Optical Rotatory Properties of the Cytochromes c_3 from Three Species of *Desulfovibrio**

Harvey Drucker,† L. Leon Campbell, and Robert W. Woody

ABSTRACT: We have studied the absorption, optical rotatory dispersion, and circular dichroism of cytochrome c_3 isolated from three species of *Desulfovibrio: D. desulfuricans*, *D. vulgaris*, and *D. salexigens*. The three proteins have similar but not identical absorption and optical rotatory properties, suggesting that the heme environment is similar in all three proteins, despite substantial differences in amino acid composition and electrophoretic and immunological behavior. Rotational strengths for the principal rotational bands in the visible and near-ultraviolet regions have been determined. The Soret band shows evidence of two circular dichroism components of opposite sign in both the oxidized and reduced states, with the positive shorter wavelength component dominant. Possible explanations of the split Soret band are considered.

We have also studied the acid denaturation of one of the three proteins in the oxidized state and observed a very sharp transition at about pH 2.6 with indications that about six protons are involved in the transition from native to denatured protein.

Cytochrome c_3 plays a key role in the terminal reductive steps of sulfate metabolism in the nonsporulating sulfate reducing bacteria of the genus *Desulfovibrio*. Recent work (Drucker and Campbell, 1969; Drucker *et al.*, 1970) has shown that the cytochrome c_3 from three different species of this genus, *D. desulfuricans*, *D. vulgaris*, and *D. salexigens* are electrophoretically and immunologically distinct and have significantly different amino acid compositions. Nevertheless, there are also striking similarities among these three proteins. Their hydrodynamic properties are quite similar, and their redox potentials are essentially identical. The number of residues and molecular weight based on amino acid analysis are also nearly the same for the three proteins. Furthermore, when amino acids are grouped into chemically similar types (basic, acidic, hydroxy, aromatic, etc.) the gross amino acid compositions become much less distinct (Drucker *et al.*, 1970), suggesting that amino acid replacements may have been largely of a conservative type (Smith and Margoliash, 1964). One of the most intriguing aspects of these cytochromes c_3 is that they contain three heme groups per molecule (Drucker *et al.*, 1970), and given the molecular weight of 13,000–14,000, the hemes account for about 15% of the protein.

We have studied the absorption, circular dichroism, and optical rotatory dispersion spectra of these three proteins. Although there are differences among the three proteins in these optical properties, the overall similarity strongly suggests, as does the identity of their redox potentials, that the environment of the hemes is substantially the same in these proteins. The circular dichroism and optical rotatory dispersion of these cytochromes c_3 is quite different from that of mammalian cytochrome *c*. We have analyzed the circular dichroism spectra of the three proteins in both the oxidized and reduced forms and obtained the rotational strengths of the Soret and visible bands.

For one of the proteins, oxidized *D. vulgaris* (8303) cytochrome c_3, we have studied the denaturation which occurs at low pH. This transition, which leads to large and parallel changes in optical rotatory dispersion, circular dichroism, and absorption spectra, occurs over a narrow pH range near pH 2.6. The sharpness of the transition suggests a cooperative transition. We estimate that about six protons are taken up in the transition.

Materials and Methods

Isolation of Proteins. VC[1] and 8303 cytochromes c_3 were purified by a variation (Drucker and Campbell, 1969) of the method of Horio and Kamen (1961). BG cytochrome c_3 was purified as described by Drucker (1967). The material used was treated (Drucker *et al.*, 1970) to remove excess iron, desalted, and lyophilized.

Absorption, Circular Dichroism, and Optical Rotatory Dispersion Spectral Studies. Lyophilized cytochrome was dissolved in 0.01 M Tris-HCl buffer, pH 8.1, at a concentration of about 0.4–0.5 mg/ml. The solution was dialyzed overnight against 200 volumes of the buffer. Exact concentrations were determined after a run from the absorbance at 552 mμ

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[1] The cytochrome c_3 from *D. vulgaris* was from strain Hildenborough, National Collection of Industrial Bacteria (NCIB) 8303, hereafter referred to as 8303 cytochrome c_3. The strain of *D. desulfuricans* used was cholincius, American Type Culture Collection 13541, referred to here as VC. The representative of *D. salexigens* was Strain British Guiana NCIB 8403, and is referred to in this work as BG.

