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Infrared Studies of Azido, Cyano, and Other Derivatives of Metmyoglobin, Methemoglobin, and Hemins*

Sue McCoy† and Winslow S. Caughey‡

ABSTRACT: Infrared spectra of aqueous solutions of metmyoglobin (Mb) and methemoglobin (Hb) exhibit a "window" of relatively low absorption from about 1750 to 2800 cm^{-1}.

Derivatives of these hemoproteins and of hemin esters with ligands (N_3, CN, OCN, SCN, and SeCN) which have absorption bands within this window have been studied. Azide derivatives of Mb and Hb each exhibit *two* narrow bands: MbN_3 at 2023 and 2045 cm^{-1}, HbN_3 at 2025 and 2047 cm^{-1}. These bands are assigned to low- and high-spin forms of each protein. The MbN_3 and HbN_3 spectra were not sensitive to pH changes (3.0–11.6)—a finding which supports n–σ donor–acceptor interaction (with histidine as donor and azide as acceptor), but not hydrogen bonding, between azido ligand and distal histidine. ν_{CN} at 2125 cm^{-1} for MbCN and HbCN and at 2110 cm^{-1} for cyanohemins in aqueous pyridine were found. With both CO and N_3 derivatives Mb shows a greater tendency to bind ligands in two ways than does Hb. CO, N_3, and O_2, but not CN, are considered to form significantly nonlinear iron–ligand bonds with tighter binding or a smaller "pocket" for ligands in Mbs than in Hbs.

Infrared spectroscopy has been used widely in the study of bonding in coordination compounds (Nakamoto, 1963; Cotton and Wilkinson, 1966). Recently this technique was successfully applied to the study of metalloproteins such as carbonylhemoglobins in aqueous solutions and within the red blood cell (Alben and Caughey, 1966; Caughey *et al.*, 1969a), carbonyl myoglobins (Caughey *et al.*, 1969b; McCoy and Caughey, 1969a,b), and carbon dioxide bound to carbonic anhydrase (Riepe and Wang, 1967, 1968). Although the technique is restricted to infrared active ligands with bands appearing within the "window" for aqueous protein solutions from about 1750 to 2800 cm^{-1} (Figure 1), a number of ligands of biochemical interest fall within this region. This paper discusses infrared studies of CO, N_3, CN, OCN, SCN, and SeCN ligands to metmyoglobins, methemoglobins, and iron(III) porphyrins. Preliminary reports have been given on portions of this work (McCoy and Caughey, 1967, 1968, 1969b).

Materials and Methods

Materials. Sperm whale and horse heart myoglobin and human hemoglobin preparations obtained from Mann Research Laboratories, bovine plasma albumin from Armour Pharmaceutical Co., sodium azide from Alpha Inorganics, potassium and sodium cyanide from J. T. Baker and Co., and deuterium oxide from Volk Radiochemicals were used as received. Pyridine and chloroform were purified and stored as previously described (Sadasivan *et al.*, 1969). Preparations of the azido and μ-oxo dimer derivatives of deuteroporphyrin IX dimethyl ester iron(III) have been reported (Sadasivan *et al.*, 1969). Preparation of the μ-oxobis(protoporphyrin IX dimethyl ester iron(III)) will be reported elsewhere (G. A. Smythe and W. S. Caughey, unpublished).

Azidoprotoporphyrin IX Diethyl Ester Iron(III). A solution of chloroprotoporphyrin IX diethyl ester iron(III) (Alben *et al.*, 1968) (510 mg) in chloroform (50 ml) was shaken thoroughly with eight 20-ml portions of saturated aqueous sodium azide and passed through a column packed with sodium azide. The column eluate was condensed to *ca.* 5 ml

* From the Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, and the Department of Chemistry, University of South Florida, Tampa, Florida 33620. *Received August 1, 1969.* This work was supported by U. S. Public Health Service Grants HE-06079 and HE-11807.

† Present address: Department of Orthopedics, University of Virginia, Charlottesville, Va. 22901.

‡ Present address: Department of Chemistry, Arizona State University, Tempe, Ariz. 85281. Reprint requests should be sent to this address.

