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Infrared Studies of Azide Bound to Myoglobin and Hemoglobin. Temperature Dependence of Ionicity†

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ABSTRACT: Recent developments in instrumentation and techniques have made possible studies of coordination of metal–protein complexes in aqueous solution by infrared spectroscopy. Thus early work with carbon monoxide complexes of hemoglobin, as well as preliminary studies with myoglobin (Mb) complexes, have now been quantitated and extended to the temperature-dependent ionicity of N_3^- bound to Mb. Titration of Mb with NaN_3 produces two peaks, one at 2046 cm^{-1} (ionic) and one at 2023 cm^{-1} (covalent). Both have narrow half-bandwidths (8 cm^{-1}) at N_3^-:Mb < 1, indicating that N_3^- is protected from the aqueous environment by the protein. The ratio of intensities is temperature dependent in agreement with the temperature-dependent spin state of Fe(III) reported by others. Thus high-spin Fe(III) is bound to ionic N_3^-, and low-spin Fe(III) to covalent N_3^-, in MbN_3 complexes. No intermediate states are detected by infrared spectroscopy. Ionic N_3^- in bulk aqueous solution has a broad half-bandwidth (25 cm^{-1}), but a frequency of maximum absorption similar to that of the bound ionic form. Titration spectra have been analyzed by comparison to computed sums of Lorentzian functions. A similar temperature dependence of ionicity is reported for the azide complex of human hemoglobin A, except that the complex is more covalent.

Techniques for infrared spectroscopy of metal proteins have been developed within the past several years (Alben and Caughey, 1966). Early experiments with carbon monoxide bound to the iron of hemoglobin or myoglobin (Alben and Caughey, 1968) indicated that the frequency of the CO stretching vibration is subject to the local molecular environment and effected by the electronic configuration and effective bond order of the carbon monoxide. The CO stretching frequency is the same for many hemoglobin derivatives but is observed at higher frequency when the distal histidine (E-7) is replaced by tyrosine or arginine in hemoglobin M_{Emory} or hemoglobin Zurich (Caughey *et al.*, 1969a) and at somewhat lower frequencies with carboxymyoglobin (Caughey *et al.*, 1969b). The Fe(II)–carbonyl and Fe(III)–cyanide complexes of hemoglobin or myoglobin are strong field ligands with low-spin iron and exhibit only one vibrational state of each ligand. We have recently studied the intermediate ligand field complexes of Fe(III)–azide which have been found by Beetlestone and George (1964) and by Iizuka and Kotani (1969a,b) to exhibit a temperature-dependent spin state of the Fe(III) by magnetic susceptibility measurements. The infrared spectra of azide bound to hemoglobin and myoglobin were first reported by McCoy and Caughey (1970) to consist of two bands which might be involved in such a temperature-dependent spin-state equilibrium. However, no quantitative studies were conducted. We have confirmed the observations of McCoy and Caughey and have obtained quantitative extinction coefficients for both of the infrared bands due to the azide bound to myoglobin. This has allowed us to demonstrate that the infrared

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TABLE I: Quantitation of Infrared Absorption Bands.[a]

		ν (cm^{-1})	a (mM)	$\Delta\nu_{1/2}$ (cm^{-1})	ϵ_{area} (mM)
Myoglobin (N_3^-)					
Horse heart	Covalent	2023.4	1.43	9–10	16.8
	Ionic	2045.8	4.8	8	*b*
Sperm whale	Covalent	2023.3			
	Ionic	2045.6			*b*
Sodium azide (aqueous)	Ionic	2048.2	1.55	25	43
Hemoglobin A (N_3^-)	Covalent	2026.1	1.66	10	22
	Ionic	2047.7			*b*

[a] Extinction coefficients for horse heart myoglobin azide (covalent and ionic) were obtained by computer-assisted analysis of titration curves, while those for the covalent band of hemoglobin azide were obtained under conditions that the ionic band was negligible. No titration was done for sperm-whale myoglobin azide, and values for horse heart myoglobin azide were assumed for studies of temperature dependence. [b] These values are assumed to be the same as that for aqueous sodium azide (see text).

