References and Notes

1. P. Alexander and R. Evans, *Nature (London) (New Biol.)* **232**, 76 (1971); J. B. Hibbs, Jr., L. H. Lambert, Jr., J. S. Remington, *ibid.* **235**, 48 (1972); J. B. Hibbs, Jr., R. R. Taintor, H. A. Chapman, Jr., J. B. Weinberg, *Science* **197**, 279 (1977); S. W. Russell, W. F. Doe, A. T. McIntosh, *J. Exp. Med.* **146**, 1151 (1977).
2. J. J. Rinehart, P. Lange, B. J. Gormus, M. E. Kaplan, *Blood* **52**, 211 (1978); J. J. Rinehart, R. Vessella, P. Lange, M. E. Kaplan, *J. Lab. Clin. Med.* **93**, 361 (1979); D. J. Cameron and W. H. Churchill, *J. Clin. Invest.* **63**, 977 (1979); G. Unsgaard, J. Hammerstrom, J. Lamvik, *Acta Pathol. Microbiol. Scand. Sect. C* **87**, 159 (1979); A. Mantovani, T. R. Jerrells, J. H. Dean, R. B. Herberman, *Int. J. Cancer* **23**, 18 (1979); A. F. LoBuglio, M. J. Solvay, S. J. Weiss, *Blood* **54** (Suppl. 1), 103a (1979).
3. J. B. Hibbs, Jr., L. H. Lambert, J. S. Remington, *Nature (London) New Biol.* **235**, 48 (1972); M. S. Meltzer, R. W. Tucker, K. K. Sanford, E. J. Leonard, *J. Natl. Cancer Inst.* **54**, 1177 (1975).
4. J. B. Hibbs, Jr., H. A. Chapman, Jr., J. B. Weinberg, *J. Reticuloendothel. Soc.* **24**, 549 (1978).
5. G. Rovera, T. G. O'Brien, L. Diamond, *Science* **204**, 868 (1979); G. Rovera, D. Santoli, C. Damsky, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2779 (1979); A. Vorbrodt, P. Meo, G. Rovera, *J. Cell Biol.* **83**, 300 (1979).
6. J. Lotem and L. Sachs, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5158 (1979).
7. S. J. Collins, R. C. Gallo, R. E. Gallagher, *Nature (London)* **270**, 347 (1977); R. Gallagher, S. Collins, J. Trujillo, K. McCredie, M. Ahearn, S. Tsai, R. Metzgar, G. Aulakh, F. Ruscetti, R. Gallo, *Blood* **54**, 713 (1979).
8. The HL60 cells were obtained from R. Metzgar, Duke University Medical Center. They were grown by seeding at 5×10^5 cells per milliliter of DMM supplemented with dextrose (1 mg/ml), streptomycin (100 μg/ml), penicillin (100 U/ml), and 20 m*M* Hepes buffer. The medium was endotoxin-free as assessed by the *Limulus* amebocyte lysate assay (*11*). Maintenance cultures were subcultured two times per week with endotoxin-free fetal bovine serum (Sterile Systems, Inc., Logan, Utah).
9. The cells HeLa, HEp2, transformed WI38, normal WI38, and Detroit 551 were from American Type Culture Collection; the mouse cell lines are maintained in continuous serial passage in this laboratory (*1*, *4*); HSB, SB, and K562 were from R. Metzgar.
10. J. B. Weinberg and J. B. Hibbs, Jr., *J. Natl. Cancer Inst.* **63**, 1273 (1979).
11. J. B. Weinberg, H. A. Chapman, Jr., J. B. Hibbs, Jr., *J. Immunol.* **121**, 72 (1978); L. P. Ruco and M. S. Meltzer, *ibid.*, p. 203.
12. R. A. Adams, L. Pothier, A. Flowers, H. Lazarus, S. Farber, G. E. Foley. *Exp. Cell Res.* **62**, 5 (1970); J. K. Anderson and R. S. Metzgar, *J. Immunol.* **120**, 262 (1978); C. B. Lozzio and B. B. Lozzio, *Blood* **45**, 321 (1975); L. C. Andersson, K. Nilsson, C. G. Gahmberg, *Int. J. Cancer* **23**, 143 (1979).
13. J. Touraine, J. W. Hadden, F. Touraine, E. M. Hadden, R. Estensen, R. A. Good, *J. Exp. Med.* **145**, 460 (1977); J. Abb, G. J. Bayliss, F. Deinhardt, *J. Immunol.* **122**, 1639 (1979).
14. C. F. Nathan, L. H. Bruckner, S. C. Silverstein, Z. A. Cohn, *J. Exp. Med.* **149**, 84 (1979); W. Grimm, E. Bärlin, H. Leser, W. Kramer, D. Gemsa, *Clin. Immunol. Immunopathol.* **17**, 617 (1980).
15. Normal human blood monocytes and lymphocytes were prepared to ≥ 95 percent purity as previously described [K. Kumagai, K. Itoh, S. Hinums, M. Tada, *J. Immunol. Methods* **29**, 17 (1979)]. The monocytes and lymphocytes were then cultured with or without TPA and assayed for cytotoxicity as described in the legend to Fig. 1.
16. L. Sachs, *Nature (London)* **274**, 535 (1978); S. J. Collins, A. Bodner, R. Ting, R. C. Gallo, *Int. J. Cancer* **25**, 213 (1980).
17. H. P. Koeffler, M. Bar-Eli, M. Territo, *Blood* (Suppl. 1) **54**, 174a (1979); *J. Clin. Invest.* **66**, 1101 (1980); L. Pegoraro, J. Abrahm, R. A. Cooper, A. Levis, B. Lange, P. Meo, G. Rovero, *Blood* **55**, 859 (1980).
18. P. E. Driedger and P. M. Blumberg, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 567 (1980); M. Shoyab and G. J. Todaro, *Nature (London)* **288**, 451 (1980).
19. J. Hughes, T. W. Smith, H. W. Kosterlitz, L. A. Fothergill, B. A. Morgan, H. R. Morris, *ibid.* **258**, 577 (1975).

