Strittmatter, P. (1960) *J. Biol. Chem. 235*, 2492.
Strittmatter, P., & Rogers, M. J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2658.
Strittmatter, P., & Enoch, H. G. (1978) *Methods Enzymol. 52*, 188.
Strittmatter, P., Rogers, M. J., & Spatz, L. (1972) *J. Biol. Chem. 247*, 7188.
Strittmatter, P., Fleming, P., Conners, M., & Corcoran, D. (1978) *Methods Enzymol. 52*, 97.
Tajima, S., & Sato, R. (1980) *J. Biochem. (Tokyo) 87*, 123.
Tajima, S., Enomato, K. I., & Sato, R. (1976) *Arch. Biochem. Biophys. 172*, 90.
Vaz, W. L. C., Austin, R. H., & Vogel, H. (1979) *Biophys. J. 26*, 415.
Visser, L., Robinson, N. C., & Tanford, C. (1975) *Biochemistry 14*, 1194.
Weber, G. (1952) *Biochem. J. 51*, 155.
Weber, G. (1953) *Adv. Protein Chem. 8*, 415.
Wehrly, J., Williams, J., Jameson, D., & Kolb, D. (1976) *Anal. Chem. 48*, 1424.

Differences in Stability against Thermal Unfolding between Trypsin- and Detergent-Solubilized Cytochromes b_5 and Structural Changes in the Heme Vicinity upon the Transition: Resonance Raman and Absorption Study†

Teizo Kitagawa,* Toshihiro Sugiyama, and Toshio Yamano

ABSTRACT: Thermal unfolding of the hydrophilic domain of cytochrome b_5 was investigated with resonance Raman and absorption spectroscopy. The trypsin-solubilized cytochrome b_5 (t-b_5) and detergent-solubilized cytochrome b_5 (d-b_5) exhibited a similar transition at definitely different temperatures (66 ± 2 °C for t-b_5 and 57 ± 2 °C for d-b_5), although such difference was unexpected from the proposed structural independence of the hydrophobic and hydrophilic domains of d-b_5. The Raman spectra of the native and initial high-spin species of t-b_5 were very close to those of the six-coordinate bis(imidazole) and five-coordinate 2-methylimidazole complexes of iron octaethylporphyrin, respectively. Accordingly, the unfolding of t-b_5 was deduced to involve, in the beginning, a change of the heme structure from the six-coordinate low-spin to five-coordinate high-spin form but later a change from the five-coordinate high-spin form to six-coordinate high-spin form presumably with a water molecule at the sixth coordination position. The changes were partially reversible. The irreversibly changed ferric high-spin t-b_5 was converted to the native-like low-spin form by addition of imidazole on the one hand, but on the other hand, by simple reduction without addition of imidazole, this was changed to the ferrous low-spin heme, probably with two histidine residues at the axial positions. The Raman spectra of the two kinds of five-coordinate ferric high-spin forms, produced by the thermal unfolding and acidification, were very alike, but the reversibility of the change was distinctly different. The acid-produced high-spin form was completely recovered to the native low-spin form in contrast with the partial recovery of the thermally produced high-spin form. Consequently, we conclude that the apparently simple thermal transition of cytochrome b_5 around 55–65 °C actually involves the reversible unfolding and the concomitant irreversible changes, and the contribution of the latter depends upon the experimental conditions such as the duration at higher temperatures. Nonetheless, the thermal transition appeared as if it were a two-state transition with an isosbestic point in the visible spectrum, because an additional change in the polypeptide structure, which is probably decisive of the reversibility, is not explicitly reflected in the heme absorption spectrum. A plausible origin for the stability difference between t-b_5 and d-b_5 is also discussed.

Cytochrome b_5 is a well characterized membrane protein involved in the microsomal electron transport system of various tissues. Intact cytochrome b_5 is known to be solubilized by treatment of microsomes with detergent (Ito & Sato, 1968; Spatz & Strittmatter, 1971), while digestion of microsomes with proteases allows the isolation of cytochrome b_5 with a smaller molecular weight (Strittmatter, 1960; Omura et al., 1967; Kajihara & Hagihara, 1968). The former and the latter preparations provide so-called detergent-solubilized cytochrome b_5 (d-b_5)[1] and trypsin-solubilized cytochrome b_5 (t-b_5), respectively, and t-b_5 has been proved to be the heme-containing hydrophilic moiety of d-b_5 (Ito & Sato, 1968; Spatz & Strittmatter, 1971). The amino acid sequences of several t-b_5 molecules from different sources are quite similar (Tsugita et al., 1970; Ozols et al., 1976), and their molecular weights are all about 11 000. The details of the molecular structure of calf liver t-b_5 have been analyzed with X-ray crystallography (Mathews et al., 1972). For rabbit liver t-b_5, the symmetry of the unit cell is found to be the same as that of calf liver t-b_5 (Kretsinger et al., 1970), while d-b_5 has never been crystallized so far. According to the recent NMR study (Keller & Wüthrich, 1980), there are two molecular conformations for t-b_5 in an aqueous solution, one of which corresponds to the

† From the Department of Molecular Physiological Chemistry, Osaka University Medical School, Nakanoshima, Kitaku, Osaka, 530, Japan (T.K.), and the Department of Biochemistry, Osaka University Medical School, Nakanoshima, Kitaku, Osaka, 530, Japan (T.S. and T.Y.). *Received May 14, 1981.* This study was supported by Grant-in-Aid for Scientific Research of the Ministry of Education, Science and Culture of Japan (No. 56480370).

[1] Abbreviations: t-b_5, trypsin-solubilized cytochrome b_5; d-b_5, detergent-solubilized cytochrome b_5; Im, imidazole; 2MeIm, 2-methylimidazole; Fe(OEP)(ClO_4), (octaethylporphyrinato)iron(III) perchlorate; Fe(OEP)($Im)_2$, (octaethylporphyrinato)iron(III)–bis(imidazole) complex; Fe(OEP)(2MeIm), (octaethylporphyrinato)iron(III)–2-methylimidazole complex; Gdn·HCl, guanidine hydrochloride; cmc, critical micelle concentration; CD, circular dichroism; Mb, myoglobin.

