Gay, S., Kresina, T. F., Gay, R., Miller, E. J., & Montes, L. F. (1979) *J. Cutaneous Pathol.* (in press).
Heathcote, J. G., Sear, C. H. J., & Grant, M. E. (1978) *Biochem. J. 176*, 283–294.
Hudson, B. G., & Spiro, R. G. (1972) *J. Biol. Chem. 247*, 4229–4238.
Kefalides, N. A. (1971) *Biochem. Biophys. Res. Commun. 45*, 226–234.
Kefalides, N. A. (1973) *Int. Rev. Connect. Tissue Res. 6*, 63–104.
Man, M., & Adams, E. (1975) *Biochem. Biophys. Res. Commun. 66*, 9–16.
Man, M., Nagle, R. B., & Adams, E. (1978) *Exp. Mol. Pathol. 29*, 144–148.
Mayne, R., Vail, M. S., & Miller, E. J. (1978) *Biochemistry 17*, 446–452.
Miller, E. J. (1971) *Biochemistry 10*, 1652–1658.
Miller, E. J. (1972) *Biochemistry 11*, 4903–4909.
Minor, R. R., Clark, C. C., Strause, E. L., Koszalka, T. R., Brent, R. L., & Kefalides, N. A. (1976) *J. Biol. Chem. 251*, 1789–1794.
Piez, K. A., Weiss, E., & Lewis, M. S. (1960) *J. Biol. Chem. 235*, 1987–1993.
Rhodes, R. K., & Miller, E. J. (1978) *Biochemistry 17*, 3442–3448.
Sato, T., & Spiro, R. G. (1976) *J. Biol. Chem. 251*, 4062–4070.
Schwartz, D., & Veis, A. (1978) *FEBS Lett. 85*, 326–332.
Timpl, R., Martin, G. R., Bruckner, P., Wick, G., & Wiedemann, H. (1978) *Eur. J. Biochem. 84*, 43–52.
Trelstad, R. L., & Lawley, K. R. (1977) *Biochem. Biophys. Res. Commun. 76*, 376–384.
Tryggvason, K., & Kivirikko, K. I. (1978) *Nephron 21*, 230–235.

Achatina fulica Hemocyanin and Its Interactions with Imidazole, Potassium Cyanide, and Fluoride as Studied by Spectrophotometry and Nuclear Magnetic Resonance and Resonance Raman Spectrometry†

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ABSTRACT: Hemocyanin from Taiwan snails, *Achatina fulica*, has not been studied previously. We have used ultraviolet spectrophotometric, proton magnetic resonance, ^{19}F NMR, and resonance Raman methods to study the interaction of several small ligands with this hemocyanin species. Addition of imidazole (up to 0.5 M) and potassium cyanide (up to 0.001 M) to oxyhemocyanin causes a relatively rapid drop in absorbance at 345 nm, a primary reaction, followed by a much slower secondary process. The primary reaction between imidazole and hemocyanin takes about 2 h, while that between cyanide and hemocyanin takes only a fraction of a second. The ^1H NMR signal of 0.20 M imidazole (4,5-H) and the ^{19}F NMR signal of 0.20 M KF at pH 9.0 are broadened by oxyhemocyanin, and the extent of broadening is linearly dependent on hemocyanin concentration. Addition of KCN (up to 0.001 M) results in reduction of paramagnetic Cu(II) to diamagnetic Cu(I), with the copper still bound to the protein. Above 0.001 M, copper begins to be removed from the protein, forming aqueous cuprous–cyanide complexes. The rate constant of the primary reaction of KCN (0.0001–0.001 M) with hemocyanin decreases with pH increase in the pH range 8.5–9.5. The data suggest that the neutral HCN, or its kinetic equivalent, is the reactive species in the primary reaction. A resonance Raman spectrum of the oxyhemocyanin, by excitation with the 514.4-nm band, shows the O_2^{2-} vibration at 752 cm^{-1}. Literature values give 744 cm^{-1} in *Cancer magister* (arthropod) and 749 cm^{-1} in *Busycon canaliculatum* (mollusc) hemocyanins for the O_2^{2-} vibration. The position of the O_2^{2-} vibration together with our NMR and spectrophotometric results suggests that *A. fulica* and *B. canaliculatum* hemocyanins have similar structures and properties.