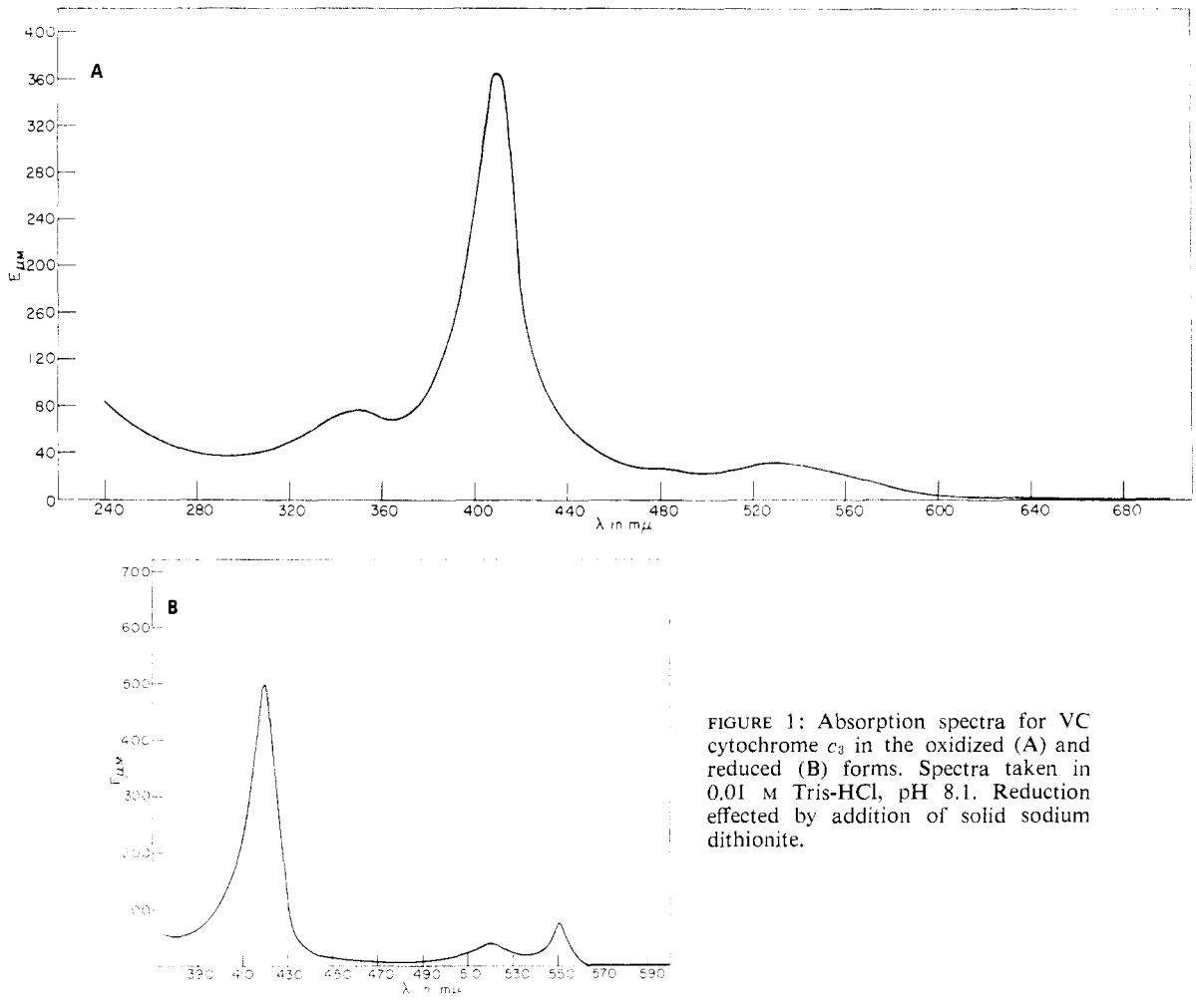

FIGURE 1: Absorption spectra for VC cytochrome c_3 in the oxidized (A) and reduced (B) forms. Spectra taken in 0.01 M Tris-HCl, pH 8.1. Reduction effected by addition of solid sodium dithionite.

in the reduced form. The reduction was carried out by addition of solid sodium dithionite. A millimolar extinction coefficient at 552 mμ of 84 was assumed for the reduced state of 8303, 74 for VC, and 76 for BG (Drucker *et al.*, 1970). Absorption spectra were run on a Cary 14 recording spectrophotometer, using 1-mm quartz cells and were performed at room temperature. Optical rotatory dispersion and circular dichroism measurements were performed on a Jasco ORD/UV-5 spectropolarimeter with circular dichroism accessory. Water-jacketed cells (Opticell) of 10-mm path length were used for wavelengths longer than 450 mμ, and 1-mm cells for wavelengths longer than 450 mμ, and 1-mm cells for wavelengths below 450 mμ. Because of the longer times required for optical rotatory dispersion and circular dichroism measurements, the cells were thermostatted at 20° with a thermostatted pump (Haake) and a refrigerated water bath (Formatemp).

Molar rotations were calculated using the relationship

$$[M] = \frac{\alpha}{l'c}\frac{M}{100}$$

where α is the observed rotation in degrees, l' is the path length in decimeters, c the concentration in grams per milliliter, and M is the molecular weight. Unless otherwise specified, the molar rotations reported here are on a per heme basis, *i.e.*, M = molecular weight of protein/3. Circular dichroism values reported as molar ellipticities:

$$(\theta) = 3300\Delta E = 3300\frac{\Delta OD}{lc}\frac{M}{1000}$$

where ΔE is the difference in molar extinction coefficient for left and right circularly polarized light, ΔOD is the observed difference in optical density for left and right circularly polarized light, and l is the light path in centimeters. Molar ellipticities reported here are on a per heme basis.

Acid Denaturation Studies. The acid titration of oxidized 8303 cytochrome c_3 was done on lyophilized material dissolved in 0.01 M $NaClO_4$ at a concentration of about 1 mg/ml. The solution was dialyzed overnight against 200 volumes of 0.01 M $NaClO_4$. The protein concentration was determined on an aliquot as described above, and the remaining solution was diluted to a concentration of 0.4–0.5 mg/ml with 0.01 M $NaClO_4$.

Acid titrations were performed at room temperature by addition of 1 M perchloric acid, previously standardized against 1 M NaOH (Acculute), with phenolphthalein as

TABLE I: Spectral Properties of Cytochrome c_3, Cytochrome c, and Hemoglobin.