0006-2960/82/0421-1680$01.25/0

structure elucidated by X-ray crystallography (Mathews, 1980).

Visser et al. (1975) proposed a two-domain structure of d-b_5, in which the hydrophilic domain, located in the NH_2-terminal side, is responsible for the catalytic activity and the hydrophobic domain, consisting of 43 amino acid residues from the COOH terminus (Fleming et al., 1978; Kondo et al., 1979), serves to bind the d-b_5 molecule to a microsomal membrane. Tajima et al. (1976, 1978) stressed that the molecular structures of the two domains are quite independent despite the fact that a peptide segment which links the two domains is very short. This may lead us to suspect that thermal unfolding of the hydrophilic domain of d-b_5 would take place at a similar temperature to that of t-b_5.

Recently Pfeil & Bendzko (1980) reported a reversible two-state transition of rabbit liver t-b_5 at about 79 °C at pH 7.4 on the basis of the scanning calorimetry, although the transition temperature depended upon pH. Sugiyama et al. (1980a), on the other hand, reported a similar transition of rabbit liver d-b_5 at 55 °C at pH 7.4. This discrepancy of the transition temperature is utterly unexpected from the two independent domain model (Visser et al., 1975; Tajima et al., 1976, 1978). Therefore, in this study, we investigated the thermal unfolding of both d-b_5 and t-b_5 simultaneously.

Resonance Raman scattering from hemoproteins reveals solely the vibrational spectra of the heme group, providing structural information on the heme proximity (Spiro, 1975; Felton & Yu, 1978; Kitagawa et al., 1978). Recent studies have enabled us to interpret another Raman spectral characteristic in terms of a coordination number of the heme iron and a coordinated atom (Teraoka & Kitagawa, 1980), core-size expansion of porphyrin (Spaulding et al., 1975; Spiro et al., 1979), a charge transfer between the heme and the protein Shelnutt et al., 1979), and a state of the bound histidyl imidazole (Stein et al., 1980; Ondrias et al., 1981; Teraoka & Kitagawa, 1981). To gain an insight into a structure change occurring in the heme vicinity of cytochrome b_5 in cooperation with the thermal unfolding, we examined resonance Raman spectra in addition to visible absorption spectra.

Materials and Methods

Detergent-solubilized cytochrome b_5 was purified from rabbit liver microsomes according to Spatz & Strittmatter (1971) with some modifications (Sugiyama et al., 1980b). In the final step of chromatography, d-b_5 bound to DEAE-cellulose was eluted with 50 mM Tris-HCl buffer, pH 7.4, containing 0.5 M KCl and 0.1% Triton X-100. For measurement of an absorption spectrum, the 0.27 mM d-b_5 solution thus obtained was diluted to 36-fold with 0.1 M phosphate buffer, pH 7.4. The trypsin-solubilized cytochrome b_5 was purified from calf liver as reported previously (Miki et al., 1980). The lyophilized t-b_5 was dissolved in 0.1 M phosphate buffer, pH 7.4, to measure the absorption and Raman spectra. The cytochrome was reduced by a small amount of solid dithionite, and the measurement of its Raman spectra was performed under an argon atmosphere. Bovine hemin was purchased from Sigma (type VI).

(Octaethylporphyrinato)iron(III) perchlorate [Fe(OEP)-(ClO_4)] (Ogoshi et al., 1980) was given by Professor H. Ogoshi (Nagaoka University). Imidazole (Im) and 2-methylimidazole (2MeIm) were recrystallized from benzene. The 2MeIm complex of Fe(OEP), Fe(OEP)(2MeIm), was obtained by adding the equimolar amount of 2MeIm to the CH_2Cl_2 solution of Fe(OEP)(ClO_4). By further addition of the equimolar amount of Im to this solution, the asymmetric six-coordinate complex [(Fe(OEP)(2MeIm)(Im)] was obtained,

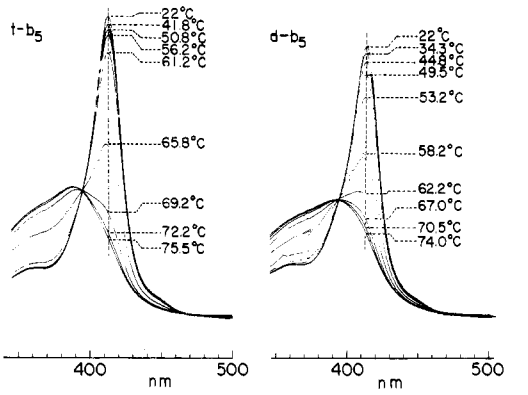

FIGURE 1: Absorption spectra of t-b_5 (left) and d-b_5 (right) at various temperatures from the series A experiments. The scanning was started always 5 min after the sample showed the indicated temperature.

while the symmetric six-coordinate complex [Fe(OEP)($Im)_2$] was obtained by adding excess Im to Fe(OEP)(ClO_4). The formation of those complexes was confirmed with absorption spectra.

Absorption spectra were measured with a Varian-Cary 119 spectrophotometer equipped with a thermojunction. The temperature of the sample was controlled by circulation of water through a water-jacketed cell holder and was measured with a copper–constantan thermocouple which was immersed directly into the sample solution in the cuvette with a stopcock. In one series of experiments (series A), the temperature of the sample was retained at a given temperature, and the scanning of the spectrophotometer was started always 5 min after the sample reached the temperature. In the other series of experiments (series B), the cuvette was first kept in a water bath at a given temperature for 5 min, second it was put into the other bath at 0 °C and kept therein for 2 min, and finally the absorption spectra were measured at room temperature.

The extent of recovery from the thermal unfolding depended upon the highest temperature to which the sample was subjected and also upon its duration. In the experiments shown in Figure 3, temperature was continuously lowered by exchanging the bath of circulating water of the cell holder with another cold one after the sample was kept at the highest temperature for 5 min. The temperatures indicated in Figure 3 denote the temperature measured at the moment when the spectrophotometer records the absorbance at 413 nm.

