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Circular Dichroism and Resonance Raman Studies of Cytochrome b_{562} from *Escherichia coli*†

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ABSTRACT: A modified procedure for the isolation of cytochrome b_{562} from *Escherichia coli* is described. The preparation obtained has the same spectral properties as the twice-crystallized preparation [E. Itagaki and P. L. Hager (1966) *J. Biol. Chem. 241,* 3687] moves as a single band on gel electrophoresis and has an amino acid composition, except for the lysines, of that of the known sequence. The CD spectra of the ferric and ferrous protein in the region 185–600 nm and the resonance Raman spectra in the region 1300–1700 cm^{-1} upon excitation with 5145 Å are reported. Resonance Raman spectra of both the ferric and ferrous forms exhibit features characteristic of the low-spin electronic configuration of heme iron. The intrinsic CD spectrum is typical of α-helical proteins, 52% α helix, no β structure, and 49% random form in the case of the ferric protein and 49% α helix and no β structure in the reduced form. The aromatic CD spectrum of the ferric form exhibits a band at 260 nm, whereas the ferrous form exhibits bands at 274, 288, and 299 nm. The Soret spectra of the two forms are distinct; the ferric form has a large negative Cotton effect at 421 nm and a small positive peak at 398 nm, and the ferrous form has only two negative Cotton effects at about 423 and 435 nm. The visible spectrum of the ferric form is negative. A low-spin electronic configuration of heme iron in both valences of the metal atom and at neutral pH is established. From a consideration of the CD spectrum it is concluded that (1) heme is localized in an asymmetric environment, (2) heme undergoes a conformational alteration upon change of valence state of the metal atom, and (3) the oxidation–reduction process involves an alteration of the environment of the aromatic chromophores.

Optical activity and resonance Raman spectroscopy provide a unique combination of probes for the elucidation of the protein conformation of hemoproteins (Myer and Pande, 1978; Myer, 1978; Blauer, 1974) and heme stereostructure and the spin and oxidation states of the metal atom (Spiro and Loehr, 1975), two complementary aspects of structure–conformation–configuration relationships. Among the *b*-type cytochromes (Hagihara et al., 1975), circular dichroism (CD)[1] studies of a variety of proteins have been reported: b_5 (Huntley and Strittmatter, 1972), b_2 (Sturtevant and co-workers, 1969; Iwatsubo and Risler, 1969), and b_{555} and b_{563} (Okada and Okunuki, 1970a,b). Comparative resonance Raman (RR) studies of cytochromes *b* and b_5 have been reported by Adar and Erecinska (1974) and Kitagawa et al. (1975). These studies have led to a better comprehension of interrelationships between protein conformation, the presence or absence of prosthetic groups, the oxidation state of the metal atom, etc. (Myer and Pande, 1978; Myer, 1978), and the mode of differentiation among these proteins (Adar and Erecinska, 1974; Kitagawa et al., 1975), but a detailed elucidation of structural–conformational or structure–heme configuration relationships has been difficult to obtain. This is because most of these proteins are poorly characterized, i.e., they are of unknown sequence, amino acid composition, spin state of heme iron, etc., for example, cytochromes b_2, b_{555} and b_{563}. Since a question has been raised regarding the integrity of membrane-solubilized preparations, e.g. cytochrome b_2 (Jacq and Lederer, 1974), the long-term significance of findings for studies of these systems is uncertain. In this report we present CD and RR spectroscopic studies of a water-soluble and non-membrane-localized cytochrome *b*, b_{562} from *Escherichia coli* (Itagaki and Hager, 1966), which is devoid of all the problems mentioned above. Its amino acid sequence is known (Itagaki and Hager, 1968), the molecular site of the prosthetic group, a single iron protoporphyrin IX, has been elaborated (Warme and Hager, 1970), and recently preliminary results from X-ray diffraction have also been reported (Czerwinski et al., 1972). The suggestion that the three-dimensional structure of this protein is similar to that of myoglobin (Warme and Hager, 1970), on the one hand, and that the protein possibly functions as a soluble electron carrier, on the other (Hagihara et al., 1975; Itagaki and Hager, 1968; Warme and

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[1] Abbreviations used: CD, circular dichroism; RR, resonance Raman; DM, debye magneton, unit for rotatory strength of Cotton effects, R_k, 1 DM = 0.927×10^{-38} cgs units.

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Hager, 1970), makes the system unique, since such a function has always been attributed to cytochromes of type *c*.

Experimental Section

Materials. Aerobically grown *E. coli* type B cell paste was purchased from the Grain Processing Co., Muscatine, Iowa, and was kept at −20 °C until used. Calcium phosphate-gel-cellulose was prepared by the method of Swingle and Tisselius (1951). Hydroxylapatite, DEAE-cellulose, Sephadex G-50 and G-25, Bio-Gel P-6, and other chromatographic materials were obtained from commercial sources and used after appropriate regeneration when needed. All chemicals used were of either reagent or analytical grade. Deionized water twice-distilled in an all-glass still was used throughout these studies.