Hemocyanins are huge, oxygen-carrying proteins in the hemolymph of many molluscs and arthropods. It is known that one oxygen molecule binds to two copper ions in the protein, and much current effort is being directed toward determining the structure of the copper–oxygen complex and the nature(s) of the copper in oxyhemocyanin (Lontie & Vanquickenbourne, 1974; Freedman et al., 1976; Guo et al., 1978). Hemocyanin from Taiwan snails, *Achatina fulica*, has not been studied previously. In this paper we report studies on this hemocyanin species, by using ultraviolet spectrophotometry, nuclear magnetic resonance, and resonance Raman methods, and study the interaction between *A. fulica* hemocyanin and the following small ligands: imidazole, potassium cyanide, and fluoride.

Study of hemocyanin from snails in Taiwan has the advantage that the snails are plentiful in the immediate vicinity of the University Laboratory, so that collection of the hemolymph together with isolation and purification of hemocyanin from the hemolymph can be carried out in 1 day. This reduces greatly the complications which arise from aging of proteins.

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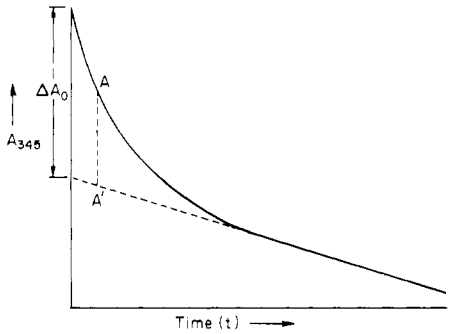

FIGURE 1: Schematic representation of the biphasic decrease in absorbance at 345 nm when imidazole is added to a buffered solution of oxyhemocyanin at pH 9.00.

Experimental Section

The shell of the snail was thoroughly cleaned. After puncturing the heart region, we placed the snail over a 10-mL beaker to collect the hemolymph. The material discolored by contamination with intestinal fluid was discarded. After being pooled, the hemolymph was centrifuged for 10 min at 10000*g* (Sorval Du Pont RC-5 superspeed refrigerated) to remove debris. The clear hemolymph was diluted with an equal volume of 0.4 M potassium acetate, pH 5.3, and the hemocyanin was precipitated by addition of an equal volume of saturated ammonium sulfate. After the mixture was centrifuged for 15 min at 10000*g*, the sediment was dissolved in 0.1 M potassium acetate, pH 5.7, and dialyzed against deionized distilled water to remove the ammonium sulfate at 4 °C. A hemocyanin pellet was obtained after ultracentrifuging for 2 h at 100000*g* and was stored in a refrigerator. Solutions were prepared from the pellet just before use.

Hemocyanin concentrations were determined spectrophotometrically by using ϵ_{345} 10000 M^{-1} (Cu) cm^{-1} and ϵ_{280} (1%, 1 cm) 15.71. The copper content was determined by activation analysis, using the Tsing Hua University reactor, to be 0.24%.

NMR spectra were obtained with a JEOL-C60HL NMR spectrometer (60 MHz for ^1H and 56.4 MHz for ^{19}F measurements, probe temperature 28 °C). In taking line width measurements, we took care to keep the radio frequency well below saturation. The observed line width ($\Delta\nu_{1/2}$) was measured from the full width at half-amplitude of the peak, as described previously (Ke et al., 1973).

Fast reactions of hemocyanin with potassium cyanide were studied by using a stopped-flow spectrophotometer, thermostated at 25 °C. Equal volumes of oxyhemocyanin, in one syringe, and potassium cyanide, at the appropriate concentration in the other syringe, were mixed, and the change in transmittance at 345 nm with time was photographically recorded on a storage oscilloscope.

Resonance Raman spectra were obtained on a Spex 1401 spectrometer and an SSR photon counter with the 514.5-nm line of a Spectra-Physics Model 170 argon ion laser as the exciting source.

Flourescence spectra were obtained with a Hitachi Model 204 fluorescence spectrometer equipped with a 150-W xenon lamp.

