SPECTROSCOPIC PROPERTIES OF LOW-SPIN FERROUS HEME COMPLEXES AND HEMEPROTEINS AT 77°K

Gerald C. Wagner and Richard J. Kassner
Department of Chemistry
University of Illinois at Chicago Circle
Chicago, Illinois 60680

Received January 2,1975

SUMMARY. The low temperature optical spectra in the region of the Q_{00} (α-band) and Q_{01} (β-band) transitions of model heme complexes for b- and c-type cytochromes were measured and the results discussed in terms of the similarities and differences to the spectra of horse heart cytochrome c and other hemeproteins. Comparisons of the resolved vibronic components of the Q_{01} and β' bands were made to the recent resonance Raman spectra of hemeproteins. Tentative assignment of the β' band to Q_{02} type transitions has been proposed.

The application of low temperature optical techniques to low-spin ferrous cytochromes (1,2) has resulted in the resolution of ambient temperature Q_{00} (α-band) and Q_{01} (β-band) transitions into a number of distinct components, the number, energy and intensity of which appear characteristic of the particular protein. However, assignments of the resolved components either to the structure or function of the protein have not been indicated, and thus discussion of these spectra has been restricted to comparisons between hemeproteins (3,4). The recent crystallographic structures of cytochromes c (5), c_2 (6) and b_5 (7) and the proton magnetic resonance spectra of cytochromes c_3 (8) have indicated the axial ligand coordination in these proteins. Based on these studies we have examined the low temperature absorption spectra of the appropriate model heme complexes for these b- and c-type cytochromes in an attempt to understand the unique protein spectra by comparison to systems of minimal structure.

EXPERIMENTAL. Horse heart cytochrome c (Type IIa and VI), equine protohemin IX (grade I), N-acetyl-DL-methionine and N-α-acetyl-L-histidine from Sigma and C. krusei cytochrome c (2X crystallized) from Calbiochem were used as received. R. rubrum cytochrome c_2 (A_{272}/A_{415} = 0.22) was a gift from Dr. R. G. Bartsch. C. ethylicium 2K cytochrome c_3 (A_{280}/A_{418} = 0.065) was a gift from Dr. T. E. Meyer. E. coli cytochrome b_{562} (A_{562}/A_{280} = 1.37) was isolated by the procedure of Itagaki and Hager (9). The heme c octapeptide of cytochrome c (Type IIa) was prepared by the method of Harbury and Loach (10).

Absorption spectra were recorded on a Cary 14R spectrophotometer equipped with a high intensity light source and an expanded scale 0-0.1, 0.1-0.2 slidewire. Liquid-nitrogen temperature spectra measured in the vitrified state of 50% aqueous glycerol were obtained with a Rho Scientific spectrodewar using spectral cells of ca. 1.0 cm optical path length. Detailed procedures for

obtaining low temperature spectra have been described elsewhere (11). Anaerobic solutions of ferrous heme complexes and hemeproteins were prepared by reduction with a minimal excess of $Na_2S_2O_4$ under an argon atmosphere. Heme concentrations were in a range of ca. 20 to 40 μM as determined by optical measurements. The c-type model heme complexes were prepared from the heme c octapeptide of cytochrome c, which provides axial coordination of one histidine residue to the heme iron (10). Addition of excess N-acetyl-methionine (12) or N-α-acetyl-histidine yielded the desired hexa-coordinate c-type model complexes (5,6,8). The desired b-type model heme complex (7) was prepared by adding excess N-α-acetyl-histidine to protoheme IX.

RESULTS. The Q_{00} transition in the three model heme complexes at 77°K is characterized by a single symmetric absorption band which is in contrast to the well-known splitting of the Q_{00} band found in mammalian cytochrome c, as seen in Figure 1, b_5 and numerous other cytochromes (2). Optical measurements of a bisimidazole mesoheme IX complex extended to liquid-helium temperatures yielded no further increase in resolution of the single symmetric Q_{00} band observed at 77°K, even though reconstituted mesoheme IX cytochrome b_5 exhibits splitting of the Q_{00} band at near ambient temperatures (13). The absence of splitting in the model spectra suggested comparisons be made to hemeproteins which have been reported to exhibit single Q_{00} bands at 77°K. However, re-examination of various proteins, as seen in Figure 1, reveals an asymmetry or splitting of the Q_{00} band in the vitrified state of 50% aqueous glycerol for R. rubrum cytochrome c_2, C. ethylicium cytochrome c_3 and E. coli cytochrome b_{562}, which have not been previously observed (1,9,14). In agreement with previous studies (1,15) the only protein examined which remained a single symmetric Q_{00} band was C. krusei cytochrome c.

