Chemistry of Anthocyanin Pigments. 6.[1] Kinetic and Thermodynamic Study of Hydrogen Sulfite Addition to Cyanin. Formation of a Highly Stable Meisenheimer-Type Adduct Derived from a 2-Phenylbenzopyrylium Salt

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Abstract: In acidic aqueous solution, the addition of the HSO_3^- anion to the AH^+ cation of flavylium or 2-phenylbenzopyrylium salts results in a Meisenheimer-type adduct S, where $K_S = [S]/[AH^+][HSO_3^-] = k_S/k_{-S}$ (eq 6). The kinetic and equilibrium constants of this reaction, measured by the concentration-jump method (either with pH jumps at constant SO_2 concentration or with SO_2 jumps at constant pH), are reported for the first time: for cyanin at 25 °C and 0.2 M ionic strength, $k_S = (4.20 \pm 0.05)10^2\ M^{-1}\ s^{-1}$, $k_{-S} = (3.85 \pm 0.05)10^{-3}\ s^{-1}$, and $K_S = (1.10 \pm 0.05)10^5\ M^{-1}$. The large equilibrium constant value clearly indicates the high stability of the colorless adduct S formed. It is also directly related to the degree of decolorization of foodstuffs and beverages of vegetable origin when treated with sulfur dioxide: the larger K_S becomes, the greater the loss of pigmentation. No evidence is found for the existence of a reaction between the chalcone form of cyanin and sulfur dioxide at any pH. Near neutrality, it seems possible that addition of the sulfite anion SO_3^{2-} to the flavylium cation occurs.

Anthocyanins are derivatives of 2-phenylbenzopyrylium (flavylium) salts and are largely responsible for the colors of flowers, fruits, fruit juices, wines, etc.[2] Sulfur dioxide is commonly used as a preservative in the processing and storage of food of vegetable origin; however, its use generally causes an undesired loss of pigmentation.[3] It is the purpose of the present paper to demonstrate, by means of kinetic and thermodynamic studies, that this loss of pigmentation is related to the nucleophilic addition of hydrogen sulfite to the flavylium anthocyanin cation resulting in the formation of a highly stable Meisenheimer-type adduct.[4] The kinetic and equilibrium constants associated with this reaction are measured and reported for the first time.[5] Cyanin chloride was chosen for study because it is one of the commonest anthocyanins.

Experimental Section

The purity of commercial cyanin chloride (Roth purum) was checked both by cellulose (Merck) and polyamide (Merck) plate chromatography in butanol/acetic acid/water mixtures and by measurement of the molecular extinction coefficient of the flavylium cation at its absorption maximum in the visible (ϵ_{AH^+} (508.5 nm) $3.5 \times 10^4\ M^{-1}\ cm^{-1}$). Similar values have been recently obtained in the case of synthetic flavylium cations.[6] The spectral characteristics of the pigment in methanolic 0.01% HCl and the bathochromic shift observed after addition of aluminum chloride agree with previously reported values.[7]

Kinetic runs were performed at 25 °C in aqueous solutions with an ionic strength adjusted to 0.2 M by the addition of KNO_3 (Merck suprapur).

Stock Solutions. The pigment stock solutions were prepared according to the following procedure. The required amount of cyanin chloride was dissolved in distilled water containing KNO_3. After complete dissolution, a few microliters of a concentrated hydrochloric acid solution (Merck suprapur) were added so as to obtain a pH value around 2 or 3. These solutions were maintained in a thermostated bath (25 °C) for several hours in order to reach equilibrium between the different cyanin species existing in this acidity range. The analytical pigment concentrations ranged from 2 to 9×10^{-5} M.