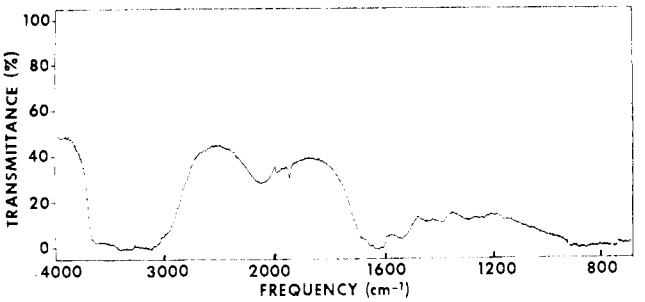

FIGURE 1: The infrared spectrum of 0.005 M carbonylhemoglobin in 0.5 M acetate buffer (pH 5). The discontinuity in the spectrum at 2015–2000 cm^{-1} was due to a grating change. CaF_2 cells; path length, 0.025 mm.

and heated nearly to the boiling point. Hot isooctane (20 ml) was added. After the solution cooled, the precipitate was collected, washed with isooctane, and dried at 50° under vacuum for 2 hr; yield, 430 mg.

Anal. Calcd for $C_{38}H_{40}FeN_7O_4$: C, 63.9; H, 5.6; Fe, 7.8; N, 13.7. Found: C, 64.3; H, 5.7; Fe, 8.1; N, 13.2.

Cyanatodeuteroporphyrin IX Dimethyl Ester Iron(III). A solution of acetatodeuteroporphyrin IX dimethyl ester iron(III) (Alben *et al.*, 1968) (100 mg) in chloroform (15 ml) was shaken thoroughly with ten 14-ml portions of saturated aqueous potassium cyanate. The chloroform solution was passed through filter paper, condensed to 4 ml, treated with 15 ml of hot isooctane, and allowed to stand at room temperature. Crystals were collected by filtration, washed with isooctane, and dried under vacuum at 30°; yield, 30 mg.

Anal. Calcd for $C_{32}H_{32}FeN_4O_4 \cdot OCN$: C, 62.5; H, 5.1; Fe, 8.8; N, 11.0. Found: C, 62.5; H, 5.4; Fe, 9.3; N, 10.5.

Thiocyanatodeuteroporphyrin IX Dimethyl Ester Iron(III). A procedure analogous to that used for preparations of the cyanate was followed except for the use of potassium thiocyanate in place of potassium cyanate; yield, 70 mg.

Anal. Calcd for $C_{32}H_{32}FeN_4O_4 \cdot SCN$: C, 60.9; H, 5.0; Fe, 8.6; N, 10.8. Found: C, 59.6; H, 5.1; Fe, 9.1; N, 10.3.

Selenocyanatodeuteroporphyrin IX Dimethyl Ester Iron(III). A solution of acetatodeuteroporphyrin IX dimethyl ester iron(III) (202 mg) in chloroform (20 ml) was washed two times with an equal volume of water, shaken thoroughly with five 30-ml portions of saturated aqueous potassium selenocyanate, and evaporated to dryness. The residue was extracted with 10 ml of chloroform. The extract was then treated at the boiling point with 10 ml of hot isooctane and allowed to stand at room temperature. The precipitate was collected, washed with isooctane, and dried under vacuum at 30°; yield, 147 mg.

Anal. Calcd for $C_{32}H_{32}FeN_4O_4 \cdot SeCN$: C, 56.8; H, 4.6; N, 10.0. Found: C, 56.5; H, 4.7; N, 10.5.

Spectral Measurements. Infrared spectra were recorded with a Perkin-Elmer spectrophotometer Model 225 equipped with a dry air purging unit. Beckman RIIC variable-temperature cell holders permitted control of cell temperatures within ±2°. Visible spectra were recorded with a Cary spectrophotometer, Model 14.

PROTEIN SOLUTIONS. Methods reported previously for infrared difference spectroscopy of carbonylhemoglobins were followed (Alben and Caughey, 1968). Metmyoglobin or methemoglobin was added to water or buffer to give a solution *ca.* 0.02 M in protein; essentially all of the protein dissolved readily except with buffers at the pH extremes. A solution containing the azido (or other anion) derivative was prepared by the addition of 0.02 ml of sodium azide (or other anion) (usually *ca.* 0.1 M) to 0.2 ml of protein solution and used to fill a calcium fluoride cell with a path length of 0.025 mm. A similar cell was filled with original protein solution for use in the reference beam. The visible spectrum of each solution was checked in the calcium fluoride cell both before and after the infrared spectrum was recorded.