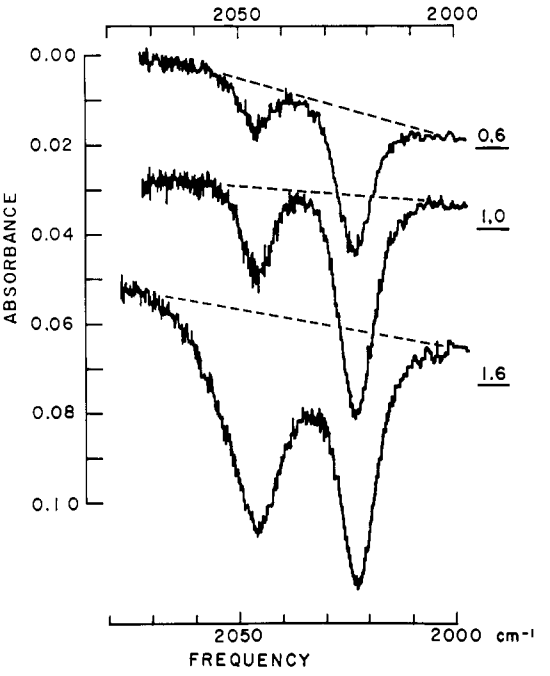


FIGURE 1: Representative infrared spectra of azide added to aqueous horse heart myoglobin (9.45 mM) at the relative azide concentrations ([azide]/Mb]) indicated for each curve. Baselines used for the measurement of maximum absorbances and areas are indicated by dotted lines. The infrared cells were cooled to about 10° during the spectroscopic measurements.

bands due to the azide bound to myoglobin are in fact due to ionic and covalent azide and that these species are involved in a temperature-dependent spin-state equilibrium in agreement with the magnetic susceptibility data of Beetlestone and George and Iizuka and Kotani.

Materials and Methods

Infrared spectroscopy was conducted with a Perkin-Elmer Model 102 double-beam spectrometer with a grating-prism double monochrometer. Quantitation was obtained in one of two ways, either from (1) independently measured concentrations of myoglobin and azide and the infrared cell path measured from interference fringes with the empty cell or (2) from the visible spectrum of a sample in the infrared cell and the known extinction coefficients for the visible region. The infrared cells were jacketed in order to maintain a predetermined temperature and in order to conduct the low-temperature experiments. For the latter, it was also necessary to blow a stream of cold dry nitrogen across the infrared cell windows. Myoglobin fractions from sperm whale or horse heart were purchased from Mann Research Co. and used without further purification. Human hemoglobin A was obtained as the hemolysate from washed red cells.

Experimental Results

Titration of Horse Heart Myoglobin with Azide. The infrared spectra of azide in the presence of horse myoglobin shown in Figure 1 are representative of those spectra that were obtained during the titration studies. The relative concentrations of azide in these studies ranged from 0.2 to 2.2, with a constant myoglobin concentration of 9.45 mM. Several points are illustrated by the spectra in Figure 1. The absorption band near 2023 cm^{-1} has a frequency that is lower than that observed for ionic azide by about 25 cm^{-1}. It has a narrow half-bandwidth similar to that previously reported for carbon monoxide complexes of myoglobin and hemoglobin, and is assigned to azide which is covalently bound to the iron and protected from the bulk aqueous solvent by the surrounding protein matrix. It should be noted that the half-bandwidth is constant even in the presence of excess azide. The higher frequency band near 2046 cm^{-1} exhibits a narrow band width only when the total azide concentration is less than that of myoglobin. The half-bandwidth becomes wider with excess azide due to the superposition of the absorption band due to azide in bulk aqueous solution. The latter form of azide absorbs at 2048 cm^{-1}, whereas the band observed at small azide concentrations absorbs at 2046 cm^{-1}, and is therefore called essentially ionic azide bound to myoglobin. The narrow half-bandwidth can only be due to a species which is not in contact with bulk aqueous solution, but rather protected from water by the protein.