Raman scattering was excited by the 441.6-nm line of a He–Cd laser (Kinmon Electrics; CDR80SG) and recorded on a JEOL-400D Raman spectrometer. About 300 μL of the 0.5 mM t-b_5 solution was put into a cylindrical Raman cell which was held in a water-jacketed cell holder. In the same way as the absorption measurements, in one series of experiments (series A) the sample was kept at a given temperature during the measurements. In the other series (series B) the sample was kept at 15 °C during the measurement, after it had been retained at a given temperature for 5 min and then promptly brought to 0 °C. The Raman spectrometer was calibrated with indene (Hendra & Loader, 1961).

Results

Figure 1 shows the temperature dependence of the absorption spectra of ferric t-b_5 and d-b_5 obtained from the series A experiments. The individual spectra are considered to reflect the equilibrium state at a given temperature. The temperature-dependent spectral changes of t-b_5 and d-b_5 were considerably alike except for the transition temperature, suggesting that a similar structural change took place in the heme moiety upon the transition. An isosbestic point was clearly observed

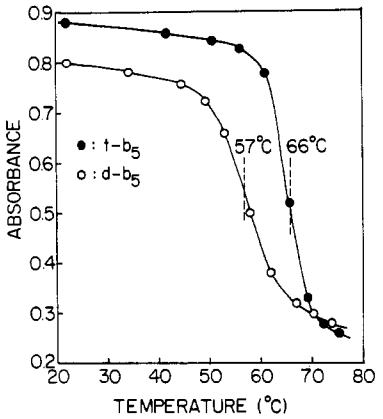

FIGURE 2: Temperature dependence of absorbance of t-b_5 (closed circles) and d-b_5 (open circles) at 413 nm.

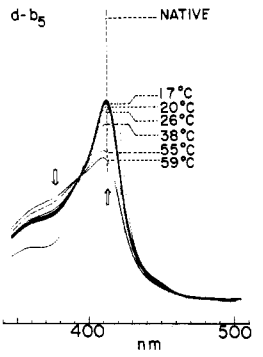

FIGURE 3: Absorption spectra of d-b_5 in the way of recovery from thermal unfolding. The spectrum of the initial state is specified by NATIVE, and the spectrum before cooling is specified as 59 °C. The method of cooling and implication of the designated temperatures are described under Material and Methods.

at 395 nm for t-b_5 and at 394 nm for d-b_5, although only below 62 °C for d-b_5. Thus the spectral changes apparently seemed to be caused by a two-state transition. The absorption spectra of the low-temperature forms of t-b_5 and d-b_5 were indistinguishable, but the absorption maxima of their high-temperature forms appeared at slightly different wavelengths (~388 nm for t-b_5 and ~392 nm for d-b_5).

The absorbances of t-b_5 and d-b_5 at 413 nm are plotted against temperature in Figure 2. The transition of t-b_5 is more sharp than that of d-b_5. From the plot, the midpoint temperature of the transition was inferred to be 57 ± 2 °C for d-b_5 and 66 ± 2 °C for t-b_5, in good agreement with the reported transition temperatures (Sigiyama et al., 1980a; Pfeil & Bendzko, 1980). Accordingly, we conclude that the thermal unfolding of the hydrophilic domain of d-b_5 takes place at a significantly different temperature from the unfolding temperature of t-b_5 when both t-b_5 and d-b_5 are dissolved in the same buffer (0.1 M phosphate, pH 7.4).

A typical result from the recovery experiment on d-b_5 is illustrated in Figure 3. Since the isosbestic point for d-b_5 upon raising the temperature became obscure above 62 °C (Figure 1), we suspected that an irreversible change participated in the reversible unfolding above 62 °C. However, the idea was not always pertinent as explained below. When the sample which had exhibited the spectrum marked by "NATIVE" in Figure 3 was kept at 59 °C for 5 min and then cooled as stated under Materials and Methods, the 413-nm band which once completely disappeared at 59 °C was restored with an isosbestic point at 394 nm as shown there. Nonetheless, the recovery was incomplete (~70%). When the rate of lowering the temperature was slower than the rate in Figure 3, the recovery was rather worse. These observations suggested the simultaneous occurrence of the reversible and irreversible changes at an identical temperature range. This feature was practically common to d-b_5 and t-b_5.

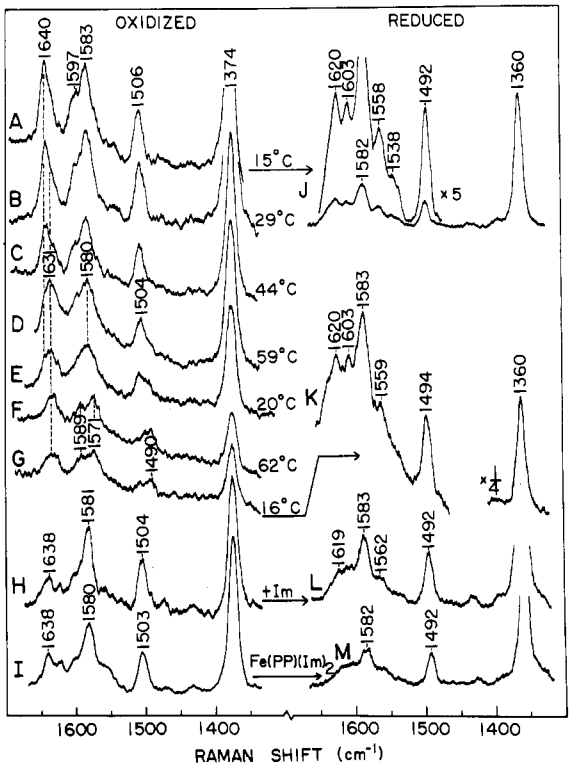

FIGURE 4: Resonance Raman spectra of t-b_5 in the oxidized (left) and reduced states (right) from the series A experiments. Spectra A and J, B, C, and D were observed at 15, 29, 44, and 59 °C, respectively. After the measurement of spectrum D, the sample was cooled and spectrum E was observed at 20 °C. The temperature was raised again for the same preparation to 62 °C where spectrum F was observed and then was lowered to 16 °C where spectrum G was observed. Half of the sample which gave spectrum G was reduced and spectrum K was observed. To the other half Im was added, and spectra H and L were observed. The spectra of ferric (I) and ferrous (M) iron protoporphyrin IX–bis(imidazole) complexes in the same buffer solution as that for cytochrome b_5 are shown at the bottom. Laser: 441.6 nm; 50 mW.