The low temperature spectra in Figure 2 represent the spectral region of the Q_{01} transition for the model heme complexes and cytochrome c. Model spectra in the Q_{01} region show a resolution of component bands similar to those seen in the hemeproteins; moreover, the degree of resolution appears dependent on the axial ligands and on the porphyrin, as seen in Figure 2. In view of the diffuse nature of these overlapping bands, which precludes a critical assignment of the small differences observed in the model spectra, analysis of the Q_{01} region is presented in terms of the dominant similarities. Resolved bands found in the model complexes can be grouped into different regions according to the energy

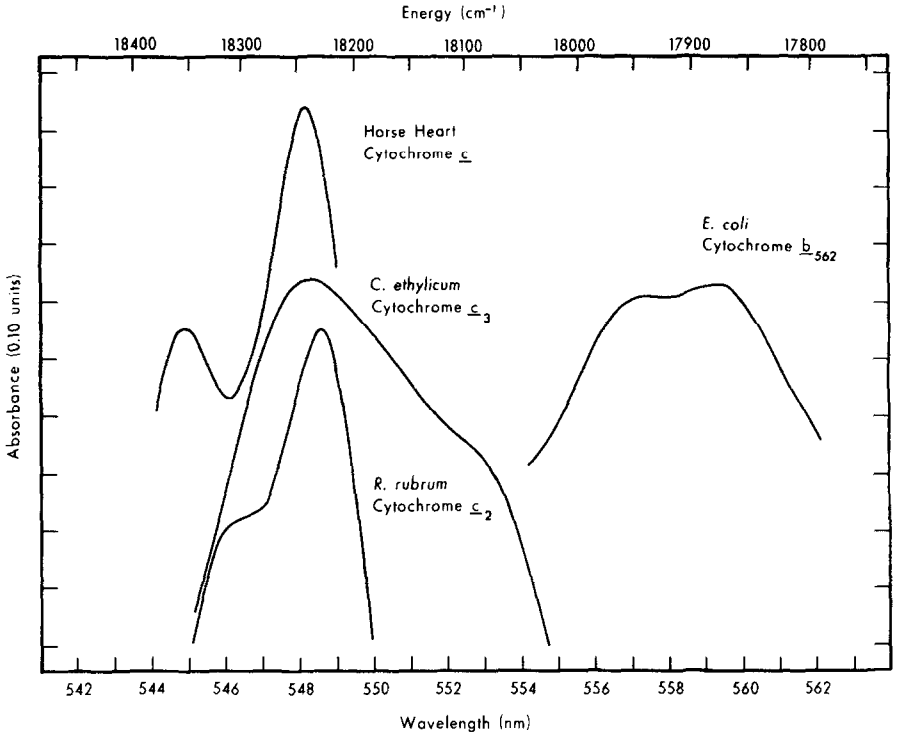

Figure 1. Hemeprotein optical spectra of Q_{00} band at 77°K. All proteins in 50% glycerol (v/v). Cytochrome c in 50 mM phosphate pH 7.4. Cytochrome c_2 in 75 mM Tris-Cl pH 7.3. Cytochrome c_3 in 5 mM phosphate pH 7.8, 0.1 M in NaCl. Cytochrome b_{562} in 0.33 M phosphate pH 8.0.

---

difference Q_{0v}-Q_{00}, which occur at ca. 360, 740, 1000, 1200, 1300, 1440, 1630 cm^{-1}. In horse heart cytochrome c there is a direct correspondence in number, relative intensity and energy to the four high energy bands at ca. 1200, 1300, 1440 and 1630 cm^{-1} in the model systems. The major difference is found in the lower energy bands where there appears to be a doubling of bands in the protein corresponding to the transitions at ca. 360, 740 and 1000 cm^{-1} in the model systems. In regards to C. krusei cytochrome c, the optical spectra in the Q_{01} region are very similar to that observed for mammalian cytochrome c with the one exception of the absence of resolved doubling corresponding to the transition at ca. 740 cm^{-1} in the model systems.