Isolation of Cytochrome b_{562}. The protein was first isolated according to the procedure described by Itagaki and Hager (1966), which was later modified for ease of preparation and better purification of the protein. The specific changes were (1) the reduction of the sonification step to 20 min using the Branson sonifier, Model S-124, at full power, (2) the centrifugation of the crude sonified suspension at 14 600*g* using a Servall SS-4 centrifuge for a period of 80 min, and (3) replacement of the chromatographic material for the first two stages of purification, calcium phosphate-gel-cellulose, by hydroxylapatite equilibrated with 0.02 M phosphate buffer, pH 7.0. All other procedures were exactly the same as used earlier. The protein concentrations at various stages of the preparation, the estimation of cytochrome b_{562}, etc., were performed as described by Itagaki and Hager (1966).

Gel Electrophoresis. Gel electrophoresis was performed in accordance with the Reisfeld method for the separation of cationic proteins (Reisfeld, 1962) using 7% polyacrylamide gel (7 × 0.5 cm) and an alanine-glacial acetic acid-water mixture (pH 4.5). Methyl green (0.2%) was used as the tracking dye and Coomassie brilliant blue as the staining dye.

Amino Acid Analysis. Amino acid analysis was performed after acid hydrolysis (6 N HCl in the presence of 0.01 mL of phenol) for 24 h. A home-made amino acid analyzer was used with either Durrum resin DC-6A using the pico buffer system-II[2] or Bio-Rad Aminex A-6 resin using a four-buffer system, pHs 3.25, 4.25, 5.9, 5.9 and citrate concentrations of 0.2, 0.2, 0.33, and 0.6 N, respectively.

Absorption Spectra. Absorption measurements were made with a Cary 15 spectrophotometer equipped with a Datex digital readout for both wavelength and absorbance. Cells varying in path lengths from 1 mm to 10 cm were used, depending upon the concentration of the solution.

Circular Dichroism Spectra. CD spectra were measured with a JASCO-J-10 dichrograph. Fused cylindrical cells with quartz windows, varying in path length from 0.2 mm to 5 cm, were used depending upon the absorbance of the solution in a given region of the spectrum. All measurements were made at room temperature, 20 °C, unless otherwise noted. The reduction of samples for both absorption and CD measurements was performed with the addition of a minimum amount of sodium dithionite while maintaining an anaerobic atmosphere over the solution. As an additional precaution, oxygen-free nitrogen was bubbled through the solution for a period of 20–25 min prior to reduction. The completion of reduction was tested by the addition of more reducing agent and measurement of the absorption spectrum. No further change in the absorption spectrum was taken as an indication of the completion of reduction. The CD spectrum above 250 nm is normalized as deg cm^2/dmol of protein, and below 250 nm as deg cm^2/dmol of amide bonds, using 109 amide bonds/molecule for this protein.

The resolution of the CD spectrum into its Gaussian constituents, the Cotton effects, was performed with the aid of a duPont 310 curve resolver. Two of the three parameters, band height and width, were adjusted for minimum deviation using least-square programming and a Univac 1110 computer. The following assumptions were made for resolution: (1) the Cotton effects were approximated by simple Gaussian bands, and (2) the high-wavelength spectrum for both the ferric and ferrous proteins, i.e., above 440 nm, was considered to be tail-end contribution from a single Cotton effect. The fitting was initiated with the generation of a Gaussian band approximating the high-wavelength end, and subsequent bands were added as deemed necessary.

Resonance Raman Spectra. Resonance Raman (RR) spectra were measured on a setup consisting of a Spectral Physics argon laser, Spex sample illuminator, Spex double monochromator, and Spex photon detection electronics. The instrument is controlled through a microprocessor, and the data are accumulated both in printed and tape form, which is later processed using a Univac 1110 computer. The samples were sealed in glass capillaries, and spectra were recorded with a 5145-Å laser line at 10–50-mW power, slit width 300 μm, in wavelength increments of 0.1 Å, the time constant depending upon the nature of the sample. The temperature of the capillary containing the sample was maintained at 2 °C by a water-jacketed capillary holder. During measurements of the oxidized form, it was observed that the sample undergoes slow photoreduction. This was prevented through the addition of a slight excess of potassium ferricyanide and checked by the absence of any abnormal intensity variations of bands in the region 1200–1650 cm^{-1} with varying light flux, from 10 to 50 mW. The integrity of the sample was established by comparison of the absorption spectrum before and after RR spectral measurement. For this purpose, the spectrum of the sample in the capillary was recorded directly using a Cary 15 spectrophotometer with a specially designed holder for the capillaries. The RR spectrum was corrected for instrumental error using indene, benzene, CCl_4, and $CHCl_3$ as references. The band positions and the peak areas were determined with the aid of a computer using 15-point first derivative convoluting function programming along lines similar to the program developed for analysis of the IR spectrum (Savitzky and Golay, 1964). No corrections were made for the intensity of the spectrum.

Results and Discussion

Isolation. The procedure employed for the isolation of cytochrome b_{562} from *E. coli* was a slight modification of the methods employed earlier (Itagaki and Hager, 1966), the major changes being (1) reduction of sonification time of the cell paste to 20 min at water-ice temperature and (2) the substitution of calcium phosphate-gel-cellulose by hydroxylapatite as the stationary phase during the first two stages of purification. The apparent effects of the modifications are (1) the ability to handle larger amounts of starting material without any alteration of either the column size or the elution patterns and (2) the rectification of the problem of clogging inherent to the calcium phosphate-gel-cellulose columns, while still yielding a preparation comparable in purity to that reported earlier. The alteration of the sonification procedure

[2] The pico buffer system II is a Durrum-patented buffer system for single-column amino acid analysis.