The SO_2 stock solutions were prepared in distilled water using Na_2SO_3 or $Na_2S_2O_5$ (Merck "pro analysi"). Their analytical concentrations were below 10^{-3} M in order to avoid formation of $S_2O_5^{2-}$ anions.[8]

Kinetic Measurements. pH and SO_2-jump experiments were performed using a classical UV–visible spectrophotometer (Cary 16 or Cary 118) whose thermostated sample cell was fitted with a fast stirring device. Both pH and SO_2 jumps must be carried out on completely equilibrated cyanin chloride solutions containing a known amount of SO_2. Therefore, to an aliquot (2.5 mL) of a pigment stock solution a weighted microquantity of a SO_2 stock solution is added, and the pH or SO_2 jumps are performed as soon as the solution has again reached equilibrium. During the pH and SO_2 jumps no new species are generated; their equilibria are only displaced. These conditions are very similar to chemical relaxation conditions but they differ from usual stopped-flow conditions where, in general, no reaction product exists before the X jump.

The molecular extinction coefficients of AH^+ and the neutral σ adduct S differ appreciably only in the visible range and S does not absorb in the visible. For this reason, the relaxation curve due to the formation of the σ adduct S can only be monitored in the visible, close to the absorption maximum of the flavylium structure (508.5 nm). In each kinetic run the pigment concentration remains constant.

SO_2-Jump Experiments at Constant pH. To an equilibrated pigment solution containing a known amount of SO_2 a weighted microquantity of a SO_2 stock solution is added through a microsyringe. In order to avoid any appreciable pH jump the pigment solution and the SO_2 stock solution were previously adjusted to the same pH value. Before the SO_2-jump experiments the analytical SO_2 concentrations ranged from 2 to 5×10^{-5} M. After the SO_2-jump experiments they ranged from 3 to 6×10^{-5} M.

pH-Jump Experiments at Constant SO_2 Concentration. To an equilibrated pigment solution containing a known amount of SO_2, a few microliters of a concentrated basic solution (NaOH Merck titrisol) are added. Before the jump the pH is between 1.85 and 3.2. The magnitude of the pH jump varies from 0.1 to 0.6. The analytical SO_2 concentrations ranged from 2 to 10×10^{-5} M.

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(4) The chemistry of Meisenheimer adducts derived from nitrobenzenes is now well known (Crampton, M. R. *Adv. Phys. Org. Chem.* **1969**, *7*, 211. Strauss, M. J. *Chem. Rev.* **1970**, *70*, 667. Bernasconi, C. F. *MTP Int. Rev. Sci.: Org. Chem., Ser. One* **1973**, *3*, 33). In contrast, very little work has been done on a pyrylium substrate which, however, is one of the fundamental heteroaromatic systems (Bersani, S.; Doddi, G.; Fornarini, S.; Stegel, F. *J. Org. Chem.* **1978**, *43*, 4112. Doddi, G.; Fornarini, S.; Illuminati, G.; Stegel, F. *Ibid.* **1979**, *44*, 4496).
(5) Timberlake and Bridle (Timberlake, C. F.; Bridle, P. *J. Sci. Food Agric.* **1967**, *18*, 479) have reported values for the $[S]/[AH^+]([SO_2] + [HSO_3^-])$ ratio for numerous flavylium salts. Unfortunately, since this ratio is pH dependent, no direct comparison with our measurements is possible.
(6) Sweeny, J. G.; Iacobucci, G. A. *Tetrahedron* **1977**, *33*, 2927.
(7) Geissman, T. A.; Jurd, L. *Arch. Biochem. Biophys.* **1955**, *56*, 259. Harborne, J. B. *Biochem. J.* **1958**, *70*, 22.
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pH Measurements. The pH was measured directly in the sample cell before and after the concentration-jump experiment, using a pH meter (Knick) fitted with a combined glass electrode (Metrohm EA 125). Buffers used for standardization were pH 7.00 and 4.00 NBS standards (Beckman) and 0.01 N hydrochloric acid (Merck titrisol).

