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Interactions of Small Ligands with *Busycon canaliculatum* and *Limulus polyphemus* Hemocyanins as Studied by Ultraviolet Spectrophotometry and ^1H and ^{19}F Nuclear Magnetic Resonance†

Jau-Min Guo, Chi Chang, Norman C. Li, and Kenneth T. Douglas*

ABSTRACT: We have used UV (ultraviolet) spectrophotometric and ^1H and ^{19}F NMR (nuclear magnetic resonance) techniques to study the interaction of small ligands with *Busycon canaliculatum* and *Limulus polyphemus* hemocyanins. Addition of thioacetamide (up to 0.02 M) to oxyhemocyanin causes a relatively rapid drop in absorbance at 340 nm, a primary reaction, followed by a considerably slower secondary process. The primary reaction between thioacetamide and *Busycon* hemocyanin commonly takes 15–30 min. The ^{19}F NMR signal of 0.125 M KF is broadened by oxyhemocyanin from both *Limulus* and *Busycon* species, and the extent of broadening is linearly dependent on hemocyanin concentration (more marked dependence for *Limulus* than for *Busycon*). Glycine, a ligand which binds to oxyhemocyanin at the copper active site without expelling oxygen, also causes a decrease in the ^{19}F line width as a function of glycine concentration which is analyzed in terms of an apparent dissociation constant (0.15 M) for glycine–hemocyanin (*Limulus*) complex(es). Addition of thioacetamide (up to 0.1 M) to solutions of oxyhemocyanin containing 0.125 M KF causes a considerable decrease in the line width of the ^{19}F signal from its paramagnetically broadened value, again with a biphasic time dependence. Our results can be interpreted in terms of thioacetamide exerting an allosteric effect, inducing a conformational change in hemocyanin leading to an altered active-site configuration, incapable not only of retaining oxygen, but also incapable of binding fluoride ion as strongly. However, at this stage, reduction of paramagnetic Cu(II) to diamagnetic Cu(I) cannot be rigorously excluded as an alternative.

For over a century the nature and function of the copper in the respiratory proteins known as hemocyanins have puzzled researchers. These huge, oxygen-carrying molecules, with molecular weights in the range of 10^6 and 10^7, occur in arthropods and molluscs, not localized in blood cells, but freely dissolved in the hemolymph (van Holde & van Bruggen, 1971). The oxygenated protein is a striking, deep blue and considerable current effort is being directed toward defining the nature(s) of the copper atoms in oxyhemocyanins (Bannister & Wood, 1972; Lontie & Vanquickenborne, 1974; Freedman et al., 1976).

One approach has been the use of small ligands which bind to hemocyanin. Proton magnetic resonance studies have shown that, with glycine derivatives, there is selective, paramagnetic line broadening of the ligand signals by oxyhemocyanin (Ke et al., 1973), providing evidence that at least part of the active site is copper(II). Ligands of hemocyanin can be divided into two classes: (a) those whose binding does not lead to expulsion of oxygen from oxyhemocyanin (e.g., glycine (Ke et al., 1973)); (b) those whose binding is accompanied by conversion of the protein to deoxyhemocyanin (e.g., thiourea (Rombauts, 1968), thioacetamide (Lee et al., 1977)).

In this paper we report studies on members of each class of ligands as complementary probes of hemocyanin activity, using both arthropod (*Limulus polyphemus*) and molluscan (*Busycon canaliculatum*) species of hemocyanin. In particular we use glycine (NMR line broadening), thioacetamide (spectrophotometric analysis of ligand binding) and fluoride ion ($^{19}F^-$ NMR line broadening).

Fluoride ions are known to bind to *Helix pomatia* hemocyanin leading to the formation of methemocyanin (Witters & Lontie, 1975) as detected by measurement of the absorbance at 340 nm (a copper-oxygen absorption band of oxyhemocyanin): it is worth noting that, by these criteria, fluoride ion was

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inactive toward *Limulus* hemocyanin (Witters & Lontie, 1975). There are several good reasons for using fluoride ion as a NMR probe in this area. The sensitivity of ^{19}F NMR is about equal to that of ^{1}H and there is a large relaxation enhancement for a $^{19}F^-$ ion bound to a paramagnetic center. Finally, there is but a single signal from $^{19}F^-$, with no further spin coupling to complicate evaluation of results.