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Structural Changes at the Heme Induced by Freezing Hemoglobin

Abstract. *A dramatic change occurs in the vibrational properties of the iron-histidine bond,* trans *to the oxygen binding site, on freezing deoxyhemoglobin. The large, quaternary structure–dependent differences in the shape and frequency of the iron-histidine mode observed in resonance Raman scattering measurements above freezing are significantly diminished by the freezing event and the scattering intensity increases substantially. On further reduction in temperature to 10 K this broad line becomes narrow and shifts to a higher frequency. These data implicate dynamical processes and protein interaction with water as contributors to the quaternary structure dependence of the iron-histidine bond and thus reflect on the role of that bond in the energetics of cooperative ligand binding.*

Resonance Raman scattering studies of deoxyhemoglobins and deoxymyoglobins have led to assignment of the mode at 216 cm^{-1} to stretching of the bond between the heme iron and the proximal histidine nitrogen (*1–4*). The properties of this mode under various states of ligation and different protein conformations will ultimately reveal the role played by the proximal histidine in controlling the energetics of cooperative ligand binding. Large differences in the frequency and shape of this mode were found when T-state human adult hemoglobin (HbA) was compared with R-state NES des-Arg-hemoglobin under solution condition at or slightly below room temperature (*4–6*). Nagai and Kitagawa (*5*) also reported recently that in T-state valency hybrid hemoglobins the α chains display a different frequency for this mode than do the β chains. In measurements at a very low temperature one might expect that the lines would narrow and one could thereby distinguish the contributions from the α and β chains in native HbA. We therefore made resonance Raman scattering measurements of this iron-histidine mode in HbA over the temperature range 10 to 300 K. We found that in the measurements on frozen samples at a very low temperature (10 K) the quaternary structure–dependent differences in this mode that are seen at room temperature are completely absent. On warming the samples from 10 K to just below freezing, the iron-histidine stretching mode in hemoglobins stabilized in both quaternary structures broadens and shifts to a lower frequency. However, the R-T differences in this mode develop only on going through the melting transition at 0°C.

