we have been conducting labeling experiments to determine which of the components in complex III are at the matrix side and which are exposed at the intracistral side of the mitochondrial inner membrane. The cross-linking and labeling data taken together can be used to develop a model of the arrangement of polypeptides in complex III. This model will be presented along with the labeling experiments in a forthcoming paper.

Acknowledgments

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Cytochrome b_{562} from *Escherichia coli*: Conformational, Configurational, and Spin-State Characterization†

Yash P. Myer* and Peter A. Bullock

ABSTRACT: The protein conformation, heme configuration, and the spin state of heme iron of cytochrome b_{562} from *Escherichia coli* have been investigated using circular dichroism (CD), optical, and resonance Raman (RR) spectroscopy as the probes. Studies are reported on the effect of ionic strength on the CD spectra of the ferric and ferrous forms, of temperature variation on the CD spectrum of the ferric form, and of pH variation in the range 3–11 on optical, circular dichroism, and resonance Raman spectra of the ferric form of the protein. Differences in conformational sensitivity to increasing ionic strength of the medium are seen between ferri- and ferrocytochrome b_{562}. The thermal denaturation of ferricytochrome b_{562} at neutral pH is found to occur in two distinct steps centered at about 35 and 67 °C, with $\Delta H°$ and $\Delta S°$ of about 74 eu and 23 kcal, and 254 eu and 74 kcal, respectively. The effect of pH on the optical spectrum of ferricytochrome b_{562} is seen in the presence of three distinct pH forms in the range 3–11 with apparent pKs of about 6 and 8.7. The acidic transition is accompanied by minimal perturbation of the optical spectrum. The basic transition, pK_a of 8.7, is accompanied by a red shift of the Soret peak and the visible spectrum and the generation of a new band at about 635 nm. The CD spectrum does not indicate any significant variation of the protein secondary structure in any of the pH transitions, but the heme symmetry is altered during the alkaline transition from a less symmetric heme to a more symmetric heme. The resonance Raman spectra of the three pH forms are found to be typical of low-spin heme iron systems. A heme configuration of methionine/histidine ligation at the two axial positions of heme iron for the acidic and neutral forms and transformation to a form with lower heme symmetry, i.e., with lysine/histidine or histidine/histidine ligation, have been concluded.

Cytochrome b_{562} from *E. coli* is thought to be a soluble electron carrier for a system located in the membrane, although the protein itself is not bound to the membrane (Lemberg & Barrett, 1973; Hager & Itagaki, 1967). In mammalian systems this physiological function is attributed to cytochrome *c*, while cytochromes of type *b* are membrane-localized (Dickerson & Timkovich, 1975; Hagihara et al., 1975; Ferfuson-Miller et al., 1978). The similarity in function of the two quite different proteins has attracted our attention, and, in order to understand this phenomenon, we have undertaken an extensive physico-

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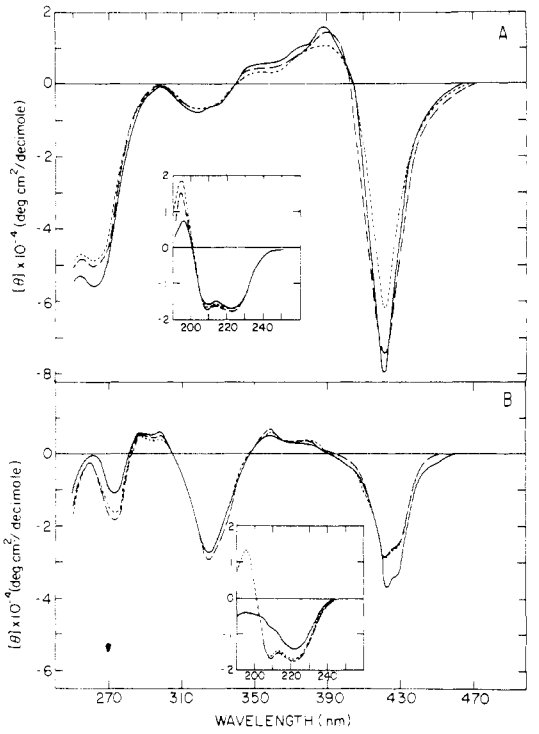

FIGURE 1: Effect of increasing phosphate buffer concentration on circular dichroism spectra of cytochrome b_{562} from *E. coli*. (A) Ferric; (B) ferrous. Conditions: pH 6.8; temperature, 20 °C. (—) 0.01 M; (— — —) 0.1 M; (- - -) 1 M.

chemical characterization of cytochrome b_{562} so as to explore any correlations between the two proteins.