Materials and Methods

Potassium fluoride dihydrate ($KF{\cdot}2H_2O$) was obtained from Matheson Coleman and Bell. Trizma base was from Sigma. Thioacetamide (Fisher, reagent grade) was purified by recrystallization to sharp melting point. Hemocyanins were obtained, as the hemolymphs, from the Marine Biological Laboratory, Woods Hole, Mass., and further purified as follows (both *Busycon* and *Limulus*). Protein pellets, obtained by centrifugation of the hemolymphs (120 000*g* at 4 °C, Spinco Model L Ultracentrifuge), were dissolved in a small amount of water and dialyzed exhaustively against water or an appropriate buffer medium for 2 days at 4 °C. All operations were conducted at 4 °C to minimize protein denaturation. Concentrations of hemocyanin solutions were determined using UV spectrophotometry at 345 nm ($\epsilon_{1cm} = 10^4$ M^{-1} (Cu) cm^{-1}) and 280 nm ($\epsilon_{1cm}^{1\%} = 15.71$). Buffers were prepared using water which was distilled off glass and deionized.

UV-visible spectrophotometry was effected either using a Cary 14 Model, or, more often, a GCA-McPherson 707-K double beam spectrophotometer, both of which were thermostated to 25 °C, in the latter case by means of a Haake FJ model thermoregulator system. Correct temperature equilibration of the multiple cuvette compartment was readily ascertained on the McPherson spectrophotoeter as the instrument was equipped with a thermocouple for immersion in the cuvette. Small quantities of reagent (protein and/or ligand) could be added to the medium in the cuvette on the flattened tip of a Teflon stirring rod. Path length was 10 mm. All pH measurements were made on either a Fisher Accumet Model 320 expanded scale research pH meter or on a Radiometer PHM 64 model pH meter (0.001 pH readability), each standardized against the appropriate Fisher standard buffers. When necessary, pD was determined by adding 0.4 to the pH meter reading (Ke et al., 1973).

^{19}F NMR measurements were performed by means of a MPC-HF 250-MHz superconducting spectrometer, interfaced with a Sigma 5 computer and using the NMR correlation technique (Dadok & Sprecher, 1970). The spectrometer was operated in the linear frequency sweep mode with an external lock on trifluoroacetic acid (TFA)[1] at a frequency sweep of 235.2 MHz. ^{1}H NMR measurements were made on a Varian A-60 NMR spectrometer interfaced with a Varian C-1024 time-averaging computer, or alternatively on an EM 360 spectrometer (for whose use we are grateful to the School of Pharmacy). ^{1}H NMR measurements were performed at 60 MHz (ambient temperature $\simeq$ 31 °C) and care was taken to keep the radiofrequency 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).

Calculations and regression analyses were performed using a programmable Wang 720 C calculator.

Results

Binding of Thioacetamide. It has been reported that addition of thioacetamide to *Busycon* or *Limulus* hemocyanins causes a decrease in the absorption band around 340 nm (Lee et al., 1977), similar to that for thiourea (Rombauts, 1968). The loss of absorbance occurs in two stages; a more or less rapid primary reaction is followed by a much slower secondary reaction. This is shown schematically in Figure 1 in which ΔA_0 is the change in absorbance caused by the primary reaction after correction for the secondary reaction. This simple subtraction procedure is obviously only possible in those cases wherein the rate constants for the two reactions are very different. The primary reaction between thioacetamide and *Busycon* hemocyanin commonly takes 15–30 min.

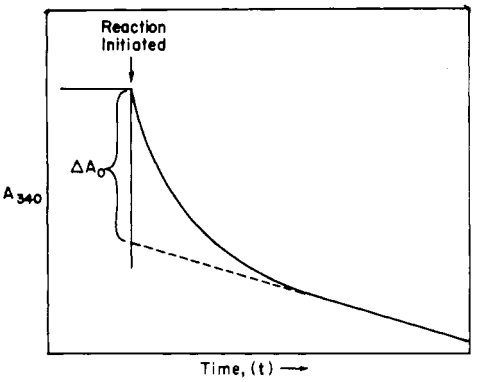


FIGURE 1: Schematic representation of the biphasic change in absorbance observed at 340 nm when thioacetamide is added to a buffered solution of oxyhemocyanin.

