al.*, 1974; Mason, 1982; Gottarelli & Collet, 1982). Here we present the application of exciton coupling theory to the CD spectra of a membrane bis-heme system, namely, cytochrome b of the mitochondrial bc_1 complex. The stereochemical relationship between the two hemes agrees well with structural conclusions drawn from sequence analysis and the location of residues responsible for resistance to inhibitors at b_H (center i) and b_L (center o). This paper is a sequel to Degli Esposti *et al.* (1989a).

MATERIALS AND METHODS

Preparations. Beef heart bc_1 complex was prepared by the method of Degli Esposti *et al.* (1986). Purified cytochrome b from beef heart was provided by C. A. Yu and L. Yu (University of Oklahoma—Stillwater). Yeast bc_1 complex and the isolated cytochrome b from the yeast bc_1 complex were prepared as described by Siedow *et al.* (1978) and by Tsai and Palmer (1982), respectively.

Spectroscopic Measurements. The spectral studies were focused exclusively on the Soret region of the heme absorption, generally between 380 and 460 nm. Optical and CD measurements were performed in Jasco Uvidec-610 and a Jasco J-500C instruments, respectively (Degli Esposti *et al.*,

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* Author to whom correspondence should be addressed.

[‡] Rice University.

[§] University of Bologna.

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[1] Abbreviations: A_m, molar absorbance; ΔA_m molar differential absorbance; CD, circular dichroism; μ_D, Debye magneton; EPR, electron paramagnetic resonance; λ_m, wavelength maximum; FWHM, full-width at half-maximum; R, rotational strength; D, dipole strength; b_H and b_L, the high- and low-potential heme centers of the mitochondrial cytochrome b.

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1989a). Spectral resolution of the contributions of cytochrome b_H and cytochrome b_L in the beef heart bc_1 complex was performed by the manipulations described previously (Degli Esposti *et al.*, 1989a). The spectra of cytochrome *b* were normalized to the total content of *b* heme determined spectrophotometrically (Degli Esposti *et al.*, 1989a; Tsai & Palmer, 1982) or by the pyridine hemochromogen method (Hauger & Coon, 1976).

Analysis of the Primary Sequences. All of the published sequences of mitochondrial and bacterial cytochromes *b* have been aligned and analyzed as described by Degli Esposti *et al.* (1993). The yeast sequence has been taken as the reference for numbering the residues, using the NH_2-terminal residue of yeast as position 1 (Degli Esposti *et al.*, 1989a, 1993). The apoprotein is assumed to have eight transmembrane helices, with the amino terminus located at the negative side of the membrane (Crofts *et al.*, 1987; Degli Esposti *et al.*, 1993).

Analysis of Optical and CD Spectra. The electronic transition responsible for the Soret band of the optical spectral of cytochrome *b* arises from a $\pi \rightarrow \pi^*$ promotion of an electron in the porphyrin ring system (Eaton & Hofrichter, 1981). The intensity of the optical band is quantified by means of its dipole strength, *D*, obtained from the integrated area of the band (Urry, 1970; Harada & Nakanishi, 1983). Because the absorption profile is not symmetric about the wavelength maximum, we elected to use the rectangular approximation to the integrated area; this is less sensitive to any asymmetry in band shape than the Gaussian approximation (Harada & Nakanishi, 1983).

$$D = (1.63 \times 10^{-2}) A_m \Delta\lambda / \lambda_m$$

where A_m is the molar absorbance, λ_m is the wavelength maximum, and $\Delta\lambda$ is 0.6× (full-width at half-height). The units of *D* are debye[2]; 1 debye = 10^{-18} cgs units.

The apparent rotational strength, *R* in Debye magnetons (μ_D), of the CD bands in the Soret region has been obtained from the integrated area of the CD absorption band using the Gaussian approximation (Myer, 1978; Harada & Nakanishi, 1983):

$$R = 0.445 \Delta A_m \Delta\lambda / \lambda_m$$

where ΔA_m is the molar differential extinction coefficient at the band maximum, $\Delta\lambda$ is the standard deviation of the CD absorption (in nm), taken as the half-bandwidth at $\Delta A_m/e$, and λ_m is the wavelength (in nm) of the band maximum.

The CD spectra of cytochrome *b* were first resolved into the minimal number of Gaussian bands of positive and negative signs which could give a reasonable fit to the experimental data. The major bands in reduced *b* cytochromes belong to the couplet centered around λ_m of the parent absorption spectrum (Degli Esposti *et al.*, 1989b). These bands substantially overlap each other, so that the longer wavelength side of the positive band and the shorter wavelength side of the negative band are distorted by mutual cancellation. The experimental values of these CD bands were therefore deduced by utilizing the $\Delta\lambda$ experimentally obtained from the side of the band which is minimally distorted by this overlap, i.e., by taking the shorter wavelength side for the positive band and the longer wavelength side for the negative band (Urry, 1970).

In the exciton calculations, it has been assumed that cytochrome b_H and cytochrome b_L are essentially identical chromophores; indeed, the spectral differences of their Soret bands are much smaller than those seen in the α-region and tend to disappear completely in the isolated cytochrome *b* (Tsai & Palmer, 1982). The two orthogonally polarized

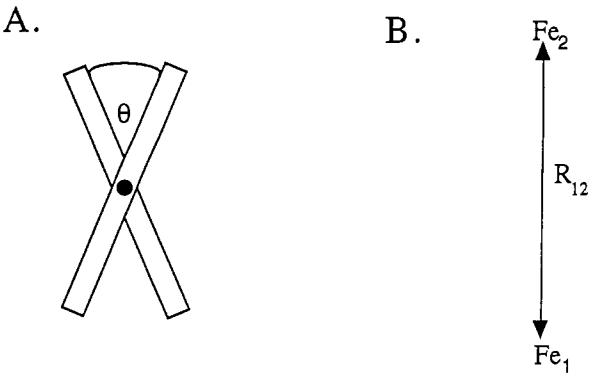

FIGURE 1: Simplified geometry of the arrangements of the two hemes in mitochondrial cytochrome *b* as depicted in Figure 4: (A) view along the Fe–Fe vector; (B) view looking down on the Fe–Fe vector.

oscillators of the Soret electronic transitions of both *b* cytochromes are assumed to have the same transition dipole moment, since they are almost isoenergetic in myoglobin and hemoglobin, which have the same prosthetic group as that of cytochrome *b* (Eaton & Hofrichter, 1981; Adar, 1978; Makinen & Churg, 1983). The value of the transition dipole moment of each oscillator of the electron transition has been taken as equal to 4.3 D for both reduced *b* hemes. All of the exciton calculations were performed assuming a point dipole approximation (Shellman, 1968; Harada & Nakanishi, 1983; Woody, 1985); as the distance between the two hemes is much larger than the size of the porphyrin ring, this is an acceptable approximation.