The Raman measurements on solution samples from 25° to 0°C or on frozen samples from 0° to −20°C were made with Raman difference instrumentation with which very small differences (< 0.1 cm^{-1}) may be detected by simultaneously comparing two samples (*7*). The liquid samples were placed in a split rotating cell and frozen by a stream of cold nitrogen gas. Measurements on individual samples below 200 K were made with the same spectrometer without using the rotating cell. For these measurements small drops of sample were placed on a precooled cold finger of an Air Products Heli-Tran refrigerator, followed by evacuation and then adjustment to a selected temperature. Spectra were obtained with excitation frequencies of 5287 and 4579 Å from an argon ion laser and 4131 Å from a krypton ion laser. Samples were stored either at 4°C or in liquid nitrogen and were chromatographed on a column before use. The procedure of Kilmartin and Hewitt (*8*) was used to prepare NES des-Arg-HbA. A solution of 1 m*M* HbA in 10 to 80 m*M* sodium phosphate buffer (*p*H 7.0) was found to yield nonfluorescent samples for low-temperature studies.

The Raman mode at 216 cm^{-1} in HbA has been assigned as the iron-histidine stretching frequency on the basis of isotope and model compound studies (*2–4*, *6*). It displays an asymmetric line shape in T-state deoxy HbA and becomes more intense, symmetric, and shifts to higher frequency in R-state deoxy-NES des-Arg-HbA. This behavior was previously reported (*4–6*) and is illustrated in Fig. 1 (see spectra a and c). Other small differences in the low-frequency region between native HbA and the R-structure chemically modified form have also been reported (*6*). Differences in bands sensitive to electron density between 1200 and 1650 cm^{-1} have been detected as well, and interpreted as evidence for charge transfer interactions between the porphyrin macrocycle and amino acid residues of the globin (*9*).

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On freezing hemoglobin and lowering the temperature to 10 K (spectrum e in Fig. 1) several notable changes are detected. First, the iron-histidine stretching mode becomes narrow (~ 8 cm^{-1}) and shifts to 230 cm^{-1}, a frequency significantly higher than that of R-state hemoglobins in solution. In contrast, the line at 300 cm^{-1} remains broad and appears to have more than one component. Second, the broad intense band evident at room temperature at ~ 160 cm^{-1} disappears in the spectra of the frozen samples. Third, the line at 340 cm^{-1} shifts to a frequency higher by about 5 cm^{-1}. These same features are seen for all deoxyhemoglobins that we examined independent of their presumed quaternary structure. This includes HbA ± IHP, Hb Kempsey, NES des-Arg-HbA, β_4 tetramers, and isolated α chains. On warming samples toward 0°C the iron-histidine stretching mode continuously broadens, shifts to a lower frequency, and increases in intensity.

Between 0° and −10°C (see spectra b and d in Fig. 1) the frozen deoxyhemoglobin samples have the following characteristics. The mode at 160 cm^{-1} evident in the room temperature spectra is absent. The iron-histidine stretching mode of HbA is markedly more symmetric than it is in solution, but its frequency remains centered at the solution value, 216 cm^{-1}. In NES des-Arg HbA this mode is at 217 cm^{-1}, substantially lower than it is under solution conditions for this R-structure hemoglobin (222 cm^{-1}). In all cases that we examined, the intensity of the iron-histidine stretching mode relative to that of other lines in the spectrum is much stronger at −3°C than it is in solution.