The dependence of ΔA_0 for *Busycon* oxyhemocyanin as a function of thioacetamide concentration has been shown to follow an equation of the form

$$\Delta A_0 = (\Delta A_0)_{max} \frac{L_0}{(L_0 + K_{app})} \quad (1)$$

where $(\Delta A_0)_{max}$ is the limiting value of ΔA_0 at high ligand concentrations (L_0) and K_{app} is an apparent dissociation constant of the ligand–protein complex(es) and is the concentration of ligand at which the protein is half saturated with ligand (Lee et al., 1977). Equation 1 is readily linearized to eq 2, and, thus, a plot of $L_0/\Delta A_0$ vs. L_0 yields $(\Delta A_0)_{max}$ and K_{app}.

$$\frac{L_0}{\Delta A_0} = \frac{1}{(\Delta A_0)_{max}} K_{app} + \frac{1}{(\Delta A_0)_{max}} L_0 \quad (2)$$

Such a procedure is analogous to a Hanes linearization of the Michaelis–Menten equation in enzyme kinetics (Wilkinson, 1964; Johansen & Lumry, 1961). Good linear plots were obtained (correlation coefficients > 0.994). At pH 9.98, the apparent dissociation constant, K_{app}, describing the primary reaction between thioacetamide and *Busycon* hemocyanin at 25 °C and ionic strength 0.1, is 5.29 mM, determined at 340 nm. The magnitude of K_{app} is a function of pH and depends upon an ionization of $pK_a' = 7.75$ as shown in Figure 2. In view of the report of the pH-dependent oxygenation of *Jasus lalandii* hemocyanin (Moore et al., 1968), one should note that for *Busycon* hemocyanin, as used in the present study, the A_{345}/A_{280} ratio has been shown constant over the pH range 5–11 (Ke et al., 1973). The ligand binds to the protein more strongly in basic than in acidic media. Addition of glycine to solutions of thioacetamide and *Busycon* hemocyanin shows no "protective" effect on the copper–oxygen band; the absorbance at 340 nm is destroyed to the same extent by thioacetamide in the presence and absence of glycine (even up to 0.6 M); see Table I. Therefore, these absorbance data do not indicate "competition" between glycine and thioacetamide.

[1] Abbreviation used: TFA, trifluoroacetic acid.

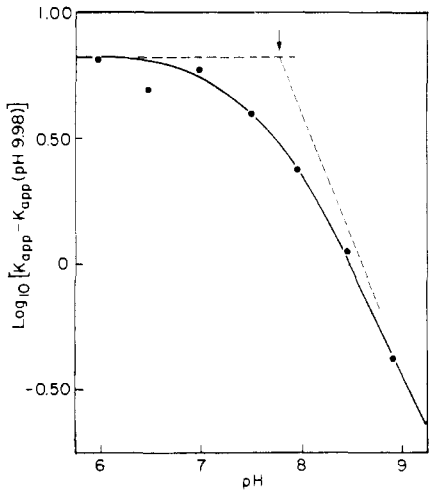


FIGURE 2: Log-log plot showing the dependence on pH of K_{app} for the primary reaction of thioacetamide with *Busycon* hemocyanin. Points are experimental and line is theoretical for an ionization of $pK_a' = 7.75$. Buffers above pH 8 were borate, below pH 8 were phosphate. The pH measured in the presence and absence of thioacetamide did not differ significantly.

^{19}F NMR Studies Using Potassium Fluoride. The ^{19}F NMR line of fluoride ion is broadened by oxyhemocyanins from both *Limulus* and *Busycon* species. The extent of line-broadening is linearly dependent on the hemocyanin concentration for both species and is some 50% greater for *Limulus* than *Busycon* hemocyanin at a given concentration (% w/v) of hemocyanin. At pH 8.60 (0.05 M Tris) the relationships between line width (NMR frequencies 39.0 ± 0.1 ppm upfield from TFA) and hemocyanin concentration are: $\Delta\nu_{1/2}$ (Hz) = α(% w/v, hemocyanin) + 1.0, where α = 2.42 for *Busycon* and 3.80 for *Limulus* hemocyanin. We have not varied fluoride ion concentration because of complications from the equilibrium, $KF_2^- \rightleftarrows KF + F^-$. The line-broadening effect is more marked for *Limulus* than *Busycon* hemocyanin. The presence of *Busycon* or *Limulus* apohemocyanin results in considerably less broadening of the fluoride ion resonance. In 0.125 M potassium fluoride solution, the ^{19}F NMR line width for *Busycon* oxyhemocyanin (2.8%) would be 7.7 Hz (see above), whereas under these conditions the observed line width for *Busycon* apohemocyanin (copper removed) is 2.1 Hz; the natural line width of free 0.125 M KF is 1.0 Hz (Figure 3a). The effects are more dramatic for 8.3% *Limulus* oxy- and apohemocyanins (line widths 32 and 4 Hz, respectively; Figure 3b).