Results

Spectrophotometric Studies. (1) Binding of Imidazole. Addition of imidazole to *A. fulica* hemocyanin causes a decrease in absorbance in the absorption around 345 nm, similar to that for addition of thioacetamide to *Busycon* and *Limulus* hemocyanins (Lee et al., 1977, Guo et al., 1978) and for thiourea (Rombants, 1968). The loss of absorbance occurs in two stages: a relatively rapid primary reaction followed by a much slower secondary reaction, shown schematically in Figure 1. ΔA_0 is the change in absorbance caused by the primary reaction after correction for the secondary reaction, and this subtraction procedure is possible because the rate constants for the two reactions are very different. The dependence of ΔA_0 for *A. fulica* oxyhemocyanin on imidazole concentration follows the form of eq 1, similar to that found

$$\Delta A_0 = (\Delta A_0)_{max}\frac{(L)_0}{(L)_0 + K_{app}} \quad (1)$$

for ΔA_0 for *Busycon* oxyhemocyanin as a function of thioacetamide concentration (Lee et al., 1977; Guo et al., 1978). In eq 1, $(\Delta A_0)_{max}$ is the limiting value of ΔA_0 at high ligand concentration $[(L)_0]$, and K_{app} is the apparent dissociation constant of the ligand–protein complex(es) and is the concentration of ligand at which the protein is half-saturated with ligand. Equation 1 is linearized to eq 2, and therefore a plot

$$\frac{(L)_0}{\Delta A_0} = \frac{1}{(\Delta A_0)_{max}}K_{app} + \frac{1}{(\Delta A_0)_{max}}(L)_0 \quad (2)$$

of $(L)_0/\Delta A_0$ vs. $(L)_0$ gives $(\Delta A_0)_{max}$ and K_{app}. For solutions containing 0.89×10^{-4} M hemocyanin (expressed in terms of copper sites) and 0.1–0.5 M imidazole at pH 9.00 (Tris buffer), the following values at 25 °C are obtained: $(\Delta A_0)_{max} = 0.43$ and $K_{app} = 0.75$ M. Because of the weak binding of imidazole to hemocyanin, pH dependence of apparent dissociation constants was not determined.

Linear plots of $-\ln (A - A')$ vs. time (for definition of A and A', see Figure 1) are obtained for the primary reaction of imidazole with hemocyanin. For solutions containing 0.89×10^{-4} M hemocyanin, the rate constants at 25 °C for the primary reactions are 0.25, 0.33, and 0.37 h^{-1} for (Im) = 0.2, 0.3, and 0.4 M, respectively, at pH 9.00.

(2) Binding of Cyanide. The primary reaction between imidazole and hemocyanin takes about 2 h, whereas that between cyanide and hemocyanin takes only a fraction of a second, so that a stopped-flow technique was used for the latter. The values of K_{app}, together with the rate constants for the primary reaction of cyanide with hemocyanin at three different pH values, are listed in Table I.

Table I: Values of K_{app} and of the Apparent First-Order Rate Constant, k, for the Primary Reaction of Potassium Cyanide with *A. fulica* Hemocyanin

	pH		
	8.5	9.0	9.5
K_{app} ($M \times 10^4$)	5.53	4.31	3.77
k (s^{-1}) for			
(KCN) = 0.0001 M	1.29	1.21	0.92
(KCN) = 0.0005 M	2.75	1.74	1.72
(KCN) = 0.0010 M	3.39	3.00	2.41

The decrease in A_{345} of hemocyanin 10 min after KCN (pH 9.0) was added is shown in Figure 2. If we let KCN of different concentrations (up to 0.001 M) react with hemocyanin for 10 min and then dialyze out the KCN for 1 h, A_{345} invariably increases. After dialysis for 3 days, A_{345} increases further. For KCN concentrations greater than 0.001 M, however, there is no increase in absorbance on dialysis. This indicates that the binding of KCN to hemocyanin is reversible up to KCN = 0.001 M. Beyond this concentration, copper begins to be removed from the protein, so that A_{345} does not increase, even though KCN is removed by dialysis.

