animal in Fig. 2 that received the labeled antibody to CEA had an additional tumor growing subcutaneously below the left shoulder (arrow). This tumor, the result of the accidental deposition of tumor cells from the trocar at the insertion site, is apparent despite the high background activity in liver.

Our results demonstrate that IgG antibodies may be coupled with DTPA simply by reaction with the cyclic anhydride in aqueous solution and that the labeled protein maintains its capacity for binding to its antigen. The coupling is highly efficient when compared with other methods. The coupling of DTPA to proteins for radiolabeling has several other advantages that are not shared by methods in which radioiodine is used as the label. Specifically, the coupled antibody can be purified from the products of hydrolysis before the addition of radioactivity, thereby avoiding the need to handle radioactive samples during purification. Furthermore, the coupled and purified samples can be stored and radiolabeled only when required. The labeling by chelation with ^{111}In is extremely rapid and is, in effect, completed upon mixing of the activity and coupled protein solutions. Since a large number of metals form strong chelates with DTPA, they probably also form strong chelates with DTPA-coupled proteins. Thus, a variety of metallic radionuclides with different detection properties, radiation properties, and half-lives may be considered. We believe that the cyclic anhydride labeling method offers an attractive alternative to radioiodination for the radiolabeling of antibodies and other proteins.

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8. A Waters Associates Inc. (Milford, Mass.) HPLC was used in this study with I250 and I125 protein columns operated in series for improved resolution and with both ultraviolet detection (280 nm) and radioactivity detection. The eluant consisted of 2.5 percent triethylamine, 0.1*M* Na_2SO_4, *p*H 4.5.
9. The monoclonal antibody to PAP (an IgG 2a) was kindly supplied by New England Nuclear. The monoclonal antibody to CEA (an IgG 1k) and purified CEA were kindly supplied by Hoffmann–La Roche, Inc.
10. The animal tumor models used in this study, that is, the "tumored ear assay" and the "subrenal capsule assay," were originally developed by A. E. Bogden, Mason Research Institute, Worcester, Mass., for the rapid in vivo screening of chemotherapy drugs against a panel of human tumors transplantable in the athymic nude mouse. The cell line used in this study (designated CX-1) has previously been shown by radioimmunoassay of mouse plasma samples to excrete CEA in the range of 6 to 67 ng/ml, depending on tumor size, when implanted in the subrenal capsule. Implantation in the renal capsule is accomplished by withdrawing the right kidney of an anesthetized animal, inserting a trocar through a small slit in the capsule, and implanting 1-mm fragments of tumor under the capsule. The kidney is then returned, the body wall closed, and the animal permitted to recover. The pinnal implant is essentially a subcutaneous graft. A 2-mm tumor fragment is deposited in the pinna of an anesthetized animal by guiding the trocar along the pinnal ridge from an insertion site in the vicinity of the opposite shoulder.
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12. Radioiodinations, by the chloramine-T method, were performed by New England Nuclear. Viability was established for the labeled antibody to PAP by immunoradioactivity assay.
13. D. J. Hnatowich, unpublished results.
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Metastable Species of Hemoglobin: Room Temperature Transients and Cryogenically Trapped Intermediates

Abstract. *Resonance Raman spectra of photolyzed carbonmonoxyhemoglobin obtained with 10-nanosecond pulses are compared with the spectra of photolyzed carbonmonoxyhemoglobin stabilized at 80 K. In comparing the deoxy with the photodissociated species, the changes in the Raman spectra are the same for these two experimental regimes. These results show that at ambient and cryogenic temperatures the heme pocket in liganded hemoglobin is significantly different from that of deoxyhemoglobin. It is concluded that measurements of the properties of intermediate species from photodissociated hemoglobin stabilized at low temperatures can be used to probe the short-lived metastable forms of hemoglobin present after photodissociation under biologically relevant solution conditions.*

Binding of small molecular ligands to hemoglobin (Hb) brings about extensive changes in the structure of the heme macrocycle and the relation between the protein subunits that surround it. These changes can be studied by photodissociating a ligand and examining the resulting metastable species by time-resolved spectroscopy (*1*) or by steady-state spectroscopic examination of the metastable species trapped at cryogenic temperatures (*2–8*). However, the physiological relevance of the low-temperature studies has been an open question. We find that the low-frequency resonance Raman spectra of photolytic transients of carbonmonoxyhemoglobin (COHb) examined 10 nsec after photolysis at room temperature and those trapped at 80 K for long periods of time are equivalent (both deviate in the same ways from deoxyhemoglobin examined under the same conditions). Thus low-temperature measurements of photodissociated Hb provide a meaningful probe for the study of the dynamic character of the ligand-heme-globin interactions within the protein.