Like the mammalian cytochromes *c*, cytochrome b_{562} is a relatively small molecule, 110 amino acids (Itagaki & Hager, 1968), and contains a single noncovalently bonded iron protoporphyrin IX as the prosthetic group (Itagaki & Hager, 1966). Its oxidation–reduction potential is found to be significantly lower than that of mammalian cytochrome *c*, 113 mV (Itagaki & Hager, 1966) instead of 250 mV (Dickerson & Timkovich, 1975), and it is more acidic, with a p*I* of 7–8. Recently we reported resonance Raman (RR)[1] spectroscopic studies (Bullock & Myer, 1978) and established the low-spin electronic configuration of heme iron for both valence states of the metal atom at neutral pH. Circular dichroism (CD) studies showed that the protein is highly α-helical, about 50%, with heme localized in an asymmetric environment and undergoing symmetry alteration upon reduction, from low heme symmetry to a configuration with relatively high symmetry (Bullock & Myer, 1978). The oxidation–reduction process is also found to involve the conformational alteration of two distinct sections of the polypeptide chain, the N-terminal end containing the tyrosyl residues and the segment containing the phenylalanine residues, the center of the chain. In this report we present further evidence indicating conformational differences of the protein in the two valence states of the metal atom, the temperature stability of the protein and studies of proton-linked functions, both spectroscopic and conformational, of ferricytochrome b_{562} in order to discern the possible nature of the coordination configuration.

Experimental Section

The cytochrome b_{562} from *E. coli* used in these investigations was isolated according to a modified procedure of Iagaki & Hager (1966), described elsewhere (Bullock & Myer, 1978). The transfer of the protein to the appropriate solution conditions was performed using the Sephadex G-10 gel filtration procedure. The pH of the solution was adjusted with 6 N HCl or 6 N NaOH, and no correction for the dilution was applied.

[1] Abbreviations used: CD, circular dichroism; RR, resonance Raman.

TABLE I: Effect of Increasing Buffer Concentrations on the Conformational Composition[a] of Cytochrome b_{562}.

	%		
	α helix	β structure	random
ferric cytochrome b_{562}			
0.01 M phosphate	48	0	53
0.1 M phosphate	52	0	49
1.0 M phosphate	53	0	49
ferrous cytochrome b_{562}			
0.01 M phosphate	20	7	80
0.1 M phosphate	49	0	50
1.0 M phosphate	50	0	

[a] Based on the isodichroic method (Myer, 1970) using poly(L-lysine) as the model for α helix and β structure and poly(L-serine) in 8 M LiCl as the model for random form (Rosenkranz & Scholtan, 1971).

Absorption spectroscopic measurements were performed with a Cary 15 spectrophotometer using the specially designed apparatus permitting concurrent adjustment of pH and measurement of the absorption spectrum (Myer & Harbury, 1973). Circular dichroism measurements were performed on a JASCO-J-10 spectrodichrograph according to the procedure described earlier (Myer, 1968). Thermal denaturation was carried out in water-jacketed, cylindrical cells with fused quartz windows, of 5-mm path length in regions above 250 nm and 1-mm path length below 250 nm. The CD data are presented as deg cm^2/dmol of protein above 250 nm and as deg cm^2/dmol of amide bonds (105 amide linkages/protein molecule) below 250 nm. Concentrations of the solutions were determined spectroscopically using an extinction of 11.3 mM at pH 6.8 in 0.1 M phosphate buffer (Bullock & Myer, 1978).

Resonance Raman spectra were recorded on the spectrophotometer under conditions described earlier (Bullock & Myer, 1978). Some details of the measurement conditions are given in the legends of the figures.

Results and Discussion

Effect of Ionic Strength. The effect of increasing buffer concentrations, 0.01 to 1 M phosphate at pH 6.8, on the absorption spectrum of both ferri- and ferrocytochrome b_{562} was found to be minimal (not shown). However, significant perturbations were noticed in the CD spectra of both the systems (Figure 1). The increasing ellipticity of the positive peak below 200 nm and an increase of ellipticity of the minima at 222 and 208 nm are clear indications of the increasing degree of structural organization of the protein moiety with increasing ionic strength. The effect of ionic strength on protein conformation is more prevalent in the ferrous form than in the ferric (Table I). Based on the "isodichroic method" (Myer, 1970; Rosenkranz & Scholtan, 1971), the intrinsic CD spectrum of the ferrous form in 0.01 M phosphate buffer reflects the presence of only about 20% α helix, which increases to a value of about 49% in 0.1 M phosphate buffer and remains unchanged for the system in 1 M buffer. The corresponding values for the ferric form amount to 49, 52, and 53% α helix in media of 0.01, 0.1, and 1 M phosphate (Table I). A high degree of sensitivity of the protein secondary structure of fer-