The exciton calculation requires evaluating two quantities. The first is the energy (V_{12}) arising from the coupling of the transition dipole moments of each heme:

$$V_{12} = \frac{5040}{R_{12}^3}\{\mu_1\mu_2 - 3[(\mu_1 \mathbf{r})(\mu 2\mathbf{r})]\} \quad (1)$$

μ_1 and μ_2 are the transition dipole moments of each heme, R_{12} is the separation between these transition dipoles (taken as the Fe–Fe distance), and **r** is a unit vector in the direction of R_{12}. The numeric factor arises because energies are expressed in cm^{-1}, distances in angstroms, and transition dipole moments in debye. The shift in energy of the absorption spectrum arising from this coupling is

$$\nu_\pm = \nu_o \pm V_{12} \quad (2)$$

where ν_o is the frequency of the parent transition and $\nu_\pm$ is the frequency of the two components arising from the coupling.

The rotational strength in Debye magnetons of these two exciton transitions is

$$R_\pm = \mp\frac{170}{\lambda}\{R_{12}(\mathbf{r}\cdot\mu_1\mu_2)\} \quad (3)$$

In this equation, the numeric factor again arises because energies are expressed in cm^{-1}, wavelength maxima and distances in angstroms, and transition dipole moments in debye.

The initial calculations were performed using the model shown in Figure 1. For this geometry, only two parameters are needed. The first is R_{12}, the length of the vector connecting the iron atoms of each heme (Figure 1B). The second is θ, the magnitude of the dihedral angle subtended by the planes of the two hemes (Figure 1A). Because heme absorption consists of two degenerate transitions (the *x* and *y* components), in principle, four interactions need to be calculated. These are x_1x_2 (the *x* component of heme 1 interacting with the *x* component of heme 2) and, likewise, y_1y_2, x_1y_2, and x_2y_1. Although the problem can be simplified because the degen-

eracy of the transitions allows us to associate them with any convenient set of orthogonal coordinates in the relevant plane, we initially allowed the x and y transitions to have an arbitrary orientation specified by α_1 and $\alpha_1 + 90$ for heme 1 and α_2 and $\alpha_2 + 90$ for heme 2, where α_1 and α_2 are counterclockwise rotations from the x coordinate. Then

$$V_{12} = \frac{\mu_1\mu_2}{R_{12}^3}(f(\theta,\alpha_1,\alpha_2)) \quad (4)$$

$$R_{\pm} = \mp\frac{170}{\lambda}R_{12}\mu_1\mu_2(g(\theta,\alpha_1,\alpha_2)) \quad (5)$$

For $f(\theta,\alpha_1,\alpha_2)$,

$$f_{xx} = \cos\alpha_1\cos\alpha_2\cos\theta - 2\sin\alpha_1\sin\alpha_2 \quad (6)$$

$$f_{xy} = \sin\alpha_1\sin\alpha_2\cos\theta - 2\cos\alpha_1\alpha_2$$

$$f_{yy} = -\cos\alpha_1\sin\alpha_2\cos\theta - 2\sin\alpha_1\cos\alpha_2$$

$$f_{yx} = -\sin\alpha_1\cos\alpha_2\cos\theta - 2\cos\alpha_1\sin\alpha_2$$

For $g(\theta,\alpha_1,\alpha_2)$,

$$g_{xx} = -\cos\alpha_1\cos\alpha_2\sin\theta \quad (7)$$

$$g_{yy} = -\sin\alpha_1\sin\alpha_2\sin\theta$$

$$g_{xy} = \cos\alpha_1\sin\alpha_2\sin\theta$$

$$g_{yx} = \cos\alpha_2\sin\alpha_1\sin\theta$$

The dipole strength calculated from the area under the absorption curve represents the combined contribution of the x and y polarized transitions. Assuming that the contribution of these two components are identical, the value of $\mu_1\mu_2 = \mu^2$ used in eqs 4 and 5 is one-half the total dipole strength.

The input to the calculation is thus ν_0, μ^2, R_{12}, θ, α_1, and α_2, and the output is ν_+, ν_-, R_+, and R_-. The CD spectrum is then calculated as the sum of two absorption curves, the first at ν_+ of area R_+ and the second at ν_- with area R_-:

$$\Delta A_{\pm} = \frac{1}{0.443P}R_{\pm}\sqrt{\nu_{\pm}}\exp\left(-\left(\frac{\nu-\nu_{\pm}}{P\sqrt{\nu_{\pm}}}\right)^2\right) \quad (8)$$

in which the line width is parametrized as $P(\nu_{\pm})^{1/2}$, a modification introduced by Mason et al. (1974) to accommodate known asymmetries in the two components of the exciton couplet. The leading numeric factor is the proportionality between the area under the curve and the rotational strength expressed in Debye magnetons.

Subsequently, a more general model was examined. It was patterned after that employed by Dratz (1966) in his analysis of the CD spectrum of bacteriochlorophyll. This more general model is depicted in Figure 2 and is described in detail in the legend to that figure. Now the expressions for V_{12} and $R_{\pm}$ are

$$V_{12} = \frac{5040\mu^2}{R_{12}^3}(f(\alpha,\beta,\phi,\theta,\chi)) \quad (9)$$

$$R_{\pm} = \mp\frac{170}{\lambda}R_{12}\mu^2(g(\alpha,\beta,\phi,\theta,\chi)) \quad (10)$$

The expressions for f( ) and g( ) can be deduced from eqs 1 and 2 and are as follows:

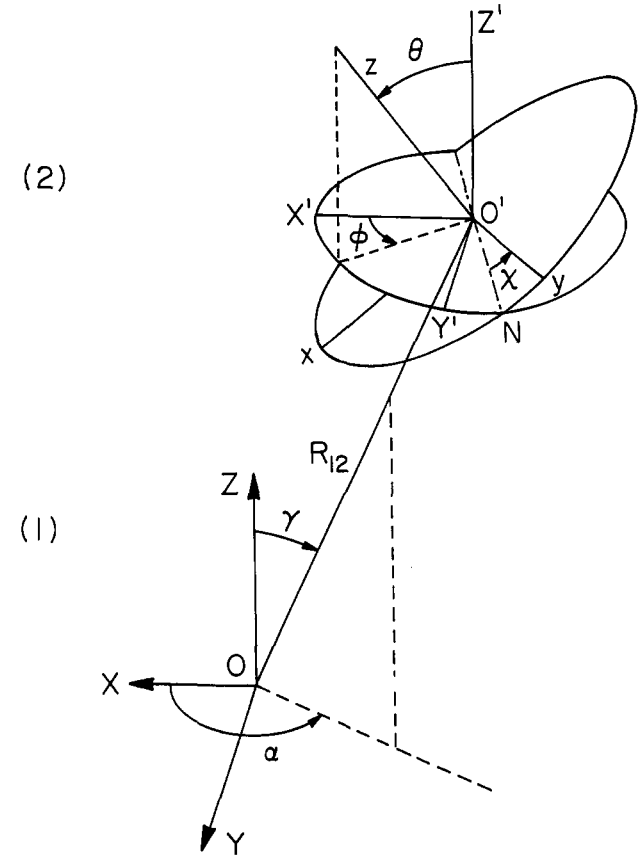

FIGURE 2: Geometric arrangements and coordinate system for two hemes arbitrarily disposed in space. O and O′ represent the two Fe atoms; XYZ and $X'Y'Z'$ are identical coordinate systems located at O and O′. A right-handed coordinate system XYZ is centered on heme 1 (O) such that the x and y polarized transitions define the X and Y coordinates; the Z axis is aligned along the normal to the XY plane. The center of heme 2 (O′) is located by the vector R_{12} originating on the iron atom of heme 1 and terminating on that of heme 2. The direction of R_{12} is defined by the cylindrical polar and equatorial angles α and γ. A second right-handed coordinate system (xyz) is centered on heme 2 with the (x) and (y) directions defined by the x and y polarized transitions of heme 2. The coordinate system $X'Y'Z'$ is identical to XYZ, except that it has been translated to be centered on heme 2. The angles ϕ, θ, and χ, which rotate (xyz) into $X'Y'Z'$, are the Eulerian angles as defined by Wilson *et al.* (1955, Table 1-1).