It could not be determined from isolated spectra whether this ionic azide bound to protein was in fact bound at the iron or rather to some other site. In order to approach this question it was necessary to develop quantitative extinction coefficients for both the covalent and ionic forms of bound azide. The extinction coefficients (Table I) for sodium azide in aqueous solution were calculated from the maximum absorbance and from the area under the absorption band. It was demonstrated that in the presence of myoglobin (Figure 1), azide was distributed into both the ionic and covalent forms. If it is initially assumed (as will be verified later) that the area under an absorption band (but not the band shape) is the same whether azide is in aqueous solution or bound to the protein, then it it is possible to use the ϵ_{area} for sodium azide in aqueous solution to estimate the total ionic azide concentrations. The difference between the total azide initially added and the calculated total ionic azide must then represent the covalently bound azide observed in the absorption band

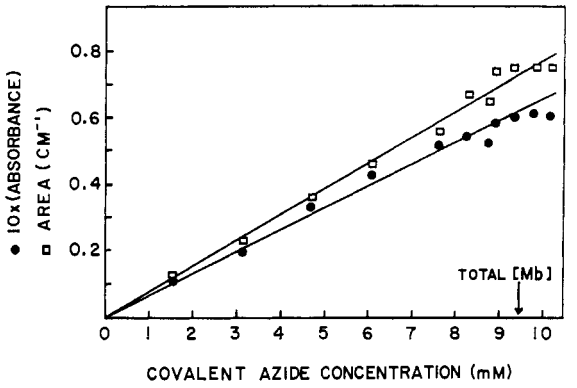


FIGURE 2: Titration of horse heart myoglobin with sodium azide. Areas (□) and maximum absorbances (○) of infrared absorption bands at 2023 cm^{-1} are compared to the concentrations of covalently bound azide. Total myoglobin concentration is indicated by the arrow.

at 2023 cm^{-1}. Thus the spectra in Figure 1 were analyzed by subtraction of the base line (indicated by the dotted line) followed by graphic analysis of each spectrum into two absorption bands using only the assumption that each band is symmetrical. The areas under the absorption curves and the absorbance at peak maximum were then measured for each curve. The calculated ionic portion of the azide was subtracted from the total to obtain the covalently bound azide. This led to the titration curves in Figure 2 for covalently bound azide. Note that the maximum calculated covalent azide concentration is in good agreement with the total myoglobin concentration as required for a 1:1 complex. However, an uncertainty of about 10% is reasonable, and the binding of a small amount of ionic azide to the Fe(III) is not excluded. The linearity of either absorbance or area with the calculated covalent azide concentration is in agreement with assumptions that were made in calculating the total ionic azide concentration from the ϵ_{area} of aqueous sodium azide. The extinction coefficient for covalent azide is listed in Table I.

Analysis of the ionic azide into the bound form and that which is free in aqueous solution was more difficult. The apparent half-bandwidth for ionic azide is not constant but varies as seen in Figure 3, from a lower limit of about 8 cm^{-1}

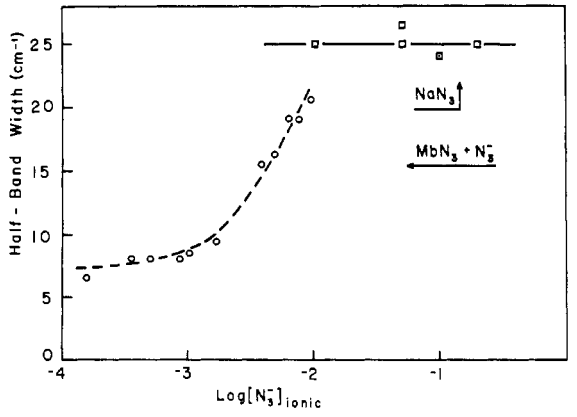


FIGURE 3: Apparent half-bandwidths ($\Delta\nu_{1/2}$) of the infrared absorptions near 2046 cm^{-1} are compared to the total ionic azide concentration during titration of horse myoglobin with sodium azide. Half-bandwidths are from total ionic azide in the presence of 9.45 mM myoglobin (○), and aqueous sodium azide in the absence of myoglobin (□).