The low temperature spectra in Figure 3 represent the spectral region of the β' band, as designated by Estabrook (16), for the model complexes and cyto-

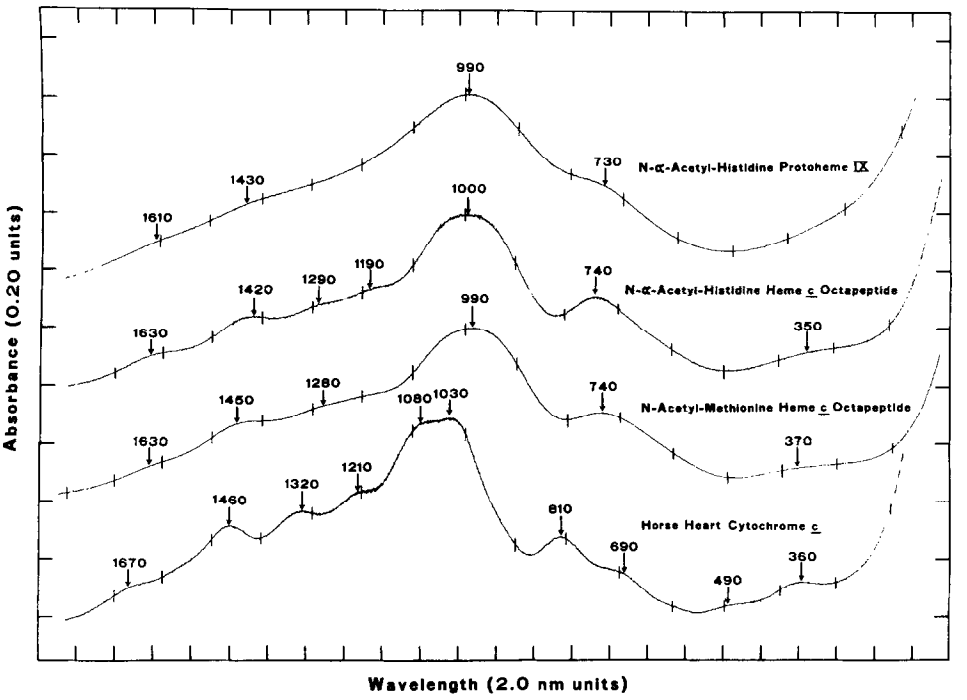

Figure 2. Optical spectra of Q_{01} band at 77°K. Model complexes in 50% glycerol (v/v), ca. 40 mM in phosphate pH 8.2. Effective concentrations: N-acetyl-methionine 2.0 M, N-acetyl-histidine 1.0 M. Cytochrome c as described in Figure 1. Vertical lines on spectra indicate 100 cm^{-1} divisions from each Q_{00} transition. Transition energies based on (Q_{0v}-Q_{00}) difference (cm^{-1}); for cytochrome c, transitions arbitrarily based on lower energy Q_{00} peak. Subtract 110 cm^{-1} for each protein transition relative to higher energy Q_{00} peak.

chrome c. Resolution in this region is comparable to that observed in the model spectra in the Q_{01} region. Thus the extent of resolution in the β' band in Figure 3 appears dependent on the axial ligands and porphyrin, which parallels that observed in the Q_{01} band in Figure 2. The resolvable bands found in the model complexes can be grouped into regions at ca. 2300, 2600, 2830, 3100 and 3450 cm^{-1} according to their energy dfiference from the Q_{00} transition. In the β' band of cytochrome c there is a direct correspondence in number, relative intensity and energy to all five resolved components in the β' band of the model complexes.

DISCUSSION. The absence of splitting in the Q_{00} band of the model complexes is the most distinguishing feature of the optical spectra when compared to most hemeproteins. The optical splitting of the Q_{00} transition, which is consistent