These data have a strong bearing on the mechanism of cooperativity and on biophysical studies of hemoglobin and other hemeproteins. Before discussing this, however, we must ask whether freezing the hemoglobin alters the quaternary structure of the protein as a whole. For the following reasons, we believe it does not. Electron paramagnetic resonance studies have shown that in methemoglobins that exhibit a spin equilibrium in solution, the spin equilibrium is "frozen in" by quick freezing (*10*). In our data we detected no differences that depended on the rate of freezing, ranging from slow freezing (several seconds) to quick freezing (direct insertion into liquid nitrogen). The Raman lines at 1471 and 1605 cm^{-1}, which are highly sensitive to the size of the porphyrin core (*11, 12*), and the Raman line at 1357 cm^{-1}, which is sensitive to the porphyrin electron density (*13*), are unshifted from their solution values by freezing at −3°C. In addition, the same quaternary structure–dependent changes (~ 1.3 to 1.4 cm^{-1}) previously reported in solution in the 1357 cm^{-1} line (*9*) are detected in the comparison of frozen HbA to frozen NES des-Arg HbA samples. We therefore assume that the quaternary arrangement of the subunits is unaltered by freezing.

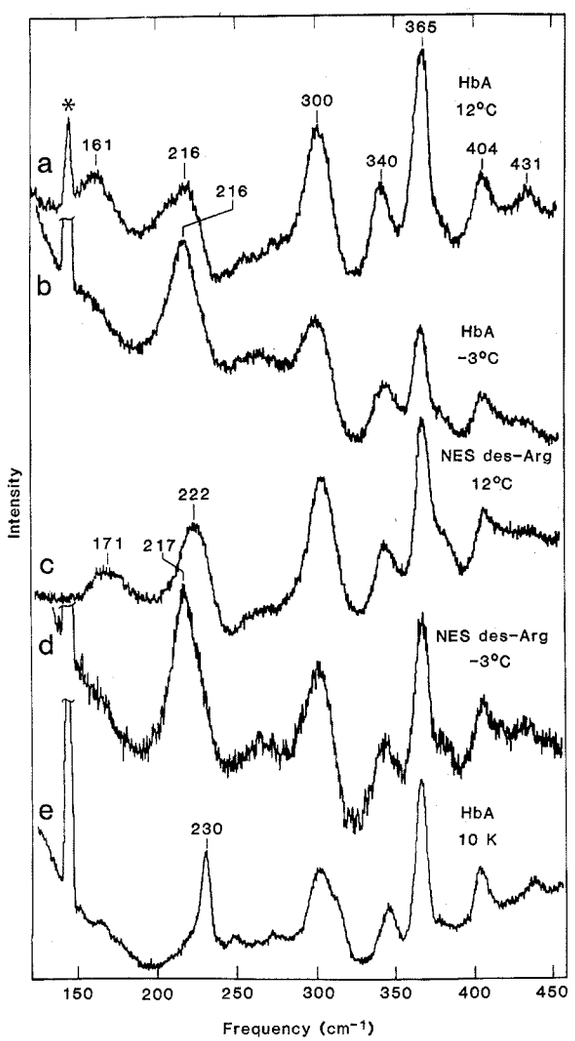

Fig. 1. Temperature effects on Raman scattering from hemoglobins. Spectra represent HbA (a) in solution at 12°C, (b) frozen at −3°C, (e) frozen at 10 K and NES des-Arg-HbA (c) in solution at 12°C, and (d) frozen at −3°C. The line marked with an asterisk at 143 cm^{-1} is a laser fluorescence line. Conditions: 1 m*M* hemoglobin, 10 to 80 m*M* phosphate buffer (*p*H 7.0 to 7.3), 4 cm^{-1} spectral slit width, 4579 Å laser excitation (125 to 150 mW).