Figure 4 shows the resonance Raman spectra of t-b_5 from the series A experiments, in which the sample was kept at the designated temperature during the measurement (1.5 h). The spin-state marker [band V in Spiro & Burke (1976); band I in Kitagawa et al. (1976); ν_{10} in Abe et al. (1978); we adopt the last notation in this paper] was shifted from 1640 cm^{-1} of the native form (A) to 1631 cm^{-1} at 59 °C (D), while the ν_4 line at 1374 cm^{-1} remained unshifted. After the measurement of spectrum D, the sample was brought to 20 °C, where spectrum E was observed. The 1640-cm^{-1} line increased slightly but the 1631-cm^{-1} feature remained. Accordingly, the irreversible process evidently proceeded even at 59 °C for t-b_5.

When the temperature of the same sample was raised again to 62 °C after the measurement of spectrum E, spectrum F was observed, and when it was cooled, spectrum G was observed. The spectra F and G are alike but distinct from spectrum D. Therefore, spectra F and G are considered to stand for the spectrum of irreversibly denatured t-b_5. We stress here that the entire Raman intensity markedly diminished at higher temperatures. This suggests a possibility that there are two kinds of irreversibly denatured t-b_5; one gives spectra F and G and the other brings no Raman line but fluorescence.

For analysis of one of the irreversibly denatured forms, the solution which had exhibited spectrum G was divided into two

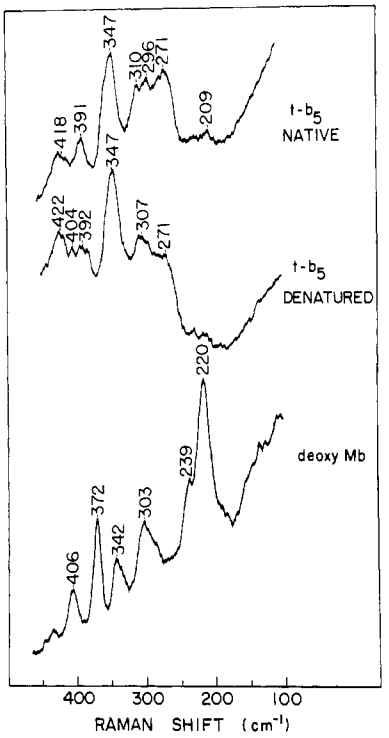

FIGURE 5: Resonance Raman spectra of native ferrous t-b_5 (top), denatured ferrous t-b_5 (middle), and deoxy-Mb (bottom) in the lower frequency region. The top and middle spectra were observed after the measurement of spectra J and K in Figure 4, respectively, for the same preparation.

portions, and Im was added to one while the other was reduced. The reduced sample showed spectrum K, which was strikingly close to spectrum J observed for the reduced form of native t-b_5. The Raman spectra in the lower frequency region of spectra J and K are compared with that of deoxy-Mb in Figure 5. In the lower frequency region, the denatured t-b_5 showed the Raman spectrum noticeably similar to that of the native form but distinctly dissimilar to the spectrum of deoxy-Mb with the five-coordinate high-spin iron. This strongly suggests that the irreversibly denatured component adopts the six-coordinate low-spin structure in the reduced state presumably with two histidine residues at the axial positions. Consequently, one of two histidine residues which were bound to the heme iron in the native form would still be retained in the ferric t-b_5 thus denatured, and the heme would not be dissociated from the polypeptide chain. Here we note that, contrary to the similarity of spectra J and K, the reduced denatured t-b_5 could not be reconverted to the native ferric form. When the sample which had brought about spectrum K was subjected to autoxidation at 10 °C, the identical spectrum with G, but not with A, was observed. Note that the intensity of the 1640-cm^{-1} line of spectrum G is less than one-tenth of that in spectrum A.

The other portion to which Im has been added gave spectrum H in Figure 4. The Raman spectrum is of a typical low-spin type (Spiro & Burke, 1976; Kitagawa et al., 1976), and therefore, one Im molecule at least was bound to the denatured t-b_5, resulting in the six-coordinate heme as in the native t-b_5. Upon reduction of it, spectrum L was observed. The Raman lines around 1620 and 1603 cm^{-1} were not well resolved. Bovine hemin, which was dissolved in an alkaline solution and then brought to pH 7.4 in the same buffer as for t-b_5, exhibited no well-defined Raman lines but strong fluorescence. However, when an excess amount of Im was added to it, spectrum I was observed, and after its reduction spectrum M was observed. The close resemblance between spectra H and I and between spectra L and M may imply an

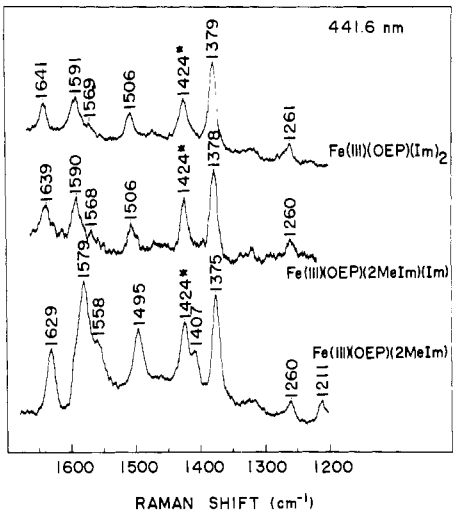

FIGURE 6: Resonance Raman spectra of Fe(OEP)$(Im)_2$ (top), Fe(OEP)(2MeIm)(Im) (middle), and Fe(OEP)(2MeIm) (bottom) in CH_2Cl_2 excited at 441.6 nm. The Raman line marked by an asterisk is due to solvent.

identity of their spectral origin.