	α Band		β Band		Soret Band	
	λ_{max}	E (μM)	λ_{max}	E (μM)	λ_{max}	E (μM)
			Oxidized			
Cytochrome c_3[a]						
8303	535	12.7			409	137
VC	535	10.3			410	122
BG	535	9.7			409	122
Cytochrome c[b]	528	11			410	106
Hemoglobin[c]	503	9			406	142
			Reduced			
Cytochrome c_3[a]						
8303	552	28.0	522	15.0	418	181
VC	552	24.7	522	13.3	420	167
BG	552	25.3	522	13.3	419	171
Cytochrome c[b]	550	28	521	16	416	129
Hemoglobin[d]	555	13.5			430	119

[a] This work. Extinction coefficients on a per heme basis. [b] Margoliash and Frohwirt (1959). [c] Lemberg and Legge (1949). Extinction coefficients on a per heme basis. [d] Rossi-Fanelli *et al.* (1959). Extinction coefficients on a per heme basis. Data are for deoxyhemoglobin, which shows only one distinct maximum in the 500–600-mμ region.

indicator. pH values were determined with a Radiometer TTT1C pH meter, using an expanded scale (Radiometer PHA 630 T) and a Radiometer combination electrode (Type C). The pH meter was calibrated with Beckman standard buffers of pH 7.0, 4.0, and 2.0 during the course of the titration. At each pH, a 0.2-ml sample was removed for optical rotatory dispersion and spectral measurements.

The optical rotatory dispersion measurements were performed as described above. The absorption spectra were run on a Cary 15 recording spectrophotometer equipped with a thermostatted cuvet holder. In both the optical rotatory dispersion and circular dichroism studies, the temperatures in the cell were determined from a calibration curve established by measurements with a copper–constantan thermocouple.

Results

Spectral Studies of Native Proteins. The absorption spectra of all three cytochromes c_3 studied here are quite similar in the oxidized and in the reduced state. The spectrum of VC cytochrome c_3 is representative and is shown in Figure 1. There are quantitative differences in extinction coefficients among the three proteins, however, and the values of the extinction coefficients at maxima are given in Table I. Also shown in Table I are the corresponding parameters for hemoglobin (Lemberg and Legge, 1949) and for horse heart cytochrome c (Marogliash and Frohwirt, 1959). All extinction coefficients are given on a per heme basis.

The Soret peak is at 408–410 mμ in the oxidized form, and there is a broad band centered at 530 mμ. There is another weak band at 350 mμ, but there is no maximum in the aromatic absorption region. The positions of the absorption bands are similar to those of cytochrome c. The Soret intensity, however, is substantially greater for cytochrome c_3 than for cytochrome c, and in the oxidized form, is comparable with that of methemoglobin.

The circular dichroism curves for the three proteins in the oxidized and reduced forms are given in Figures 2–4. The oxidized proteins all show a broad positive band in the

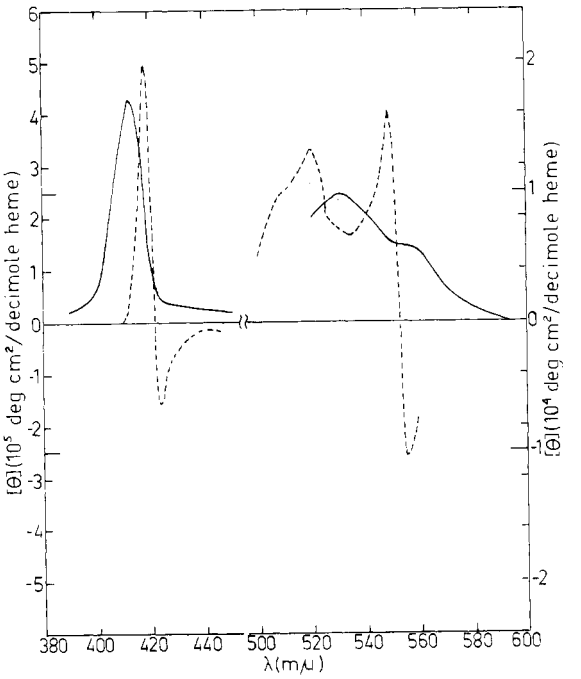

FIGURE 2: Circular dichroism curves for 8303 cytochrome c_3: oxidized (——) and reduced (– – –). Conditions as described in Figure 1.

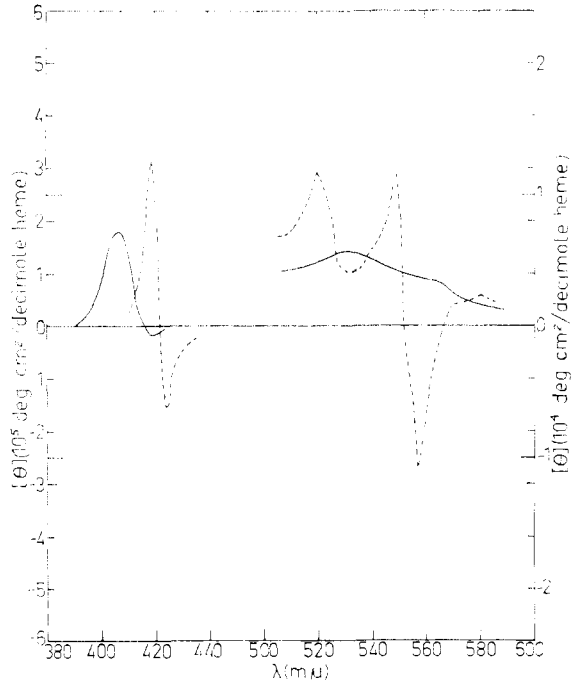

FIGURE 3: Circular dichroism for VC cytochrome c_3: oxidized (—) and reduced (– – –). Conditions as described in Figure 1.