$$f_{xx} = \{(\cos\phi\cos\chi\cos\theta - \sin\phi\sin\chi) - 3(\sin\gamma\cos\alpha)[\sin\gamma\sin\alpha(\cos\phi\sin\chi + \sin\phi\cos\chi\cos\theta) - \sin\gamma\cos\alpha(\sin\phi\sin\chi - \cos\phi\cos\chi\cos\theta) - \cos\gamma\sin\theta\cos\chi]\} \quad (11)$$

$$f_{yy} = \{(\cos\phi\cos\chi - \sin\phi\sin\chi\cos\theta) - 3(\sin\gamma\cos\alpha)[\sin\gamma\sin\alpha(\cos\phi\cos\chi - \cos\theta\sin\phi\sin\chi) - \sin\gamma\cos\alpha(\sin\phi\cos\chi + \sin\phi\cos\chi\cos\theta) + \cos\gamma\sin\theta\sin\chi]\}$$

$$g_{xx} = \cos\gamma(\cos\theta\sin\phi\cos\chi + \cos\phi\sin\chi) - \sin\gamma\sin\alpha\sin\theta\cos\chi \quad (12)$$

$$g_{yy} = \cos\gamma(\cos\theta\cos\phi\sin\chi + \sin\phi\cos\chi) + \sin\gamma\cos\alpha\sin\theta\sin\chi$$

f_x and f_y are for the interactions of the x and y polarized transitions on the two hemes; g_x and g_y are analogous. As the expressions for f_{xy} and f_{yx} evaluate to zero, the corresponding expressions for g_{xy} and g_{yx} are irrelevant. These expressions

Table 1: Parameters of the Optical and CD Spectra of Various *b*-Type Cytochromes in the Soret Region[k]

cytochrome	redox state	λ_m (nm)	A_m (mM^{-1} cm^{-1})	Γ[l] (nm)	D (debye^2)	crossover (nm)	λ_m (nm)	ΔA_m (M^{-1} cm^{-1})	$\Delta\lambda$ (nm)	R (μ_D)[m]
cytochrome *b*562 *E. coli*	OX	419	117	33	7.65	403	398	4.8	12.2	0.065
							421	−23.7	9.1	−0.22
	RED	427	182	17	7.09	399	422	−8.4	9.2	−0.08
cytochrome b_2 yeast	OX	411	164	30	11.71		375	−10.0	25.0	−0.30
	RED	422	232	71	9.03	415	378	4.0	22.0	0.11
							425	−20.6	10.0	−0.22
cytochrome b_5 calf liver	OX	413	118	27	7.55	403	416	−9.0	12.6	−0.12
	RED	423	171	17	6.72					
cytochrome *b*555[a] housefly larva	OX	414	120	26	7.37	402	416	−27.0	7.5	−0.22
	RED	424	179	18	6.70	417	427	−31.0	5.0	−0.16
cytochrome *b*563 housefly larva	OX						418	−25.5	15.0	−0.41
	RED						429	−30.0	10.0	−0.31
cytochrome *b* (*b*562)[b] bc_1	OX	414	171	31	12.54		416	48.5	16.0	0.83
complex *Rh sphaeroides*	RED	428	210	20	9.60		430	56.4	9.0	0.53
cytochrome *b* isolated[c] yeast	OX	415	142	31	10.37	416	409	22.6	20.0	0.50
							422	−28.7	8.4	−0.26
	RED	428	205	16.6	7.78	428	424	50.8	8.4	0.45
							433	−80.4	4.0	−0.33
cytochrome *b* isolasted[d] beef heart	OX	416	115	31	8.39	415	409	9.3	13.7	0.14
							422	−10.5	10.3	−0.11
	RED	429	132	19	5.72	429	424	11.7	9.1	0.11
							432	−15.8	4.6	−0.08
	OX	416	112	36	9.48	419	413	27.5	12.0	0.36
							425	−25.0	7.3	−0.19
cytochrome *b* in situ bc_1 complex beef heart	RED	429	210	17	8.14	429	413	14.4	9.0	0.14
							426	40.5	8.0	0.34
							435	−70.5	4.6	−0.33
cytochrome *b*557[f] NO_3 reductase	OX	412	117	32	8.89	392	375	1.5	8.3	0.002
Chlorella vulgaris							415	−5.0	12.0	−0.008
	RED	423	161	21	7.82	421	415	1.0	6.5	0.006
							426	−11.0	6.2	0.070
cytochrome *b*562 fumarate reductase[g]	OX	416	132	36	11.17		442	23.5	17.5	0.34
W. succinogenes	RED	428	182	16.5	6.86	426	422	−14.0	6.0	−0.09
							430	28.0	5.0	0.14
cytochrome *b*561 chromaffin granules	OX	415	124	36	10.52	409	403	15.0	6.8	0.11
							420	−25.6	11.4	−0.43
	RED	427	178	17	7.17	423.5	421	31.5	8.8	0.29
							423	−72.0	6.0	−0.44
cytochrome[h] *b*558 NADPH oxidase	OX	413.5	139	31	10.18	413	406	121.0	18.0	2.34
human neutrophils							426	−119.0	7.0	−0.99
	RED	426	167	18	6.90	424.5	420	102.0	7.0	0.74
							429	−150.0	4.0	−0.61
cytochrome *b*558[i] isolated from succinate:	OX	412.5	153	29.5	10.70	412	404	41.2	19.0	0.848
Q reductase *B. subtilis*							417	−23.7	10.0	−0.206
	RED	425	233	16.5	8.85	423	418	91.5	9.0	0.862
							427	−58.4	5.5	−0.329
cytochrome b_L in situ[j] bc_1 complex beef heart	RED	431	181	10.0	4.11	432	427	83.0	6.3	0.505
							435.5	63.2	4.0	−0.258