To elucidate a structural change which occurred during the temperature change, we carried out some experiments on model compounds. Figure 6 shows the Raman spectra of Fe(OEP)$(Im)_2$, Fe(OEP)(2MeIm)(Im), and Fe(OEP)(2MeIm) excited at 441.6 nm. It is well-known that 2MeIm of the 2MeIm complexes pulls the Fe ion away from the porphyrin plane due to the repulsive interaction between the CH_3 group of 2MeIm and porphyrin nitrogens, restraining the coordination of another 2MeIm to the other axial position (Goff & LaMar, 1977; Jameson et al., 1978), while Fe(OEP)$(Im)_2$ adopts a typical six-coordinate low-spin structure (Takenaka et al., 1972). The Raman lines of Fe(OEP)$(Im)_2$ at 1641 (ν_{10}) and 1506 cm^{-1} (ν_3) were shifted to 1629 (ν_{10}) and 1495 cm^{-1} (ν_3), respectively, for Fe(OEP)(2MeIm), in good agreement with the frequency shifts from 1641 and 1506 cm^{-1} of native t-b_5 to 1631 and 1490 cm^{-1} of the unfolded form. The Raman spectrum of the asymmetric complex, Fe(OEP)(2MeIm)(Im), which aimed to mimic a hypothetical structure, that is, one of the Fe–histidine bonds was loosened but not cleaved, was rather close to the spectrum of Fe(OEP)$(Im)_2$. Accordingly, the initial part of the thermal unfolding of t-b_5 was presumed to involve a change of the coordination number of the heme iron from six to five.

If the irreversible process for the structural change followed the reversible unfolding, the transition temperature for the irreversible change would be higher than the unfolding temperature. So that the possibility could be examined, the series B experiments were undertaken. The absorption spectra of t-b_5 from the series B experiments are shown in Figure 7, where the temperatures at which the sample was incubated for 5 min are designated along with the individual spectra. In the inset figure, the absorbances at 413 and 387 nm are plotted against the incubated temperature. Unexpectedly, the midpoint temperature of the spectral change was identical with that from the series A experiments. This may possibly imply freezing of the high-temperature structure of t-b_5 upon rapid cooling and thus recording of the spectra of the high-temperature form at a room temperature. If this were the case, the equilibrium state could be reproduced by appropriate annealing. When the sample which had shown spectrum 6 was annealed by keeping it at 62 °C for 10 to ~30 min and then cooling slowly, the absorbance at 413 nm slightly increased but its spectrum was quite different from spectrum 2. Furthermore, prolonged

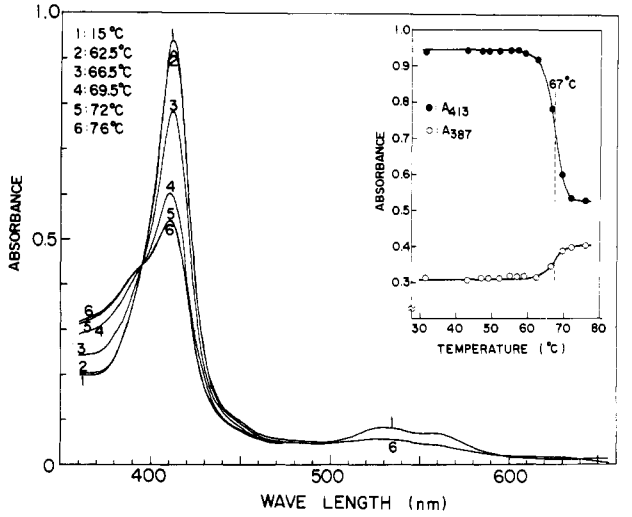

FIGURE 7: Absorption spectra of t-b_5 from the series B experiments. Spectra 1, 2, 3, 4, 5, and 6 were observed for the samples which were incubated at 15, 62.5, 66.5, 69.5, 72, and 76 °C, respectively, for 5 min and then promptly cooled. The inset figure plots the absorbances at 413 nm (closed circles) and at 387 nm (open circles) against the incubation temperatures.

incubation as long as 10 min resulted in a shift of the apparent transition temperature to a lower side (65 °C). Therefore, it is more likely that the reversible portion of unfolded t-b_5 was promptly folded to the native form during the rapid cooling and that the observed spectral differences of the incubated t-b_5 from the native form reflected the contribution of the irreversibly altered portions. This observation also supports the idea of the simultaneous occurrence of the reversible and irreversible processes at the identical temperature range.

Figure 8 shows the resonance Raman spectra of t-b_5 from the series B experiments. The spectral characteristics of native t-b_5 (A) remained unaltered at 46 °C (B), although in the series A experiments (Figure 4) the 1631-cm^{-1} line was recognized even at 44 °C. In the series B experiments, however, the 1631-cm^{-1} line became recognizable at 56 °C and dominant at 66 °C (D). The frequency shifts of ν_3 from 1506 to 1491 cm^{-1} and of ν_{10} from 1640 to 1631 cm^{-1} are obvious in spectrum E (75 °C). Since t-b_5 is known to adopt a high-spin form in an acidic solution (Ikeda et al., 1974), the Raman spectrum of high-spin t-b_5 was examined in the acidic solution for comparison. Although an isoelectric precipitation occurred in the midst of the pH change, the solution became transparent at pH 2.4, exhibiting spectrum F in Figure 8, and its neutralization gave rise to spectrum G. Restoration of spectrum A in spectrum G is evident. The ν_{10} and ν_3 lines of the reversible high-spin form were identified at 1630 and 1491 cm^{-1}, respectively. These are close to the ν_{10} (1629 cm^{-1}) and ν_3 (1495 cm^{-1}) frequencies of Fe(OEP)(2MeIm) shown in Figure 6. Consequently, spectrum F can be regarded as a typical spectrum of the five-coordinate high-spin protoheme-containing proteins with a histidine residue at the axial position of the ferric iron.