It has been reported that both oxy- and apohemocyanins from *Octopus vulgaris* have similar structures, differing only in the oxygen-binding site (Salvato et al., 1974). Thus, the excess broadening of oxy- over apohemocyanin is most likely caused by interaction between the fluoride ion and the paramagnetic Cu(II) at the oxyhemocyanin active site. Broadening of the ^{19}F signal caused by a paramagnetic metal has previously been reported (Navon et al., 1970) for solutions of CPA(Mn) and F^-, where CPA(Mn) is carboxypeptidase A in which the single zinc ion is replaced by the paramagnetic Mn. By determining the line width of ^{19}F signal at different temperatures and frequencies along with T_1 data, they determined that the mechanism of ^{19}F relaxation caused by CPA (Mn) is predominantly chemical exchange rather than dipolar interaction. The broadening caused by apohemocyanin (4 Hz for 8.3% *Limulus*) probably results from F^- binding to the gross structure of the huge, slow-tumbling hemocyanin molecule. It is known that the other halide ions bind to *Helix pomatia* oxyhemocyanin (causing slight oxygen expulsion)

TABLE I: Thioacetamide–Glycine Competition (pH 8.91) with *Busycon* Hemocyanin.

(i) Spectrophotometric Studies[a]

[Thioacetamide] (M)	[Glycine] (M)	ΔA_0[b]
0.01	0	0.165
0.01	0.60	0.160
0.003	0	0.051
0.003	0.02	0.054

(ii) NMR Studies[c]

[Thioacetamide] (M)	[HCO_2] (M)	$\Delta\nu_{1/2}$(Gly)[d] (Hz)	$\Delta\nu_{1/2}$(TA)[e] (Hz)
	−	0.82	
	+	7.50	
0.18	+	0.78[f]	0.80[g]
0.18	+		0.79[h]
0.18	−		0.80

[a] As measured at 340 nm. Glycine was added to hemocyanin in borate buffer and then thioacetamide added. [b] The initial absorbance at 340 nm of the hemocyanin in 3.0 mL of buffer was 0.56 for all of these determinations. [c] 2.59% *Busycon* hemocyanin; glycine concentration was 0.24 M throughout. [d] $\Delta\nu_{1/2}$ is for glycine $-CH_2-$ signal. [e] $\Delta\nu_{1/2}$ is for thioacetamide (TA) $-CH_3$ signal. [f] $\Delta\nu_{1/2}$ measured 53 min after addition of thioacetamide to glycine–hemocyanin solution. [g] $\Delta\nu_{1/2}$ measured 3.5 h after addition of thioacetamide to glycine–hemocyanin solution. [h] $\Delta\nu_{1/2}$ measured 7 min after addition of glycine to thioacetamide–hemocyanin solution.

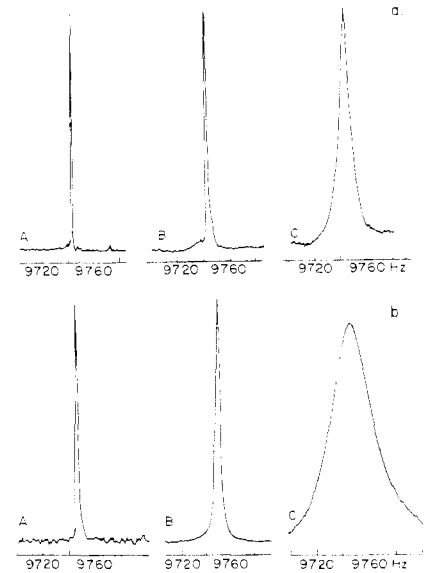


FIGURE 3: (a) $^{19}F^-$ NMR spectra of 0.125 M KF in pH 8.60, 0.05 M Tris buffer. Signals are upfield from TFA. (A) No additive, $\Delta\nu_{1/2}$ = 1.0 Hz. (B) With 2.8% *Busycon* apohemocyanin, $\Delta\nu_{1/2}$ = 2.1 Hz. (C) With 3.8% *Busycon* oxyhemocyanin, $\Delta\nu_{1/2}$ = 10.6 Hz. (b) ^{19}F NMR spectra of 0.125 M KF in pH 8.60, 0.05 M Tris buffer. Signals are upfield from TFA. (A) No additive, $\Delta\nu_{1/2}$ = 1.7 Hz. (B) With 8.3% *Limulus* apohemocyanin, $\Delta\nu_{1/2}$ = 4 Hz. (C) With 10% *Limulus* oxyhemocyanin, $\Delta\nu_{1/2}$ = 38.7 Hz.