[a] Actually a cytochrome b_5-like heme protein (Myer, 1985). [b] Preparation containing only the cytochrome b_H heme in a nearly native state (Salerno et al., 1986). [c] Optical data from Tsai and Palmer (1982). The parameters refer to preparations containing more than 1.5 mol of *b* per mole of apoprotein. [d] Preparation of C. A. Yu, largely denatured [see Degli Esposti *et al.* (1989a)], containing ≥1.4 mol *b* heme/mol. [e] Resolved spectra, obtained as described in Degli Esposti *et al.* (1989a). [f] A member of the cytochrome b_5 family in the purified preparation reported by Kay *et al.* (1988). [g] Resolved spectra in the native reductase complex containing two hemes per apoprotein (Degli Esposti *et al.*, 1991). [h] Purified NADPH oxidase almost certainly containing two heme *b* per complex, possibly as a heterodimer (Yamaguchi *et al*, 1989; Quinn *et al.*, 1992; Segal & Abo, 1993). [i] Purified cytochrome from an *E. coli* strain overexpressing the *B. subtilis* apoprotein gene, containing two *b* hemes per mole of apoprotein (Friden *et al.*, 1990; Hagerhall *et al.*, 1992; M. Degli Esposti and L. Herestadt, unpublished data). [j] Optical and CD data from the reduced − oxidized difference spectra obtained by subtracting the succinate-reduced spectrum from the dithionite-reduced spectrum of the beef heart enzyme. [k] The parameters were calculated by rectangular approximation of the optical bands and Gaussian approximation of the CD bands. Data are from Myer (1985) and references therein for the first five proteins. [l] Γ, half-width at half-height. [m] μ_D, Debye magnetons.

differ slightly from those given by Dratz because (i) the usage here follows Wilson, Decius, and Cross precisely, while Dratz rotated χ by 90°, and (ii) several terms in sin χ, were omitted from Dratz' formulae. In this geometrically general model, the quantities input to the calculation are μ^2, R_{12}, λ, α, γ, ϕ, θ, and χ.

RESULTS AND DISCUSSION

Optical and CD Properties of Mitochondrial Cytochrome b and Other b-Type Cytochromes. Native mitochondrial cytochromes *b* characteristically show intense derivative-like CD spectra in the Soret region (Degli Esposti *et al.*, 1987, 1989a), usually called a couplet (Schellman, 1968). Because the positive lobe of the couplet is at higher energy, the couplet is said to be positive and this implies that the chiral relationship between the two hemes is that of a left-handed screw (Figure 1A).

Only a few other heme proteins exhibit CD couplets that are qualitatively comparable to that of mitochondrial cytochrome *b*; an example is flavocytochrome *c*-552 from *Chromatium vinosum* (Myer, 1985). However, the intensity of the CD of mitochondrial cytochrome *b* is larger than that found in all other heme proteins studied so far, with the exception of a preparation of cytochrome *b*-558 of human

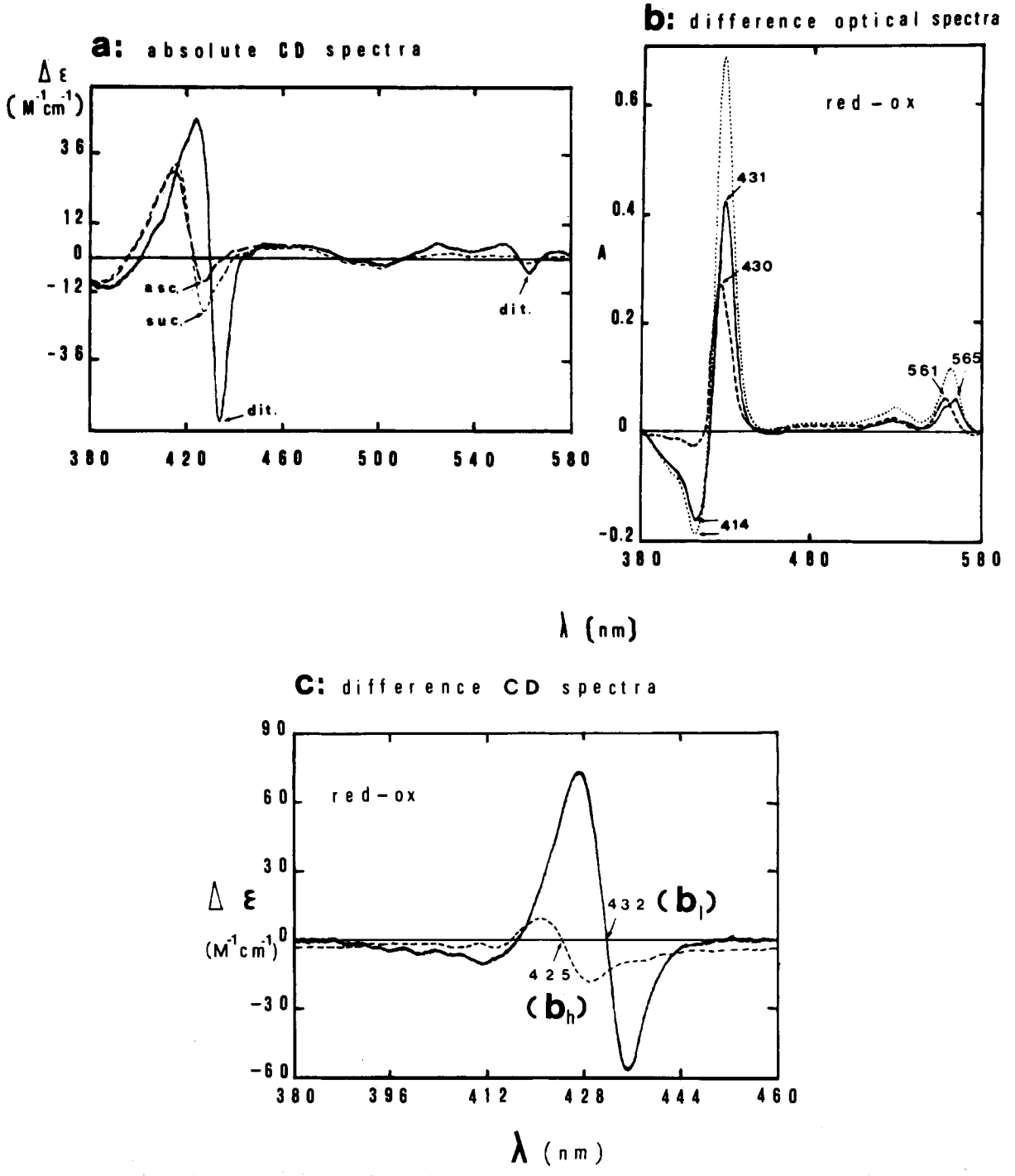

FIGURE 3: (A) Absolute CD spectra of isolated beef heart bc_1 complex (4.14 μM cyt *b*) reduced with solid ascorbate (asc,- - -), then with 10 mM succinate followed by 5 mM malonate (suc,—), and finally with solid dithionite (dit,—). (B) Difference absorption spectra: Succinate – ascorbate (- - -); dithionite – ascorbate (···); dithionite – succinate (—). (C) Difference CD spectra in the Soret region: succinate – ascorbate (- - -); dithionite – ascorbate (—). The conditions are those of Degli Esposti *et al.* (1989a).