Figure 3 gives the fluorescence spectra of (a) oxyhemocyanin, HcO_2 (excitation at 280 nm), $A_{345} = 0.96$ at pH 9.0

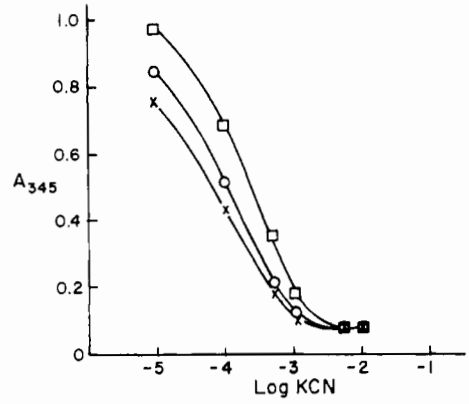

FIGURE 2: Dependence of absorbance at 345 nm of HcO_2 on the concentration of KCN for a 10-min reaction time and then dialysis out of KCN against buffer solution, pH 9.0. (×) A_{345} before dialysis; (○) A_{345} measured after dialysis for 1 h; (□) A_{345} measured after dialysis for 3 days.

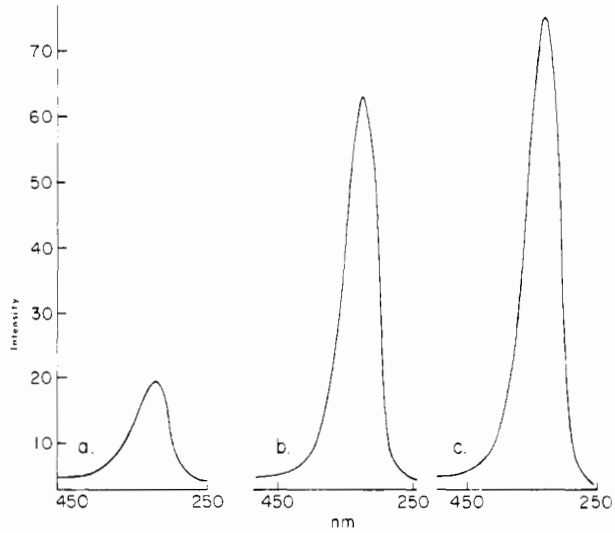

FIGURE 3: Fluorescence spectra of (a) oxyhemocyanin (excitation at 280 nm), A_{345} = 0.96 at pH 9.0 (Tris buffer), (b) the same solution + 10^{-4} M KCN, and (c) solution (a) + 10^{-2} M KCN.

(Tris buffer), (b) the same solution + 10^{-4} M KCN, and (c) solution (a) + 10^{-2} M KCN. In (b) deoxyhemocyanin is formed while in (c) Cu is removed to form apohemocyanin (see Figure 2). The ratios of the fluorescence intensities at 325 nm for deoxyhemocyanin (b)/pure oxyhemocyanin (a) and for apohemocyanin (c)/pure oxyhemocyanin (a) are 4 and 5, respectively. It is interesting to compare these findings for *A. fulica* with the fluorescence findings of *Levantina hierosolima* by Shaklai & Daniel (1970), who reported that when the hemocyanin is deoxygenated, or when copper is removed, the fluorescence yield at pH 6.6 increases fourfold in each case. Bannister and co-workers (Bannister & Wood, 1971; Bannister et al., 1973) reported that for *Murex trunculus* the fluorescence is attributable to tryptophan, and the fluorescence is enhanced by 513% when the apohemocyanin is prepared. The fluorescence data thus complement absorbance data of the copper band at 345 nm, on the effect of adding KCN to hemocyanin.

(*3*) *Binding of Fluoride.* Addition of potassium fluoride to *A. fulica* oxyhemocyanin causes a decrease in absorbance at 345 nm, at pH 5.0 (acetate buffer), with observed rate constants of 0.21, 0.23, and 0.27 for (KF) = 0.2, 0.4, and 0.6 M, respectively. At pH 9.0, however, fluoride has no effect on the absorbance. Witters & Lontie (1975) have previously