The variations in frequency and width of the iron-histidine mode demonstrate the effect of protein dynamics on the vibrational spectrum. At the lowest temperature (10 K) the position of the proximal histidine with respect to the porphyrin macrocycle and the vibrational potential of the histidine-iron bond are independent of quaternary structure. At this temperature this vibrational mode has the same width as the other vibrational modes associated with the porphyrin macrocycle. As the protein is warmed, due to the temperature dependence of the interatomic interactions, many amino acid residues undergo progressively larger mean square displacements. Such displacements have been found in studies of the temperature dependence of the x-ray diffraction patterns in biomolecules (*14*). In particular, it has been reported (*14*) that in myoglobin, residues on the proximal side of the heme, residues on the protein surface, and the entire F helix undergo large displacements at room temperature. Such displacements can result from large vibrational amplitudes or from actual conformational substates in which there are several nearly isoenergetic minima that atoms may occupy at a given temperature. These effects may significantly influence vibrational lines, such as the iron-histidine stretching frequency, that reflect motions between molecular units linked by a single bond. With increasing temperature large average displacements of the molecular units, resulting from increased population of low-lying vibrational or rotational states or from the population of many conformational substates of the histidine-porphyrin complex, can account for the decreased frequency and increased broadening of the iron-histidine stretching mode. An additional contribution to the broadening

may result from Raman scattering from vibrational hot bands which are thermally populated in anharmonic potentials of low energy such as that of the iron-histidine bond (~ 10 kcal) (*4*).

The changes on going through the melting transition are very striking and bring about the distinction in the iron-histidine mode between the two quaternary structures. The other surprising effect is the appearance of a line at 160 cm^{-1} that is absent below 0°C. At room temperature this mode is absent in myoglobin but present in HbA (*15*), β_4 tetramers, and α chains. Its normal mode origin has not been assigned. When the excitation frequency is changed to 4131 Å it becomes exceedingly strong in comparison to other modes in the spectrum. The changes in frequency (5 to 10 cm^{-1}) and intensity of this mode with changing quaternary structure (*6*), which parallel the behavior of the iron-histidine mode, strongly suggest that the 160 cm^{-1} mode also involves the proximal histidine. A definitive determination of the origin of this mode must await more extensive studies. Accompanying the appearance of the 160 cm^{-1} mode is the development of a quaternary structure dependent difference in the iron-histidine stretching mode. In deoxy HbA this mode remains at 216 cm^{-1}, but it weakens and the line shape becomes asymmetric. In contrast, in NES des-Arg HbA this mode shifts to higher frequency while decreasing in intensity. The expression of quaternary structure differences in the iron-histidine bond is therefore dependent on an interaction with water. It is attractive to postulate that water molecules situated in the heme crevice interact directly with the heme or the proximal histidine in vitro. However, we cannot exclude a long-range effect in which the tertiary conformation at the heme is controlled by hydrogen-bonding interactions with water at the surface of the protein. Also, the effect of thermal contraction of the globin on the tertiary structure of the protein remains to be explored.

There have been very few reports of conformational changes induced in biomolecules on freezing. In heme proteins changes in the spin equilibrium of methemoglobins (*16*), in ligand orientation in oxycobalt myoglobin (*17*), and in intermolecular order in cytochrome c peroxidase (*18*) have been reported. Those results, coupled with our observations that freezing can alter the properties of the biologically active site in deoxyhemoglobin, demonstrate that the utmost care must be utilized in drawing functional implications from studies in which heme proteins have been probed under nonphysiological conditions. On the other hand, the recognition of these differences may allow the factors that influence biological function to be determined.