TABLE I: Comparison of Purification Procedures for Cytochrome b_{562} from *E. coli* B.

	earlier procedure (ref 9)			modified procedure[a]			
stage of purif	total protein[b] (mg)	cytochrome b_{562} (μM)	net purif (folds)	total protein[b] (mg)	cytochrome b_{562} (μM)	net & comp. purif (folds)	rel level of purif[c] (folds)
crude extract	94 000	3.06	1	44 165	4.8	1 (3.34)[d]	3.34
stage I $CaHPO_4$-gel-cellulose hydroxylapatite	5 520	3.03	17	5580	4.7	7 (23)	1.53
stage II $CaHPO_4$-gel-cellulose hydroxylapatite	1 260	2.48	62	528	2.4	42 (140)	2.30
stage III DEAE-cellulose	53	1.78	1050	107	1.3	112 (375)	0.36
stage IV concn on DEAE-cellulose	47	1.64	1090	39	1.3	307 (1025)	0.96
crystallization (2nd)	34	1.12	1000	ND[e]	ND	ND	ND

[a] Based on use of 2 kg of *E. coli* cell paste. [b] Total protein estimated by the procedure of Lowry et al. (1951). [c] The relative level of purification is the ratio of cytochrome b_{562}/mg of total protein for the modified procedure to cytochrome b_{562}/total protein reported by Itagaki and Hager (1966). [d] The value in parentheses is the comparative level of purification at each step of the modified procedure. [e] ND, not determined.

TABLE II: Spectral Characteristics of Ferric and Ferrous Cytochrome b_{562} from *E. coli*.

absorption	position,[a] nm (extinction, mM) ferric	ferrous
α band	*564.0* (9.3)	561.8 (32.1)
β band	530.9 (11.3)	531.0 (17.6)
Soret band	419.0 (117.0)	426.7 (180.2)
δ band	*374* (33.9)	318.5 (65.4)
extinction ratio $\epsilon_{561.8}$ (reduced)/ϵ_{280} (oxidized)		1.49 ± 0.01

CD	position, nm ($\theta_{max,min} \times 10^{-4}$)[b] ferric	ferrous
Soret region	421.0 (−7.3)	*432.5* (−2.55)
	389.8 (+1.5)	423.0 (−2.87)
δ-band region	*355* (+0.45)	360.0 (+0.51)
	319.0 (−0.7)	326.0 (−2.96)
aromatic region		299.0 (+0.48)
		288.0 (+0.50)
		274.0 (−1.62)
	260.0 (−5.1)	
intrinsic region	222.0 (−1.86)	222.0 (−1.73)
	208.0 (−1.75)	208.0 (−1.68)
	194.6 (+1.70)	

[a] Italicized positions reflect shoulders or plateaus. [b] Ellipticities expressed as deg cm^3/dmol of protein above 250 nm and as deg cm^2/dmol of amide bonds (109 in this protein) below this region.

TABLE III: Amino Acid Composition of Cytochrome b_{562} from *E. coli*.

amino acid	no. of residues/molecule prep with modified proced[a]	est from sequence[b]
Asp	18.9	19
Thr	3.7	4
Ser	1.9	2
Glu	14.9	15
Pro	4.4	4
Gly	3.4	3
Ala	17.2	17
Cys	0.0	0
Val	4.0	4
Met	3.0	3
Ile	2.7	3
Leu	10.1	10
Tyr	1.9	2
Phe	1.9	2
Lys	11.2	16
His	1.8	2
Arg	3.8	4
Trp	ND	0

[a] Represents average of results from three lots, three analyses of each. Calculated with reference to 19 aspartic acids, 15 glutamic acids, and 2 histidines. The amino acid composition of the crystallized preparation was found to be identical to that of the preparation obtained with the modified procedure. [b] From Itagaki and Hager (1968).

produces a crude extract which is about 3.3-fold purer, but with an approximate loss of 20% in total protein. Although the substitution of chromatographic material results in an increase in purity of only 42-fold (Table I, row 3, data column 6), a comparative purity of about 140-fold is, however, the net result, as the crude extract was about 3.3-fold purer. Similarly, although the net purity of the preparation obtained by this procedure is only about 307-fold when compared with the characteristics of the starting material of the earlier procedure, it amounts to about 1025-fold. The critical aspect of the modified procedure is the higher purity of the crude extract and the increased manageability, thus making the procedure suitable for handling larger amounts of starting material and lending it to bulk preparation of the protein.