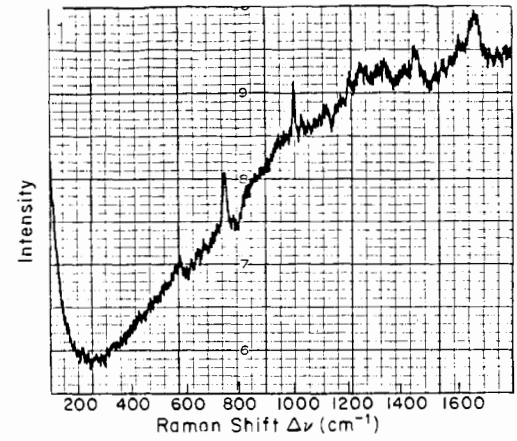

FIGURE 4: Raman spectrum of *A. fulica* oxyhemocyanin using 514.5-nm excitation, 60 cm^{-1}/min scanning rate, and a 1-s time constant. The protein is in the pellet form. Peaks at 1240 and 1675 cm^{-1} are amides III and I, respectively. For the peak at 752 cm^{-1}, see text.

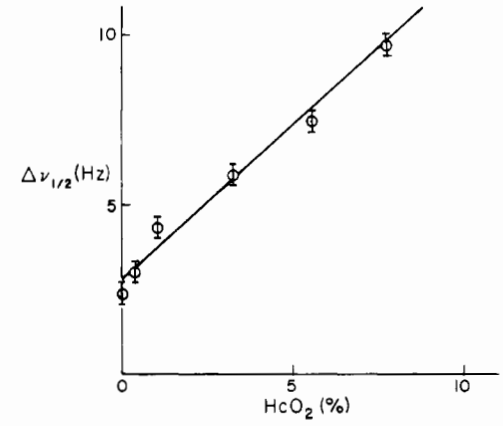

FIGURE 5: ^1H NMR line width of 0.2 M imidazole (4,5-H) as a function of oxyhemocyanin concentration (% w/v) in pH 9.00 (Tris buffer) solutions.

reported a considerable decrease of the copper band (340 nm) on adding fluoride to *Helix pomatia* oxyhemocyanin at pH 4.90 and that the decrease of the copper band diminishes with increasing pH. For *Limulus polyphemus* hemocyanin, they reported no reaction with fluoride even at pH 4.90.

Resonance Raman Study of A. fulica Oxyhemocyanin. Freedman et al. (1976) report that resonance Raman spectroscopy of hemocyanins, by excitation within the ~570-nm band, has permitted the identification of bound oxygen as O_2^{2-} and, hence, the oxidation states of copper as Cu(II). They found the frequency of the O_2^{2-} vibration at 744 cm^{-1} in *Cancer magister* (arthropod) and at 749 cm^{-1} in *Busycon canaliculatum* (mollusc hemocyanins). Figure 4 shows a resonance Raman spectrum of *A. fulica* oxyhemocyanin, by excitation with the 514.5-nm band. The strong peak at 752 cm^{-1} is identified as due to the O–O stretching vibration in O_2^{2-} and, hence, the copper in *A. fulica* oxyhemocyanin is also Cu(II). It must be mentioned that when the protein was purified in the ways described by Freedman et al. (1976), fluorescence under the laser light was so intense that no resonance Raman spectra could be obtained. A good resonance Raman spectrum, Figure 4, could be obtained only when the protein is precipitated by addition of saturated ammonium sulfate and a pellet is used.

Nuclear Magnetic Resonance Studies. The line widths, $\Delta\nu_{1/2}$, of the ^1H NMR signals of the imidazole 4,5-H and 2-H protons (0.2 M, pH 9.0 Tris buffer) are 2.3 and 3.0 Hz,

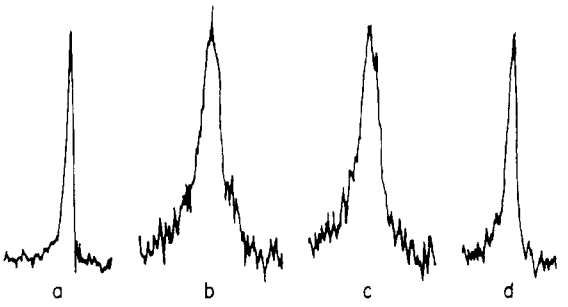

FIGURE 6: ^{19}F NMR spectra of 0.2 M KF in 0.1 M Tris buffer, pH 9.0. Signals are 39.6 ± 0.1 ppm upfield from F_3AcOH. (a) No additive, $\Delta\nu_{1/2}$ = 1.2 Hz; (b) with 2.9% HcO_2, $\Delta\nu_{1/2}$ = 4.1 Hz; (c) with 2.9% HcO_2, 10^{-4} M KCN, $\Delta\nu_{1/2}$ = 3.5 Hz; (d) with 2.9% HcO_2, 10^{-3} M KCN, $\Delta\nu_{1/2}$ = 1.7 Hz.