Reversible binding of small diatomic ligands such as O_2, CO, and NO to the heme in Hb is regulated by the surrounding protein configuration, but the specific interactions responsible for this regulation are not known. Indeed, it has not yet been determined whether the critical interactions occur near the ligand binding site (*9*) or throughout the protein (*10*). However, in metastable species generated immediately after binding or release of ligands, energetically important disturbances may be manifested, at least transiently, at the interface between the binding site and the surrounding protein. Under physiological conditions these metastable species persist on time scales ranging from picoseconds to milliseconds and are inaccessible to conventional steady-state spectroscopic techniques.

The relation between the two methods used to isolate and examine the transient species (Hb*) involved in ligand binding—time-resolved spectra of transients (*1*) or trapping and examination of metastable species at low temperature (*2–8*)—has been unexplored. Time-resolved studies of transients have been limited to optical absorption (*11–14*) and resonance Raman spectroscopy (*1*), while the low-temperature studies have primarily centered on electron paramagnetic reso-

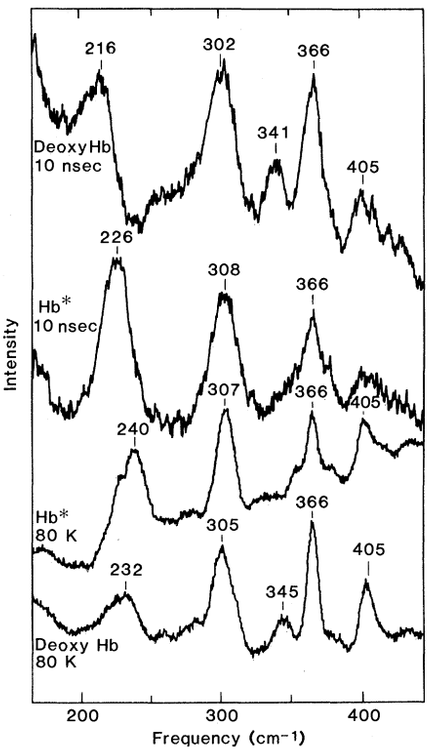

Fig. 1. Resonance Raman spectra of deoxy Hb and Hb*. In descending order from the top: deoxy Hb at 35°C; Hb* generated by photolysis of COHb at 35°C; Hb* generated by photolysis of COHb at 80 K; and deoxy Hb at 80 K. The samples for the 80 K spectra were 2 to 3 m*M* in 0.05*M* phosphate buffer, *p*H 7.0 to 7.2. The samples for the 35°C spectra were ~ 100 μ*M* in 0.10*M* phosphate, *p*H 7.0 to 7.2.

nance (EPR) (*2, 3*), Mössbauer (*5, 6*), and infrared absorption spectroscopy (*4*). Optical absorption studies (*4*) of low-temperature metastable species have not revealed differences between deoxy species and photolyzed species owing to incomplete sample photolysis. Resonance Raman studies of low-temperature intermediates have only recently been reported (*8*). We present here resonance Raman spectra obtained from transient Hb species generated by photolysis of COHb. We compare spectra obtained at room temperature 10 nsec after photolysis to spectra resulting from photolyzed COHb at 80 K. These spectra are qualitatively equivalent, showing that the hemes in Hb* produced transiently under solution conditions and those stabilized at cryogenic temperatures undergo similar interactions with the surrounding protein.

Low-frequency spectra of Hb generated by photolysis of COHb are presented in Fig. 1. The upper trace and the one below it are, respectively, a spectrum of deoxy Hb and a spectrum of photolyzed COHb, both obtained at 35°C with 10-nsec pulses. An excimer-pumped dye laser with an average power of ~ 10 to 20 mW (10 Hz) and a slit width of ~ 10 cm^{-1} operating at 435 nm was used to photolyze the COHb and to generate the resonance Raman spectrum. The bottom trace is a spectrum of frozen deoxy Hb at 80 K obtained with 441.6-nm continuous-wave excitation. The third trace was generated by photolyzing COHb at 80 K and examining the resulting transient with the 441.6-nm excitation from a He-Cd laser, which was used as both the photolysis beam and the probe beam. The low-temperature spectra were obtained with 180° backscattering geometry at relatively low laser power (~ 15 mW) and a slit width of ~ 5 cm^{-1}.