Discussion

Difference between t-b_5 and d-b_5. Tajima et al. (1976) investigated the denaturation of cytochrome b_5 by guanidine hydrochloride (Gdn·HCl) and concluded that the unfolding of the hydrophilic and hydrophobic domains takes place quite independently. They also noted the similarity in the CD change at 413 nm between t-b_5 and d-b_5 and the presence of a small but reproducible difference in the concentration of Gdn·HCl at which the unfolding occurred: 2.9 M for t-b_5 and

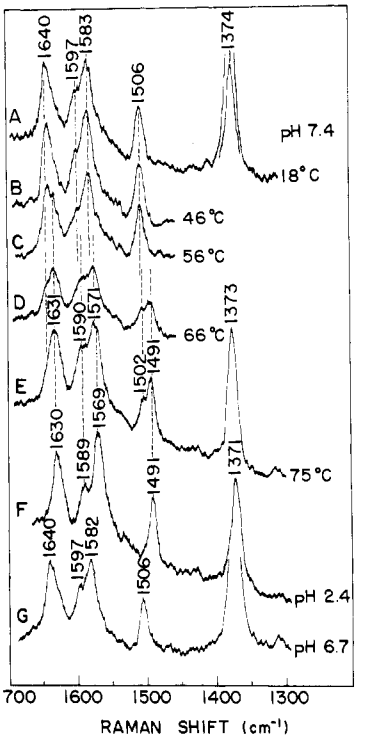

FIGURE 8: Resonance Raman spectra of t-b_5 from the series B experiments and acid t-b_5 at pH 2.4. Spectrum A stands for the initial state. Spectra B, C, D, and E were observed at 18 °C for the sample which was incubated at 46, 56, 66, and 75 °C, respectively, for 5 min and then promptly cooled. Spectrum F was observed for the native t-b_5 at pH 2.4, and after the neutralization of this preparation to pH 6.7, spectrum G was observed.

2.6 M for d-b_5. This implied higher stability of the tertiary structure of t-b_5 against unfolding, and this trend is consistent with the higher transition temperature of t-b_5 shown in Figure 2.

Triton X-100 is bound to the hydrophobic domain of d-b_5 in the concentration above the cmc (Robinson & Tanford, 1975). However, the concentration of Triton X-100 in this experiment (5×10^{-5} mol/L) is about one-fifth of the cmc (2.5×10^{-4} M for $I = 0.1$ and 2.6×10^{-4} for $I = 0.01$; I = ionic strength) (Robinson & Tanford, 1975). Therefore, under the present experimental conditions, the binding of the detergent molecules to d-b_5 is presumably negligible. On the other hand, the experiments of gel filtration from Sephadex G-200 demonstrated an apparent molecular weight of d-b_5 in the presence of 0.4% (w/w) deoxycholate to be 68 000, indicating the aggregation of four molecules (T. Sugiyama et al., unpublished results). In the present preparation which lacks deoxycholate would occur a larger size of molecular aggregation.

Tajima et al. (1976) found that the aggregation number of d-b_5 in such an aggregated condition was retained after unfolding of the hydrophilic domain by Gdn·HCl. When several d-b_5 molecules are indeed aggregated together by the hydrophobic interactions among the hydrophobic domains having 43 residues, the hydrophilic domains with 89 residues would not be sufficiently solvated by water, because the peptide segment which links the two domains is extremely short [2 amino acid residues (Tajima et al., 1978)]. The insufficient hydration would cause unstability of the secondary structure of the hydrophilic domain of d-b_5 in the aggregated assembly. When one or two of the composing molecules were unfolded, the remaining molecules would then have more room, be sufficiently hydrated, and thus be more stabilized than in the initial assembly. This would result in a widening of the temperature range of the transition for d-b_5. On the other hand,

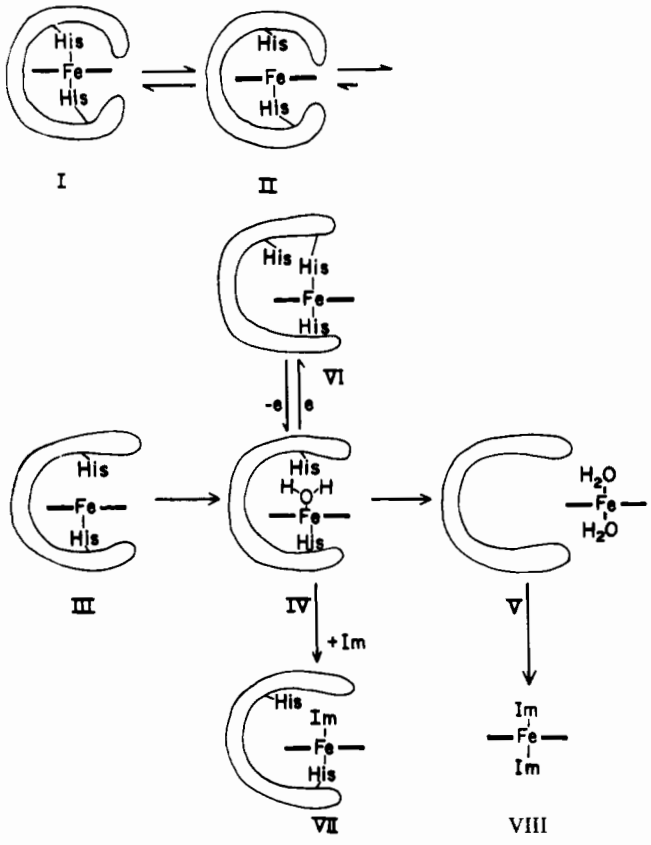

FIGURE 9: Schematic illustration of the structural changes of t-b_5 upon raising temperature and upon treatment performed in the experiments of Figure 4: I, native form; II, acid-induced high-spin form and the reversible high-spin form which appears at the initial stage of the thermal unfolding; III, irreversible high-spin form; IV, six-coordinate high-spin form; V, dissociated heme; VI, reduced form of the irreversibly denatured t-b_5 in which the histidine residue at the sixth coordination position may be the original one or a different one; VII, imidazole-coordinated denatured t-b_5; VIII, free bis(imidazole) complex of iron protoporphyrin IX.

as all the t-b_5 molecules are sufficiently hydrated, it gives the sharp transition curve. If this interpretation were accepted, the origin of the destabilization of the hydrophilic domain of d-b_5 would be ascribed to an insufficient protein–water interaction rather than to interdomain interactions. In this sense the present interpretation reserves consistency with the proposed two independent domain model (Visser et al., 1975; Tajima et al., 1978).