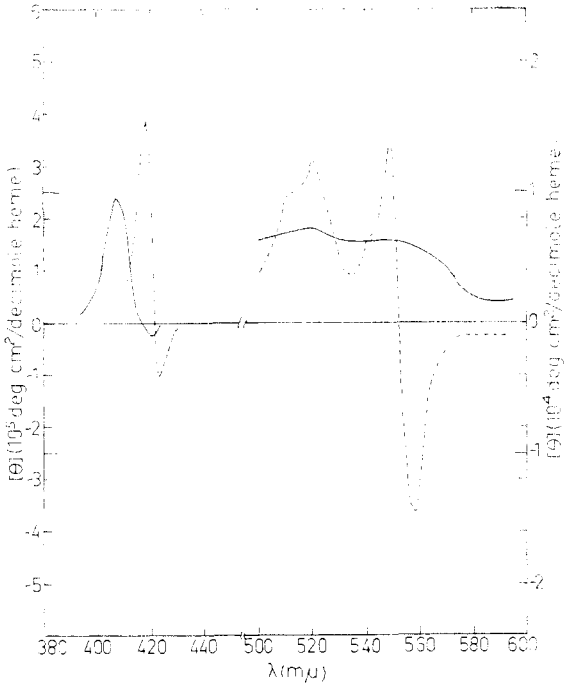

FIGURE 4: Circular dichroism curves for BG cytochrome c_3: oxidized (——) and reduced (– – –). Conditions as described in Figure 1.

500–600-mμ region. Measurements in this region proved to be quite difficult because of the small signals, and there is appreciable uncertainty in our data for this region. From data on the 8303 cytochrome c_3, it is not clear how many rotational bands occur in this region. The data on the BG and VC proteins, however, indicate at least two positive bands.

In the Soret region, the 8303 protein shows what at first sight is a single positive band. On closer examination, this band appears to be asymmetric. The VC and BG proteins show a distinct negative component at longer wavelengths.

In the reduced proteins, the bands are strikingly sharpened. In the 550–560-mμ region, two sharp bands appear—a short-wavelength positive band and a long-wavelength negative band. The crossover point is near the absorption maximum of the α band at 552 mμ, suggesting that the α band is split into two components. Corresponding to the β band is a broad positive band with a maximum at about 520 mμ. There is some indication that this band consists of two positive bands in the 8303 protein, and this is more clearly the case in VC and BG. The Soret band in the reduced form shows a definite splitting with a positive short-wavelength component and a negative long-wavelength component.

We have attempted to analyze these data by fitting a minimal number of Gaussian circular dichroism bands to the observed circular dichroism curves. A computer program using a nonlinear least-squares method (Deming, 1938) for optimizing the parameters was employed. The contribution of the ith band to the circular dichroism, $[\phi_i]$, is assumed to be of the form

$$[\theta_i] = [\theta_i^\circ] \exp\left[-\left(\frac{\lambda - \lambda_i}{\Delta_i}\right)^2\right]$$

where $[\theta_i^\circ]$ is the amplitude in molar ellipticity units, λ_i is the wavelength of the band center, and Δ_i is the half-width of the band between the two points where $[\theta_i] = [\theta_i^\circ]/e$, e being the base of natural logarithms. Once a satisfactory fit is obtained, the rotational strengths can be readily calculated from the amplitudes and the half-widths (Moscowitz, 1960). As a further check, the rotational strengths were used to generate a calculated optical rotatory dispersion curve using the expression given by Moscowitz (1960) for the Kramers–Kronig transform of a Gaussian circular dichroism band.

Figure 5 shows a comparison of calculated and observed circular dichroism spectra for oxidized and reduced 8303 cytochrome c_3. In the oxidized form, two bands of opposite sign were used to fit the Soret circular dichroism and two positive bands to fit the long-wavelength region. The agreement is quite satisfactory for the Soret band. There are discrepancies in the wings of the long-wavelength band, but here the experimental uncertainties are quite large. The agreement for VC and BG is equally good.

For the reduced form, six bands were assumed—pairs of positive and negative bands in the Soret and α region and two positive bands in the β region. Although minor discrepancies remain, our calculated curve provides a good fit over most of the wavelength range considered. Again, comparably good fits were achieved for the reduced VC and BG data.

In Figure 6, the calculated and observed optical rotatory dispersion curves are compared for 8303 cytochrome c_3. Since the calculated curves do not include contributions from far-ultraviolet transitions, which would be expected to provide a negative background monotonically increasing in magnitude with decreasing wavelength, it is the shape of the curve, not the absolute value, which is significant. It is clear that in general our circular dichroism parameters are adequate for reproducing the optical rotatory dispersion curves for both oxidized and reduced forms of 8303 cytochrome c_3. They

TABLE II: Circular Dichroism Bands of Cytochrome c_3 from Three Species of *Desulfovibrio*.

	8303			VC			BG		
	λ (mμ)	*R* (DBM)[a]	Δ (mμ)	λ (mμ)	*R* (DBM)	Δ (mμ)	λ (mμ)	*R* (DBM)	Δ (mμ)
Oxidized	408.4	0.751	8.4	408.8	0.631	8.7	408.0	0.755	7.0
	413.9	−0.141	4.2	414.1	−0.263	6.1	411.1	−0.326	7.8
	529.7	0.055	25.1	526.6	0.024	18.6	528.3	0.065	41.8
	560.3	0.008	10.8	560.0	0.020	25.8	564.1	0.005	14.3
Reduced	418.3	0.528	3.25	418.9	0.435	3.63	418.0	0.765	4.40
	422.8	−0.327	5.21	423.2	−0.302	4.99	420.6	−0.422	5.00
	509.1	−0.0002	4.61	516.2	0.044	2.00	517.6	0.036	21.50
	518.0	0.054	15.4	520.9	0.004	3.81	519.1	0.010	7.08
	552.4	0.060	6.30	549.7	0.021	8.25	551.6	0.028	7.21
	554.4	−0.058	5.38	556.0	−0.020	5.24	555.7	−0.035	6.38

[a] The unit of rotational strength is the Debye–Bohr magneton (DBM) = 0.9273×10^{-38} cgs unit.

also suffice for the oxidized forms of the VC and BG proteins. In the case of reduced VC and BG, however, a significant discrepancy occurs in the region of the α band. The calculated optical rotatory dispersion curve has approximately the correct shape but an amplitude which is too small by a factor of 3 for VC and 7 for BG. We believe that these discrepancies are due to inadequate resolution in measuring the very sharp overlapping circular dichroism bands of opposite sign associated with the α band. The amplitude of the circular dichroism band will be reduced much more strongly by this effect than will that of the optical rotatory dispersion Cotton effect. Thus, the rotational strengths reported for the α-band components of reduced VC and BG are lower limits.