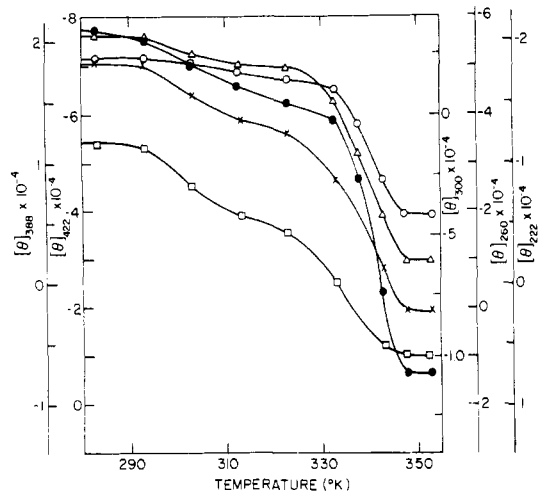

FIGURE 2: Thermal denaturation ellipticity profiles at various Cotton effects of ferricytochrome b_{562} from *E. coli*. Conditions: 0.1 M phosphate buffer, pH 6.8. Positions of Cotton effects used: (○) 222 nm; (△) 260 nm; (□) 300 nm; (X) 388 nm; (●) 422 nm.

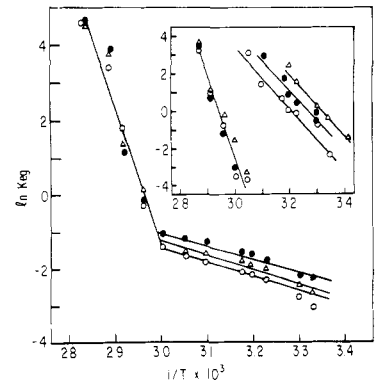

FIGURE 3: Van't Hoff plots for thermal denaturation of ferricytochrome b_{562} from *E. coli*. Conditions and description same as in Figure 2. Inset: plots after resolution of the two steps.

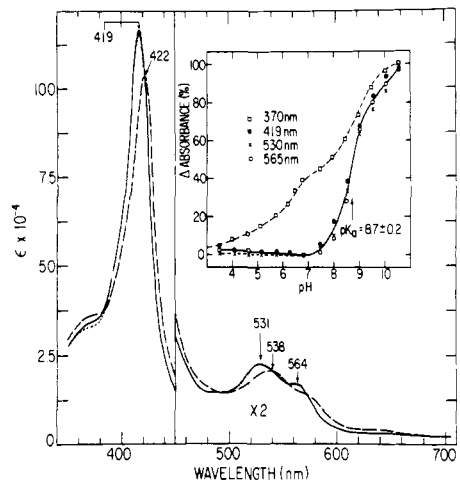

FIGURE 4: pH-optical spectroscopic behavior of ferricytochrome b_{562} in 0.1 M phosphate, 20 °C. Inset: absorbance-pH profiles at various absorption peaks. Main body: Absorption spectra of three pH-stable forms at: (- - -) pH 4.02; (—) pH 6.8; (— — —) pH 9.98. Abscissa on right = ½ of scale on left.

rous cytochrome b_{562} to increasing ionic strength is thus apparent. A similar conclusion is also drawn for the dichroic changes in the region 250–300 nm, reflecting the conformation of the aromatic chromophores. Consideration of the dichroic changes in the region above 300 nm, the heme contribution region, however, indicates that heme in the ferric form is more sensitive to increasing ionic strength than the ferrous form. The CD spectra of the ferrous form in 0.1 and 1 M buffers above 300 nm are almost indistinguishable, whereas the major alteration of the Soret spectra of the ferric forms occur primarily in this range of concentration variation (Figure 1). The differential behavior of the protein secondary structure and the heme group to increasing ionic strength is consistent with the idea that the two valence forms must have differences in the nature of the secondary structural organization of the polypeptide chain (Bullock & Myer, 1978). Since the effect of salts is to shield electrostatic interaction, the increasing organization of the ferrous form with increasing ionic strength is a reflection of the operational nature of destabilizing electrostatic interactions among the protein charged groups and, conversely, the absence thereof in the ferric form of the protein. The clustering of identically charged groups in distinct surface domains in the ferrous form, while randomly dispersed in the ferric form, may well be the situation. The preponderance of acidic amino acids in the amino terminal end and of 33% of the basic amino acids in the C-terminal chain from 98 to 110 (ITAGAKI & Hager, 1968) does indeed suggest the likelihood of the clustering of identically charged groups on a discrete surface location of the protein.