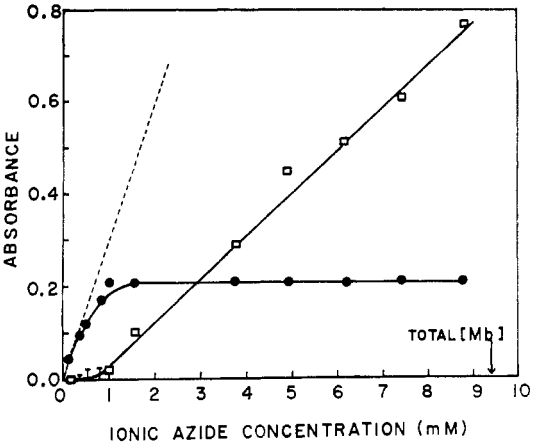


FIGURE 4: Titration curves computed for the composite infrared absorption band at 2046 cm^{-1}. Curves for ionic azide bound to horse heart myoglobin (○) with $\Delta\nu_{1/2}$ = 8 cm^{-1}, and ionic azide free in aqueous solution (□) with $\Delta\nu_{1/2}$ = 25 cm^{-1}, are obtained by comparison of the observed infrared absorption bands near 2046 cm^{-1} due to total ionic azide with the computed sum of two Cauchy (Lorentzian) functions.

to an upper limit approaching the half-bandwidth for aqueous sodium azide. This is reasonable if this band indeed is that of a mixture of bound and free azide. However, the apparent half-bandwidth of the total ionic azide is not a simple function of the mole fractions of the two components,[1] and it was therefore necessary to determine the fraction of azide which was bound to the iron by comparison of the observed spectra to the computed sum of bands represented by Cauchy (Lorentzian) functions (Ramsey, 1952) (eq 1). Each fraction in eq 1

$$\log (I_0/I) = \frac{a_1}{(\nu - \nu_0)^2 + b_1^2} + \frac{a_2}{(\nu - \nu_0)^2 + b_2^2} \qquad (1)$$

represents one species of azide that is either bound to protein or free in aqueous solution.[2] Equation 1 was computed with a variety of values for a_1 and a_2 while b_1 was held constant at 12.5 and b_2 at 4 for the limiting half-bandwidths of 25 and 8 cm^{-1}, respectively. No restrictions were placed on the sum of the computed absorbances, so that the base line was free to adjust without restriction. The computed curves were compared to the observed spectra until reasonable fits were obtained. This led to the titration curves in Figure 4.

It is now possible to test whether the assumption was true, which we made earlier, that the area under an absorption band for ionic azide was independent of solvation (*i.e.*, whether azide was in free aqueous solution or bound to iron within the protein matrix). Since from the Beer-Lambert law, A =

[1] The half-bandwidth of a Cauchy function for a single band is $\Delta\nu_{1/2} = 2b$; for the sum of two bands, it is

$$\Delta\nu_{1/2} = 2\sqrt{(1/2a)[-b \pm (b^2 - 4ac)^{1/2}]}$$

where a, b, and c each have the form, $(\alpha k_1 + [1 - \alpha]k_2)$, k_1 and k_2 are products of a_1, a_2, b_1, and b_2, from eq 1, and α and $1 - \alpha$ are the fractional amounts of each band.

[2] Each absorption band is represented by an equation, $\log (I_0/I) = A$ (or absorbance), and $A = a/[(\nu - \nu_0)^2 + b^2]$; the maximum absorbance of a band (A_{max}) at the center frequency (ν_0) is equal to a/b^2, the half-bandwidth ($\Delta\nu_{1/2}$) = $2b$ (= the bandwidth at $A_{max}/2$); the area of the absorption band = $\int_{-\infty}^{\infty} A d\nu = \pi a/b = (\pi/2)A_{max}\Delta\nu_{1/2}$; and ν is the frequency in cm^{-1}.

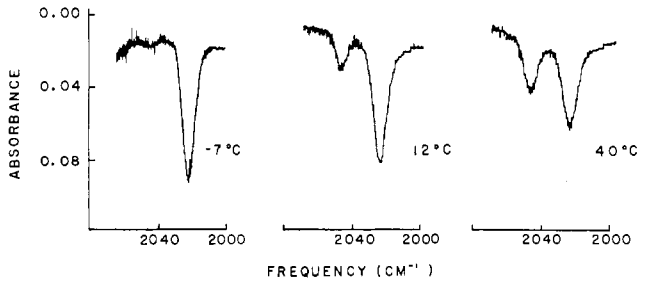


FIGURE 5: Typical infrared absorption spectra of horse myog obin azide complexes, illustrating the temperature dependence of covalency of the bound azide. Covalently bound azide absorbs near 2023 cm^{-1} and ionically bound azide near 2046 cm^{-1}. $[Mb^+]$ = 9.4 mM, and $[N_3^-]/[Mb^+]$ = 0.9.