respectively. Figure 5 shows a linear plot of the line width of 0.20 M imidazole (4,5-H) as a function of *A. fulica* oxyhemocyanin concentration in pH 9.0 Tris buffer solutions. NMR measurements were taken within 20 min after the solutions containing hemocyanin and imidazole were prepared. The results of Figure 5 suggest that the copper in oxyhemocyanin is Cu(II) and that imidazole binds to the copper site.

The ^{19}F NMR line of fluoride is broadened by oxyhemocyanins from both *Limulus* and *Busycon* species (Guo et al., 1978). The extent of line broadening is linearly dependent on the hemocyanin concentration for both species. At pH 8.60 (0.05 M Tris) the relationship between the line width of 0.125 M KF (NMR frequencies 39.0 ± 0.1 ppm upfield from F_3AcOH) and hemocyanin concentration is $\Delta\nu_{1/2}$ (Hz) = α(% w/v hemocyanin) + 1.0, where α = 2.42 for *Busycon* and 3.80 for *Limulus* hemocyanin. For *A. fulica*, we find that the line width of the ^{19}F NMR signal of 0.2 M KF, pH 9.0 (0.1 M Tris) at an NMR frequency of 118.4 ppm upfield from CCl_3F, is also linearly dependent on HcO_2 concentration up to 6.9% HcO_2, with $\Delta\nu_{1/2}$ (Hz) = 1.48(% w/v hemocyanin) + 1.1. The broadening of the ^{19}F signal is due to the interaction between the fluoride ions and the paramagnetic Cu(II) at the HcO_2 active site. The present data suggest that there is a greater steric constraint at the Cu(II) site of *A. fulica* than that of the *Busycon* and *Limulus* species since "α" for the *A. fulica* is smaller (α = 1.48).

The addition of KCN to solutions containing 0.2 M KF and 2.86% HcO_2, pH 9.0, results in an increasing narrowing of the ^{19}F signal as KCN concentration is increased from 10^{-4} to 10^{-3} M, as shown in Figure 6. This is as expected since Cu(II) in oxyhemocyanin is being reduced by diamagnetic Cu(I).

The line width of the ^{19}F signal of a solution 0.5 M KF and 5×10^{-5} M $CuSO_4$ (equivalent to Cu content of 0.12% HcO_2), at pH 9.0, is 8.0 Hz. When the solution contains in addition 0.5 M imidazole, the ^{19}F line width increases to 37.0 Hz. This dramatic broadening may be ascribed to a positive cooperative effect of imidazole binding to Cu(II) on fluoride binding to Cu(II). A similar cooperative effect of imidazole on fluoride binding to *A. fulica* oxyhemocyanin has been observed in our experiments, again demonstrating that the copper in oxyhemocyanin is Cu(II) and that imidazole is bound at the copper site.

Discussion

The change in absorbance at 345 nm which occurs when oxyhemocyanin (HcO_2) and imidazole (L) are mixed and reach equilibrium can be calculated as

$$HcO_2 + L \rightleftarrows HcL + O_2$$

Defining $K_{app} = (HcO_2)(L)/(HcL)$, then

$$\Delta A_0 = (HcO_2)_0(\epsilon_{HcO_2} - \epsilon_{HcL})\frac{(L)_0}{K_{app} + (L)_0} \quad (3)$$

wherein $(HcO_2)_0$ = initial hemocyanin concentration, $(L)_0$ = initial imidazole concentration, and ϵ_{HcO_2} and ϵ_{HcL} are the extinction coefficients of oxyhemocyanin and imidazole–protein complex, respectively. Equation 3 is of the same form as eq 1. Obviously, several equilibria may be involved, and K_{app} is readily shown to be the concentration of ligand at which hemocyanin is half-saturated and is an apparent dissociation constant. The value of K_{app} for the primary reaction of imidazole with *A. fulica* oxyhemocyanin is 0.75 M. A search of the literature shows that imidazole has not previously been studied as a ligand to *any* species of hemocyanin. A value of K_{app} for the primary reaction of thioacetamide with *Busycon* hemocyanin at pH 8.91 has been reported to be 5.7×10^{-3} M (Guo et al., 1978). Hence, with imidazole as ligand, a very marked diminution in the extent of the primary reaction with hemocyanin occurs, compared to a sulfur–nitrogen ligand such as thioacetamide.