Since a phosphate buffer concentration of 0.1 M is the limiting condition for the conformation of the ferrous form and is also the point after which only a slight alteration of protein conformation in the ferric form of the protein occurs (Table I), these conditions were selected for the studies reported here as well as in the earlier publication (Bullock & Myer, 1978).

Effect of Temperature. The thermal denaturation of ferricytochrome b_{562} at neutral pH exhibits two distinct steps, one centered at 35 °C and the other at 67 °C, irrespective of the Cotton effect under observation (Figures 2, 3). The dichroic changes at the 222- and 260-nm bands, both reflecting changes in the polypeptide conformation, are relatively minor during the first denaturation step, i.e., less than 5% of the total change, whereas much greater proportional changes are noticed for the heme Cotton effects during the same step of the denaturation process. The selectivity of the dichroic changes, the protein Cotton effects vs. the heme Cotton effects, is like that seen in the unfolding process of cytochrome *c*, in which the first step is shown to be a reflection of the unfolding or relaxation of the heme environment, and the second, a composite of the unfolding of the protein and exposure of the heme group (Myer, 1968). The near agreement of the thermodynamic quantities associated with the second denaturation step (Figure 3) for cytochrome b_{562}, $\Delta S°$ of 254 eu and $\Delta H°$ of 74 kcal, and cytochrome *c*, values of 220 eu and 75 kcal (Myer, 1968), and proportional values for the thermodynamic quantities associated with the first step, 74 eu and 23 kcal vs. 150 eu and 50 kcal, respectively, supports the idea that the two steps of the denaturation process for the two proteins are similar in nature. Since heme is deeply buried in the protein crevice in cytochrome *c* (Dickerson & Timkovich, 1975), the values of one-half for both the standard enthalpy and entropy changes during the first thermal denaturation step of cytochrome b_{562} can be related in a comparative manner to the extent of heme shielding. The heme in cytochrome b_{562} thus seems to be only partially buried in comparison to heme in cytochrome *c*.

Effect of pH. The presence of three spectroscopic forms of ferric cytochrome b_{562} in the pH range of 3–10, interconverting with apparent p*K*s of about 6 and 8.7, is clearly seen in the optical density vs. pH profiles shown in the inset of Figure 4.

TABLE II: Absorption Characteristics of Various pH Forms of Ferric Cytochrome b_{562}.

	position in nm[a] (extinction, mM) at pH:		
	4.02	6.80	9.96
near-IR bands	ND	710 (1.6) |235|[b]	
	ND	650 (2.2) |274|	
			630 (2.9)
α band	564 (9.2)	564 (9.3)	568 (8.1)
β band	531 (11.4)	531 (11.3)	538 (10.4)
soret band	419 (116.2)	419 (117.0)	422 (103.2)
soret shoulder	370 (33.1)	370 (34.5)	370 (36.3)

[a] Underlined positions are of shoulders and plateaus: solid line for shoulders, dotted line for plateaus. [b] Values in bars are the absolute molar extinctions of the bands estimated according to the procedure outlined by Kaminsky et al. (1973). Measurements reported at pH 6.68.

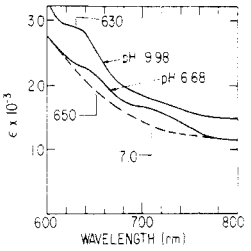

FIGURE 5: Absorption spectrum of neutral and alkaline pH forms of ferricytochrome b_{562} in the region 600–800 nm. (—) Observed spectrum; (- - -) exptrapolated spectrum according to procedure outlined by Kaminsky et al. (1973). Spectrum recorded of solution containing protein concentration of 3.35×10^{-5} M/L; 1-cm path length and 0.1-OD expansion of Cary 15 spectrophotometer.

The transformation of the neutral pH form to the alkaline pH form is clearly discerned from alterations of absorptivity of all the bands in the visible region of the spectrum, whereas the neutral pH form and the acidic pH form can be differentiated primarily from absorptivity changes in the region below the Soret band, i.e., at the 370-nm shoulder. Small but definite spectral changes accompanying the acid transition are seen in the Soret peak and the visible peaks (Figure 4), but the magnitude of change is relatively small (Table II), prohibiting an analysis similar to that of the changes at 370 nm. Below pH 3 and above pH 11 the protein seems to undergo irreversible transformation to forms distinct from the acidic and the alkaline forms. Since the irreversible nature of the transitions at extreme pHs prohibits the characterization of the resulting form, further consideration of the extreme pH forms is not dealt with in this communication.