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References and Notes

1. Abbreviations: T, low oxygen affinity; R, high oxygen affinity; NES, *S*-(*N*-ethylsuccinimido)-cysteinyl; Arg, arginine; IHP, inositol hexaphosphate.
2. T. Kitagawa, K. Nagai, M. Tsubaki, *FEBS Lett.* **104**, 376 (1979).
3. H. Hori and T. Kitagawa, *J. Am. Chem. Soc.* **102**, 3608 (1980).
4. K. Nagai, T. Kitagawa, H. Morimoto, *J. Mol. Biol.* **136**, 271 (1980).
5. K. Nagai and T. Kitagawa, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2033 (1980).
6. D. L. Rousseau, J. A. Shelnutt, M. R. Ondrias, J. M. Friedman, E. R. Henry, S. R. Simon, in *Proceedings of the Symposium on Interaction between Iron and Proteins in Oxygen and Electron Transport*, C. Ho, Ed. (Elsevier North-Holland, New York, in press).
7. D. L. Rousseau, *J. Raman Spectrosc.* **10**, 94 (1981).
8. J. V. Kilmartin and J. A. Hewitt, *Cold Spring Harbor Symp. Quant. Biol.* **36**, 311 (1971).
9. J. A. Shelnutt, D. L. Rousseau, J. M. Friedman, S. R. Simon, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4409 (1979).
10. W. E. Blumberg, unpublished results.
11. A. Warshel, *Annu. Rev. Biophys. Bioeng.* **6**, 273 (1977).
12. T. G. Spiro, J. D. Strong, P. Stein, *J. Am. Chem. Soc.* **101**, 2648 (1979).
13. T. G. Spiro and T. C. Strekas, *ibid.* **96**, 338 (1974).
14. H. Frauenfelder, G. A. Petsko, D. Tsernoglou, *Nature (London)* **280**, 558 (1979).
15. J. Kincaid, P. Stein, T. G. Spiro, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 549 (1979).
16. C. Messana, M. Cerdonio, P. Shenkin, R. W. Noble, G. Fermi, R. N. Perutz, M. F. Perutz, *Biochemistry* **17**, 3652 (1978).
17. H. Hori, M. Ikeda-Saito, T. Yonetani, *Nature (London)* **288**, 501 (1980).
18. T. Yonetani and H. Schleyer, *J. Biol. Chem.* **242**, 3919 (1967).
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The Posterior Pituitary: Regulation of Anterior Pituitary Prolactin Secretion

Abstract. *Removal of the posterior pituitary from anesthetized male rats results in a prompt and significant increase in circulating prolactin that is reversed by the injection of dopamine. Posterior pituitary extracts, which contain high concentrations of endogenous dopamine, inhibit prolactin secretion from isolated anterior pituitary cells. This inhibition is prevented by incubation of the cells with the dopamine receptor antagonist (+)-butaclamol. The data show that posterior pituitary dopamine reaches the anterior pituitary via the short hypophysial portal vessels and participates in the regulation of prolactin secretion.*

Dopamine is widely accepted as a physiological prolactin-release inhibiting hormone (PIH) for two reasons: the presence of dopamine in hypophysial portal blood at higher concentrations than in the systemic circulation and the demonstration of a reciprocal relation between hypothalamic dopamine secretion and pituitary prolactin release under a variety of endocrine states (*1*). Indeed, dopamine inhibits prolactin secretion in vitro at a range of concentrations found in hypophysial portal blood (*2*). Further supporting evidence that dopamine acts directly on the pituitary comes from the identification of specific high-affinity dopaminergic receptors in anterior pituitary homogenates (*3*).

The source of dopamine affecting the anterior pituitary is believed to be the tuberoinfundibular dopaminergic tract whose cell bodies are located in the

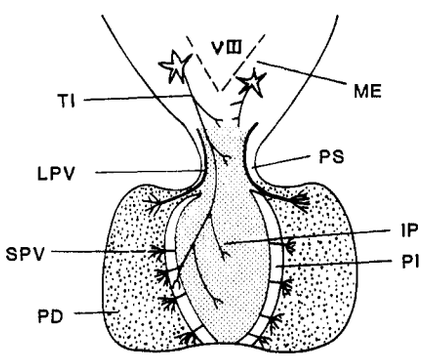

Fig. 1. Diagrammatic presentation of selected vascular and neural connections of the rat pituitary gland, shown here from a dorsal aspect. The pars distalis (*PD*) or anterior pituitary is supplied with long portal vessels (*LPV*) from the median eminence (*ME*) of the hypothalamus via the pituitary stalk (*PS*), and short portal vessels (*SPV*) from the infundibular process (*IP*) or neurohypophysis, via the pars intermedia (*PI*). The tuberoinfundibular (*TI*) dopaminergic pathway has cell bodies in the arcuate nucleus near the third ventricle (*VIII*), and nerve terminals in the median eminence as well as in the neurointermediate lobe, commonly referred to as the posterior pituitary. Redrawn and modified from Daniels and Prichard (*7*).

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