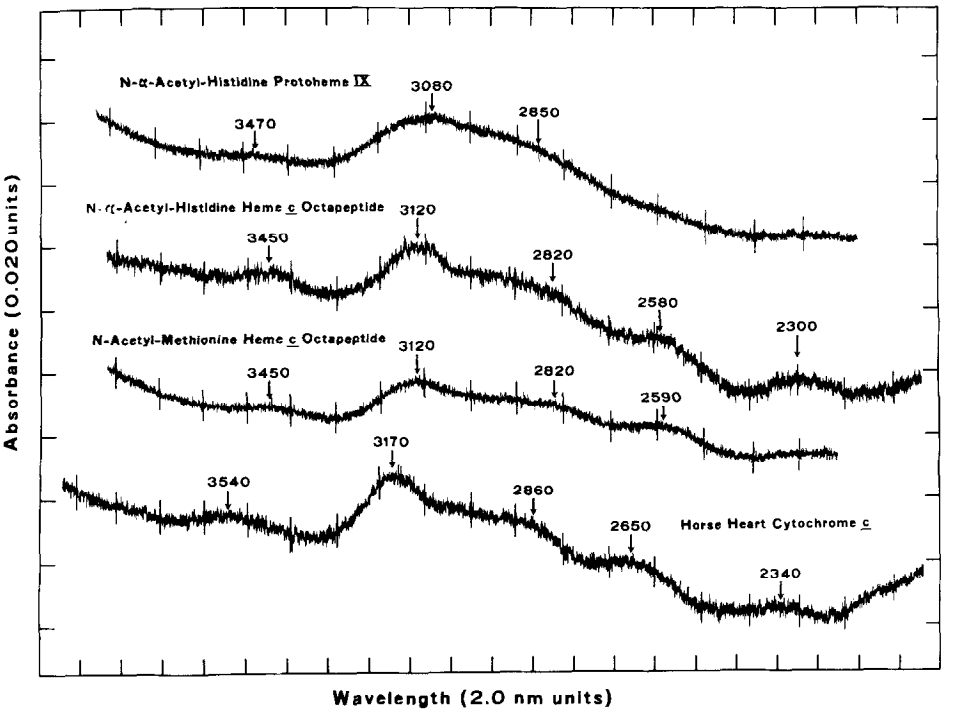

Figure 3. Optical spectra of β' band at 77°K. Identical conditions and descriptions as indicated in Figure 2.

with the removal of the double degeneracy (17) of the lowest excited state of the prosthetic group, appears to be influenced by the protein environment around the heme and its axial ligands. Recent X-ray determinations (5,6,7) do in fact indicate a preferred orientation of the imidazole rings of the histidine residues with respect to the heme plane, which appears stabilized by the tertiary structure of the protein. Such preferred stereochemical orientations of axial ligands would not be predicted in the simple model systems, and may reflect the inability to resolve the Q_{00} transition into components in the model heme complexes. In regards to the spectrum of C. krusei cytochrome c, it should be noted that although the axial ligands appear to be identical to mammalian types, the heme environment is distinguishingly unique from numerous mammalian type cytochormes c (18).

The similarity of resolved Q_{01} vibronic transitions in the model complexes and hemeproteins is evident when the spectra are normalized in energy units

relative to the Q_{00} transition; the similarity extends into the β' region and when comparing spectra derived from different porphyrins. The numerous resonance Raman spectra (19) reported for cytochrome c have indicated that the vibrational modes that provide vibronic intensity in the electronic absorption spectrum will show increased intensity as resonance is approached in the Raman spectrum (20). The excited state vibronic character of the Q_{01} band and possibly the β' band allows comparison with the ground state vibronic spectra, whereby analogous assignments from ground state vibrations are considered for the transitions in the electronic spectra. Fundamental vibrations in the region of ca. 1100-1650 cm^{-1}, which dominate the Raman spectrum of cytochrome c, have been attributed to numerous in-plane stretching modes of the porphyrin skeleton (19). Using the nomenclature of Spiro (19) these frequencies have been assigned to porphyrin bond stretching modes of the relative order (21) $C_b-C_b > C_a-C_m > C_a-N > C_a-C_b$ as well as δC_m-H bond bending modes, whose energies appear on both sides of the C_a-N bond stretches. In both the hemeprotein and model complexes the Q_{01} band draws significant intensity in this vibronic region of the excited state as indicated by the transitions at ca. 1200, 1300, 1440 and 1630 cm^{-1}. The Raman spectra also indicate three other regions of fundamental vibronic intensity at ca. 350, 750 and 900-1000 cm^{-1} (19,22), to which there is an evident correlation of optical transitions in the protein and model complexes at ca. 360, 740 and 1000 cm^{-1}. Low energy ground state vibrations at ca. 350 and 750 cm^{-1}, which have been tentatively assigned to iron-pyrrole nitrogen (23) or iron-histidine nitrogen (24) stretching modes and to out-of-plane heme bending modes (23) respectively, correlate with transitions at ca. 360 and 740 cm^{-1} in the Q_{01} band. The β' band corresponds to energy differences (2200-3500 cm^{-1}) that have been reported for combination and overtone vibronic modes (between ca. 2200 to 3000 cm^{-1}) in the Raman spectrum of cytochrome c (22). The low intensity of this band and the apparent correlation of fundamental vibrations at ca. 1200, 1300, 1440 and 1630 cm^{-1} in the Q_{01} band to overtone vibrations at ca. 2300, 2600, 2830 and 3100 cm^{-1} respectively in the β' band