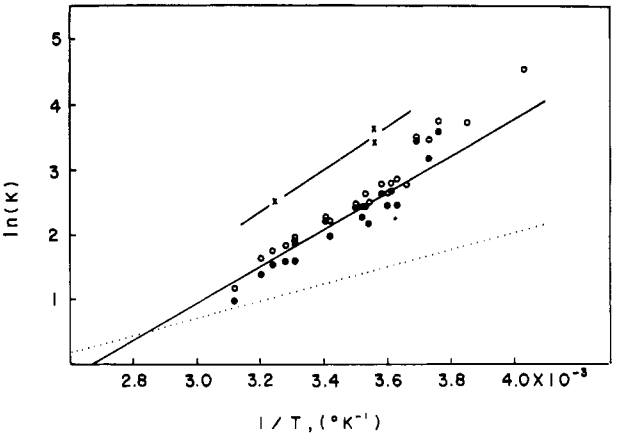


FIGURE 6: Temperature dependence of covalency of horse heart myoglobin-azide complex determined by infrared spectroscopy: ln K was calculated from $(area)_{cov}/(area)_{ion}$ (●), from the absorption bands at 2023 cm^{-1} (covalent) and 2046 cm^{-1} (ionic), and from A_{cov}/A_{ion} (○), with $[Mb^+]$ = 9.4 mM and $[N_3^-]/[Mb^+]$ = 0.9. Equilibrium constants for human hemoglobin A (×) were calculated from $(area)_{cov}/(area)_{ion}$, with $[HbA^+]$ = 18 mM, and $[N_3^-]/[HbA^+]$ = 0.56. The curve for horse heart myoglobin azide complex, calculated from the magnetic susceptibility data of Beetlestone and George (1964), (.....), is included for comparison.

ϵlc, and area = $\epsilon_{area} lc$, then for unit concentration and path length

$$\frac{\text{area}_1}{\text{area}_2} = \frac{(\pi/2)A_1(\Delta\nu_{1/2})_1}{(\pi/2)A_2(\Delta\nu_{1/2})_2} = \frac{(\epsilon_{area})_1}{(\epsilon_{area})_2} = \frac{\epsilon_1(\Delta\nu_{1/2})_1}{\epsilon_2(\Delta\nu_{1/2})_2} \quad (2)$$

and $area_1/area_2$ will equal unity if our assumption is correct. The ratio of extinction coefficients for absorbance at the peak maximum is obtained from the computed titration curves in Figure 4, and compared to the ratio of half-bandwidths for aqueous (2) and bound (1) ionic azide. Thus ϵ_1/ϵ_2 = 3.08, and $(\Delta\nu_{1/2})_2/(\Delta\nu_{1/2})_1$ = 3.12, so that $(\epsilon_{area})_1 = (\epsilon_{area})_2$, and our assumption is indeed valid. The "measured" extinction coefficient for bound ionic azide is determined as the ratio, ϵ_1/ϵ_2, from Figure 4, times the measured extinction coefficient (at maximum absorbance) for aqueous sodium azide. This value is listed in Table I.