For the infrared measurements, an unexpanded scan was first obtained rapidly (less than 2) min over the 2300–1850-cm^{-1} region to find absorption bands of interest and to permit comparison with spectra obtained later to detect possible changes as a result of exposure to the conditions of measurement; under these conditions wave numbers were determined only within ±5 cm^{-1}. Four to six hours was usually required to obtain a spectrum expanded two to ten times in both ordinate and abscissa with optimal resolution and accuracy of wavelength and band-width measurements; adjacent water vapor bands were included for wave number calibration to permit determination of wave numbers within ±1 cm^{-1}. Similar spectra were obtained whether the cells were maintained at a temperature between 10 and 15° or at 40°.

HEMINS AND INORGANIC SALTS. Infrared spectra of hemin derivatives, sodium azide, and potassium cyanide were measured in KBr cells of 0.1-mm path length for solutions with organic solvents and in CaF_2 cells of 0.025-mm path length for aqueous solutions. Here, satisfactory spectra for the region of interest could be obtained in less than 5 min. The cyanohemin derivatives were prepared in aqueous pyridine solutions (Wüthrich *et al.*, 1969). Either azido or μ-oxo dimer hemin derivative (2 mg) was added to a saturated solution of KCN (~1 mg) in pyridine–water (4:1) (0.1 ml). The immediate formation of the cyano derivative could be detected by the change in visible spectrum. When azidohemins were used to prepare the cyanohemin solutions, the infrared spectra were complicated by bands from free azide which are much stronger bands than those for CN species.

Results and Discussion

Infrared Evidence for Two Forms of Azide Bound to Metmyoglobin. Unbuffered aqueous solutions of metmyoglobin (*e.g.*, horse heart or sperm whale protein) exhibit visible spectra characteristic of the presence of both acidic and basic forms (Hanania *et al.*, 1966). Upon addition of sodium azide, the visible spectrum ascribed to the azide derivative can be observed (Goldsack *et al.*, 1965). The infrared difference spectrum of two such solutions has *two* bands characteristic of iron-bound azido ligands at 2045 and 2023 cm^{-1} (Figure 2).

Assignment of *both* bands in the difference spectrum to the asymmetric stretch of an iron-bound azide was supported by several observations. Each band differed in wavelength and in band width from the band observed for free azide (Figure 2). Thus, when the protein to azide ratio was 2 to 1, the visible spectrum was consistent with incomplete conversion into the azide derivative and in the infrared there were only the two bands at 2045 and 2023 cm^{-1}. With excess azide

TABLE I: Stretching Frequencies of Azides.

Compound	Solvent	ν (± 1 cm^{-1})
NaN_3	H_2O	2047
NaN_3	D_2O	2041
NaN_3	0.05 M citrate, pH 3.0	2147, 2048
NaN_3	0.1 M glycine, pH 11.6	2047
MbN_3	H_2O	2045, 2023
MbN_3	D_2O	2042, 2021
MbN_3	0.05 M citrate, pH 3.0	2045, 2023
MbN_3	0.1 M glycine, pH 11.6	2045, 2023
MbN_3	0.05 M phosphate, pH 9.4	2045, 2023
Albumin[a] + N_3	H_2O	2043
Albumin[a] + N_3	0.05 M citrate, pH 3.0	2146, 2045
HbN_3	H_2O	2047, 2025

[a] Bovine plasma.

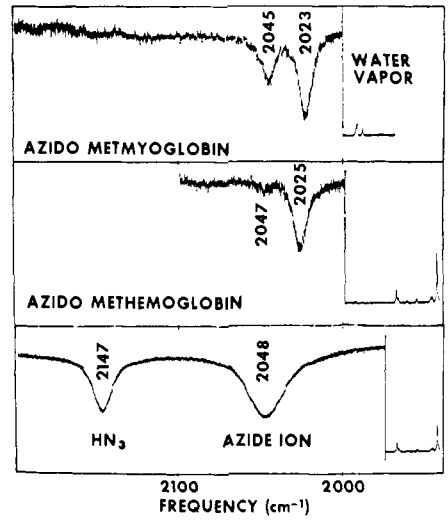

FIGURE 2: The infrared difference spectra. Top: of azidometmyoglobin *vs.* metmyoglobin (0.02 M). Middle: of azidomethemoglobin *vs.* bovine plasma albumin (0.01 M). Bottom: of sodium azide in 0.05 M citrate buffer (pH 3) *vs.* buffer.