Results and Discussion

Mechanistic Considerations. Scheme I represents the structural

Scheme I

$$AH^+ \rightleftarrows A + H^+ \quad \text{proton transfer reaction} \quad (1)$$

$$AH^+ + H_2O \rightleftarrows B + H^+ \quad \text{hydration reaction} \quad (2)$$

$$B \rightleftarrows C \quad \text{tautomerism} \quad (3)$$

transformations of anthocyanins in aqueous acidic media.[9]

In dilute aqueous solutions ($<10^{-3}$ M), there are three forms of sulfur dioxide in equilibrium according to Scheme II.[8]

Scheme II

$$SO_2 + H_2O \rightleftarrows HSO_3^- + H^+ \quad K_1 = 1.6 \times 10^{-2} \text{ M (25 °C)}^{10} \quad (4)$$

$$HSO_3^- \rightleftarrows SO_3^{2-} + H^+ \quad K_2 = 10^{-7} \text{ M (25 °C)}^{10} \quad (5)$$

Of the four cyanin structures existing in aqueous acidic solutions (Figure 1), only AH^+ and C may react with sulfur dioxide; AH^+ has two nucleophilic addition sites (positions 2 and 4) and C bears a carbonyl function. For natural anthocyanins the quinonoid base A and the chalcone C generally do not exist below pH 4.[11] Moreover, we previously demonstrated that after 1 h C does not react with sulfur dioxide, regardless of pH.[12] Therefore, for an acidic aqueous solution ($1 < pH < 4$) containing an anthocyanin and sulfur dioxide, we postulate the following mechanism (Scheme III).

Scheme III

$$SO_2 + H_2O \rightleftarrows HSO_3^- + H^+ \quad K_1 = [HSO_3^-][H^+]/[SO_2] \quad (4)$$

$$AH^+ + H_2O \rightleftarrows B + H^+ \quad K_h = [B][H^+]/[AH^+] \quad (2)$$

$$AH^+ + HSO_3^- \underset{k_{-S}}{\overset{k_S}{\rightleftarrows}} S \quad K_S = [S]/[AH^+][HSO_3^-] \quad (6)$$

Relaxation Kinetics. When an aqueous acidic solution of cyanin containing sulfur dioxide is subjected to a suitable *pH jump*, and when the wavelength is set to the absorption maximum of AH^+ (for cyanin, 508.5 nm), two relaxation processes are observed (Figure 2). The fastest relaxation is due to the covalent addition of water to the flavylium cation (eq 2).[9] The slow relaxation is ascribed to the equilibration of the hydrogen sulfite addition to the same cation (eq 6). Indirect perturbation of this pH-independent equilibrium is made possible by the changes in the concentrations of species HSO_3^- and AH^+ brought about by the shifts of equilibria 4 and 2, respectively. Also, since no species involved in equilibrium 4 absorbs in the visible range, the spectroscopic amplitude for this fast diffusion-controlled[13] reaction is zero. Now, if the same solution is subjected to *a SO_2 jump, at constant pH*, only the slow relaxation is observed (Figure 2). We explain this in the following manner: since equilibration of reaction 2 is much faster than equilibration of reaction 6, and since reaction 2 does not involve any sulfur dioxide species, reaction 2 is always in a *state of equilibrium* and under these conditions its relaxation amplitude is zero.

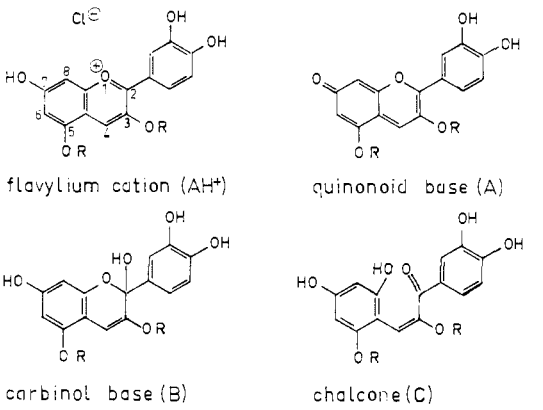

Figure 1. The different structures of cyanin existing in aqueous acidic solutions (R = glucose).