Once the extinction coefficients for azide were available, it became possible to calculate the concentration of each species from the ir spectra, determine concentration ratios, and compare the spectrally observed total concentrations to the amounts of azide initially added. The latter procedure yielded estimates of precision for each total azide concentration and $([N_3^-]_{added} - [N_3^-]_{obsd})/[N_3^-]_{added}$ ranged from 1 to 6% with a mean of 3.2%. At the smallest azide concentrations (where the concentration of aqueous azide was very small), the concentration ratios for (total ionic/covalent) azide were 0.088, 0.085, and 0.106 for 1.7, 3.5, and 5.2 mM total added azide, respectively. The concentration ratios for bound ionic/covalent azide derived from Figure 4 are 0.09 ± 0.01 from the smallest total azide concentration of 12.2 mM, and 0.07 at higher concentrations. The accuracy of the latter value is limited by the insensitivity of the band at 2048 cm^{-1} to a relatively small contribution from bound ionic azide in the presence of a large contribution from azide in aqueous solution. However, it is clear that the ratio of bound ionic/covalent azide is constant throughout the titration. Such an observation would be unusual if these two forms of azide were bound to the protein at independent sites, but would be required for azide bound to Fe(III) which was in a temperature-dependent spin-state equilibrium. At saturation, the sum of the bound ionic azide (0.7 mM) and bound covalent azide (9.7 mM) equals 10.4 mM, which is in satisfactory agreement with the estimated myoglobin concentration (9.43 mM).

During the infrared titration of horse acid metmyoglobin with azide, visible absorption spectra were obtained of a few samples in the infrared cells after measurement of the azide vibrational bands. Visible absorption bands at 497 and 628 nm, due to high-spin iron(III) porphyrin, decrease in intensity with an increase in the intensity of absorption bands at 540 nm and 570 nm due to the low-spin azido-iron(III) porphyrin complex. With excess azide, the low-spin bands at 540 and 570 nm predominate, but some absorption intensity at 628 nm due to high-spin Fe(III) complex remains at room temperature. These observations are consistent with our infrared data and with spectral and magnetic susceptibility data of Beetlestone and George (1964), and Iizuka and Kotani (1969a).

Temperature Dependence of Covalency of Bound Azide. If the ionic and covalent forms of protein-bound azide are in fact associated with a temperature-dependent spin-state equilibrium of the iron, then at constant total azide and myoglobin concentration the concentrations of ionic and covalent azide must be reciprocally related when temperature is varied. This is illustrated by the spectra in Figure 5. The absorption band due to ionic azide increases with temperature with a corresponding decrease in the peak due to covalently bound azide. This reciprocal relation between the concentration of ionic and covalent azide is required by the postulated reaction: $Mb(N_3^-)_{ionic} \rightleftharpoons Mb(N_3^-)_{cov}$, with $K_{equil} = [N_3^-]_{cov}/[N_3^-]_{ionic}$. Concentrations were calculated from the measured areas under the absorption bands and from absorbances at peak maxima. The reasonably good agreement of these two types of data, and the observation of constant half-bandwidths over the temperature range studied, indicate that band shapes were independent of temperature.

Data from two sets of experiments with horse myoglobin are combined in Figure 6. Values for ln K calculated from absorption band areas (closed circles) are very close to those calculated from absorption maxima (open circles). Corresponding symbols (squares) are used in Figure 7 for sperm-whale myoglobin. The solid lines in Figures 6 and 7 are calculated from combined measurements of band areas and absorbances, which were obtained above the freezing temperature. Data obtained below this temperature are included in the figures, but may contain greater error. No significant differences are observed in the infrared data from sperm-whale

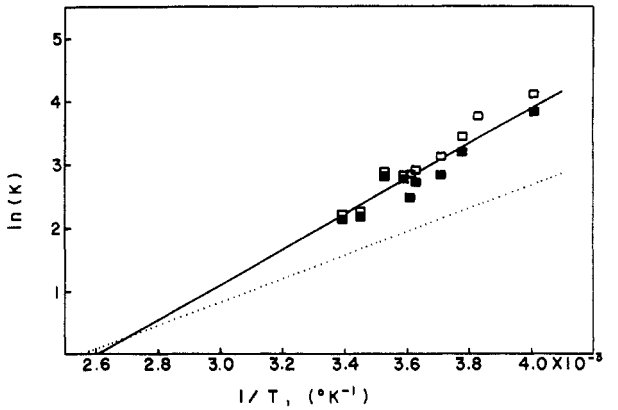
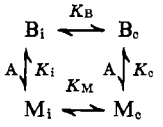