SCHEME I

$$\underline{\text{below pH 3}}\ A \overset{pK_a \simeq 6}{\longleftrightarrow} B \overset{pK_a = 8.7}{\longleftrightarrow} C\ \underline{\text{above pH 11}}$$

The pH-spectroscopic behavior of ferric cytochrome b_{562} can be represented by Scheme I, where A, B, and C (to be referred to as the acidic, neutral, and alkaline forms) are the three stable spectroscopic forms interconverting with apparent pKs of about 6 and 8.7, respectively. Since the positions of the absorption bands are the same for the acidic and the neutral forms, and since the pH-induced changes are merely slight variations of extinctions of the Soret and the visible bands (Table II), the similarity, if not the identity, of the heme electronic configurations for the two forms is evident. The distinct red shift of the Soret peak and of the visible spectrum (Figure 4) and the elimination of weak bands at 650 and 715 nm with the generation of a new band at about 630 nm upon

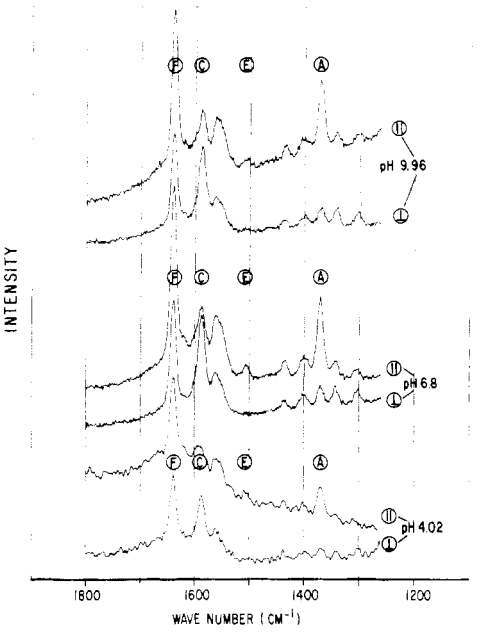

FIGURE 6: Resonance Raman spectra of the three pH forms of ferricytochrome b_{562} from *E. coli*. Conditions: excitation wavelength, 5145 Å; 30 mW for samples at pH 6.8 and 9.98 and 15 mW for sample at pH 4.02; slit width, 6 cm^{-1}, integration time 1 and 2 s, respectively, and wavelength stepping, 0.1 Å. Peak positions and polarization ratios calculated using 21-point slope-detection procedure and fitting of the bands (Bullock & Myer, 1978). A, C, E, and F are the oxidation and/or spin-state markers (Spiro & Loehr, 1975).

transformation of the neutral form to the alkaline form (Figure 5; Table II) are, on the other hand, all indicative of significant alteration of the electronic configuration of heme iron accompanying the alkaline transition of the protein. Since the red shift of the Soret peak is known to occur with ligation of groups of increasing ligand-field strength at the axial position of heme iron (Smith & Williams, 1970), the change from form B to C could be a reflection of such an occurrence. A similar consideration leads one to conclude that the transition from form A to B is thus non-heme-linked, i.e., reflecting effects of protonation or deprotonation of a functional group or groups distinct from the heme coordination sphere groups.

The spectroscopic characteristics of the three pH forms of ferricytochrome b_{562}, a Soret peak above 410 nm and well-developed α and β bands (Figure 4 and Table II), are typical of low-spin ferric hemoproteins (Smith & Williams, 1970). The low-spin electronic configuration of heme iron in all three forms is indeed clearly discerned by the resonance Raman spectra of these systems (Figure 6 and Table III). Spiro & co-workers (1974, 1975) have correlated the RR bands of a variety of hemoproteins and heme models and have shown a number of vibrational modes serve as excellent markers for determining the oxidation and/or spin state of the metal atom for these systems. The presence of a depolarized band between 1636 and 1642 cm^{-1} (band F), two polarized bands, one between 1502 and 1508 cm^{-1} (band E) and the other between 1373 and 1374 cm^{-1} (band A), and an anomalously polarized band betwen 1582 and 1588 cm^{-1} (band C) are the characterizing features of low-spin ferric heme systems. As shown in Figure 6 and Table III, all three pH forms exhibit distinct vibrational modes with appropriate polarization at the expected frequencies. A low-spin form of heme iron for the three pH forms is thus unquestionable, and, consequently, the heme iron electronic configurations of the three pH forms must be identical. The low-spin configuration of heme iron dictates that the nature of the groups occupying the axial position of heme

TABLE III: Ferric Heme Oxidation and Spin State Resonance Raman Markers[a] and Observed Bands for the Three pH Forms of Ferric Cytochrome b_{562} from *E. coli*.