Heme Structure in the Unfolded and Denatured Forms. Structural changes of t-b_5 occurring during the temperature change are illustrated schematically in Figure 9. The Raman spectrum of native low-spin t-b_5 was characterized by two prominent lines at 1640 (ν_{10}) and 1506 cm^{-1} (ν_3) (spectrum A in Figure 8). The corresponding Raman lines of the acid-induced high-spin form of t-b_5 (Ikeda et al., 1974) were observed at 1630 (ν_{10}) and 1491 cm^{-1} (ν_3) as seen in spectrum F in Figure 8. As spectra B–E would be reproduced by weighted superposition of those two typical spectra, the t-b_5 sample which underwent the higher temperatures (46–75 °C) contained both the high- and low-spin molecules.

The ν_{10} and ν_3 frequencies of native t-b_5 were remarkably close to those of Fe(OEP)(Im)$_2$. On the other hand, the ν_{10} and ν_3 frequencies of the acidic high-spin form were close to those of Fe(OEP)(2MeIm) with the five-coordinate structure. Furthermore, the six-coordinate high-spin hemes give the ν_{10} line around 1615–1625 cm^{-1} as demonstrated for aquomet-Mb (Kitagawa et al., 1976), ferric cytochrome *c* at pH 2.0 (Lanir et al., 1979), and model compounds (Teraoka & Kitagawa, 1980). Other lines of native t-b_5 at 1597 (ν_2) and 1583 cm^{-1} (ν_{19}) exhibited slightly different frequencies from those of Fe(OEP)(Im)$_2$ due to different types of porphyrin, but the trend of frequency changes upon the conversion of the spin state of t-b_5 appeared consistent with that observed for the model system. Consequently, we conclude that the spectral change from A to F in Figure 8 is caused by a structural change of the heme from the six-coordinate low-spin (I in Figure 9) to the five-coordinate high-spin form (II).

Although the high-spin forms specified by spectra E and F in Figure 8 were irreversible and reversible, respectively, to the native low-spin form, the heme environments of these two species would be alike for those amino acid residues which interact directly with the heme sufficiently strongly to modulate ligand binding and spin state. Hence, their difference should lie in the polypeptide structure at the region which little affects the heme environments. In accord, the high-spin form which gave spectrum E is represented as III in Figure 9 and is distinguished from II. With regard to this, it is worth noting that the temperature dependence of the far-ultraviolet CD spectrum of d-b_5 (Sugiyama et al., 1980a) is quite dissimilar to that shown in Figure 2.

Previously Tajima et al. (1976) pointed out that the concentration of Gdn·HCl required to induce a far-ultraviolet CD spectral change is much higher for the hydrophobic domain of d-b_5 (5.0–5.5 M) than for the hydrophilic domain as well as for t-b_5 (2.6–2.9 M). This strongly suggests that the hydrophobic domain possesses the higher stability of the secondary structure and thus the higher unfolding temperature in comparison with those of the hydrophilic domain. Recalling that a structural change of the hydrophobic domain is not reflected in the visible absorption spectrum due to lack of a chromophore, we may be allowed to attribute the apparent discrepancy between the temperature dependence of the ultraviolet CD spectra of d-b_5 and that of the curve shown in Figure 2 to the appearance of the two kinds of changes in the CD spectra, that is, superposition of two effects which are caused by the unfolding of the hydrophilic domain and the successive unfolding of the hydrophobic domain at higher temperatures.

Even if this were taken into consideration, the temperature dependence of the far-ultraviolet CD spectrum of d-b_5 around 55 °C (Sigiyama et al., 1980a) would still differ from the curve shown in Figure 2. Hence it seems likely to assume that a structural change of the polypeptide chain takes place in addition to the change in the heme coordination and causes an appreciable change of the far-ultraviolet CD spectrum without any effect on the visible absorption spectrum. In other words, II and III in Figure 9 are considered to exhibit an identical visible absorption spectrum but different far-ultraviolet CD spectra. Then we can understand the existence of an isosbestic point in the visible absorption spectrum despite the fact that the transition is partly irreversible.

The Raman spectral difference between spectrum C in Figure 8 (56 °C) and spectrum E in Figure 4 (59 °C → 20 °C) manifests the effect of keeping t-b_5 at higher temperatures for a longer time. Although it was below the temperature of the onset of the transition, the ν_{10} (1631 cm^{-1}) and ν_3 (1490 cm^{-1}) lines of the high-spin form were more intensified in spectrum E in Figure 4, indicating development of the irreversibly denatured form with the five-coordinate high-spin heme (III in Figure 9).

Spectrum G in Figure 4 exhibited broadening of the ν_{10} line to a lower frequency. Since the ν_{10} line of the six-coordinate high-spin form is usually located around 1615–1625 cm^{-1}

(Kitagawa et al., 1976; Spiro et al., 1979; Teraoka & Kitagawa, 1980), the broadening of ν_{10} would indicate accommodation of a water molecule to the axial position of the heme iron (IV in Figure 9). This may suggest a collapse of the heme pocket. At this stage an exogeneous Im molecule, if present, is accessible to the heme iron (VII), displacing the coordinated water (see H in Figure 4).

When t-b_5 was kept at 62 °C for 1.5 h, the Raman intensity decreased seriously, and it was no more improved by lowering the temperature (spectra F and G in Figure 4). Here we point out that the acidic form of t-b_5 gave the Soret and charge-transfer bands at 366 and 649 nm, respectively, but no absorption features around 413 and ~390 nm, where the thermally denatured t-b_5 exhibited the absorption peaks. On the other hand, free hemin in aqueous solutions exhibited a broad Soret band around 360–390 nm at pH 7.4 and two resolved maxima at 365 and 385 nm at pH 11.8. It is therefore likely that the absorption spectrum of thermally denatured t-b_5 (spectrum 6 in Figure 6) consists of three components, namely, the renatured low-spin form which gives the 413-nm feature (I in Figure 9), the high-spin form which gives the 360-nm feature (III), and a dissociated free heme which gives the 390-nm feature (V). Recalling that a free heme without a strong ligand did not show a prominent Raman line, we can ascribe the intensity decrease in spectra F and G (Figure 4) to the dissociation of heme from the protein.