Both the He-Cd and dye laser systems operate at frequencies that are more resonant with the Soret band of deoxy Hb than with the Soret band of COHb. Thus the low-frequency spectra generated are exclusively those of the deoxy transients independent of the extent of photolysis. The amount of COHb in the spectra of the samples can be assessed by the presence of either the Fe–C stretching mode at ~ 505 cm^{-1} or the ν_4 mode of COHb at ~ 1372 cm^{-1}. In all our spectra the contributions from COHb are negligible. Instrumental details of both the time-resolved (*15*) and low-temperature studies (*8*) have been described.

Previous low-temperature studies showed that the resonance Raman spectrum of the heme in Hb* generated by COHb photolysis differs qualitatively from that of low-temperature deoxy Hb obtained at the same temperature (*8*). Time-resolved studies have, up to this point, been confined to narrow regions of the Hb* spectrum, namely, the regions of the iron-histidine (Fe–His) stretching vibration at 215 to 230 cm^{-1}; the heme mode (ν_4) at ~ 1350 to 1380 cm^{-1}, which is sensitive to π-electron density; and the core size–sensitive modes in the region 1550 to 1650 cm^{-1} (*1*). The present spectra include the heme modes in the range 300 to 500 cm^{-1}. We find that with the exception of the Fe–His frequency (discussed below), the 10-nsec, 35°C spectrum of Hb* matches that of Hb* trapped at 80 K. Specifically, both deviate from deoxy Hb spectra taken under similar conditions in that (i) the intensity of the Fe–His stretching mode increases dramatically and shifts to higher frequency; (ii) the mode at 302 cm^{-1} shifts to higher frequency and narrows; (iii) the mode at 345 cm^{-1} shifts to 354 cm^{-1}, becoming a shoulder on the 366-cm^{-1} mode; (iv) the mode at 366 cm^{-1} decreases in intensity; and (v) the mode at 405 cm^{-1} becomes asymmetric.

Upon further temperature reduction to 10 K the spectrum of Hb* displays a new weak mode at ~ 330 cm^{-1} and an additional 2- to 3-cm^{-1} shift to higher frequency of the ~ 307-cm^{-1} mode (*8*). These changes may be ascribed to a slight relaxation of the heme environment at 80 K compared to that at 10 K. The fact that the Hb* species generated 10 nsec after photolysis resembles the Hb* trapped at the elevated temperature (80 K) suggests the existence of a rapidly relaxing component of the heme's metastable environment. Picosecond studies might be able to detect this species.

In the comparison of solution spectra of deoxy Hb and frozen deoxy Hb, as reported previously (*16*), there are several notable changes. The largest change is in the frequency of the Fe–His stretching mode. However, the modes at 341 and 302 cm^{-1} also shift to 345 and 305 cm^{-1}, respectively, at cryogenic temperatures. Thus freezing substantially perturbs the heme of the deoxy protein. Ligand binding to Hb causes a major structural change in the porphyrin, which becomes flatter; the core size, which becomes smaller; and the π-electron density, which is depleted to an extent similar to that of a ferric heme. Thus the heme pocket may reorganize to accommodate the the liganded heme complex. The data presented here show that such a restructuring does occur and certain features of this reorganization significantly influence the heme vibrational modes and induce the same changes in both the transient form of the heme (at 10 nsec) and the frozen Hb at 80 K. These ligand-induced changes are readily detected in the frozen state in spite of the temperature-dependent changes that are also present.

The only substantive difference between the time-resolved and low-temperature spectra is the frequency of the line assigned to Fe–His stretching mode (*17*). In solutions of T-state deoxy Hb the frequency of this mode is ~ 216 cm^{-1} and in the R state it is ~ 222 cm^{-1} (*16, 18, 19*). Previous time-resolved studies (*15*) showed that within a given quaternary structure the Fe–His stretching frequency increases in going from deoxy Hb to the photolyzed transient at 10 nsec. For low-affinity (T-state) hemoglobins the frequency increases from ~ 216 to ~ 220 cm^{-1} in going from deoxy to transient species, whereas the analogous frequencies for high-affinity (R-state) structures are ~ 222 and 226 to 231 cm^{-1}, respectively. The range of values

for the transient species originates from allosteric effects on this mode.