(Witters and Lontie, 1975) so that such line broadening is indeed to be expected.

Ligands of hemocyanin can be classified into two types, those which bind with concomitant expulsion of oxygen (e.g., thiourea (Rombauts, 1968), thioacetamide (Lee et al., 1977)) and those which, on binding to the protein do not drive out oxygen (e.g., glycine derivatives (Ke et al., 1973)) as measured by the A_{340}. Fluoride ion (12 mM with 0.1% *Limulus* oxyhemocyanin; 10 mM with 0.2% *Busycon* hemocyanin) caused

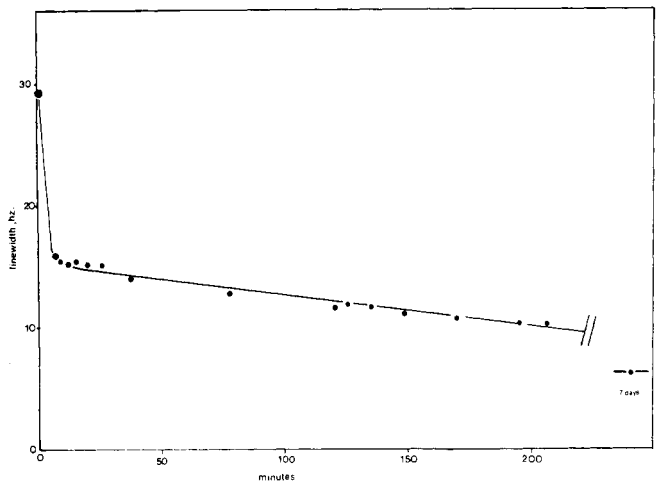


FIGURE 4: Time dependence of the ^{19}F line width of 0.125 M KF and 7.5% *Limulus* oxyhemocyanin at pH 8.60 in the presence of 24 mM thioacetamide.

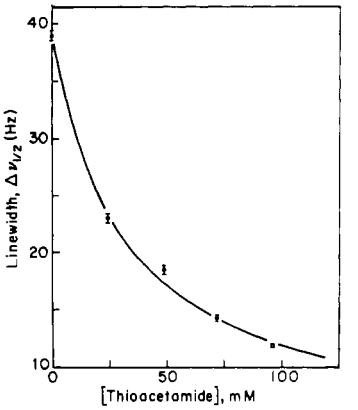


FIGURE 5: $^{19}F^-$ NMR line widths of 0.125 M KF–10% *Limulus* oxyhemocyanin solution at pH 8.60 in the presence of different concentrations of thioacetamide. Data were obtained within 20 min of mixing and NMR frequencies were 38.7 ± 0.1 ppm upfield from TFA. Points are experimental; line is theoretical for an apparent dissociation constant, K_{app}, of 32.8 mM, $\delta(\Delta\nu_{1/2})_{max}$ 35.9 Hz obtained by treatment analogous to eq 1.

no significant spectral change at 340 nm in pH 8.60 Tris buffer (0.05 M) over the first hour. In the light of the ^{19}F NMR line broadening of KF caused by hemocyanin, we must put fluoride ion in the latter category.

Effects of Thioacetamide on ^{19}F NMR. In the reaction of thioacetamide with hemocyanin, followed by the decrease in absorbance at 340 nm, two stages are readily seen (Lee et al., 1977). A relatively fast, primary reaction is followed by a much slower secondary reaction. In Figure 4 we have plotted the line width of the ^{19}F NMR signal of 0.125 M KF as a function of time in the presence of thioacetamide for *Limulus* oxyhemocyanin. There is a progressive sharpening of the line as time passes. The line width drops sharply from the value in the absence of thioacetamide and then slowly changes over a period of several hours—again a biphasic dependence. The line width becomes approximately constant within 10–20 min of mixing (the change over the first measurable 10–20 min, after the initial rapid drop, is only some 3%). A plot of the line widths after 20 min of 0.125 M potassium fluoride–10% *Limulus* oxyhemocyanin solutions in the presence of different concentrations of thioacetamide at pH 8.60 is shown in Figure 5. There is an apparent leveling effect, similar to the saturation shown in the change in absorbance at 340 nm caused by the primary reaction of *Busycon* hemocyanin with thioacetamide (see Figure 1). If $\delta(\Delta\nu_{1/2})$ is the change in line width caused by a given thioacetamide concentration, one can obtain an apparent dissociation constant (K_{app}) to describe thioacetamide binding to *Limulus* hemocyanin in the presence of fluoride ion by plotting, for example [thioacetamide]/$[\delta(\Delta\nu_{1/2})]$ vs. [thioacetamide]. The value of K_{app} (pH 8.60) is 3.28 mM $[\delta(\Delta\nu_{1/2})]_{max}$. At a similar pH, in the absence of potassium fluoride, thioacetamide binds to *Busycon* hemocyanin with an apparent dissociation constant of ~6 mM (see Figure 2). One cannot compare directly these apparent dissociation constants for different species of hemocyanin.