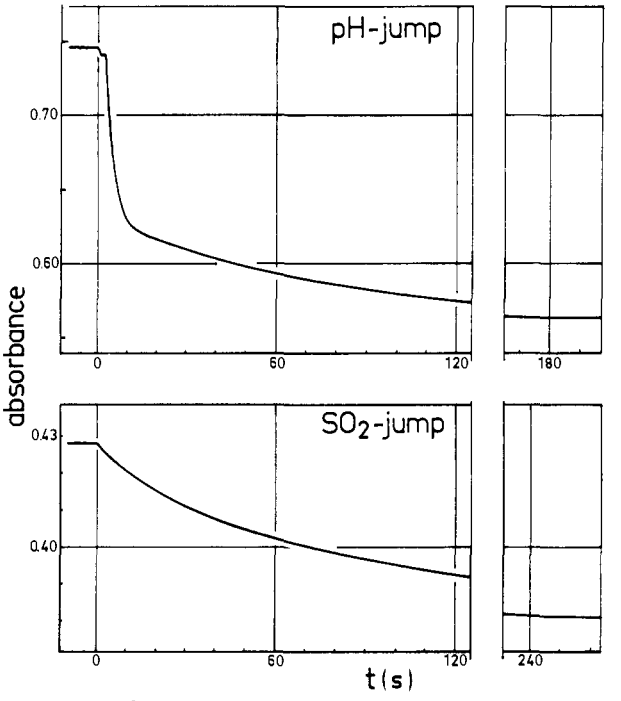

Figure 2. pH-jump: absorbance change at 508.5 nm of a cyanin solution containing sulfur dioxide at 25 °C and pH 2.02 when subjected to a rapid pH jump (final pH 2.19). The fast phenomenon is related to the flavylium–carbinol transformation. The slow decrease of the absorbance is related to the reversible addition of hydrogen sulfite to the flavylium cation. $C_0 = 6.7 \times 10^{-5}$ M; $C_0' = 7.4 \times 10^{-5}$ M; $l = 1$ cm; $\tau_S^{-1} = 1.48 \times 10^{-2}$ s^{-1}. SO_2-jump: absorbance change at 508.5 nm of a cyanin solution containing sulfur dioxide at 25 °C and pH 2.63 when subjected to a rapid SO_2 jump at constant pH (initial concentration in sulfur dioxide is 2.6×10^{-5} M; $C_0' = 3.9 \times 10^{-5}$ M). The decrease in absorption is due to the reversible formation of the σ adduct S. $C_0 = 6.3 \times 10^{-5}$ M; $l = 1$ cm; $\tau_S^{-1} = 1.03 \times 10^{-2}$ s^{-1} (C_0 and C_0' are the analytical concentrations of cyanin chloride and sulfur dioxide, respectively).

The reciprocal of the relaxation time τ_S for equilibrium 6 has been calculated both by the matrix method[14] and by the substitution method,[15] on the assumption that $[H^+] \gg [HSO_3^-]$, [B]:

$$\tau_S^{-1} = k_{-S} + k_S\left\{\frac{[HSO_3^-]}{1 + K_h/[H^+]} + \frac{[AH^+]}{1 + [H^+]/K_1}\right\} \quad (7)$$

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Concentrations refer to equilibrium concentrations. Since $[HSO_3^-]$ cannot be measured directly, it is convenient to rewrite eq 7 in the form

$$\tau_S^{-1} = k_{-S} + k_S\{a(C_0' - C_0) + b[AH^+]\} \quad (8)$$

where

$$C_0 = [AH^+] + [B] + [S] \qquad C_0' = [SO_2] + [HSO_3^-] + [S]$$

$$a = \frac{[H^+]K_1}{(K_1 + [H^+])(K_h + [H^+])}$$

$$b = \frac{2K_1}{K_1 + [H^+]}$$

$K_h = (1.1 \pm 0.1)10^{-2}$ M for cyanin at 25 °C.[12] The plot of τ_S^{-1} vs. $\{a(C_0' - C_0) + b[AH^+]\}$ is linear (Figure 3). The slope gives $k_S = (4.20 \pm 0.05)10^2$ M^{-1} s^{-1}, and the intercept $k_{-S} = (3.85 \pm 0.05)10^{-3}$ s^{-1} at 25 °C. Thus the stability constant $K_S = k_S/k_{-S}$ for the Meisenheimer-type adduct is $(1.10 \pm 0.05)10^5$ M^{-1}. This latter value is high but normal[16] and shows that the competitive binding of hydrogen sulfite and water for the flavylium structure strongly favors the sulfite adduct S.