NADPH oxidase (Yamaguchi *et al.*, 1989). A summary of the optical properties of the *b*-type cytochromes whose CD spectra have been reported emphasizes the peculiarity of the CD properties of the mitochondrial cytochrome (Table 1).

Most *b*-type cytochromes, including that isolated from the bc_1 complex of *Rhodobacter sphaeroides* R26 (which is reported to contain only one heme per mole, namely, cytochrome b_H; Salerno *et al.*, 1986), show a substantial (≥30%) decrease of both the dipole strength of the Soret absorbance and the rotational strength of the CD following reduction of the heme iron (Table 1). However, a few bis-heme proteins, including mitochondrial cytochrome *b*, exhibit an increase in rotational strength upon reduction, even though the dipole strength of the absorbance decreases upon reduction as with all other *b*-type cytochromes (Table 1).

Cytochrome b_L, which is completely reduced only by dithionite, appears to account for most of the increase in CD intensity upon reduction of the bc_1 complex (Table 1; Degli Esposti *et al.*, 1989a). The resolved CD spectrum of dithionite-reduced minus succinate-reduced bc_1 complex shows that the CD couplet is centered at approximately the maximum of the optical spectrum (Figure 3). This difference spectrum of b_L is rather similar to the difference spectra of the fully reduced minus fully oxidized bis-heme cytochrome *b* (Degli Esposti

et al., 1989a). The rotational strength of the positive bandof the couplet increases by about 0.5 μ_D, upon reduction of b_L alone (Table 1).

The simplest explanation for the increase in the rotational strength of the CD spectrum of cytochrome b_L upon reduction is that a conformational change occurs in the *b* apoprotein when the low-potential heme is reduced. This change in conformation produces an interaction of the two hemes that is much stronger than that which exists when either or both hemes are oxidized. Spectroscopically, this heme–heme interaction can be visualized as an exciton coupling of the electronic transition of the two *b* hemes leading to the nearly symmetric CD couplet having a zero-crossing coincident with λ_m of the Soret absorbance. This exciton couplet is not seen in the CD spectrum of preparations having one *b* heme per mole, such as that of *Rhodobacter* (Table 1). The CD couplet originating from the exciton coupling of the two hemes overlaps and obscures the CD signals that each *b* heme possesses independently of the other. These latter CD features derive from the coupling of the *b* hemes with nearby aromatic residues of the apoprotein (Degli Esposti *et al.*, 1987, 1989a) and are less intense in the reduced than in the oxidized cytochromes, presumably because the dipole strength of the reduced heme is smaller than that of the oxidized heme (Table 1; Hsu & Woody, 1971) and the wavelength maximum has shifted to the red.

A Structural Model of the Heme-Coordinating Domain of Cytochrome b. Although the precise structure of mitochondrial cytochrome *b* is not known, important information on the domain(s) which binds the two hemes has been obtained from EPR and linear dichroism spectroscopy (Erecinska & Wilson, 1979; Palmer, 1985; Von Jagow *et al.*, 1986; Ohnishi *et al.*, 1989) and from the analysis of the primary sequence deduced from the gene from diverse species (Saraste, 1984; Crofts *et al.*, 1987, 1992; Degli Espos ti *et al.*, 1989b, 1993; Tron *et al.*, 1991). It is commonly agreed that the two *b* hemes are buried within the transmembrane portion of the protein and are coordinated to four invariant histidines, His_{82}, His_{96}, His_{183}, and His_{197} (Figure 4). The presence of three highly conserved, positively charged residues at both sides of the membrane (i.e., Arg_{79}, Arg_{99}, and Arg_{178}) suggest that they may form salt bridges or hydrogen bonds with the two negatively charged propionyl groups present in each heme (Saraste, 1984; Widger *et al.*, 1984). Each *b* heme, therefore, should be inserted into the protein with its propionyl groups directed toward the water–membrane interface and with the β substituents at positions 1–4 directed toward the center of the lipid bilayer. The plane of each heme is oriented approximately perpendicular to the membrane plane (Erecinska & Wilson, 1979), so that the axial coordination of the iron with the histidines is perpendicular to the normal of the membrane plane (Saraste, 1984; Widger *et al.*, 1984). The structural constraints of the α-helix, which is the conformation preferred for the transmembrane segments of the apoprotein of cytochrome *b* (Saraste, 1984; Rao & Argos, 1986; Crofts *et al.*, 1987; Degli Esposti *et al.*, 1989b), and the location of the histidines lead to an estimate of about 20 Å for the distance between the two heme iron atoms.

The observation that reduced cytochrome *b* exhibits a CD spectrum largely domianted by a contribution due to exciton coupling of the two hemes (cf. Figure 3) indicates that the relative orientation of the two *b* hemes must be chiral. This implies that the *b* hemes do not lie in the same or parallel planes as is commonly assumed (Saraste, 1984; Widger *et al.*, 1984; Von Jagow *et al.*, 1986; Link *et al.*, 1986), but must lie

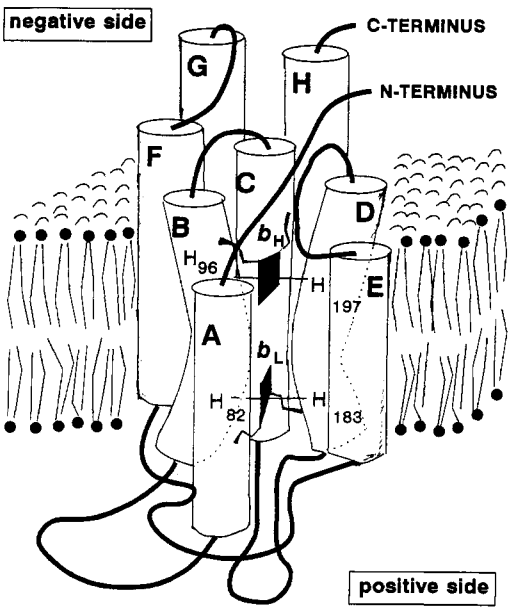

FIGURE 4: Hypothetical structure of mitochondrial cytochrome *b* located in the membrane (Degli Esposti *et al.*, 1993). The cylinders represent the presumed transmembrane helices using the nomenclature of Crofts *et al.* (1992). The hemes are represented as the solid trapezoids attached to the totally conserved histidines. The zig-zag lines attached to the hemes represent the propionic acid side chains. From the separation of the pairs of histidine residues, it is estimated that the Fe–Fe distance is approximately 20 Å (Saraste, 1984; Widger *et al.*, 1984).

in two different planes subtending a discrete angle (Shellman, 1968; Mason, 1982; Degli Esposti & Palmer, 1988; Degli Esposti *et al.*, 1989a). This prediction is supported by inspection of the mutual orientation of the hemes in all of the multiheme proteins whose refined three-dimensional structure is known, such as hemoglobin (Bolton & Perutz, 1970), cytochrome c_3 from *Desulfovibrio* (Higuchi *et al.*, 1984), and the cytochrome subunit of the reaction center from *Rhodopseudomonas viridis* (Deisenhofer *et al.*, 1985). In each case, the planes of the various hemes are never parallel to each other, but always form an angle $\geq 20°$. Moreover, the six porphyrin-derived cofactors contained in the transmembrane portion of bacterial reaction centers are all oriented in different planes (Deisenhofer *et al.*, 1985; Allen *et al.*, 1987).