Effects of Glycine and Fluoride Ion. Glycine is a member of the class of ligands which binds to oxyhemocyanin at the copper active site without expelling oxygen (Ke et al., 1973). Thus, although the A_{340} values of hemocyanin solutions are not affected by the presence of glycine or derivatives, there is a (selective) broadening of the glycyl $-CH_2-$ protons in the presence of oxyhemocyanin (Ke et al., 1973). The addition of glycine also results in a decrease in the line width of the ^{19}F NMR signal of 0.125 M KF in the presence of *Limulus* hemocyanin in pH 8.60 Tris buffer (Figure 6). This decrease

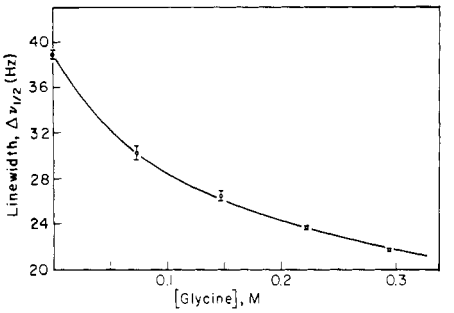


FIGURE 6: $^{19}F^-$ NMR line widths of 0.125 M KF solutions in the presence of 10% *Limulus* oxyhemocyanin at pH 8.6 (0.05 M Tris buffer) at various concentrations of glycine. NMR frequencies were 38.7 ppm upfield from TFA. Points are experimental; line is theoretical for an apparent dissociation constant, K_{app}, of 0.15 M, $\delta(\Delta\nu_{1/2})_{max}$ 26.0 Hz.

in line width as a function of glycine concentration has been analyzed (as previously) in terms of an apparent dissociation constant with a value of 0.15 M, $\delta(\Delta\nu_{1/2})_{max}$ 26.0 Hz.

In contrast to the above results demonstrating the effect of added glycine on ^{19}F line width, addition of 0.077 M potassium fluoride to 5.7% *Limulus* oxyhemocyanin and 0.33 M glycine solution (pH 8.60) decreased the glycine $-CH_2-$ ^1H NMR line width from 2.9 Hz to 2.2 Hz possibly indicating a competitive effect but the significance of this remains to be evaluated.

Discussion

The interactions of thioacetamide (and other nitrogen-sulfur ligands) are characterized by a relatively rapid drop in A_{340}, a primary reaction, followed by a considerably slower secondary process. The observed primary reaction between thioacetamide and *Busycon* hemocyanin commonly took 15–30 min depending on concentrations. Although the primary reaction was reversible by dialysis, the secondary reaction was not fully reversible, in terms of regeneration of the 340-nm band.

An equation of the correct mathematical form to explain the dependence on ligand concentration of the absorbance change corresponding to the primary reaction for thioacetamide is readily derived based simply on a 1:1 binding process with concomitant oxygen displacement (eq 3).

$$HcO_2 + L \underset{K}{\rightleftharpoons} HcL + O_2 \qquad (3)$$

Such a situation is likely to be an oversimplification for a number of reasons, not the least of which is that the rate of ligand exchange on Cu(II) is unlikely to be as slow at pH 8-9 as is observed in the primary reaction with thioacetamide. Further, the observation of oxygen expulsion by thioacetamide binding need not indicate that this molecule binds at the active site: an allosteric process is possible. It is interesting also in this case that there is no broadening of the CH_3-^1H NMR signal of thioacetamide when *Busycon* oxyhemocyanin is added (Table I, ii).

Three reasons have been offered for this (Lee et al., 1977), viz., reduction of paramagnetic Cu(II) to diamagnetic Cu(I) by thioacetamide or, sulfur being a weakly binding group so that the chelate is not strong enough for detectable broadening or, an allosteric (noncopper site) binding of thioacetamide.