present the visible spectrum indicated complete conversion into azidometmyoglobin and the infrared difference spectrum gave in addition to the narrow band at 2023 cm^{-1} a much broader band centered at about 2047 cm^{-1}. The latter band was more intense the greater the excess of azide and was due to a combination of the narrow 2045-cm^{-1} band for iron-bound azide and the broad band for free azide. (Aqueous solutions of sodium azide exhibited one broad band centered at about 2047 cm^{-1}; see Table I.) Upon dialysis against water for 24 hr, the broad 2047-cm^{-1} band disappeared whereas the 2045- and 2023-cm^{-1} bands remained. Aqueous solutions of bovine plasma albumin containing sodium azide exhibited one very broad absorption band at 2043 cm^{-1} (Table I) which disappeared after dialysis against water for 8 hr. Similarly Riepe and Wang (1968) observed a single broad band at 2046 cm^{-1} for azide in the presence of carbonic anhydrase. Furthermore, solutions of MbN_3 at low pH (citrate buffer pH 3) do not exhibit a band for hydrazoic acid (HN_3) unless excess NaN_3 is added. With excess azide, a band does appear at *ca.* 2150 cm^{-1}. Solutions of NaN_3 in citrate buffer, pH 3, exhibit two bands at 2047 cm^{-1} (azide ion) and 2148-cm^{-1} (HN_3) (Figure 2). Thus neither the 2045-cm^{-1} band nor the 2023-cm^{-1} band found with limited azide at pH 3 can represent free azide ion since at this pH free azide, if present, would be in equilibrium with HN_3 which would be observed in the infrared spectrum as was the case for bovine plasma albumin solutions at pH 3 (Table I).

Nature of Azide Binding to Metmyoglobin. Present evidence indicates that azido ligand can assume two distinctly different modes of bonding to a given metmyoglobin molecule. The possibility that the two different forms of azide arose from myoglobins of different structure was considered and rejected. Numerous cases of the separation of myoglobins into several components have been reported, *e.g.*, Quinn *et al.* (1964), DuFresne (1964), and Hardman *et al.* (1966). However, several different sperm whale myoglobin preparations including an electrophoretically homogeneous sample and one preparation of horse heart protein were used with similar results: two narrow bands of the same relative intensity in the spectrum of each preparation.

One azide, represented by the 2023-cm^{-1} band is about four times as abundant as the other (the 2045-cm^{-1} band) based upon the relative areas of the infrared bands and an assumption of similar extinction coefficients. We conclude the 2023-cm^{-1} band corresponds to a low-spin form and the 2045-cm^{-1} band to a high-spin form.[2] Magnetic susceptibility measurements of Beetlestone and George (1964) also indicated a ratio of low-spin to high-spin forms of about 4 to 1 in the 0–20° temperature range. The frequencies appear reasonable in that the high-spin form is near that found for "ionic azides" and the low-spin form is at significantly lower frequency as noted with model hemin azides (*vide infra*).

The nature of any differences in location for two forms is not clear. Only one location was suggested by an X-ray study (Stryer *et al.*, 1964) and it is likely to represent the most favored location. However since relatively subtle changes in structure could significantly affect azide–heme and azide–histidine interactions, a second location need not be far removed from the first. The relative intensities of the two infrared bands were not influenced by azide concentration. Thus a sufficient explanation is simply that an azide ligand may bind at either of two locations with one location favored somewhat energetically over the other without evoking the concept of a thermal equilibrium of the type suggested by Beetlestone and George (1964) where no structural differences accompany spin-state changes.

The relative position of azide and distal histidine found in the X-ray study (Stryer *et al.*, 1964) led the authors to suggest hydrogen bonding between azide and histidine.

[1] Abbreviations used are: Mb, myoglobin; Hb, hemoglobin.

[2] Although the analogy to the hemeproteins is not very close, it is of interest to note that the high- and low-spin forms of $Fe(2,2'\text{-dipyridyl})_2(NCS)_2$ exhibited different ν_{CN} values, at 2109 and 2060 cm^{-1}, respectively (Konig *et al.*, 1968).

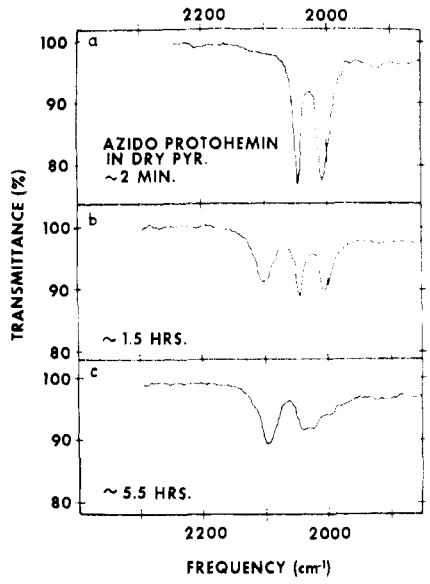

FIGURE 3: The infrared spectra of azidoprotoporphyrin IX diethyl ester iron(III) (1 mg) in dry pyridine (0.1 ml) *vs.* solvent. The discontinuity at 2000 cm^{-1} was due to a grating change.