Our data lead to the conclusion that copper in oxyhemocyanin is Cu(II) and that imidazole, fluoride, and cyanide (up to 1 mM) bind to the hemocyanin at the active site. When the KCN concentration exceeds 0.001 M, copper begins to be removed from the protein, forming aqueous cuprous cyanide complexes.

Table I shows that the values with K_{app} decrease with increasing pH. This means that cyanide ion, rather than HCN, is the species bound to hemocyanin. On the other hand, the observation of a decreasing rate constant, k, with increasing pH must indicate that the rate process being observed is not a simple nucleophilic attack and that the unionized HCN, or its kinetic equivalent, rather than cyanide ion, is more reactive in the primary reaction with hemocyanin. We have recalculated the rate constants in Table I by taking HCN as the reactive species and K_a(HCN) = $10^{-9.19}$ and obtained decreasing values of k_{HCN} with increasing pH. Thus, for (KCN) = 0.0001 M, k_{HCN} = 1.07, 0.74, and 0.30 for pH 8.5, 9.0, and 9.5, respectively. If cyanide ion is taken to be the reactive species, then the values of k_{CN^-} increase with pH increase, which is contrary to the trend of k with the pHs shown in Table I.

It is interesting to note that with *Busycon* hemocyanin, higher rate has also been observed in acidic buffers than in basic buffers for potassium cyanide and thioacetamide (Chang, 1978; K. T. Douglas, C. Chang, and N. C. Li, unpublished experiments). These authors suggest that ligand-induced conformational changes or aggregational changes in hemocyanin may account in some way for the pH dependence. Indeed, treatment of *A. fulica* oxyhemocyanin with 0.001 M KCN must have drastically reduced the molecular weight of the protein since in the presence of 0.001 M KCN it was not possible to obtain a hemocyanin pellet even after ultracentrifuging at 100000*g* for more than 3 h.

Addition of fluoride to *A. fulica* oxyhemocyanin causes a decrease in A_{345} at pH 5.0, but has no effect at pH 9.0. This influence of the pH is reminiscent of the effect of azide in decreasing the A_{340} of the copper band of *H. pomatia* oxyhemocyanin (Witters & Lontie, 1975). At pH 9.22 azide decreases A_{340} only very slightly, and there is a dramatic decrease in A_{340} at pH 5.01. The authors suggest that at low pH the action of azide or fluoride on the oxyhemocyanin of *H. pomatia* corresponds to a displacement of peroxide from the active site which contains $1O_2^{2-}$ for $2Cu^{2+}$. Since the pK values of hydrogen azide and hydrogen fluoride are 4.76 and 3.2, respectively, the displacement of O_2^{2-} by the neutral HAz

or HF molecules, taken as the reactive species, would occur to a lesser extent at high pH (9.2).

Acknowledgments

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References

Bannister, W. H., & Wood, E. J. (1971) *Comp. Biochem. Physiol. B 40*, 7.

Bannister, W. H., Cammilleri, P., & Chantler, E. N. (1973) *Comp. Biochem. Physiol. B 45*, 325.

Chang, C. (1978) Ph.D. Thesis, Duquesne University.

Freedman, T. B., Loehr, J. S., & Loehr, T. M. (1976) *J. Am. Chem. Soc. 98*, 2809.

Guo, J. M., Chang, C., Li, N. C., & Douglas, K. T. (1978) *Biochemistry 17*, 452.

Ke, C. H., Schubert, J., Lin, C. I., & Li, N. C. (1973) *J. Am. Chem. Soc. 95*, 3375.

Lee, I. Y. Y., Li, N. C., & Douglas, K. T. (1977) in *Proceedings in Life Sciences* (Bannister, J. V., Ed.) p 231, Springer-Verlag, Heidelberg.