supports the assignment of overtone (or combination) transitions to the β' band. More descriptive correlations of the Q_{01} and β' band transitions must await the resonance Raman spectra of these model heme complexes, but in view of the apparent vibronic nature of the β' band to combination and overtone modes in the Q transition, we propose that these transitions be collectively represented as the Q_{02} band.

ACKNOWLEDGEMENT. This work was supported by a grant from the National Institutes of Health (HL-16575). Proteins from Dr.'s Bartsch and Meyer, and the measurement of the liquid-helium spectra by Dr. A. H. Francis are gratefully acknowledged.

REFERENCES

1. Estabrook, R. W. (1956) J. Biol. Chem. 223, 781-794.
2. Estabrook, R. W. (1961) in "Haematin Enzymes," Falk, Lemberg and Morton, eds., Int'l. Union of Biochemistry Symposium, 19, Pergamon Press, Oxford, 436-460.
3. Estabrook, R. W. (1966) in "Hemes and Hemeproteins," Chance, Estabrook and Yonetani, eds., Academic Press, New York, 405-411.
4. Elliott, W. B. and Margoliash, E. (1971) Devel. Appl. Spectry. 9, 125-140.
5. Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O. B., Samson, L., Cooper, A., and Margoliash, E. (1971) J. Biol. Chem. 246, 1511-1533.
6. Salemme, F. R., Freer, S. T., Xuong, Ng, H., Alden, R. A., and Kraut, J. (1973) J. Biol. Chem. 248, 3910-3921.
7. Mathews, F. S., Argos, P., and Levine, M. (1971) Cold Spring Harbor Symp. Quant. Biol. 36, 387-395.
8. McDonald, C. C., Phillips, W. D., and LeGall, J. (1974) Biochemistry 13, 1952-1959.
9. Itagaki, E. and Hager, L. P. (1966) J. Biol. Chem. 241, 3687-3695.
10. Harbury, H. A. and Loach, P. A. (1960) J. Biol. Chem. 235, 3640-3645.
11. Wagner, G. C. and Kassner, R. J., submitted for publication.
12. Harbury, H., Cronin, J., Fanger, M., Hettinger, T., Murphy, A., Myer, Y., and Vinogradov, S. (1965) Proc. Nat. Acad. Sci. 54, 1658-1664.
13. Ozols, J. and Strittmatter, P. (1964) J. Biol. Chem. 239, 1018-1023.
14. Meyer, T. E., Bartsch, R. G., and Kamen, M. D. (1971) Biochim. Biophys. Acta. 245, 453-464.
15. Kawai, K. and Mizushima, H. (1973) J. Biochem. (Tokyo) 74, 183-185.
16. Estabrook, R. W. and Sacktor, B. (1958) Arch. Biochem. Biophys. 76, 532-545.
17. Zerner, M., Gouterman, M., and Kobayashi, H. (1966) Theoret. Chim. Acta 6, 363-400.
18. McDonald, C. C., Phillips, W. D., and Vinogradov, S. N. (1969) Biochem. Biophys. Res. Commun. 36, 442-449.
19. Spiro, T. G. (1974) Accounts Chem. Res. 7, 339-344.
20. Spiro, T. G. and Strekas, T. C. (1972) Proc. Nat. Acad. Sci. 69, 2622-2626.
21. Spiro, T. G. and Strekas, T. C. (1974) J. Amer. Chem. Soc. 96, 338-345.
22. Friedman, J. M. and Hochstrasser, R. M. (1973) Chem. Phys. 1, 457-467.
23. Brunner, H. and Sussner, H. (1973) Biochim. Biophys. Acta. 310, 20-31.
24. Ogoshi, H., Watanabe, E., Yoshida, Z., Kincaid, J., and Nakamoto, K. (1973) J. Amer. Chem. Soc. 95, 2845-2849.