	expected[b] position (cm^{-1})	obsd positions, $\nu_\perp/\nu_\parallel$ (cm^{-1}) acidic (4.02)	neutral (6.8)	alkaline (9.96)
oxidation state marker, band A (p)	1373–1374	1369/1371 (p)	1370/1370 (p)	1369/1371 (p)
spin state marker low-spin, band C (ap)	1582–1588	1587/- - - (ap)	1588/1587 (ap)	1587/1588 (ap)
oxidation & spin state marker, ferric low spin band E (p)	1502–1508	- - - -/1508 (p)	- - - -/1507 (p)	- - - -/1508 (p)
band F (dp)	1636–1642	1639/1639 (dp)	1639/1639 (dp)	1640/1639 (dp)

[a] Characterized by Spiro & Strekas (1974). [b] Data taken from Spiro & Strekas (1974) and Spiro & Loehr (1975).

iron of the three pH forms must be of strong ligand-field strength. In this regard, the imidazole side chains of the two histidyl residues, the sulfur moieties of the three methionyl residues, the ϵ-amino group of a variety of lysyl residues, and the α-amino group are the only strong-ligand groups available in this protein.

Further characterization of the three pH forms in terms of molecular conformation, the conformation of the aromatic chromophores, of the heme environment and of heme symmetry stems from a consideration of the CD spectra of the three forms (Figure 7). The lack of any significant differences among the intrinsic CD spectra, i.e., below 250 nm, the region reflecting the protein secondary structure (Myer, 1970; Fasman, 1973), reflects the insensitivity of the protein conformation to the pH transformations of the molecule. In contrast, the three forms can be discerned by their CD spectra in the regions above 250 nm, especially the heme optical activity region, i.e., above 300 nm (Figure 7). The dichroic differences between the acidic and the neutral forms—a lowering of the ellipticities of the Cotton effects in the region above 300 nm, without alteration of either the position of the bands or the complexity of the spectrum—are consistent with the conclusion that the acidic transition involves only small but definite variations in the heme-protein conformation, without other alterations, such as of the coordination configuration of the metal atom. Hsu & Woody (1971) have shown that the Soret optical activity of the heme transition in proteins with noncovalently bonded heme groups, hemoglobin and myoglobin, is determined mainly by transition-dipole interactions between porphyrin and the surrounding aromatic chromophores. The magnitude of the resultant Cotton effect from the dipole coupling mechanism is dependent upon the magnitude of the interacting dipoles, their relative orientations, and the distance between them (Schellman, 1968; Myer, 1978). The alteration of the relative orientation of the aromatic chromophore or spatial displacement by about 1–2 Å from heme could easily account for the observed dichroic differences between the acidic and the neutral forms of the protein. The conformational nature of the acid transition is also consistent with the observation of two first-order pH-dependent rates with pK_as in the region 4–6 during the reconstitution of the protein from holocytochrome b_{562} (Itagaki et al., 1967).

The CD characteristics of the alkaline form, when compared with those of the neutral pH form, give added strength to the conclusion that this pH transition involves the alteration of the electronic configuration of the heme group and a change in the axial ligand to a group of greater ligand-field strength. The red shift of the Soret Cotton effects with $\frac{1}{3}$ the rotatory strength of the major band and about $\frac{2}{3}$ the rotatory strength of the minor band (Figure 7), with a concurrent variation of the spectrum in the aromatic region, are consistent with the conclusion that the transition with an apparent pK_a of 8.7 produces significant alterations of heme configuration. In addition to

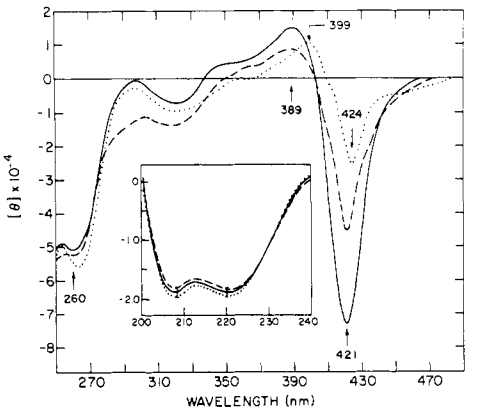

FIGURE 7: Circular dichroism spectra of the three pH forms of ferricytochrome b_{562} from *E. coli*. (— — —) Acidic form, pH 4.02; (—) neutral pH form, pH 6.8; (- - -) alkaline form, pH 9.84. Ellipticity as deg cm^2/dmol of protein above 250-nm main bond and as deg cm^2/dmol of amide bonds below 250 nm for data in inset.