In deoxy Hb at cryogenic temperatures the Fe–His stretching mode shifts to a much higher frequency (235 cm^{-1} at 10 K). In this regard the frozen deoxy Hb spectrum is very different from the solution spectrum. However, the magnitude of the change in frequency of this mode between deoxy and photolyzed low-temperature samples is similar to that displayed by the comparison of time-resolved room temperature spectra. The mode is detected at 232 cm^{-1} in deoxy Hb (80 K) and at 240 cm^{-1} in photolyzed COHb (80 K). The effect of the lowered temperature, therefore, is to increase the frequency of the Fe–His stretching mode, but the change in the frequency due to the previous state of ligand binding is similar at both high and low temperatures.

The data presented here and other independent data (*18, 20, 21*) show that the Fe–His stretching mode is very sensitive to structural variations. With the available data the origin of these changes cannot be unequivocally determined, but a consideration of the influence of nonbonded repulsions yields a consistent interpretation of a large body of data. The temperature dependence of the Fe–His stretching frequency may be accounted for by these repulsions. As the temperature is lowered a suppression of the mean vibrational fluctuations of the heme-histidine bond length due to a decrease in the thermal population of excited vibrational levels and a concomitant decrease in the vibrational amplitudes would be expected to reduce nonbonded repulsions and lead to a stronger Fe–His bond. Thus for a fixed heme-histidine orientation the observed increase in frequency of the Fe–His mode with decreasing temperature would be predicted. The change in frequency of the Fe–His stretching mode in the photodissociated state may be explained in terms of a ligand-induced change in the proximal heme pocket geometry that reduces the nonbonded repulsive forces between histidine and heme. Decrease in the tilt of the histidine relative to the heme plane has been suggested (*15*) as the most likely origin of the reduction in repulsive forces.

There have been many studies of metastable forms of Hb stabilized at low temperatures (*2–8*). However, the physiological applicability of these studies has been uncertain owing to the possible constraints imposed on the protein and heme by the frozen solvent. The analogous behavior of Hb* species generated at room temperature and 80 K confirms the physiological relevance of the low-temperature spectra. Furthermore, preliminary studies at higher temperatures ($\geq$ 100 K) in the frozen state indicate some additional relaxation of Hb* toward a deoxy species. Thus the parallel examination of time-resolved and temperature-resolved spectra could lead to a determination of the dynamic pathways by which the globin, heme, and ligand interact.

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Endogenous Pyrogen Activity in Human Plasma After Exercise

Abstract. *Plasma obtained from human subjects after exercise and injected intraperitoneally into rats elevated rat rectal temperature and depressed plasma iron and zinc concentrations. The pyrogenic component was heat-denaturable and had an apparent molecular weight of 14,000 daltons. Human mononuclear leukocytes obtained after exercise and incubated in vitro released a factor into the medium that also elevated body temperature in rats and reduced trace metal concentrations. These results suggest that endogenous pyrogen, a protein mediator of fever and trace metal metabolism during infection, is released during exercise.*

Infection causes a stereotyped physiological response in vertebrates characterized by fever and depression of plasma iron and zinc concentrations. The febrile response appears to be mediated by endogenous pyrogen (EP), a protein released from monocytes and macrophages. The reduction of plasma iron and zinc occurs through the action of leukocyte endogenous mediator (LEM), another macrophage product. In addition, LEM mobilizes neutrophils from the bone marrow into the circulation and stimulates the synthesis of certain plasma proteins (ceruloplasmin, C-reactive protein, and many others) (*1*). There is increasing evidence that EP and LEM are the same molecule or are at least members of a class of proteins that share many biochemical characteristics (*2*). The actions mediated by EP and LEM (hereafter called simply "EP") are termed the "acute-phase" response to infection and are thought to create an internal environment that is less favorable for the growth and multiplication of pathogens (*3*).

After lengthy aerobic exercise, body core temperature can remain elevated for many hours (*4*), the number of circulating neutrophils increases (*5*), and plasma concentrations of the acute-phase proteins (such as ceruloplasmin and C-reactive protein) often rise (*6*). Epidemiological studies indicate that long-term physical training may lead to chronic depression of plasma iron and zinc (*7*). The remarkable number of similarities between the physiological responses to exercise and to infection led us to hypothesize that the mediator released during infection, EP, may also be released during exercise.

We report that human plasma collected after exercise elevated rectal temperature and depressed plasma iron and zinc when injected into rats, indicating that a factor is present in human plasma after exercise that exhibits EP activity. Supernatant from incubated mononuclear leukocytes obtained after exercise also had EP activity, indicating that these cells are a source of the plasma factor.