The parameters obtained from this analysis—rotational strengths, band positions, and widths—are given in Table II. The most interesting points to be noted are: (1) there is a substantial splitting of the Soret band. The splittings are of the order of 3–5 mμ. (2) The total rotational strength of the split components is not zero. (3) The rotational strengths of the

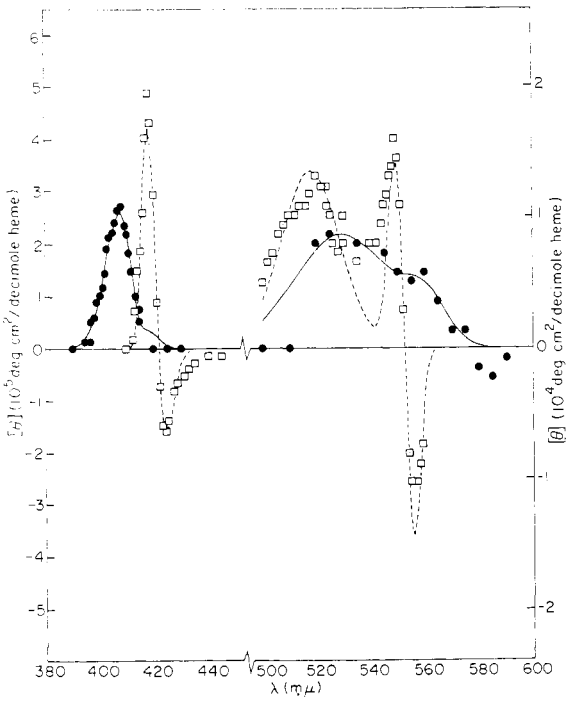

FIGURE 5: Comparison of observed 8303 cytochrome c_3 circular dichroism curves with that calculated from Gaussian parameters: calculated curves: oxidized (——), reduced (– – –); experimental points: oxidized (●), reduced (□).

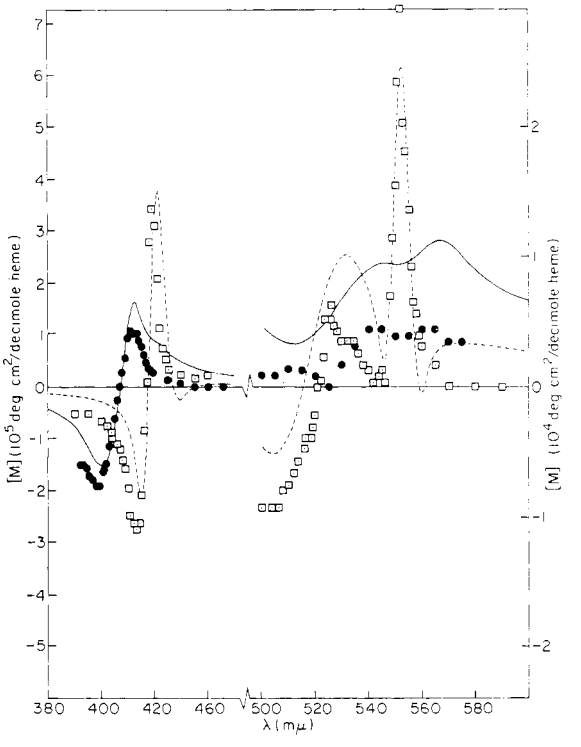

FIGURE 6: Comparison of observed 8303 cytochrome c_3 optical rotatory dispersion curve with that calculated from Gaussian parameters: calculated curves: oxidized (——), reduced (– – –); experimental points: oxidized (●), reduced (⊡).

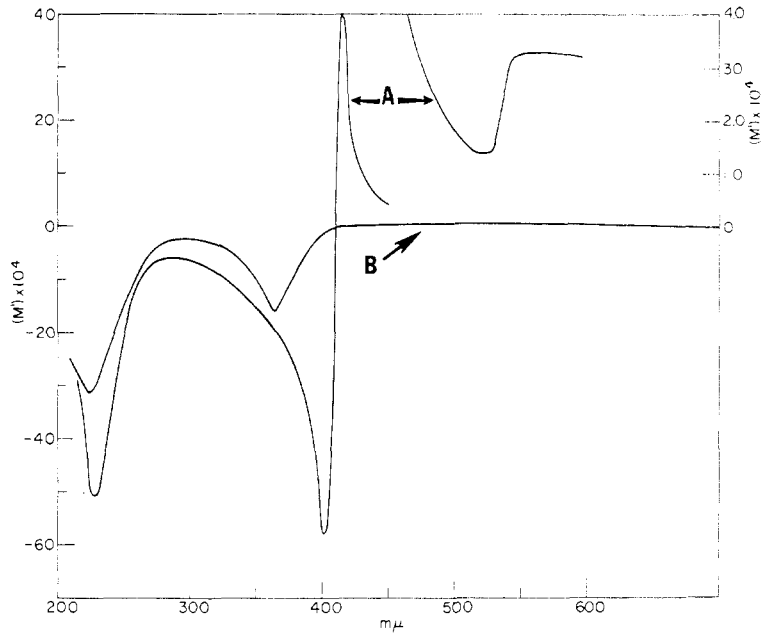

FIGURE 7: Comparison of optical rotatory dispersion curves for native (A) and acid-denatured (B) 8303 cytochrome c_3. Native optical rotatory dispersion spectrum taken at pH 6.0 in 0.01 M $NaClO_4$. Acid-denatured curve taken at pH 2.35 in 0.01 M $NaClO_4$.