FIGURE 7: Temperature dependence of covalency of sperm-whale myoglobin azide complex determined by infrared spectroscopy: ln K was calculated from $(\text{area})_{cov}/(\text{area})_{ion}$ (■), and from A_{cov}/A_{ion} (□). The curve included for comparison is calculated for sperm-whale myoglobin azide complex from the magnetic susceptibility data of Iizuka and Kotani (1969a) (.....).

or horse myoglobin, and indeed the data appear to be from the same population. The dotted lines are calculated from magnetic susceptibility data from Beetlestone and George (1964) (horse myoglobin azide, Figure 6) and Iizuka and Kotani (1969a) (sperm-whale myoglobin azide, Figure 7). Values for ln K obtained from magnetic susceptibility are clearly different from each other and from those obtained by infrared spectroscopy. No errors have been found in any of these calculations, and the differences appear to be either real or the result of experimental bias. The apparent thermodynamic constants are summarized in Table II.

A possible solution to differences between observations from infrared spectroscopy and magnetic susceptibility appeared during recent discussions with Dr. Alan Adler (New England Institute). Magnetic susceptibility measures contributions from all paramagnetic species, so that for the system



where A is free azide, M is free myoglobin, B is the azide-bound complex, i and c refer to ionic (high spin) and covalent (low spin), respectively, and the K's are the corresponding equilibrium constants. The apparent equilibrium constant

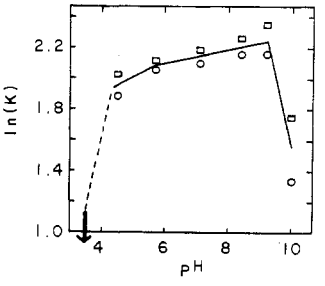


FIGURE 8: Dependence of azide covalency upon pH in horse heart myoglobin azide complexes determined by infrared spectroscopy at 22°: ln K is calculated from $(\text{area})_{cov}/(\text{area})_{ion}$ (○) and from A_{cov}/A_{ion} (□). $[Mb^+]$ = 4–7 mM, and $[N_3^-]/[Mb^+]$ = 0.9.

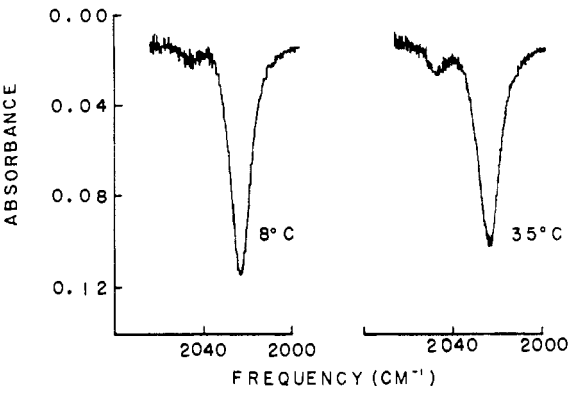


FIGURE 9: Infrared spectra of azide bound to human hemoglobin A in aqueous solution at 8 and 35°. $[HbA^+]$ = 18 mM, and $[N_3^-]/[HbA^+]$ = 0.56

(K') observed by susceptibility measurements equals the total low-spin iron $M_c + B_c$ divided by the total high-spin iron $M_i + B_i$ leading to the expression $K' = K_B(K_c + A)/(K_i + A)$. Thus K' approaches K_B (the true equilibrium constant for ionic and covalent azide bound to myoglobin, *i.e.*, the spin-state equilibrium constant) as free azide concentration becomes very large.

In similar fashion the present infrared studies have measured total covalent azide and total ionic azide under conditions where the free azide concentration is thought to be negligibly small. The apparent infrared equilibrium constant is $K_{ir} = B_c/(A + B_i)$, so that $K_B/K_{ir} = A \cdot K_B/B_c + 1$, and $K_B = K_{ir}$ when free azide concentration becomes negligible. Further studies are in progress to clarify these relations.

The pH dependence of azide covalency was determined with horse myoglobin (Figure 8). Azide covalency increases slightly from pH 4.5 to 9.2. All azide is converted to the aqueous ionic form at pH 3.5, accompanied by protein denaturation and loss of the heme, while at pH 10, azide covalency is less than maximal with no protein insolubility. The covalency of azide bound to myoglobin closely parallels the pH dependence of ellipticity of hemoglobins recently reported by Ruckpaul *et al.* (1970), and is thus sensitive to changes in protein conformation.