Degree of Purity. The degree of purity of the protein was established using three criteria: the gel-electrophoretic pattern, the absorption characteristics (Figure 1, Table II), and the amino acid composition (Table III). The homogeneity of the preparation is ascertained by a single detectable band upon gel electrophoresis (not shown), and its purity is ascertained by the identity of the spectral characteristics (Table II) to those

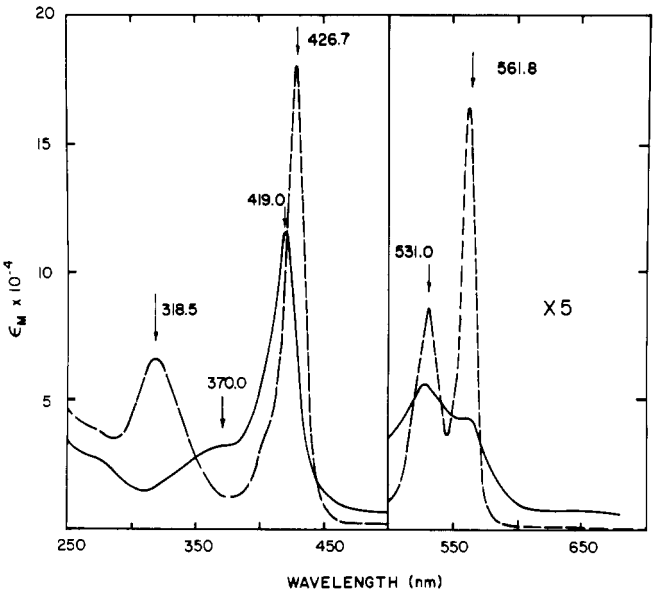

FIGURE 1: Absorption spectra of cytochrome b_{562} from *E. coli* in 0.1 M phosphate, pH 6.8: (—) ferric; (- - -) ferrous.

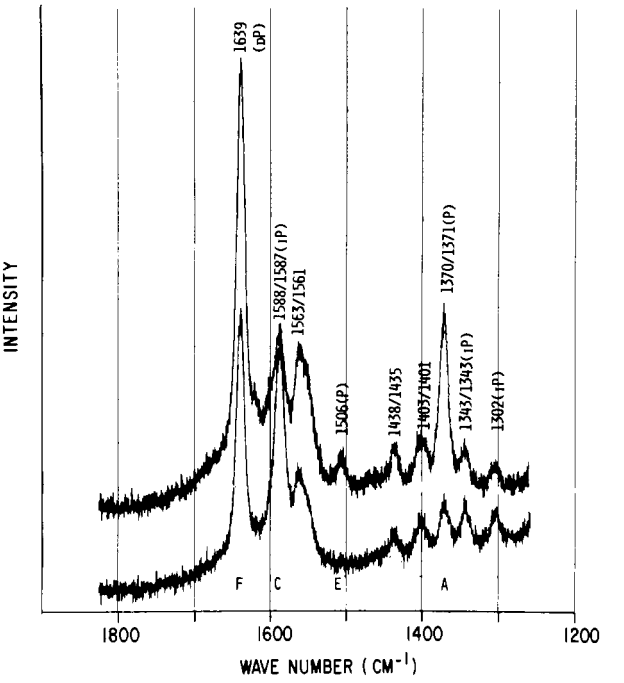

FIGURE 2: Resonance Raman spectra of ferric cytochrome b_{562} from E. *coli* in 0.1 M phosphate, pH 6.8. Conditions: excitation wavelength 5145 Å; flux, 20 mW; slit width, 300 μm; wavelength steps, 0.1 Å; integration time, 1 s; p, polarized; dp, depolarized; ip, inversely polarized: classified by Spiro and Strekas (1974). Protein concentration, 0.23 mM.

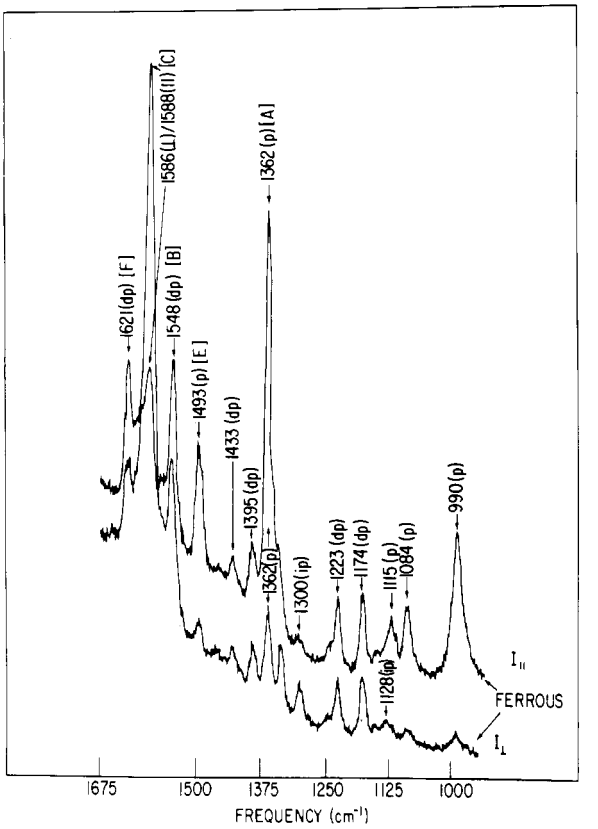

FIGURE 3: Resonance Raman spectra of ferrous cytochrome b_{562} from *E. coli* in 0.1 M phosphate, pH 6.8. Conditions and other details are the same as in Figure 2.