TABLE II: Stretching Frequencies of Several Ligands Bound to Hemins and as Sodium or Potassium Salts.

Hemins	Medium	ν (cm^{-1})
Deuteroporphyrin IX dimethyl ester iron(III)		
Azido	KBr	2055
Azido	$CHCl_3$	2060
Cyanato	$CHCl_3$	2197
Thiocyanato	$CHCl_3$	2040
Selenocyanato	$CHCl_3$	2041
Protoporphyrin IX dimethyl ester iron(III)		
Azido	KBr	2065
Azido	$CHCl_3$	2065
Salts		
KNCO	H_2O	2167
NaSCN	H_2O	2063
KSeCN	H_2O	2075
NaN_3	Pyridine–H_2O (4:1)	2025
NaN_3 (? suspended)	$CHCl_3$	2170
NaN_3 (? suspended)	Dry pyridine	2170
KCN	Pyridine–H_2O (4:1)	2075

However, the infrared data do not support this speculation. Neither frequencies nor relative intensities of the bound azide bands were significantly affected either by variations in pH from 3 to 11.6 or by replacement of H_2O with D_2O. Over the pH range studied a change in protonation of the more remote nitrogen of the distal histidine is expected and such a change would significantly affect both the strength of a hydrogen bond between azide and histidine and the intensity (and/or the frequency) of the infrared bands. The insensitivity of the infrared spectrum to pH changes is, however, consistent with the more remote histidine nitrogen retaining a proton over the pH range and the closer nitrogen serving as an electron donor to azide, *e.g.*, at the iron-bound nitrogen. As a consequence of bonding to heme iron this nitrogen will assume positive character and can therefore serve as an acceptor in an n–σ donor–acceptor interaction. Dipole–dipole-type interactions between azide and histidine are also possible.

Azidomethemoglobin. Methemoglobin solutions from which excess azide had been removed by dialysis against water for 24 hr gave strong absorption at 2025 cm^{-1} and only a very weak absorption at 2047 cm^{-1} (Figure 2). Here the low-spin form (band at 2025 cm^{-1}) was even more strongly favored over the high-spin form (2047-cm^{-1} band) than was the case for MbN_3. Magnetic susceptibility data had indicated such a difference between the Mb- and Hb-azides (Beetlestone and George, 1964). A smaller "pocket" for ligand binding for Mb than for Hb can explain the different ratios of high and low-spin forms for the two proteins since the high-spin forms could accommodate a smaller Fe–N_3 angle more readily than the low-spin form.

Infrared and Electron Paramagnetic Resonance Studies of Azidohemins. As solids or in chloroform solutions azidohemins were found to give only one infrared band near 2060 cm^{-1} (Table II). Solid azidodeuterohemin ester was exclusively high-spin in terms of electron paramagnetic resonance and Mössbauer spectra (Caughey *et al.*, 1969b; Moss *et al.*, 1969) with a directly measured zero field splitting of 14.8 cm^{-1} (Richards *et al.*, 1967). Chloroform solutions exhibited absorption spectra in the visible and near-infrared regions (Caughey *et al.*, 1969b) and electron paramagnetic resonance spectra at 103°K similar to those found for the chloride, a typical high-spin hemin. The infrared bands observed for azidoproto- and deuterohemins in KBr or in $CHCl_3$ (Table II) were therefore due to the high-spin forms.

The azidohemins in dry pyridine solutions exhibited both low- and high-spin forms at 103°K in electron paramagnetic resonance spectra as well as two infrared bands at 2010 and 2045 cm^{-1} which we assign to low- and high-spin forms, respectively (Figures 3 and 5).