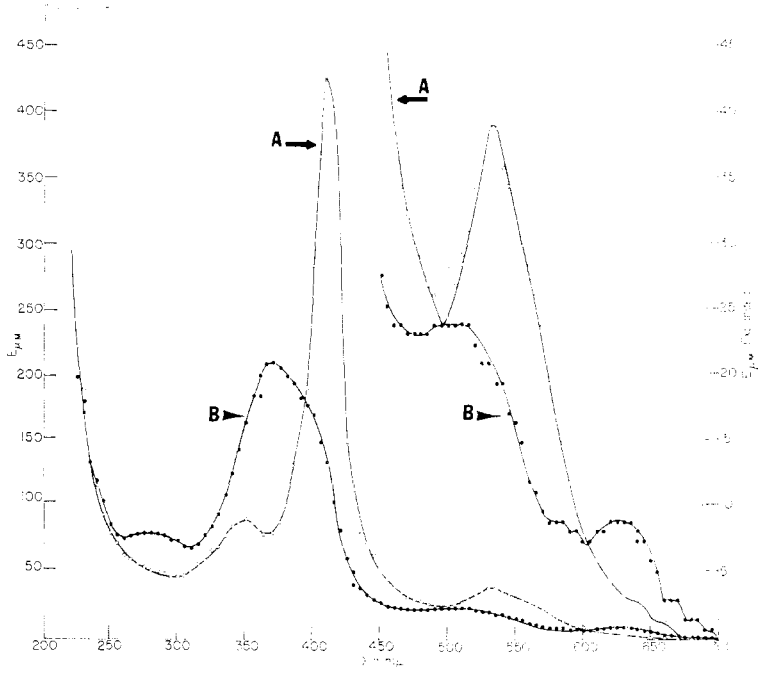

FIGURE 8: Comparison of absorption spectra for native (A; ○) and acid-denatured (B; ●) 8303 cytochrome c_3. Conditions as described for Figure 7.

Soret bands are large, of the order of 0.1–0.8 DBM.

Acid Denaturation of Oxidized 8303. The optical rotatory dispersion spectra of native and denatured 8303 cytochrome c_3 show marked differences (Figure 7). The native protein (pH 6.0) shows a large positive Cotton effect in the Soret region, with a maximum at 416 mμ and a minimum at 400 mμ. There is also a complex optical rotatory dispersion feature in the α,β-band region, and a trough at 233 mμ. On lowering the pH to 2.35, the optical rotatory dispersion curve becomes essentially featureless in the visible, the positive Soret Cotton effect is replaced by a complex feature with a minimum at 365 mμ and the far-ultraviolet trough is shifted to shorter wavelengths and less negative rotations. These large changes, the most striking of which are associated with heme absorption bands, indicate a major change in the environment of the hemes.

Absorption spectral changes also reflect this change in heme environment upon acid denaturation (Figure 8). The most striking spectral effect is the disappearance of the sharp 409-mμ peak of the native protein. In the acid-denatured protein, the Soret band is much broader, with a maximum at 365 mμ and a shoulder at 395 mμ. In the visible region, the 530-mμ band is replaced by two broad bands of lower intensity at about 500 and 630 mμ.

These observations suggest three useful measures of the

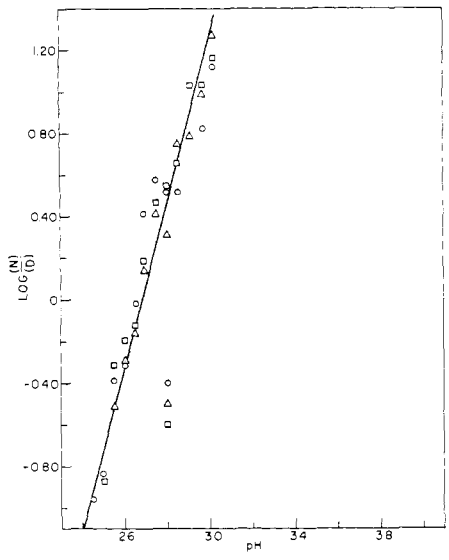

FIGURE 9: Double logarithmic plot for acid denaturation of 8303 cytochrome c_3 at 25°. Rotation per heme (⊙), E_{409} (⊡), E_{362} (△).

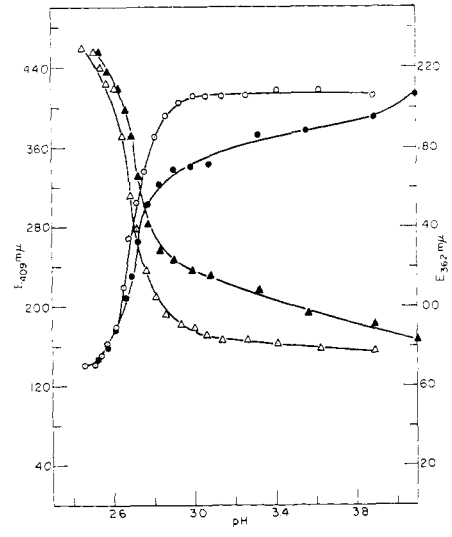

FIGURE 10: Acid denaturation of 8303 cytochrome c_3 of 25° as measured by changes in absorption spectra: E_{409}' forward titration (⊙); E_{409} back-titration (●); E_{362} forward titration (△); E_{362} back-titration (▲).

degree of denaturation: the amplitude of the Soret Cotton effect in optical rotatory dispersion, and the absorbance at 409 and 362 mμ. The amplitude of the Soret Cotton effect was defined by the difference between the rotation per heme at the peak (416 mμ) and trough (400 mμ), $[M']_{416} - [M']_{409}$. When this amplitude, or the millimolar extinction coefficients at 362 or 409 mμ (E_{362} and E_{409}, respectively) are plotted *vs.* pH, a sigmoidal curve results.