of the twice-crystallized preparation (Table III in Itagaki and Hager, 1966), including the ratio of the extinction of the reduced protein at the 561.8-nm band (heme absorption band) to the extinction at 280 nm (aromatic chromophores) of the oxidized form, 1.49 ± 0.01 (Table II). The same amino acid composition, except for the lysines, as that of the published sequence (Itagaki and Hager, 1968) (Table III) further establishes the purity of the preparation obtained by the modified procedure. The lower proportion of lysines,[3] the single variation in Table III, is however found to be a consistent feature of the analysis of all the lots obtained (four in all) and, furthermore, it prevails after crystallization of the protein as well. Since the variation is of only a single amino acid, and further, since the observed proportion is less than that expected for the known sequence, the possibility of contamination by a protein, polypeptide, or homonuclear poly(amino acid) is ruled out, as such an occurrence should yield a higher proportion of the contaminant amino acid or acids. Whether the disparity is the result of error in the earlier estimates (Itagaki and Hager, 1966, 1968) or is an indication of a protein different from that characterized earlier can only be discerned through reelucidation of sequence. Since the preparation obtained in this work exhibits spectral characteristics indistinguishable from those of the twice-crystallized preparation of Itagaki and Hager (Table II), and since the proportion of all other amino acids, except the lysines, is identical to that expected from the known sequence (Table III), the preparation reported here is taken to be cytochrome b_{562}, and of a purity comparable to, if not better than, that earlier reported.

Resonance Raman Spectra. The resonance Raman spectra of oxidized and reduced cytochrome b_{562}, at a pH of 6.8 in 0.1 M phosphate buffer, are shown in Figures 2 and 3, and in Table IV are listed the positions of the bands characterizing the spin state and the oxidation state of the metal atom, along with those expected for the various possibilities as characterized by Spiro and co-workers (1974, 1975).

[3] The possibility that the lower estimate of lysines is the result of instrumental abnormality or error in the color constant used for these studies is ruled out, since correct proportions of all the amino acids were obtained for horse heart cytochrome *c* and ribonuclease, both of which contain a rather high proportion of lysines.

The RR spectrum of ferrous cytochrome b_{562} in gross detail is very similar to those of cytochromes *b* and b_5 (Adar and Erecinska, 1974; Kitagawa et al., 1975), but it exhibits variations which differentiate this protein from other *b*-type cy-

TABLE IV: Resonance Raman Characteristics of Ferric and Ferrous Cytochrome b_{562} Compared with Those Expected for Various Spin Forms and Oxidation States of Heme Iron.

band[a]	valence state	spin state	exptd[b] posit (cm^{-1})	obsd posit ferric (cm^{-1})	obsd posit ferrous (cm^{-1})
oxidation state marker					
band A[c] (polarized band)	ferric	na	1373–1374	1370	
	ferrous	na	1358–1362		1362
band B[d] (depolarized)	ferric	na	1562–1565	1563	
	ferrous	na	1546–1548		1548
spin-state marker					
band C (anomalously polarized)	ferric	low	1582–1588	1588	
	ferric	high	1555	none	
	ferrous	low	1584–1586		1586
	ferrous	high	1552		none
oxidation and spin-state marker					
band E (polarized)	ferric	low	1502–1508	1506	
	ferric	high	1482	none	
	ferrous	low	1493		1493
	ferrous	high	1473		none
band F (depolarized)	ferric	low	1636–1642	1639	
	ferric	high	1608	none	
	ferrous	low	1620		1621
	ferrous	high	1607		none

[a] Classification according to Spiro and Strekas, (1974). [b] Data taken from Spiro and Strekas (1974); Spiro and Loehr (1975). [c] Bands, labels, and polarization as defined by Spiro and Strekas (1974). [d] Bands B and D may be unreliable because of interference from nearby bands, especially in protoheme derivatives (Spiro and Loehr, 1975). Band D not included for above reason.

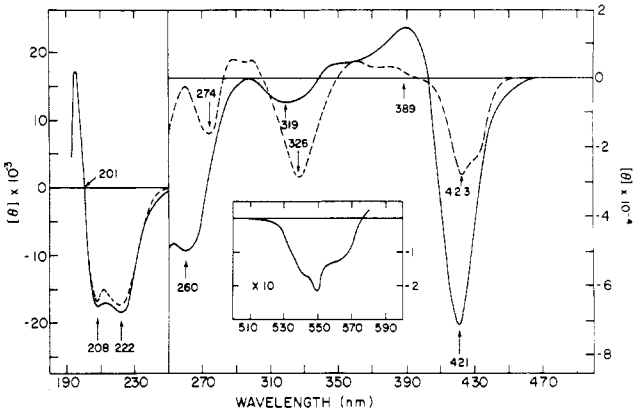

FIGURE 4: Circular dichroism spectra of cytochrome b_{562} from *E. coli* in 0.1 M phosphate, pH 6.8: (—) ferric; (- - -) ferrous. Ellipticity above 250 nm as deg cm^2/dmol of protein and below 250 nm as deg cm^2/dmol of amide bonds (109 amides/protein molecule).

tochromes. The polarized bands at 1084, 1362, and 1493 cm^{-1}, the depolarized bands at 1174 and 1621 cm^{-1}, the strongly inversely polarized bands at 1128 and 1586 cm^{-1}, and a band at 1340 cm^{-1} are features common to all *b*-type cytochromes, while the inversely polarized band at 1300 cm^{-1} is 5 cm^{-1} lower with respect to cytochrome b_5 and 15 cm^{-1} lower with respect to cytochrome *b*; the depolarized band at 1223 cm^{-1} is lower in position by 12 and 3 cm^{-1} and the 1548-cm^{-1} depolarized band is higher by 13 and 9 cm^{-1}, respectively. In addition, a band at 1562 cm^{-1} is reported for ferrous cytochrome b_5; no such band is seen for ferrous cytochrome b_{562} (Figure 3). Conversely, there appear to be no counterparts to the b_{562} bands at 1433 and 1115 cm^{-1} in the RR spectrum of cytochrome b_5. Since the spectrum in the region above 1000 cm^{-1} reflects in the main the stretching vibrational modes of the porphyrin-conjugated double bond system, the spectral differences clearly reflect differences in the stereoconfiguration of the heme group among the three cytochromes of type *b*.