We have no evidence for the addition of HSO_3^- to the 2 or 4 position and we only observe one σ adduct. However, on the basis of our relaxation experiments we cannot completely exclude the possibility that small amounts of a second neutral σ adduct S′ exist. In the Appendix we show that the relaxation amplitude associated with the formation of S′ may be, in unfavorable cases, too small to give rise to a measurable signal.

Scheme III is essentially valid in acidic media (pH <4). Near neutrality, we observe that, if the acidity of a solution with no sulfur dioxide and containing a large amount of the AH^+ cation is abruptly changed toward pH 6–7, the blue quinonoid base A appears immediately.[9] On adding sulfur dioxide to this solution, the blue color disappears. In this case, since large amounts of the sulfite anion SO_3^{2-} are now present, the discoloration process is probably related to the competitive reactions of HSO_3^- and SO_3^{2-} for the flavylium structure, although this last species is no longer stable at pH 7.[11]

Conclusion

In this work we have described a method for measuring both the kinetic and thermodynamic stability constants related to the covalent addition of a nucleophile to derivatives of 2-phenylbenzopyrylium salts (anthocyanins). Such physicochemical measurements are important, not only for determining mechanisms in organic chemistry, but also for agriculture and the food industry.

Acknowledgments. We wish to express our gratitude to Professor J. E. Dubois for fruitful discussions.

Appendix

Relaxation Amplitudes Associated with the Formation of Two Neutral σ Adducts S and S′ in the SO_2-Jump Experiments. For an anthocyanin not forming appreciable amounts of carbinol B and chalcone C and in the presence of HSO_3^-, the following mechanism (eq I) applies if two σ adducts S and S′ are formed.[17]

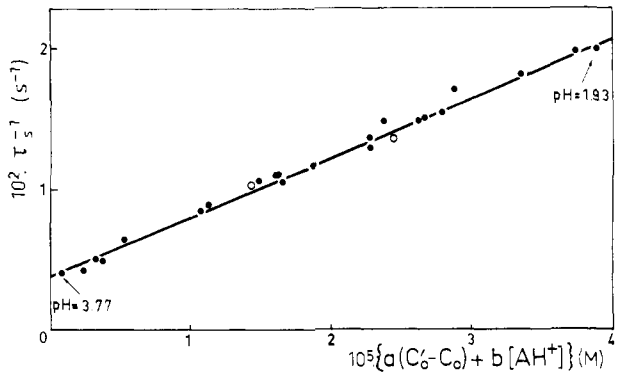
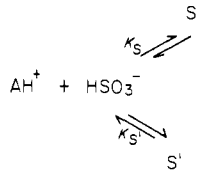


Figure 3. Plot of τ_S^{-1} vs. $\{a(C_0' - C_0) + b[AH^+]\}$ at 25 °C. Intercept = $(3.85 \pm 0.05)10^{-3}$ s^{-1}; slope = $(4.20 \pm 0.05)10^2$ M^{-1} s^{-1}; $r = 0.996$. All data but two have been obtained by pH jump (○, SO_2 jump).



(I)

Such a mechanism is characterized by a relaxation spectrum consisting of two relaxation times, τ_S and $\tau_{S'}$.[14] Since it has been well established for 2,4,6-trinitrobenzene derivatives and for 2,6-diphenylpyrylium cations that the rate of addition of a nucleophile at a substituted ring carbon is markedly different from the rate of addition at a ring carbon carrying hydrogen,[4] we do not have to consider the case where $\tau_S \simeq \tau_{S'}$ (for anthocyanins position 2 always bears a phenyl group and position 4 always bears a hydrogen atom). Thus we only examine the case where the two relaxation times are sufficiently separated (for instance, τ_S lower than $\tau_{S'}$) so as to allow separate equilibration of both steps after a suitable increase of the HSO_3^- concentration has occurred.