In pyridine solutions other reactions were observed particularly with the protohemin. A freshly prepared solution of azidoprotohemin ester in dry pyridine gave only two azide bands, at 2010 and 2045 cm^{-1}, but upon standing at room temperature a new band at 2100 cm^{-1} intensified (Figure 3). In aqueous pyridine (pyridine–H_2O, 4:1, v/v) bands first appeared at 2010 and 2045 cm^{-1} along with another band due to free azide ion, but on standing a broad band near 2100 cm^{-1} intensified as the 2010- and 2045-cm^{-1} bands lost intensity (Figure 4). The azidodeuterohemin ester in dry pyridine exhibited infrared bands at 2010 and 2045 cm^{-1} and in aqueous pyridine at 2010, 2025, and 2045 cm^{-1} (Figure 5); however, on standing for several hours at room temperature no change in spectrum was observed in either solvent system. The formation of free azide ion in aqueous pyridine is readily explained; azide may be displaced by an aquo or hydroxo ligand to give free azide ion and FeOFe hemin dimers (Sadasivan *et al.*, 1969).

The species with absorption at 2100 cm^{-1} which formed slowly from the azidoprotohemin ester on standing in dry or aqueous pyridine does not form in chloroform and may

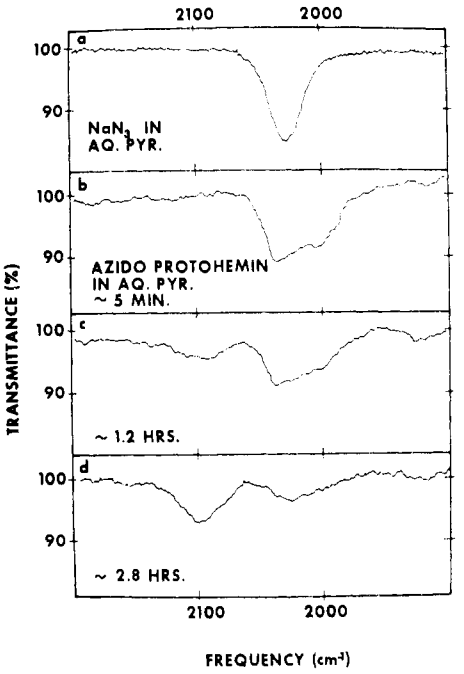

FIGURE 4: Infrared spectra: (a) sodium azide (4 mg) in pyridine–water (4:1, v/v, 3 ml); (b–d) azidoprotoporphyrin IX diethyl ester iron(III) (1 mg) in pyridine–water (4:1, v/v, 0.1 ml) *vs.* solvent. The times indicated were measured from the time the hemin crystals were added to the solvent. Dissolution required several minutes.

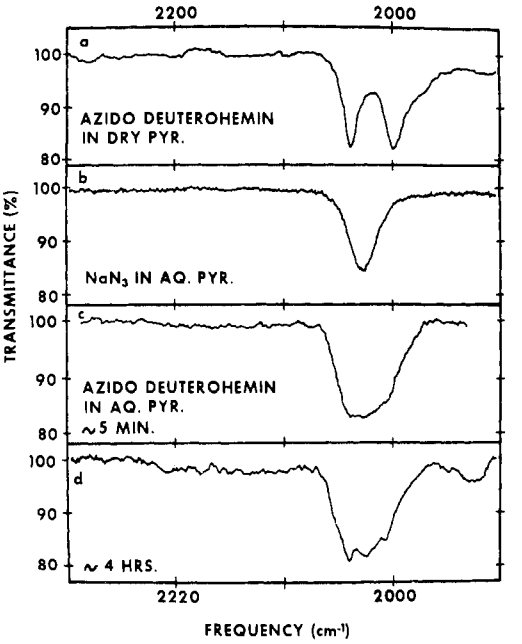

FIGURE 5: Infrared spectra: (a) azidodeuteroporphyrin IX dimethyl ester iron(III) (1 mg) in dry pyridine (0.1 ml), the spectrum did not change in 80 min; (b) sodium azide (4 mg) in pyridine–H_2O (4:1, v/v, 3 ml); (c,d) azidodeuteroporphyrin IX dimethyl ester Fe(III) (1.7 mg) in pyridine–H_2O (4:1, v/v, 0.1 ml). Absorption below 1950 cm^{-1} is due to solvent.