A comparison of information in the ferric state of the protein is not possible, since there are no studies reported of ferric cytochrome *b* from which precise information on the positions and polarization of bands can be extracted.

Regarding the spin state of the metal atom and its oxidation state, the presence of bands such as the polarized bands at 1370 and 1362 cm^{-1}, the inversely polarized bands at 1588 and 1586 cm^{-1}, polarized bands at 1506 and 1493 cm^{-1}, and, finally, depolarized bands at 1639 and 1621 cm^{-1} corresponding to the positions expected for low-spin ferric and low-spin ferrous heme systems (Spiro and Strekas, 1974; Spiro and Loehr, 1975) (Table IV) directly confirm the suggestion, based on analogy of the absorption characteristics of the protein (Itagaki and Hager, 1966, 1968), that cytochrome b_{562} at neutral pH exists as a low-spin hemoprotein in both valence states. Thus, the hypothesis that cytochrome b_{562} may have a coordination configuration of histidine/histidine, the only two histidines present in the molecule (Itagaki and Hager, 1966; 1968), could well be true. Since it is well established that a coordination configuration of methionine/histidine could also generate a low-spin electronic configuration of heme iron, as in the cytochromes *c* (Fanger et al., 1967; Harbury, 1966) and heme *c* model systems (Harbury et al., 1965), this possibility cannot be ignored for the present.

CD Spectra. The intrinsic CD spectrum of a protein provides a most sensitive probe for conformational characterization in terms of both the nature and the magnitude of the secondary structures. The characteristic 222- and 208-nm minima, a positive peak at lower wavelengths, and a cross-over point at about 201 nm in the CD spectrum below 250 nm for both ferric and ferrous cytochrome b_{562} (Figure 4) are typical of highly α-helical proteins and polypeptides (Myer, 1970). The estimation of the secondary structure based on the isodichroic procedure (Myer, 1970; Rosenkranz and Scholtan, 1971) yields values of about 52–53% α helix, no β structure, and about 49% random form for the ferric protein and 49–50% α helix, no β form, and the remaining random form for the fer-

TABLE V: Conformation Composition[a] of Cytochrome b_{562} and Other Hemoproteins.

% of	α helix	β structure	random	ref
ferric cytochrome b_{562}	52	0	49	this work
ferrous cytochrome b_{562}	49	0	50	this work
cytochrome b_5	19	36	45	Huntley & Strittmatter (1972)
cytochrome b_2	34–40[b]			Sturtevant & Tsong (1969)
ferric cytochrome *c*	11	21	66	Myer (1968a)
ferrous cytochrome *c*	17	20	63	Myer (1968a)
metmyoglobin	66	0	40	Rosenkranz & Scholten (1971)
hemoglobin	72[c]			Kendrew (1962)

[a] Based on isodichroic method (Myer, 1970) and using poly(L-lysine) as model for α-helical and β-form and poly(L-serine) in 8 M LiCl for random form (Rosenkranz and Scholten, 1971). [b] Based on ellipticity at 222 nm. [c] From X-ray diffraction data.

rous protein (Table V). As the above numbers total 100 ± 5%, this indicates internal consistency and thus lends validity to the above estimates. In relation to other hemoproteins, the relatively high proportion of α-helical structure and little or no β structure puts this protein in a class with hemoglobin and myoglobin rather than other cytochromes *b* or *c* (Table V). The hypothesis that cytochrome b_{562} may have a three-dimensional structure like that of myoglobin (Warme and Hager, 1970) may well be true, but only in terms of the nature of the organized structure, and to some extent in terms of the magnitude, as the 66% α-helical nature of the myoglobins is significantly different from the 50% seen in cytochrome b_{562}. Furthermore, the two proteins differ by as much as 40–50 amino acids in chain length.

The small difference in the magnitude of the secondary structure between the ferric and the ferrous forms, however, could not be ascribed to the oxidation–reduction phenomenon, firstly, because the magnitude of difference is relatively small, within experimental error (6%), and, secondly, this region of the spectrum contains contributions from chromophores other than the polypeptide backbone, the aromatic side chains, etc., which adds a certain degree of ambiguity to the estimations. This is especially true for heme systems, as heme transitions in this region may not only add to the ellipticities, but, since these Cotton effects are valence-state sensitive (Myer, 1968b; Myer and Harbury, 1966), the small alterations could easily be those of the heme chromophore rather than reflections of alteration of the protein secondary structure. The secondary structure of cytochrome b_{562} is thus about 50% α-helical and the remaining is random form, without any indication of dependence of the secondary structure on the valence state of the metal atom.