The results in Table I indicate that the ΔA_0 value for the thioacetamide reaction with *Busycon* hemocyanin is unaffected even by the presence of a 60-fold molar excess of glycine, whereas addition of thioacetamide to a mixture of glycine and hemocyanin causes (instantaneous) line sharpening of the $-CH_2-$ NMR signal to the value for unbound glycine (a tenfold change in $\Delta\nu_{1/2}$). Such a dominance of thioacetamide could be caused by a very much lower dissociation constant for the initially formed thioacetamide–oxyhemocyanin complex than for the glycine–oxyhemocyanin complex. However, K_{app} for glycine with *Busycon* hemocyanin is 6 mM, calculated from literature data (Ke et al., 1973) at pD 9.4. If we consider glycine and thioacetamide to bind, mutually exclusively, to the same site on hemocyanin with dissociation constants K_1 and K_2, respectively, the situation is closely analogous to that of competitive inhibition in enzymology, leading to an equation of the form of eq 4 describing ΔA_0 in the presence of glycine.

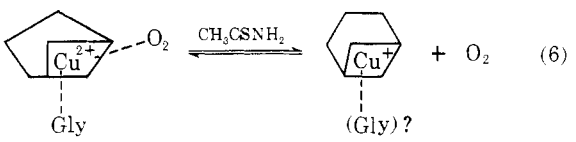
$$\Delta A_0 = \frac{(\Delta A_0)_{max}(TA)}{K_2\left[1 + \frac{(G)}{K_1}\right] + (TA)} \quad (4)$$

Thus, the ratio of the values of A_0 in the presence and absence of glycine is

$$r = \frac{(\Delta A_0)_{+G}}{(\Delta A_0)_{-G}} = \frac{K_2 + (TA)}{\left[(K_2\left[1 + \frac{(G)}{K}\right] + (TA)\right]} \quad (5)$$

Equation 5 has a value of $\simeq 0.025$ under the conditions described in Table I (0.6 M glycine, 0.01 M thioacetamide). In other words, the measured ΔA_0 for thioacetamide would have been reduced by 97.5% and thioacetamide and glycine are unlikely to be competing simply for the same site.

A second explanation of the observed results is a thioacetamide-induced conformational change of oxyhemocyanin to a form favoring the deoxy active site, resulting in expulsion of oxygen (eq 6).



(6)

If, in the presence of thioacetamide, this equilibrium is forced to the right-hand side, one can easily explain the observed primary reaction, its reversibility on dialysis and the dominance of thioacetamide over glycine. The reversibility of the primary reaction on dialysis is more readily explained by this allosteric approach than by reduction of Cu(II) to Cu(I) on sulfur-ligand binding, although this latter option cannot yet be ruled out.

The line-broadening of $^{19}F^-$ shown in Figure 3 for *Busycon* (Figure 3a) and *Limulus* (Figure 3b) hemocyanins is a dramatic demonstration that fluoride ion does indeed bind to both molluscan and arthropod hemocyanins. Moreover, this binding is specific and active-site directed as considerably less broadening is observed for $^{19}F^-$ in the presence of apohemocyanin. Witters & Lontie (1975) have reported that fluoride ion accelerates the rate of formation of *Helix pomatia* methemocyanin, determined from the absorbance at 340 nm. However, they detected no such effect with *Limulus* hemocyanin leading them to the conclusion that the active sites of arthropodan and molluscan hemocyanins differ. It is apparent from our observations of paramagnetic broadening that *both* arthropodan and molluscan hemocyanins bind fluoride ion at their copper sites. When thioacetamide is added, fluoride ion is also expelled from the active sites (Figure 4), the extent of ejection being a function of time: there appears to be a rapid decrease in linewidth followed by a much slower decrease over several hours, a situation reminiscent of the primary and secondary reactions of *Limulus* hemocyanin as studied spectrophotometrically (Lee et al., 1977). Again these results are explained readily by a thioacetamide-induced conformational change in hemocyanin leading to an altered active-site configuration, incapable not only of retaining oxygen (primary reaction in the A_{340} experiments) but also incapable of binding fluoride ion as strongly (initial rapid decrease in fluoride ion line width). The secondary reaction with thioacetamide, whatever its nature, leads to further removal of oxygen or fluoride ion, as the case may be, from the active site.

The secondary reactions of the various ligands evidently involve more extensive architectural changes in the protein as they cannot be fully reversed by dialysis. Observations of secondary reactions similar to those reported here for thioacetamide have been published for thiourea as a ligand. Rombauts (1968) suggested that the secondary reaction of thiourea may involve an oxidative action of thiourea toward disulfide linkages in the neighborhood of the copper active sites. There is indeed biochemical and chemical precedent in the literature for such a view (Walker & Walker, 1960). An alternative explanation of the secondary reactions of thiourea and thioacetamide might be nucleophilic attack by the nitrogen–sulfur ligand on disulfide linkages, leading to stabilization of a conformation favoring the deoxy form of the active-site. Thiourea is well known as a sulfur nucleophile in synthetic organic chemistry and, indeed, it is commonly used in the preparation of mercaptans (Reid, 1958).