represent a molecular nitrogen–heme complex or dimer with azides serving as bridging ligands. Azides represent one route to nitrogen metal complexes (Collman *et al.*, 1967; Allen *et al.*, 1967) which typically exhibit weak infrared absorption in the 2100-cm^{-1} region; Bancroft *et al.* (1969) assigned a band for an iron(II)–nitrogen complex which appeared at 2090 cm^{-1} to ν_{NN}. Formation of an iron(II)N_2–heme complex would require reduction of iron(III) azide. That pyridine promotes the reaction and protohemin azide reacts under conditions wherein deuterohemin azide does not are in accord with such a reduction process because pyridine is known to facilitate hemin reductions and to stabilize hemes against oxidation (Caughey *et al.*, 1966; Alben *et al.*, 1968) and because of the greater electron-withdrawing effect of 2,4 vinyl groups of protohemin compared with 2,4-hydrogens of deuterohemin (Caughey *et al.*, 1966; McLees and Caughey, 1968). Azides can also give rise to dimeric or higher polymeric species using azide as a bridging ligand (Lange and Dehnicke, 1966; Beck *et al.*, 1967; Nelson and Nelson, 1969). The isolation, structure, and properties of this species is currently under study.

Infrared Studies of Cyanohemins. As KCN was added to an aqueous pyridine solution of μ-oxobis(deuterohemin dimethyl ester), a broad band at 2070 cm^{-1} (free cyanide ion) gradually decreased in intensity as a sharper band at 2110 cm^{-1} (bound cyanide) intensified (Figure 6). With azido-deuterohemin ester under similar conditions the 2110-cm^{-1} band developed more rapidly; displacement of azide by cyanide was noted as the 2025-cm^{-1} band due to free azide ion appeared and the bands near 2010 and 2045 cm^{-1} for bound azide disappeared. Similar results were obtained with azido- and oxo-bridged derivatives of protohemin ester. These infrared data as well as the nuclear magnetic resonance spectra of such solutions (Wüthrich *et al.*, 1969) are consistent with the formation of only one species. However, these data do not demonstrate which ligands other than cyanide may be bound to iron: possibilities include monoaquomonocyano and monopyridinemonocyano as well as dicyano species. That the ligands present interact strongly with water was indicated by the unusually high water solubility of cyano derivatives observed here and in earlier studies; also, attempts to isolate solid cyano derivatives *via* methods found satisfactory for other derivatives provided only FeOFe dimers (Sadasivan *et al.*, 1969). Solutions of deuterohemins after standing developed another species of cyanohemin with a weak band at 2155 cm^{-1}. Although bridging CN can absorb in such a high-frequency range (Dows *et al.*, 1961) and bidentate–cyanides in crystals have been well documented (*e.g.*, Britton, 1967), identification of the 2155-cm^{-1} species requires further study.

Cyano Derivatives of Methemoglobin and Metmyoglobin. Cyano stretch bands are much weaker and therefore more difficult to detect than those for azide and carbon monoxide. Solutions of metmyoglobin and methemoglobin with enough NaCN to give visible spectra which indicated complete conversion into the cyano derivative were dialyzed against water for 20 hr and then concentrated to about 700 mg/ml. The metmyoglobin solution absorbed weakly at 2125 cm^{-1} ($T \sim 1\%$) and at 2150 cm^{-1} ($T < 0.5\%$). The methemoglobin solution only gave a band at 2125 cm^{-1} ($T \sim 1\%$). Spectra of undialyzed solutions were variable with several bands, *e.g.*, at 2145, 2125, 2090, 2080, and 2040 cm^{-1}. NaCN in water absorbs at 2078 cm^{-1}. Albumin solutions plus NaCN

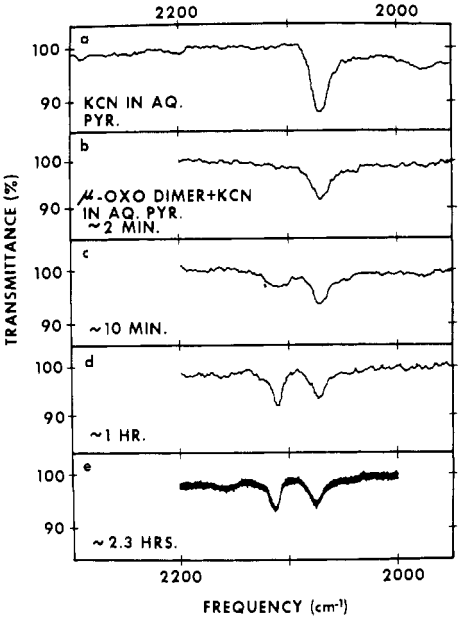

FIGURE 6: The formation of cyanodeuterohemin dimethyl ester iron(III). (a) The infrared spectrum of potassium cyanide (saturated) in pyridine–H_2O (4:1, v/v). (b–d) The infrared spectra of KCN (0.9 mg) in aqueous pyridine (4:1, v/v, 0.1 ml) to which had been added μ-oxobis(deuteroporphyrin dimethyl ester iron(III)) (2 mg). (e) The spectrum of the solution used in b–d, recorded more slowly in order to detect the high-frequency band at 2155 cm^{-1}.

absorb at 2090 and 2080 cm^{-1}; bands at these frequencies for hemoglobin and myoglobin solutions disappear upon dialysis so it is unlikely they reflect covalently bound CN. However, the possibility of some cyanide binding to protein at sites other then heme iron cannot be excluded.