the red shift of the spectrum, there is a definite indication of the alteration of the complexity of the Soret spectrum. The splitting between the two components constituting the Soret CD spectrum is significantly reduced, 31 nm to 24 nm, and the relative magnitudes of the two Cotton effects are also significantly different from those of the protein at neutral pH. The magnitude of splitting of the Soret Cotton effect is a measure of the splitting of the porphyrin π–π^* doubly degenerate transition (Gouterman, 1961). Since a decrease in splitting is indicative of a higher degree of symmetry of the heme group, and since studies with model systems have shown that the rotatory strength of the Soret Cotton effects is determined by the difference in the ligand-field strengths of the two axial ligands (Myer & Pande, 1978; Myer, 1978; Pande et al., 1975), the observed decrease in the magnitude of splitting as well as the rotatory strength of both components agree with the idea of the alteration of the axial coordinating ligands from significantly different ligand-field strength groups at neutral pH to ligands with relatively similar liganding properties in the alkaline form.

Coordination Configuration of Heme. The low-spin electronic configuration of heme iron throughout the pH range 4–10 of ferricytochrome b_{562} reflects the presence of two strong ligand-field groups at the two axial positions of the heme group. Of the various possible coordinating groups in this protein, several are capable of generating the low-spin configuration of heme systems: the imidazole of the two histidines at positions 66 and 106, the sulfur of the methionine residues at positions 7, 35, and 61, one or more of the ϵ-amino groups of the lysines (11–12 lysines/molecule) and the α-amino group (Itagaki & Hager, 1968; Bullock & Myer, 1978). Of the various possible permutations of the available strong ligand-field groups,

coordination configurations with histidine/histidine, methionine/histidine, lysine/histidine, and the α-amino group/histidine are the four possibilities known to produce low-spin ferric heme states in hemoproteins and heme–peptide models, for instance, the histidine/histidine configuration in cytochrome b_5 (Hagihara et al., 1975), the methionine/histidine configuration in cytochrome c (Dickerson & Timkovich, 1975) and heme–peptide systems (Harbury et al., 1965), and α-amino/histidine and lysine/histidine in heme c models (Harbury & Loach, 1959, 1960).

Of the above four possibilities, we propose a coordination configuration of methionine/histidine for forms A and B and lysine/histidine or histidine/histidine for form C. This conclusion is based on the following considerations.

(a) The possibility of lysine/histidine coordination configuration for form A and for form B can be ruled out simply on the basis of the pK_a expected for such a case. The transition from form A to B is shown to be a conformationally linked transition not involving the alteration of the coordination configuration of heme iron; the presence of lysine as an axial ligand at a pH as low as 4 would necessitate a shift of as much as 6–7 pH units in its intrinsic pK of 10.5. Since such an occurrence is as yet unknown, and, as the lowest values thus far observed are in the pH range 9–10 (Harbury & Loach, 1959, 1960; Stellwagen & Cass, 1974; Lambeth et al., 1974), this possibility is most unlikely.

(b) A similar consideration of the α-amino group leads to its elimination as one of the axial ligands of heme iron. The lowest value thus far reported for the apparent pK_a of a heme iron-ligated α-amino group is only about 1 pH unit lower than its intrinsic pK_a of about 7.4, and that too, for simple heme–peptide systems (Harbury & Loach, 1959, 1960). It is, however, well established that appreciable additional lowering of the apparent pK could result from the protein environment, a classical case being the ligation of histidine in a variety of hemoproteins (Dickerson & Timkovich, 1975; Hagihara et al., 1975), but the maximum variation thus far found is in the range of 3–4 pH units (Walba & Isensee, 1956; Myer & Harbury, 1973; George et al., 1961). If a similar situation is applied to the ligation of the α-amino group, the maximum expected value for its apparent pK_a would be in the pH region of 4–5. Since there is no indication of an apparent pK involving the alteration of the coordination configuration of heme iron in the pH region 3–6 for this protein, the possibility of the α-amino group being coordinated can also be ruled out.