All of this structural information has been incorporated in the model of cytochrome *b* shown in Figure 4 (Degli Esposti *et al.*, 1993). This general model has been utilized for a semiquantitative description of the CD spectra of cytochrome *b* by applying exciton coupling theory to the geometric representation of this model shown in Figure 1.

Exciton Coupling between b_H and b_L. Our initial attempt to analyze the CD spectrum of reduced cytochrome *b* was based on the approach utilized by Hasselbacher *et al.* (1988) in their analysis of the CD spectrum of halorhodopsin. These workers attempted to extract the exciton interaction energy [(V_{12}, called Δ by Hasselbacher *et al.* (1988)] and spectral bandwidth (called *B*) by fitting the experimental data to a pair of Gaussian peaks of opposite sign. In using the same approach to fit the cytochrome data, our normally very robust, nonlinear optimization routines consistently failed to converge. On closer inspection it became clear that the approach of Hasselbacher *et al.* is invalid when the exciton splitting is much smaller than the spectral bandwidth, for under these conditions the values of Δ and *B* are coupled and an infinite number of solutions exist.

Table 2: Experimental Parameters from the Absorption and Circular Dichroism Spectra of Reduced Cytochrome[a] *b*

parameter	physical meaning	numerical value[b]
ν_0	wavenumber of the optical peak maximum ($10^7/\lambda_m$)	23310 cm^{-1}
FWHM	full bandwidth at $1/2\lambda_m$	983 cm^{-1}
D^*	$(\mu_{1(x,y)}\cdot\mu_{x,y})$ dipole strength of the coupling of a transition dipole on each heme[c]	21.5 $debye^2$
$\mu_{(x,y)}$	transition dipole moment of each oscillator of the double-degenerate electronic transition of the hemes, taken as identical for both cytochrome b_H and cytochrome b_L	4.6 debye
R^+	experimental value of the positive band of the exciton couplet in the CD spectrum of cytochrome b_L(redox) (cf. Figure 3)[d]	0.46 μ_D

[a] The values were determined from the optical spectra of isolated yeast cytochrome *b* and of cytochrome *b* within the beef heart bc_1 complex (cf. Table 1). [b] For the parameters derived from the optical spectra, the values are the mean between the values of yeast cytochrome *b* and beef heart cytochrome *b* within the enzyme. [c] This value of *D* is one-half the value measured in the optical spectra of reduced cytochrome *b* (cf. Table 1) since these latter values are equal to the sum of the *x* and *y* components of the transition (Hsu & Woody, 1971). [d] Approximated as the mean of the *R* of the positive band and the *R* of the negative band to account for the imbalance of the couplet due to the asymmetry of the Soret transition.

We consequently changed our strategy and, as an alternative, have calculated the exciton contribution to the CD of a bis-heme system assuming a specific geometry, as shown in Figure 1. In this calculation the only variables are the Fe–Fe distance (R_{12}), the dihedral angle subtended by the planes of the two porphyrins (θ), and *P*, a parameter which controls the line width of the computed curve (see eq 8, Materials and Methods); as discussed below, α_1 and α_2 prove to be irrelevant.

As the dihedral angle which gives the largest rotational strength is 45° (see below), we first set θ equal to 45° and examined the fit of the calculated spectrum to the experimental data as a function of R_{12}; this yields the largest distance that is consistent with experiment. This distance was found to be 19.5 Å (Figure 5). As noted above, the existing structural model suggests a heme–heme distance of about 20 Å. As the width of the positive and negative lobes of the experimental data differed, it was not possible to accurately reproduce both lobes with a single value for the FWHM, despite use of the modification of Mason *et al.* (1974). The value selected, 600 cm^{-1}, provided the best compromise. Increasing the FWHM to 650–700 cm^{-1} gave a very accurate fit to the positive lobe at the expense of degrading the fit to the trough. With this larger value of FWHM it was necessary to decrese R_{12} to 17–18 Å. Conversely, a good fit to the trough required a FWHM of 500 cm^{-1} together with an Fe–Fe distance of 23 Å. The Fe–Fe distance thus seems to lie in the range 17–23 Å, with 19.5 Å being the most plausible value. (With the FWHM of 983 cm^{-1} extracted from the absorption spectrum, the computed CD was some 60% broader than that observed experimentally; the distance necessary to reproduce the experimental amplitudes was 12.5 Å.)

As expected, the amplitude of the coupled CD spectrum depends accurately on $1/R_{12}^2$ (Figure 6A). This dependence arises from the cancellation of two effects: a linear increase in $R_\pm$ as R_{12} is increased (eq 2) and a cubic decrease in V_{12} with increasing R_{12} (eq 1). The range of acceptable distances is shown by the horizontal bar in Figure 6, while the most probable distance is denoted with an X. Similarly, when the dihedral angle is varied at a fixed value of R_{12}, the amplitude of the CD spectrum is maximal at 45° and falls to zero at both 0° and 90° (Figure 6B). The data can be accurately fit by $\sin\theta\cos\theta$ (equivalent to $\sin 2\theta$); this variation is also to be expected. In the rigid-shift limit ($V_{12} << \Delta\lambda$), the difference of two Gaussians slightly displaced from one another resembles the derivative of an unshifted Gaussian, and the amplitude of this difference spectrum is determined by (i) the amplitude of the parent Gaussian curve (linearly proportional to $R_\pm$), (ii) the separation between the individual Gaussians (linearly proportional to V_{12}), and (iii) the line width of the Gaussian curve. Thus, the amplitude is proportional to the product $R_\pm V_{12}$ and, hence, to $\sin\theta\cos\theta$, as is found by simulation.

In the original simulations, the orientations of the two transition dipoles on each heme were arbitrary and determined by the angles α_1 and α_2 (see Materials and Methods). In fact, systematic variation of α_1 and α_2 yielded values for the various components of *R* and *V*, which changed as required by eqs 6 and 7. However, while the net values of $R_\pm$ and V_{12} were found to depend on the values of α_1 and α_2, the net CD (which depends on the product of $R_\pm$ and V_{12}) was totally insensitive to the values of α_1 and α_2. This empirically established result was subsequently verified by general analysis (P. Palmieri and G. Palmer, unpublished calculation). The shallow dependence of the optical activity on dihedral angle when θ is in the neighborhood of 45° implies that this angle is determined only approximately.

The angular and distance dependence of the optical activity leads to the following expression relating the CD amplitude (M^{-1} cm^{-1}) of the cytochrome *b* couplet to distance and angle:

$$CD = \pm 32131 \sin(2\theta) R_{12}^{-2}$$

Subsequently, the CD was analyzed using a geometric system that made no assumptions about the relative orientations of hemes b_H and b_L (Figure 2, eqs 11 and 12). Using values for the several angles which reproduced the geometry of Figure 1 led to calculated CD spectra identical with that shown in Figure 5. Deviations from this geometry, which resulted in a small canting of the two hemes, also led to acceptable fits but required a reduction in the Fe–Fe distance. The results from this more general model make it clear that deviations from the idealized geometric arrangement of Figure 1 must be small.