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Heterogeneity in the 3′-Terminal Sequence of Ribosomal 5S RNA Synthesized by Isolated HeLa Cell Nuclei in Vitro†

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ABSTRACT: Isolated HeLa cell nuclei synthesize ribosomal 5S RNA of very nearly correct sequence and size. The in vitro product was resolved according to size on formamide-containing polyacrylamide gels and the fractions were subsequently hybridized to recombinant DNA containing the 5S genes from *Xenopus mulleri.* It could be shown that the 5S RNA synthesized in vitro differed only very slightly in size from the mature species labeled in vivo and contained a few extra nucleotides in some of the molecules. Analysis of the 3′-terminal base of molecules synthesized independently with four different nucleotides showed that the chains were almost exclusively terminated with uridine. Digestion of the in vitro product with ribonuclease T_1 and analysis of the oligonucleotides on DEAE-Sephadex A-25 in the presence of 7 M urea revealed that the molar yield of the internal fragments agreed well with the expected theoretical values. The 3′-terminal fragments, however, were found to be present in three different species with the sequences CUU_{OH}, $CUUU_{OH}$, $CUUUU_{OH}$ which occurred with a frequency of about 60%, 20%, and 20%, respectively. From these data we conclude that 5S RNA synthesis in isolated HeLa cell nuclei was correctly initiated but that termination occurred with a slight ambiguity, adding either one or two uridine residues to some of the chains.

To analyze regulatory mechanisms of transcription, it is necessary to establish in vitro systems in which a given RNA product is synthesized with acceptable fidelity in primary sequence and size. Conventional sequencing and/or hybridization techniques can be used for these studies provided the exact size of the initial product (precursor molecule) is known for a given RNA species. In a previous report (Yamamoto and Seifart, 1977) we have shown that isolated HeLa cell nuclei provide a suitable system to study the transcription of ribosomal 5S RNA. Although the system itself is crude, it has been shown that 5S RNA is transcribed with high fidelity both in sequence and size.

Analysis of 5S rRNA synthesized in vivo has provided evidence that most of these molecules have retained the β or even γ-phosphate (Denis and Wegnez, 1973), indicating that the 5′ terminus of the mature molecule probably represents that of the initial transcription product. On the contrary, the 3′ end is heterogeneous both in sequence and in length (Denis and Wegnez, 1973; Forget and Weissmann, 1967), indicating that the 3′ terminus of the mature RNA is different from the primary transcript. It is not known, however, whether this end is generated by trimming of larger precursor molecules, or by the addition of nucleotides. Precursor molecules of 5S RNA were initially shown to occur in cells of *E. coli* (Freunteun et al., 1972) and bacilli (Pace et al., 1973; Stoof et al., 1974). The existence of precursor molecules to 5S rRNA has not definitively been shown for eukaryotic cells, although suggestive evidence to this extent has been put forward by Denis and Wegnez (1973) and Rubin and Hogness (1975) for *Xenopus* oocytes and cultured *Drosophila* cells, respectively. In both cases the molecules contained approximately 15 additional nucleotides at the 3′ end. Especially in *Drosophila* cells such molecules were accumulated following the exposure of the cells to higher than physiological temperatures, presumably due to a temperature-sensitive processing step (Rubin and Hogness, 1975). We have investigated this question by analyzing the 3′-terminal sequence of 5S rRNA synthesized by isolated nuclei from HeLa cells.

Materials and Methods

Isolation of Nuclei and Synthesis of RNA. HeLa cells were grown in suspension culture and nuclei were isolated by the procedure described previously. They were suspended as described (Yamamoto and Seifart, 1977) at a concentration of about 1×10^8/mL and subsequently incubated at 25 °C for 20 min in a volume of 120 μL containing: 0.5 μg of α-amanitin/mL, 50 mM NH_4Cl, 5 mM $MgCl_2$, 12.5% glycerol, 25 mM Tris-HCl, pH 7.9, 2.5 mM dithiothreitol, 0.1 mM EDTA, 1 mM each of ATP, GTP, CTP, 0.044 mM and 100 μCi of [^{3}H]UTP (Radiochemical Centre, Amersham). The reaction was terminated by the addition of 10 μL of a solution of *E. coli*

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