Lontie, R., & Vanquickenborne, L. (1974) in *Metal Ions in Biological Systems* (Sigel, H., Ed.) Vol. 3, Marcel Dekker, New York.

Rombauts, W. A. (1968) in *Physiology and Biochemistry of Haemocyanins* (Ghiretti, F., Ed.) pp 75–80, Academic Press, London.

Shaklai, N., & Daniel, E. (1970) *Biochemistry 9*, 564.

Witters, R., & Lontie, R. (1975) *FEBS Lett. 60*, 400.

Myoglobin Semisynthesis: Removal of the NH_2-Terminal Valine of Sperm Whale Myoglobin and Its Subsequent Reincorporation†

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ABSTRACT: A chemical procedure for selectively removing the NH_2-terminal valine residue of sperm whale myoglobin has been developed. Reaction of N^{ϵ}_{19}-acetimidoapomyoglobin with 3-sulfophenyl isothiocyanate yielded the N^{α}3-sulfoPhNHCS,N^{ϵ}_{19}-acetimidoapomyoglobin. The heme was reintroduced after cleavage of the 3-sulfophenylthiohydantoin of valine in anhydrous trifluoroacetic acid. The des-Val[1],-N^{ϵ}_{19}-acetimidomyoglobin was purified and the homogeneity of the protein was ascertained through electrophoresis before and after reaction with 3,4,5,6-tetrahydrophthalic anhydride, time-course amino acid analysis, and automated stepwise Edman degradation. Investigation of the physical properties of the des-Val[1],N^{ϵ}_{19}-acetimidomyoglobin included near- and far-ultraviolet circular dichroism, potentiometric titration, and ultraviolet–visible absorption measurements. Reconstitution of the sperm whale myoglobin sequence was accomplished through coupling of the *N*-hydroxysuccinimide ester of methylsulfonylethyloxycarbonyl-protected L-valine to the des-Val[1],N^{ϵ}_{19}-acetimidomyoglobin. Removal of the amino-protecting groups yielded a molecule identical with the starting material, contaminated with only a small amount of readily distinguishable byproduct.

Prior to the substitution of any amino acid in a protein, it must be clearly shown that the synthetic procedure employed has in no way altered the structure of the final product. Consequently, all physical changes noted in the final product can be attributed to the substituted residue. In this light, we report an unperturbing chemical procedure for removing the NH_2-terminal valine residue of sperm whale myoglobin and its subsequent covalent reintroduction.

Chemical experimentation directed at replacing any one amino acid in a protein is dependent first on a method of removing it. Ideally the method should be site specific and quantitative while being structurally unperturbing in and of itself. Removal of the NH_2-terminal residue is commonly achieved with phenyl isothiocyanate (Edman, 1950) and its use in preparing semisynthetic proteins is well documented (Offord & DiBello, 1978). Sequence studies have shown this reagent to be highly quantitative with nearly all peptides (Edman & Henschen, 1975). However, in protein semisynthesis its inability to couple selectively with the α-amino group in the presence of several ϵ-amino groups is a severe disadvantage. Furthermore, its extremely low water solubility and the necessary presence of a strong anhydrous acidic solvent to effect cleavage are two additional drawbacks. Three milder methods of removing the NH_2-terminal valine of sperm whale myoglobin have been explored (Garner, 1974). Cleavage with aminopeptidase M (Wachsmuth et al., 1966) or (triethylenetetramine)cobalt(III) ion (Bentley & Creaser, 1973) or through transamination (Dixon & Fields, 1972) was found not as efficient or reliable as the Edman procedure.

As a consequence of the irreversible denaturation of myoglobin in nonaqueous media (Herskovitz & Solli, 1975; Herskovitz et al., 1977), a hydrophilic Edman reagent, 3-sulfophenyl isothiocyanate (Dwulet & Gurd, 1976) was used in the present work. Selective coupling to the α-amino group of the apoprotein was directed through acetimidyl protection of the ϵ-amino groups (DiMarchi et al., 1978b). The 3-sulfophenylthiohydantoin formed simplifies the subsequent purifications following coupling and cleavage by virture of its

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