Before the SO_2 jump, the concentrations of the four species involved in eq I are $[HSO_3^-]_0$, $[AH^+]_0$, $[S]_0$, and $[S']_0$ (equilibrium conditions). After the SO_2 jump and at $t = 0$, they are $([HSO_3^-]_0 + [HSO_3^-]_{added})$, $[AH^+]_0$, $[S]_0$, and $[S']_0$ (nonequilibrium conditions). As soon as the fast reaction has reached equilibrium again and before the relaxation of the slow reaction starts, they become $[HSO_3^-]_1$, $[AH^+]_1$, $[S]_1$, and $[S']_0$ (partial equilibrium conditions). After complete equilibration the new equilibrium concentrations are $[HSO_3^-]_f$, $[AH^+]_f$, $[S]_f$, and $[S']_f$. Since the experiments are conducted at constant temperature and pressure and since the pigment concentration C_0 remains constant, eq II–V are valid:

$$K_S = [S]_0/[AH^+]_0[HSO_3^-]_0 = [S]_1/[AH^+]_1[HSO_3^-]_1 = [S]_f/[AH^+]_f[HSO_3^-]_f \quad (II)$$

$$K_{S'} = [S']_0/[AH^+]_0[HSO_3^-]_0 = [S']_f/[AH^+]_f[HSO_3^-]_f \quad (III)$$

$$C_0 = [AH^+]_0 + [S]_0 + [S']_0 = [AH^+]_1 + [S]_1 + [S']_0 = [AH^+]_f + [S]_f + [S']_f \quad (IV)$$

$$[AH^+]_1 - [AH^+]_f = [HSO_3^-]_1 - [HSO_3^-]_f \quad (V)$$

It is easy to demonstrate that

$$[AH^+]_0 = \frac{C_0}{1 + (K_S + K_{S'})[HSO_3^-]_0} \quad (VI)$$

$$[AH^+]_f = \frac{C_0}{1 + (K_S + K_{S'})[HSO_3^-]_f} \quad (VII)$$

(16) Since the heteroaromatic 2-phenylbenzopyrylium (flavylium) cation bears a full positive charge the pyrylium nucleus is probably more electron deficient than the benzene nucleus in 2,4,6-trinitrobenzene derivatives. Consequently, the stability constant K_S for a given σ adduct should be larger in the case of flavylium compounds than in the case of 2,4,6-trinitrobenzene derivatives. Indeed, for adducts derived from 2,4,6-trinitrobenzene derivatives in water, SO_3^{2-} being the nucleophile, the highest stability constant measured is 5.4×10^4 M^{-1} (M. R. Crampton, ref 4). Though SO_3^{2-} is a stronger nucleophile than HSO_3^-, this last value is still lower than the value found in this work.

(17) Though the mechanism of eq I is a simplified one, since we neglect the existence of the carbinol and chalcone structures as well as the existence of the neutral SO_2 species, the conclusions reached in the Appendix are valid for anthocyanins in general.

$$[AH^+]_1 = \frac{(1 + K_S[HSO_3^-]_0)C_0}{(1 + K_S[HSO_3^-]_1)\{1 + (K_S + K_{S'})[HSO_3^-]_0\}} \quad \text{(VIII)}$$

Thus, the amplitude for the fast step (formation of the σ adduct S) is given by $[AH^+]_0 - [AH^+]_1$:

$$[AH^+]_0 - [AH^+]_1 = \frac{K_S([HSO_3^-]_1 - [HSO_3^-]_0)C_0}{(1 + K_S[HSO_3^-]_1)\{1 + (K_S + K_{S'})[HSO_3^-]_0\}} \quad \text{(IX)}$$