It should be noted that the system under study exhibits an exciton coupling significantly smaller than the bandwidth of the parent absorption curve and thus falls into the category of weak coupling as defined by Simpson and Peterson (1957); in such a circumstance, classical polarizability theory (Devoe, 1965) is more appropriate. By contrast, the form of exciton theory employed here assumes that the chromophores are strongly coupled. However, Woody (1985) has shown that strong coupling theory predicts intensities and splittings comparable to these from polarizability theory, at least in the cases of cytochrome c_3, hemoglobin, and deoxyhemoglobin, although the strong coupling approach tends to underestimate the intrinsic interaction, which suggests that our estimate of R_{12} may be too small, θ may be too large, or both.

A competing interpretation of the biphasic nature of the CD spectrum is that it arises from a single heme with a split Soret, i.e., the energies of B_x and B_y are slightly different. Cytochrome b_L is known to have a split α-band due to the inequivalence in energy in Q_x and Q_y. The magnitude of this zero-field splitting is around 300 cm^{-1} (Kamensky & Palmer, 1992). It was previously deduced from group theory (Gale *et al.*, 1972) that the splitting of the Soret is very much less than that of the α-band, typically no more than one-tenth with one-fifth being the upper limit. This puts the Soret

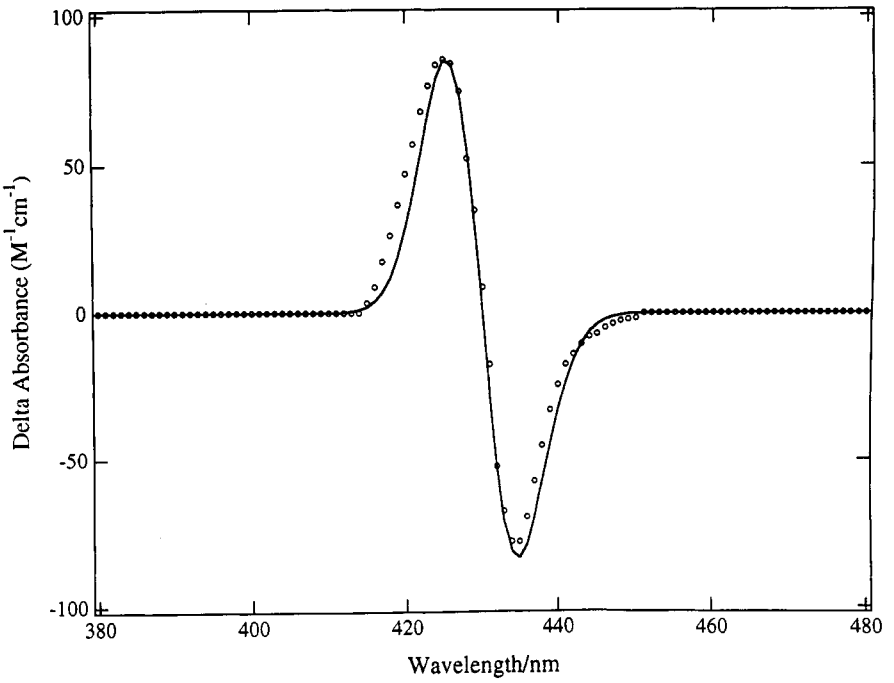

FIGURE 5: Fit to the observed reduced – oxidized CD spectrum of yeast cytochrome *b*. The data are represented by the open circles, and the line is the CD spectrum calculated as described in Materials and Methods using a value for the dihedral angle of 45° and a Fe–Fe separation of 19.5 Å. The line-width parameter was set to 600 cm^{-1}.

splitting at no more than 60 cm^{-1}, equivalent to a ±0.5-nm displacement about a mean value of 429 nm. If an amino acid with a suitable $\pi \rightarrow \pi^*$ transition (e.g., tyrosine) were located close to the heme of cytochrome b_L and were oriented such that the transition dipole of this $\pi \rightarrow \pi^*$ transition bifurcated the B_x and B_y transition dipoles, then one component of the Soret transition would have a positive Cotton effect while the other would exhibit a negative Cotton effect of comparable intensity. As the absorption extremes of these transitions are close to one another, the net CD from the combination of these two transitions will be a biphasic spectrum with zero amplitude at the apparent maximum of the unresolved absorption spectrum.

In this competing interpretation, the intensity of the rotational strength that would be developed is reduced from that of two identical chromophores by the quantity $V_{12}/\Delta E$ (Pearlstein, 1982), where ΔE is the difference in energies (λ_m) of the heme and the tyrosine, and V_{12} is the strength of the coupling between them; ΔE is about 13 000 cm^{-1}. To develop a rotational strength comparable to that obtained with the coupled hemes, V_{12} must approach ΔE; this requires that the heme and the phenol ring of the tyrosine be very close. For example, if we assume that the transition dipole of the tyrosine is the same as that of the heme (essentially the maximum allowed value), the calculated value for V_{12} at a separation of 3.5 Å is about 5000 cm^{-1} and $V_{12}/\Delta E = 0.4$ (3.5 Å is the typical interplanar distance of aromatic systems). But at this distance the rotational strength is reduced additionally by a factor of 5 via its dependence on R_{12} (eq 3). Thus at this distance, and even with the optimum angle, the calculated rotational strength would be more than an order of magnitude smaller than that observed, and even this value requires a closeness of approach between the tyrosine and the heme which is difficult to envision given the presumed bis-histidine coordination which exists in these proteins. Furthermore, with this large value of V_{12} the position of the Soret maximum would be red-shifted by ca. 80 nm (Mason, 1982). While the λ_m for the mitochondrial cytochrome *b* is larger