We have assumed that the denaturation can be treated as a two-state equilibrium between native and denatured protein, the two forms differing in degree of protonation

$$N + n(H^+) \rightleftharpoons D$$

where N denotes native protein, D is the denatured protein, and n is the number of extra protons bound by the denatured form. The two-state assumption allows us to formulate an equilibrium constant

$$K = \frac{(D)}{(N)(H^+)^n}$$

The fraction of native protein is calculated from the experimental data

$$f_N = \frac{I - I_D}{I_N - I_D}$$

where f_N is the fraction of native protein, I is the observed value of the optical property at a given pH, and I_N and I_D are, respectively, the values of this property for the native and denatured forms. Using the two-state hypothesis,

$$f_D = 1 - f_N.$$

A plot of log f_N/f_D against pH should then yield a straight line of slope n and intercept pH_0, the pH of the midpoint of the titration, which corresponds to an apparent pK.

A typical plot is shown in Figure 9, again for data at 25°. Within experimental error, all three spectral parameters show the same pH dependence. Further, there is no evidence of any inflections, suggesting that all three hemes titrate simultaneously. The data for 5, 15, 40, and 50° give essentially the same kind of agreement among the various spectral parameters.

It was found, however, that these titrations were not completely reversible. Only a few points were taken in the reverse direction, and there was appreciable scatter in these points, which are not shown in Figure 9. On returning to the original pH, it was found that some of the protein was irreversibly denatured, judging from the spectral parameters. Such irreversibility is not surprising, given that each titration required some 3–5 days for completion, and thus involved long exposure to low pH at room temperature.

To reduce the extent of irreversibility, we carried out a second series of titrations measuring only the absorption spectra. These experiments required only about 12 hr, and thus greatly decreased the exposure of the protein to low pH. Figure 10 shows a plot of the forward and back-titrations at 25°, as measured by the absorption spectra. There is clearly a substantial amount of hysteresis. However, if one assumes that a fixed amount of irreversibly denatured material (approximately 25%) is present throughout the back-titration, one can correct the points for the reverse titration. This correction, which is determined from the plateau in the back-titration curve, does indeed suffice to bring the two titration curves into very good agreement. This is illustrated in Figure 11, where the data for log f_N/f_D are plotted on the same curve for both forward and back-titrations, after the reverse titration points have been corrected. It can be seen that the agreement among the four separate titrations is quite good. Similar results were found at 12 and 40°.

The data for these three temperatures were subjected to a

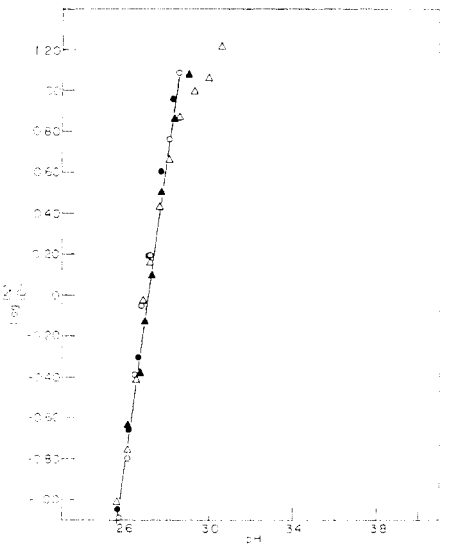

FIGURE 11: Double logarithmic plot of absorption data on the acid denaturation of 8303 cytochrome c_3 at 25° after correction for a fixed amount of irreversible denaturation at the low pH extreme: E_{409} forward titration (⊙); E_{409} back-titration (●); E_{362} forward titration (△); E_{362} back-titration (▲).

linear least-squares treatment, to find the best values for n and for pK. These values are listed in Table III, together with the percentage irreversible character assumed. At all three temperatures, it appears that 6 ± 1 protons are involved, and that the titratable groups have a pK of 2.7 ± 0.1. There does not appear to be any substantial temperature dependence of these results.

Since these titrations were done at low total ionic strength, the ionic strength changes significantly during the course of the experiment. Therefore a control experiment in which the ionic strength was varied by adding solid $NaClO_4$ to vary the concentration of $NaClO_4$ from 0.01 to 0.20 M. None of the optical properties were affected over this range of concentration.

Discussion

A comparison of the spectral and optical rotatory properties of the three proteins considered here leads to the conclusion that the heme environment in all three proteins is very similar. Thus in spite of a large number of amino acid replacements, the heme environs must be substantially conserved. This is not unexpected since the hemes are the active site or sites of the molecule. This conservation appears also in the identity of the redox potentials for the three proteins, and also, more directly, in the composition and sequence of heme peptides (Drucker, 1967; E. B. Trousil and L. L. Campbell, unpublished data).

The absorption spectra of the cytochromes c_3 studied here are similar to mammalian cytochrome c spectra with respect to band positions and absorption intensities in the visible region (Margoliash and Frohwirt, 1959). However, the maximum extinction coefficient per heme in the Soret band is about 30% larger for the cytochromes c_3 than for mammalian cytochrome c (See Table I). This suggests that the heme

TABLE III: Results of Acid Titration of Oxidized 8303 Cytochrome c_3.