Cyanide usually shifts to higher frequency on binding to a metal (Nakamoto, 1963) and this generalization is followed when CN in aqueous pyridine (2070 cm^{-1}) binds to hemins (2110 cm^{-1}). Thus the 2125-cm^{-1} band can be reasonably assigned to heme-bound CN in Mb and Hb. The weak 2150-cm^{-1} band remains unexplained; however a similar weak band appears in the spectrum of cyanodeuterohemin solutions (Figure 6) and may represent a contaminant.

Infrared Studies of OCN, SCN, and SeCN Derivatives. OCN, SCN, and SeCN derivatives of deuterohemin dimethyl ester in chloroform each exhibited one broad (half-band width 40 cm^{-1}) adsorption within the range of the window for aqueous protein solutions (Table II). In pyridine the derivatives initially each gave only one band at 2193, 2055, and 2065 cm^{-1} for OCN, SCN, and SeCN, respectively. Studies in progress indicate results of value can also be obtained with hemoprotein derivatives with these complexes.

Interpretation of Infrared Data. The potential usefulness of infrared spectra in the study of metalloprotein–ligand systems is shown by the significant differences in several parameters (*i.e.*, frequency, intensity, band width, and number of absorption bands) found in the few infrared studies carried out thus far with such systems. The infrared technique is strengthened by its applicability to aqueous protein solutions at or near room temperature and to intact cells (*e.g.*, the red blood cell), by the use of isotopic labels to identify absorption bands, by the use of infrared cells which permit the determination of optical spectra on the same solution, and by comparison of protein spectra with spectra obtained with simpler metal complexes. The value of infrared data of course depends upon the extent to which the observed parameters can be interpreted in terms of structure and bonding.

The spectra presented here as well as in earlier hemoprotein studies have shown ligands to exhibit significantly narrower band widths in the protein than for protein-free iron porphyrins in solution. This narrowness can be ascribed to differences in effective "solvent" polarity and to more stable (less random) environments about the protein-bound ligand.

The protein environment was also shown to affect the CO stretching frequency in hemoglobins, where replacement of the distal histidine by tyrosine or arginine resulted in shifts of 20 and 7 cm^{-1}, respectively (Caughey *et al.*, 1969a). The frequency differences noted between COHb (*e.g.*, 1951 cm^{-1}) and COMb (1944 and 1931 cm^{-1}) must also result from differences in protein structure. On the other hand, the appearance to two CO bands in COMbs is not reasonably explained by differences in amino acid substitution (*i.e.*, by two different myoglobins) but apparently arises from two different ways in which CO may bind to a given Mb molecule (McCoy and Caughey, 1969a). (It should also be pointed out that the unlikely possibility exists that vibrational interactions of the CO with other groups in the protein with vibrations of suitable energy could result in a single CO species giving two infrared bands. This possibility is amenable to test and is being examined.) The infrared spectrum is the only physical property which has indicated different modes of CO binding to myoglobin. Gibson (1969) has considered the observation as a possible explanation for previously unexplained kinetics for CO binding to myoglobin.

Both CO and N_3 show a greater tendency to give two forms in Mb than in Hb. This finding as well as the infrared frequency and affinity differences suggest a tighter binding or smaller pocket for ligands in Mb than in Hb. Ligand locations for N_3, CO, and O_2 appear similar with the X-ray data for azide (Stryer *et al.*, 1964), and with far less certainty for oxygen (Watson and Nobbs, 1968), in support of a bent Fe-ligand bond (Caughey, 1967, 1970). However, neither the relatively small difference in ν_{CN} between cyanohemins and cyanohemoproteins nor X-ray evidence suggest bending of the FeCN from linearity. Interpretations of ligand infrared band frequencies, Fe–ligand bond angles, and effects of distal histidine–ligand interactions will be considered in greater detail elsewhere (Caughey, 1970).

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