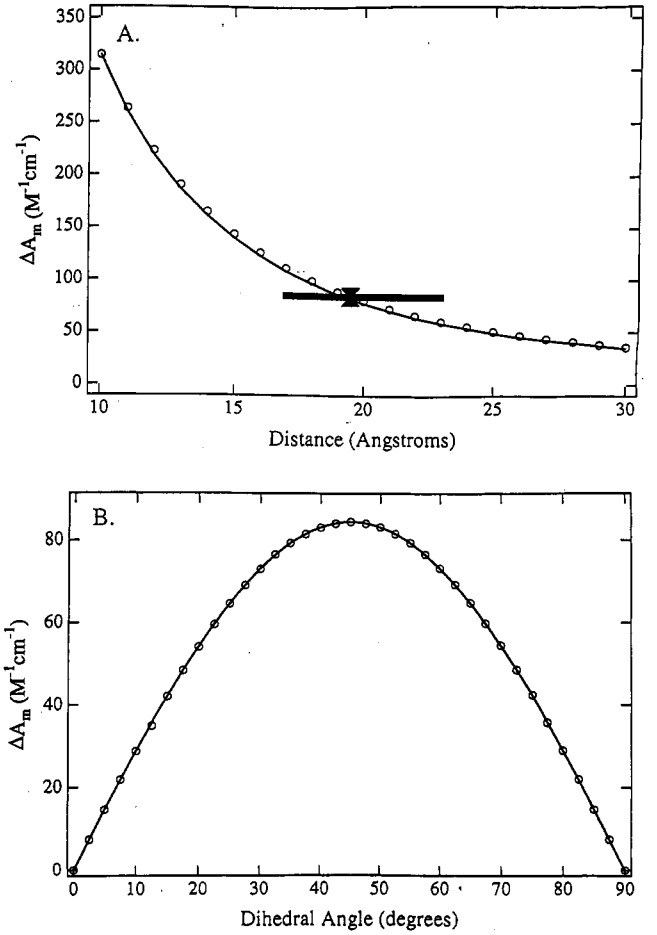

FIGURE 6: (A) Dependence of calculated CD intensity (O) of either band of the CD couplet on R^{-2}. The solid line is the theoretical decay for a R^{-2} dependence. The bar denotes the range of possible distances deduced from fitting the experimental data, and the dumbbell indicates the most probable distance (cf. Figure 5). (B) Dependence of calculated CD intensity (O) on the dihedral angle (θ) at a fixed distance of 19.5 Å; the solid line is calculated as sin (2θ).

than those for prototypic bis-imidazole heme proteins (for example, cytochrome b_5) and analogous model compounds, which typically have a maximum at ca. 423 nm, the 6-nm red shift observed with the mitochondrial cytochrome *b* would require that V_{12} be no larger than 1500 cm^{-1} if this shift were indeed the result of a dipole–dipole interaction.

Molecular Aspects of the Redox-Linked Conformational Change of Mitochondrial Cytochrome b. The substantial increase in the rotational strength of the CD spectra of mitochondrial cytochrome *b* upon reduction of its low-potential heme (Figure 3 and Table 1) can be rationalized within the framework of the above exciton calculations as a large modification of the mutual orientation of the two hemes. A simple explanation would be that the chiral angle between the coupled electronic transitions of the two hemes is between 0 and 10 deg where b_L is oxidized and increases to ca. 45 deg when b_L is reduced. In other words, upon reduction the plane of one heme would rotate by over 30 deg with respect to that of the other.

How can we envisage this change in heme geometry in the light of the present knowledge of the cytochrome *b* structure? It is likely that the heme-coordinating domain of cytochrome *b* is organized as an antiparallel bundle of transmembrane helices (Link *et al.*, 1986; Degli Esposti *et al.*, 1989b, 1993; Tron *et al.*, 1991; Crofts *et al.*, 1992). Although the helical bundles of metalloproteins of known atomic structure form rather compact domains, in the case of heme proteins such as *E. coli* cytochrome *b*-562, a spatial divergence at one side of the bundle is present to create the internal binding pocket for the heme (Mathews *et al.*, 1979; Weber & Salemme, 1980). Hence, the transmembrane bundle of cytochrome *b* should present a spatial divergence of the helices at both sides of the membrane (Degli Esposti *et al.*, 1989b) or have helices with a longitudinal axis substantially tilted out of the membrane normal (Farid *et al.*, 1993) so as to be able to accommodate the binding pockets of the two hemes.

There are conserved amino acids within the ligand helices B (previously II) and D [previously V, cf. Crofts *et al.* (1992)], especially Asn_{83}, Gly_{84}, Ser_{87}, and Pro_{187}, which have strong helix-breaking character (Richardson & Richardson, 1989; Degli Esposti *et al.*, 1989b). These residues lie between the middle and the positive side of the membrane and most likely induce bends or heterogeneity in the secondary structure of the transmembrane segments. These features may be essential for creating the spatial divergence to accommodate the b_L heme and, additionally, could confer flexibility to the protein structure, e.g., via exchange of hydrogen bonds between the amino acid side chain and the backbone main chain. A flexibility of the cytochrome *b* structure at the positive side of the membrane is required to permit the redox-linked change in the orientation of one heme with respect to the other that is deduced from the exciton coupling calculations.

A lesser change in the chiral orientation of the two hemes, for example, a canting of one with respect to the other, would also lead to the appearance of optical activity, but as the chiral relationship would be less than optimal, the heme–heme separation would have to be much smaller if the experimental values were to be reproduced. In view of the disposition of the four invariant histidines, it seems most unlikely that this decrease in Fe–Fe distance could be possible.

Other Applications of Exciton Analysis of Bis-heme Systems. The present study has provided a strategy for deriving structural information for a membrane bis-heme protein on the basis of exciton analysis of CD spectra. In principle, similar applications should be possible for other membrane bis-heme systems whenever their CD spectra provided evidence for heme–heme interactions. An example could be cytochrome oxidase for which structural models with a chiral configuration of the hemes have been proposed (Holm *et al.*, 1987; Brown *et al.*, 1993). However, in agreement with earlier data of Myer (1978), we have been unable to detect any evidence for exciton interaction in the CD spectra of cytochrome oxidase in any oxidation state, even though the a–a_3 distances would support such an interaction (Holm *et al.*, 1987; Brown *et al.*, 1993). This absence of exciton interaction in cytochrome oxidase is consistent with a coplanar disposition of the two hemes, as suggested by a recent model (Hosler *et al.*, 1993).

Cytochrome *b*-558 of the succinate:quinone reductase of the soil bacterium *Bacillus subtilis* (Hagerhall *et al.*, 1992) provides a recently discovered example of another bis-heme membrane protein. The cytochrome *b*-558 protein isolated from an *E. coli* strain overexpressing the gene of *B. subtilis* basically retains the spectroscopic properties that are seen in the parent reductase complex purified from *B. subtilis* membranes (Friden *et al.*, 1990; Hagerhall *et al.*, 1992). Two *b* hemes are present with different EPR spectra and midpoint redox potentials (Hagerhall *et al.*, 1992), and the CD spectra in the Soret region indicate heme–heme interactions since they resemble those of cytochrome *b* within the *Rhodobacter* bc_1 complex [M. Degli Esposti, unpublished results; cf. Table 1 and Degli Esposti *et al.* (1989a)]. In particular, the CD spectrum of reduced *B. subtilis b*-558 has a couplet with a positive band stronger than the negative band and also stronger than that of mitochondrial cytochrome *b* (Table 1).

By assuming a geometrical configuration for the two hemes similar to that we which use for cytochrome *b*, we have fit the intensity of the positive band of the reduced cytochrome according to our exciton analysis (see Materials and Methods). As with the mitochondrial cytochrome the positive and negative lobes are of different amplitudes and widths, and fitting the lobes individually led to values for R_{12} in the range 16.5–23 Å. It thus seems that the bis-heme structure of *B. subtilis* cytochrome *b* is similar to that of mitochondrial cytochrome *b*. The geometry deduced from these CD exciton calculations is compatible with both the structural model of cytochrome *b*-558 proposed by Degli Esposti *et al.* (1991) and that recently suggested by Hederstedt and co-workers (Hederstedt & Ohnishi, 1992; Hagerhall *et al.*, 1992).

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