The Soret CD spectrum of cytochrome b_{562} does not conform in detail to the pattern observed for all *b*-type cytochromes (Myer and Pande, 1978; Myer, 1978). The double-banded Soret CD spectrum of ferric cytochrome b_{562}, with a large negative Cotton effect and a small positive Cotton effect at lower wavelengths (Figure 4), is similar to cytochrome b_{555} (Okada and Okunuki, 1970a) and cytochrome b_5 (Huntley and Strittmatter, 1972), and it is also more or less like the spectrum of cytochrome b_{563} (Okada and Okunuki, 1970b) but distinct from native cytochrome b_2 (type I), DNA-free cytochrome b_2 (type II) (Sturetevant and Tsong, 1969), or apocytochrome b_2, i.e., free of both DNA and FMN (Tsong and Sturtevant, 1969). The Soret CD spectrum of reduced cytochrome b_{562}, a spectrum with two negative Cotton effects (Figure 4), on the other hand, is different from those of all the *b*-type cytochromes, as all exhibit Soret spectra like that of the ferric form shown in Figure 4, i.e., with a large negative and a small positive band. It should be pointed out that the Soret spectrum of reduced cytochrome b_{562} is rather unique among the hemoproteins, as no comparable system is yet known, although the intrinsic feature, the presence of two Cotton effects, is common to a large number of hemoproteins (Myer and Pande, 1978; Myer, 1978).

The Soret absorption region contains contributions from the porphyrin π–π^* transition (Smith and Williams, 1970), which, in the fundamental chromophoric moiety, porphyrin with D_{4h} symmetry, is a doubly degenerate transition (Gouterman, 1961). The incorporation of iron (if not in the porphyrin plane), the introduction of side chains as in protoporphyrin IX, and the localization of the group in the protein environment and the coordination of the axial ligands (if not identical) will lower the symmetry of the chromophore, resulting in splitting of the degenerate band. In such an instance, if each resulting transition gains optical activity through one or another of the possible mechanisms, dipole–dipole interaction (Kuhn, 1930; Kirkwood, 1937; Hsu and Woody, 1971), the μ–m coupling mechanism (Schellman, 1969), etc., the effect will be the generation of a Soret CD spectrum of any possible complexity, as all permutations of sign and magnitude of the two Cotton effects are possible. A complex Soret CD spectrum with positive and negative bands could also be a result of the dipole–dipole coupling mechanism involving identical chromophores, a classical case of exciton splitting (Moffitt, 1956). However, this requires the presence of two heme groups in relatively close proximity, which, as detailed below, is not the case in this system. The third possibility producing a complex Soret CD spectrum is the presence of contributions from vibrational components, and the fourth possibility is contributions from transitions other than the porphyrin transitions, such as metal d–d or charge-transfer bands.

Of the above four possibilities, we tend to favor the first explanation. Exciton splitting can be easily ruled out, since the protein is known to have one heme/polypeptide chain and it is shown to be monomeric (Itagaki and Hager, 1966, 1968; this work). In addition, the observed Cotton effects do not conform to the expectation of equal but opposite optical activity, as should be the case (Figure 4, and the parameters listed in Table VI from Gaussian resolution of the CD spectrum as shown in Figure 5). Significant vibrational contributions for an electrically allowed transition like the Soret band (Smith and Williams, 1970; Myer and Pande, 1978) are highly unlikely. Similarly, overlapping contributions from either a metal d–d transition or a charge-transfer complex seem unlikely, since the splitting of the Soret CD spectrum is persistent in both the ferric and the ferrous forms of iron (Figures 4 and 5), which would not be the case, as the energy of such transitions should be strongly dependent on the oxidation state of the metal atom.

TABLE VI: Gaussian Band Constituents of CD Spectra of Ferric and Ferrous Cytochrome b_{562} from *E. coli*.

ferric cytochrome b_{562}			ferrous cytochrome b_{562}		
position (λ_{max}, nm)	max ellipticity ($[\theta]_{max}$, deg cm^2/dmol)	rotatory strength[a] (DM)	position (λ_{max}, nm)	max ellipticity ($[\theta]_{max}$, deg cm^2/dmol)	rotatory strength[a] (DM)
421.0	−79 000	−0.39	435.0	−15 500	−0.03
398.0	+16 000	+0.12	422.5	−28 000	−0.09
			382.0	+3 500	+0.01
360.0	+4 000	+0.04	360.5	+5 000	+0.02
327.0	−6 000	−0.03	326.6	−29 000	−0.15
314.0	−4 200	−0.02			
			298.0	+5 000	+0.02
			285.0	+6 000	+0.02
			273.5	−16 700	−0.07
260.0	−48 000	−0.45			

[a] Rotatory strength calculated using the expression: $R_k = 1.23 \times 10^{-42}$ ($[\theta]_{max} \times \Delta_{max}/\lambda_{max}$), where $[\theta]_{max}$ and λ_{max} are the maximum ellipticity and its position, and Δ_{max} is the half-bandwidth at the eth height. The unit is the debye magneton (DM), which equals 0.927×10^{-38} cgs units.