In conclusion, such irreversible changes take place at the same time as the reversible unfolding when we keep the protein at higher temperatures. This conclusion might be partially inconsistent with the results of the thermodynamic investigation by Pfeil & Bendzko (1980), who stressed the transition of t-b_5 around 70 °C is completely reversible even upon raising the temperature to 97 °C at the rate of 1 K/min. However, their value of the unfolding free energy (ΔG = 25 kJ/mol) might be modified when our conclusion is accepted.

Acknowledgments

We thank Professor H. Ogoshi of Nagaoka University for the courtesy of giving us Fe(OEP)(ClO_4). We express our gratitude to the Yamada Science Foundation for providing the JEOL-400D Raman spectrometer.

References

Abe, M., Kitagawa, T., & Kyogoku, Y. (1978) *J. Chem. Phys. 69*, 4526–4534.

Felton, R. H., & Yu, N.-T. (1978) in *The Porphyrins* (Dolphin, D., Ed.) Vol. 3, Chapter 8, Academic Press, New York.

Fleming, P. J., Dailey, H. A., Corcoran, D., & Strittmatter, P. (1978) *J. Biol. Chem. 253*, 5369–5372.

Goff, H., & LaMar, G. N. (1977) *J. Am. Chem. Soc. 99*, 6599–6606.

Hendra, P. J., & Loader, E. J. (1961) *Chem. Ind. (London)*, 718–719.

Ikeda, M., Iizuka, T., Takao, H., & Hagihara, B. (1974) *Biochim. Biophys. Acta 336*, 15–24.

Ito, A., & Sato, R. (1968) *J. Biol. Chem. 243*, 4922–4923.

Jameson, G. B., Molinaro, F. S., Ibers, J. A., Collman, J. P., Brauman, J. J., Rose, E., & Suslick, K. S. (1978) *J. Am. Chem. Soc. 100*, 6769–6770.

Kajihara, T., & Hagihara, B. (1968) *J. Biochem. (Tokyo) 63*, 453–461.

Keller, R. M., & Wüthrich, K. (1980) *Biochim. Biophys. Acta 621*, 204–217.

Kitagawa, T., Kyogoku, Y., Iizuka, T., & Saito, M. I. (1976) *J. Am. Chem. Soc. 98*, 5169–5173.

Kitagawa, T., Ozaki, Y., & Kyogoku, Y. (1978) *Adv. Biophys. 11*, 153–192.

Kondo, K., Tajima, S., Sato, R., & Narita, K. (1979) *J. Biochem. (Tokyo) 86*, 1119–1128.

Kretsinger, R. H., Hagihara, B., & Tsugita, A. (1970) *Biochim. Biophys. Acta 200*, 421–422.

Lanir, A., Yu, N.-T., & Felton, R. H. (1979) *Biochemistry 18*, 1656–1660.

Mathews, F. S. (1980) *Biochim. Biophys. Acta 622*, 375–379.

Mathews, F. S., Levine, M., & Argos, P. (1972) *J. Mol. Biol. 64*, 449–464.

Miki, N., Sugiyama, T., & Yamano, T. (1980) *J. Biochem. (Tokyo) 88*, 307–316.

Ogoshi, H., Sugimoto, H., & Yoshida, Z. (1980) *Biochim. Biophys. Acta 621*, 19–28.

Omura, T., Siekevitz, P., & Palade, G. E. (1967) *J. Biol. Chem. 242*, 2389–2396.

Ondrias, M. R., Rousseau, D. L., & Simon, S. R. (1981) *Science (Washington, D.C.) 213*, 657–658.

Ozols, J., Gerard, C., & Nobrega, F. G. (1976) *J. Biol. Chem. 251*, 6767–6774.

Pfeil, W., & Bendzko, P. (1980) *Biochim. Biophys. Acta 626*, 73–78.

Robinson, N. C., & Tanford, C. (1975) *Biochemistry 14*, 369–377.

Shelnutt, J. A., Rousseau, D. L., Friedman, J. M., & Simon, S. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4409–4413.

Spatz, L., & Strittmatter, P. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1042–1046.

Spaulding, L. D., Chang, C. C., Yu, N.-T., & Felton, R. H. (1975) *J. Am. Chem. Soc. 97*, 2517–2518.

Spiro, T. G. (1975) *Biochim. Biophys. Acta 416*, 169–187.

Spiro, T. G., & Burke, M. J. (1976) *J. Am. Chem. Soc. 98*, 5482–5489.

Spiro, T. G., Stong, J. D., & Stein, P. (1979) *J. Am. Chem. Soc. 101*, 2648–2655.

Stein, P., Mitchell, M., & Spiro, T. G. (1980) *J. Am. Chem. Soc. 102*, 7795–7797.

Strittmatter, P. (1960) *J. Biol. Chem. 235*, 2492–2497.

Sugiyama, T., Miura, R., Yamano, T., Shiga, K., & Watari, H. (1980a) *Biochem. Biophys. Res. Commun. 97*, 22–27.

Sugiyama, T., Miki, N., & Yamano, T. (1980b) *J. Biochem. (Tokyo) 87*, 1457–1467.

Tajima, S., Enomoto, K., & Sato, R. (1976) *Arch. Biochem. Biophys. 172*, 90–97.

Tajima, S., Enomoto, K., & Sato, R. (1978) *J. Biochem. (Tokyo) 84*, 1573–1586.

Takenaka, A., Sasada, Y., Watanabe, E., Ogoshi, H., & Yoshida, Z. (1972) *Chem. Lett. 12*, 1235–1238.

Teraoka, J., & Kitagawa, T. (1980) *J. Phys. Chem. 84*, 1928–1935.

Teraoka, J., & Kitagawa, T. (1981) *J. Biol. Chem. 256*, 3969–3977.

Tsugita, A., Kobayashi, M., Tani, S., Kyo, S., Rashid, M. A., Yoshida, Y., Kajihara, T., & Hagihara, B. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 443–447.

Visser, L., Robinson, N. C., & Tanford, C. (1975) *Biochemistry 14*, 1194–1199.