(c) The resolution between the possibilities of histidine/histidine and methionine/histidine configurations stems from a number of observations. One of the characteristics of the methionine/histidine configuration of ferric heme iron systems is the presence of a weak band at 695 nm (Dickerson & Timkovich, 1975) with an absolute molar extinction of about 198 only (Kaminsky et al., 1973). Although ferricytochrome b_{562} at neutral pH does not exhibit a band at the same wavelength, there is a weak band centered at about 710 nm with an absolute molar extinction of about 235 (Figure 5 and Table II). If this band is indeed the counterpart of the 695-nm band of the cytochromes c,[2] then this protein most likely contains a coordination configuration of methionine/histidine, and not histidine/histidine as proposed by Itagaki & Hager (1968). Additional support, although indirect, for the methionine/histidine configuration of the protein at neutral pHs stems from a consideration of the pH dependence of the protein in the alkaline region of the scale. The apparent pK for the transformation of systems containing the histidine/histidine coordination configuration of heme iron to another coordination form, whether in simple heme systems such as heme c systems (Myer & Harbury, 1973) or hemoproteins, i.e., cytochrome b_5 (Hagihara et al., 1975; Ikeda et al., 1974), is found to be in the pH region 10–11. A value of 8.7 for the change of the neutral pH form to another low-spin form at alkaline pH for this protein (Figure 4) is significantly different, which raises doubts regarding the possibility of the histidine/histidine configuration of the system at neutral pHs. The closeness of the observed pK_a for the alkaline transition of ferricytochrome b_{562} to that observed in the cytochromes c, 8.7 vs. 9.2 respectively, where the latter is known to contain the Met/His configuration at neutral pHs, is added support for the proposed configuration of the protein. A consideration of the known sequence of the protein, on the other hand, and the conclusions from the CD studies of the oxidized and reduced forms of the protein at neutral pHs also support the conclusion that the coordination configuration of heme iron is not likely to be His/His. The two histidine residues of the protein are located at positions 66 and 106; both the phenylalanines of the protein are located in a sequence surrounding His-66, locations 64 and 68; and both the tyrosyl residues surround the second histidine residue, locations 104 and 109, and they constitute the terminal end of the polypeptide chain (Itagaki & Hager, 1968). The CD studies of the protein have led to the conclusion that the protein fragment containing the two tyrosyl side chains, i.e., the C-terminal end of the molecule, is exposed to the solvent in the ferric form of the protein (Bullock & Myer, 1978). Since the studies of thermal denaturation reported in this article lead to the conclusion that the heme moiety is at least partially buried in the protein environment, it seems unlikely that the solvent-exposed histidine localized in the C-terminal end could be an axial ligand to heme iron. Since the protein contains two histidines only, the possibility of the His/His configuration seems remote.

Some insight into the nature of the coordination configuration of heme iron of the alkaline form is evident from the characterization of the alkaline transition. This transition is shown to involve a change of heme symmetry from lower symmetry at neutral pH to a configuration with higher symmetry at alkaline pH. The spectral characterization of the transition, on the other hand, is described as a change of liganding group to a group of higher ligand-field strength at alkaline pH. Of the available groups, the deprotonated lysine ϵ-amino group and/or the histidine side chains are the only two possible candidates with ligation characteristics stronger than the sulfur of the methionine side chains (Harbury & Mark, 1973). Whether the group replacing methionine is an ϵ-amino group of lysine or the side chain of the unligated histidine is difficult to discern at this time.

In view of the above considerations, Scheme I can now be rewritten to show the coordination configuration of the protein,[3] i.e.

$$\xrightarrow{<3} \left|\frac{\text{Met}}{\text{His}}\right| \overset{*\ pK_a \simeq 6}{\longleftrightarrow} \frac{\text{Met}}{\text{His}} \overset{pK_a = 8.7}{\longleftrightarrow} \frac{\text{L}}{\text{His}} \xrightarrow{>11}$$

where L is either an ϵ-amino group of lysine or the imidazole

[2] The Soret band of ferricytochrome b_{562} as well as the α and β bands of the reduced form are shifted by 10–12 nm to the red. A similar shift in the 695-nm band would not be entirely unexpected.

[3] Added at the revision of the manuscript: At the time of the first review of the manuscript, it was brought to my attention that a coordination configuration of Met/His has been detected through X-ray diffraction of this protein by Dr. F. Scott Mathews, Department of Physiology and Biophysics, Washington University Medical School, St. Louis, Mo. It has been confirmed that the conclusion of the Met/His coordination configuration of ferricytochrome b_{562} is consistent with data from both X-ray diffraction and NMR studies (personal communication).

side chain of the unligated histidine residue and the asterisk indicates a form conformationally different from the neutral pH form. Which of the two histidines provides the axial ligand and which of the three methionines provides the second coordinating group are subjects of investigation. It should be noted that the identity of the coordination configuration of a *b*-type cytochrome to that of the cytochromes *c*, on the one hand, and the possibility that the physiological function of the two proteins may be the same, i.e., soluble electron carrier, may have far-reaching implications regarding the evolutionary aspects of electron transport in bacterial vs. mammalian systems.

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