Temp (°C)	n	pK	% Irreversible Component
12	6.4	2.62	26
25	7.0	2.70	24
40	5.6	2.72	23

environment differs between these bacterial cytochromes c_3 and mammalian cytochrome c.

More striking still are the differences in optical rotatory properties. Mammalian cytochromes c show exceedingly complex but relatively weak circular dichroism and optical rotatory dispersion spectra in the visible and Soret regions (Myer, 1968; Flatmark and Robinson, 1968). In particular, the Soret circular dichroism band shows two overlapping bands of opposite sign but nearly equal magnitude. The amplitude of these ellipticity bands is approximately 3–4 × 10^4 deg cm²/dm in the oxidized form. Reduction leads to an inversion in sign, *i.e.*, the long-wavelength component, which is negative in the oxidized form, becomes positive in the reduced form, while the short-wavelength component undergoes a change in the opposite direction.

The cytochromes c_3 which we have studied show circular dichroism bands which are an order of magnitude larger than those of mammalian cytochromes c. Further, although the Soret band definitely exhibits a splitting, reduction does not lead to a reversal in the signs of the two components.

Of the heme protein circular dichroism spectra reported thus far, only three systems show Soret circular dichroism spectra resembling those of cytochrome c_3. Urry *et al.* (1967) and Urry and van Gelder (1968) have reported that reduced cytochrome oxidase shows a split Soret Cotton effect, but that the oxidized form shows a single positive circular dichroism band in the Soret region. Bartsch *et al.* (1968) have reported that cytochrome c-552 from *Chromatium* D′, which contains two hemes per molecule as well as a flavin, has a nearly symmetrical split Soret Cotton effect with an amplitude of about 10^5 deg/cm² dmole of heme. Reduction leads to a wavelength shift and a broadening of the bands but the oxidized and reduced spectra are qualitatively similar. Willick *et al.* (1969) have found that the carbon monoxide complex of reduced horseradish peroxidase shows a split Soret circular dichroism band, although the unliganded reduced protein and the oxidized protein and its cyanide derivative show only a positive Soret band.

There are several possible explanations for this splitting. (1) The Soret band in porphyrins with square-planar D_{4h} symmetry is doubly degenerate (Gouterman, 1961). Protoporphyrin and heme c have much lower symmetry, and interaction with the protein will reduce this still further. One therefore expects some splitting of this degenerate band and this has been clearly demonstrated by the work of Eaton and Hochstrasser (1967, 1968) on cytochrome c and metmyoglobin. Unfortunately, their measurements do not permit one to determine the size of the splitting. (2) Exciton coupling (Moffitt, 1956) due to the interaction of two or more hemes

can lead to splitting of the Soret band. This could be operative in cytochrome c_3, cytochrome oxidase (Urry *et al.*, 1967; Urry and van Gelder, 1968), and cytochrome *c*-552 (Bartsch *et al.*, 1968), each of which contains two or more hemes per molecule and has been invoked in each of the latter two cases. It cannot account for the case of the carbonyl derivative of reduced horseradish peroxidase which contains only one heme per molecule and which has been shown by Willick *et al.* (1969) to be monomeric. Furthermore, exciton coupling alone leads to a net rotational strength of zero, *i.e.*, the two or more split components must have rotational strengths of equal magnitude but opposite sign. This may be the case for cytochrome *c*-552 but it does not hold for cytochrome c_3 or for cytochrome oxidase. (3) A given electronic transition will in general have numerous vibrational components which may or may not be well resolved. It is possible that these may not all have rotational strengths of the same sign and Weigang (1965) has interpreted $n\pi^*$ circular dichroism bands of certain ketones which show sign changes in the middle of the band in this way. (4) Another transition (metal d→d or charge transfer) may overlap the Soret transition. The persistence of this splitting in both oxidized and reduced forms of cytochrome c_3 makes this seem quite unlikely however, for one would expect the energy of such a transition to depend strongly on oxidation state of the metal.

At present we cannot decide which of these alternatives is correct in the case of cytochrome c_3. However, we favor alternatives 1 or 2. The presence of a distinct electronic transition seems implausible, and a sign reversal among vibrational subbands for an electrically allowed transition does not appear probable. In a molecule as small as these cytochromes, and containing three hemes, the heme–heme distance cannot be very large and some kind of coupling would be expected. Theoretical work by R. W. Woody on the spectral effects of such heme–heme interaction is in progress.

The process of acid denaturation studied in this work leads to a change in the visible spectrum from a ferricytochrome *c* type to an acid methemoglobin type (Lemberg and Legge, 1949). The optical rotatory dispersion changes which occur simultaneously suggest that the heme goes from an asymmetric environment to a more nearly symmetrical one. This denaturation is largely reversible. Indeed, if the corrections we have described are based on correct assumptions, we have obtained thermodynamically significant results for that fraction of the material which behaves reversibly. Unfortunately, these corrections are somewhat *ad hoc* in character. Due to lack of material in the final stages of this study, we have not been able to obtain independent evidence for heterogeneity in the titrated protein, or ascertain the time and temperature dependence of the suspected irreversible reaction. Further studies are required to characterize the irreversible component and to find procedures which will eliminate it.

The sharpness of the titration and the parallelism of the three spectral parameters strongly imply that all three hemes titrate simultaneously in a cooperative transition. Our analysis of the data indicates that about six protons are taken up in the transition. In every heme protein for which there is any information on liganding, one coordination position of the iron is occupied by an imidazole. The uptake of three protons could therefore be utilized in protonating three coordinating imidazoles. The remaining protons are probably taken up by carboxylate groups—perhaps heme propionate side chains, or glutamate or aspartate side chains.

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