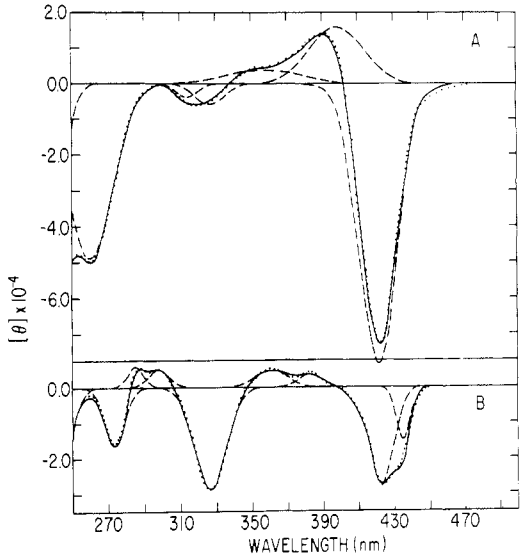

FIGURE 5: Gaussian components of CD spectra of cytochrome b_{562}: A, ferric; B, ferrous. (- - -) Gaussian bands; (—) summation of Gaussian bands; (• • •) observed spectrum.

Further consideration of the Soret Cotton effects in relation to the heme conformation, once a plausible mechanism for their origin is accepted, lies in resolution of the Cotton effects and correlation of the magnitude of splitting and their relative strengths. The results of Gaussian fitting over the entire region from 250 to 500 nm of the ferric and ferrous forms are shown in Figure 5, and Table VI lists the appropriate parameters of the bands constituting the CD spectra. The two Cotton effects making up the Soret CD spectrum of ferric cytochrome b_{562} are separated by about 23 nm, while the ferrous form exhibits a separation of 12–13 nm only, which indicates point-group symmetry differences of the heme group in the two valence states, low symmetry in the ferric, and relatively higher symmetry in the ferrous. The significant variation in the magnitude and the sign of the two Cotton effects in each valence state (one negative and one positive with rotatory strengths of 0.39 and 0.12 DM for the ferric and both negative with rotatory strengths of 0.03 and 0.09 DM; Table VI), when taking into consideration the possible operational mechanism for the origin of optical activity, further indicates that, accompanying the alteration of the symmetry of the heme group, significant alterations of dipolar interactions between heme and the protein chromophores must occur, most probably with the aromatic chromophores: groups known to be prominently involved in determining the Soret optical activity in other hemoproteins (Hsu and Woody, 1971).

The presence of distinct Cotton effects in the region 300–250 nm, the aromatic contribution region (Timasheff, 1970; Strickland, 1975), distinguishes the two valence forms of the protein in reference to the conformation of the polypeptide side-chain chromophores. Interpretation of the data in terms of the nature of the protein structures involved requires structural assignment to various Cotton effects. The problem inherent to the resolution and characterization of Cotton effects in this spectral region (Timasheff, 1970) is much simplified in this case, as of all the possible contributing chromophores cytochrome b_{562} contains only[4] two tyrosines and two phenylalanines (Table III). Also, studies of model systems show that the Cotton effects of the above two chromophores are localized in two distinct regions of the spectrum, 255 to 269 nm for the phenylalanine Cotton effects and 270 to 295 for the tyrosyl Cotton effects (Strickland, 1975). Therefore, the 260-nm band of ferric cytochrome b_{562} is a reflection of contributions of one or both the phenylalanine side chains, and the Cotton effects at 274 nm and possibly the 285-nm band[4] in the spectrum of ferrous cytochrome b_{562} must have their origin in one or both tyrosyl side-chain chromophores. The distinct phenylalanine Cotton effect with a rotatory strength of 0.45 DM [orders of magnitude greater than that observed for any model (Strickland, 1975)] in the ferric CD spectrum and the tyrosyl Cotton effects in the CD spectrum of the ferrous form must be a consequence of a conformational rearrangement of the two types of chromophores upon reduction of heme iron.

[4] Heme Cotton effects are also known to exist in this region of the spectrum (Urry, 1967; Myer and Harbury, 1966). Since the heme Cotton effects have been shown to be positive, the type of interference one could expect is a decrease in the negative ellipticities and possibly a shift in the position of the minima. Since the observed Cotton effects at 260 and 274 nm are negative, and positionally they coincide with the Cotton effects of the phenylalanine and tyrosyl side chains, there seems to be little reason to doubt this assignment. As both the positive bands in the CD spectrum of the reduced form, the 285- and 298-nm bands, have counterparts in the CD spectrum of heme, a definitive assignment is difficult.

An examination of the sequence indicates that the only two phenylalanyl residues of the protein are located at positions 64 and 68, in the middle of the polypeptide chain, whereas both the tyrosyl residues are located at the C-terminal end of the polypeptide chain, residues 104 and 109 (Itagaki and Hager, 1968). The almost equidistant location of the two types of aromatic chromophores, on the one hand, and their separation on the peptide chain, on the other hand, structurally localizes the sites of the oxidation–reduction-linked conformational alteration of the protein. Although the consideration of the intrinsic CD spectrum failed to provide an indication of oxidation–reduction-linked conformational alteration of the protein moiety, the above structural interpretation is not contradictory. A simple reorganization of the C-terminal end, which is apt to occur in a disordered form, resulting in proximity to the heme group, and displacement or merely alteration of the relative orientation of the phenolic groups are the only factors needed to produce the observed CD effects. This type of reorganization of the polypeptide chain would not be expected to show up in the intrinsic CD spectrum, since it does not necessarily involve either the generation or the elimination of protein secondary structure.

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