Hemoglobin azide complexes have a similar temperature dependence as myoglobin complexes (Figures 5 and 9) but are considerably more covalent. The extinction coefficients for covalently bound HbN_3 (Table I) were determined by azide titration after correction for the small amounts of ionic form present. The extinction coefficient for area for covalent HbN_3 was used with that for aqueous ionic sodium azide (in place of the undetermined value for bound ionic HbN_3) to calculate the thermodynamic constants in Table II. While hemoglobin azide complexes show similar differences between infrared and magnetic susceptibility measurements to those observed with myoglobin, both types of measurements show the azide complexes in hemoglobin to be considerably more covalent at 25° than those of myoglobin.

Discussion

The quantitation of infrared absorption bands due to ionic and covalent azide bound to myoglobin has made it possible to define a temperature-dependent equilibrium between the two states. This equilibrium corresponds reasonably well with the temperature-dependent spin-state equilibrium of the

TABLE II: Thermodynamic Constants for Azide Covalency Determined by Infrared Spectroscopy Are Compared to Those for the Spin-State Equilibrium of the Iron in Myoglobin and Hemoglobin Azide Complexes.[a]

	$\Delta H°$ (cal/mole) $\pm \sigma$	$\Delta S°$ (cal/(mole deg)) $\pm \sigma$	T_c (°K)	$K_{equil}^{25°}$[b]	Ref[c]
Horse myoglobin					
Infrared spectroscopy	−5600 ± 290	−15.1 ± 1.0	375	7.0	1
Magnetic susceptibility	−2740 ± 400	−6.8 ± 1.4	403	3.3	2
Sperm-whale myoglobin					
Infrared spectroscopy	−5600 ± 1700	−14.6 ± 6.0	383	8.1	1
Magnetic susceptibility	−3740	−9.6	391	4.5	3
Hemoglobin A (human)					
Infrared spectroscopy[d]	−6700	−17	400	18	1
Horse hemoglobin					
Magnetic susceptibility	−5094	−13.6	374	5.8	4

[a] Thermodynamic constants were calculated from least-squares fits of data to the equations of state, $-RT \ln K = \Delta G = \Delta H - T\Delta S$, in which $\Delta H = -R[\Delta \ln K/\Delta(1/T)]$, and $\Delta S = \Delta H/T_c$, where $T_c = T$ at $\ln K = 0$. Combined data from infrared band absorbances and areas at temperatures above 273 °K were used for these calculations. [b] $K_{equil} = [N_3^-]_{cov}/[N_3^-]_{ionic}$ from infrared data, and $K_{equil} = [Fe_{1/2}^{3+}]/[Fe_{5/2}^{3+}]$ from magnetic susceptibility data. [c] References: (1) this work; (2) Beetlestone and George (1964); (3) Iizuka and Kotani (1969a); (4) Iizuka and Kotani (1969b). [d] Thermodynamic constants for hemoglobin A were estimated from data at three temperatures only.

iron from magnetic susceptibility studies. Differences between the magnetic susceptibility and infrared measurements may be due to as yet undefined experimental bias, or may be a function of the equilibria between bound and free azide and the spin states of the iron. In either case, this appears to be the first demonstration of a correspondence between high-spin iron with an ionic ligand and low-spin iron with a covalently bound ligand, where ionic azide is defined as a group having essentially the same infrared absorption frequency (*i.e.*, azide bond order) as the sodium salt in aqueous solution, and covalently bound azide is defined as a group that absorbs at a lower frequency (*i.e.*, with a lower azide bond order, presumeably with greater Fe–N bond order than is found with the ionic form).

Azide covalency appears to be critically poised so as to be very sensitive to small differences in the local molecular environment. It may be especially sensitive to differences in the size of the protein cavity at the heme binding site resulting from differences in the positions of the E and F helices. Thus, the highest azide covalency is observed in the pH region of maximum protein stability, which is also the region for which maximum ellipticity was reported from circular dichroism measurements at 222 nm. A small difference in protein conformation (cavity size) may also explain the difference in azide covalency between myoglobin and hemoglobin.

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