The amplitude for the slow step (formation of the σ adduct S') is given by $[AH^+]_1 - [AH^+]_f$:

$$[AH^+]_1 - [AH^+]_f = [K_{S'}([HSO_3^-]_f - [HSO_3^-]_0)C_0]/[\{1 + (K_S + K_{S'})[HSO_3^-]_0\}\{K_SC_0 + [1 + (K_S + K_{S'})[HSO_3^-]_f][1 + K_S[HSO_3^-]_1]\}] \quad \text{(X)}$$

For a typical SO_2-jump experiment C_0 is 3×10^{-5} M and $[HSO_3^-]_1 \simeq [HSO_3^-]_f = 2 \times 10^{-5}$ M. If we first assume that $K_{S'} = K_S = 1.1 \times 10^5$ M^{-1}, the ratio of the fast to the slow relaxation amplitudes is about 6. In this case, the amplitude of the slow relaxation signal is high enough and can be measured. If now $K_{S'}$ is much lower than K_S, for instance, $K_{S'} = K_S/10 = 1.1 \times 10^4$ M^{-1}, the above ratio increases to about 40. Since the amplitude of the fast signal remains constant, the slow relaxation is no longer detectable.

Chemical and Spectroscopic Comparison of the Binuclear Copper Active Site of Mollusc and Arthropod Hemocyanins

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Abstract: A series of active-site derivatives of hemocyanin have been reported which allow the binuclear copper active site to be chemically varied in a reasonably controlled manner. These include the met apo [Cu(II)-], half-met [Cu(II)Cu(I)], dimer (EPR-detectable met) [Cu(II)Cu(II)], and met (EPR-nondetectable met) [Cu(II)···Cu(II)] forms. Detailed spectroscopic study of these has demonstrated that the binuclear cupric active site of oxyhemocyanin contains both an endogenous and exogenous ligand bridge with the peroxide binding in a μ-dioxo fashion between the equatorial planes of both tetragonal coppers. Extension of these chemical and spectral studies to a series of five molluscs (*Busycon canaliculatum*, *Lunatia heros*, *Megathura crenulata*, *Octopus bimaculatus*, and *Helix pomatia*) and five arthropods (*Cancer borealis*, *Cancer irroratus*, *Cancer magister*, *Homarus americanus*, and *Limulus polyphemus*) has shown the active sites in both phyla to be quite similar; however, significant differences are observed. All the arthropod forms exhibit similar spectral features which quantitatively differ from those of the molluscs. These electronic structural differences indicate that the arthropod active site is distorted from that of the mollusc. This distortion strongly affects exogenous ligand binding at the binuclear copper site and, by extension, the peroxide regeneration of met to oxy (the arthropods have a much lower catalase activity than the molluscs). Further, the arthropods (excluding *Limulus*) are found to have an unstable active site which is irreversibly disrupted by group 2 ligands (those which break the endogenous bridge by binding the coppers with a >5 Å M-M distance). This active-site instability seems to be associated with a strain induced by the protein ligand. Finally, the *Limulus* oxy active site is found to differ from that of molluscs and other arthropods in terms of access to an axial coordination position for peroxide displacement reactions ($k_{\text{arthropods}} > k_{\text{molluscs}} \gg k_{Limulus}$).

Hemocyanin is the binuclear copper-oxygen binding protein (10_2:2Cu)[1] found in arthropods and molluscs. In the oxy form the oxygen has been shown to bind as peroxide,[2] and therefore the coppers are formally copper(II). However, this active site exhibits rather unique spectral features compared to simple inorganic cupric complexes (λ 345 nm, $\epsilon \approx 20000$ M^{-1} cm^{-1}; λ 570 nm, $\epsilon \approx 1000$ M^{-1} cm^{-1}; no EPR signal). These have recently been interpreted based on spectroscopic studies of a series of active-site derivatives[3,4] (vide infra), the 345- and 570-nm absorption features being peroxide to copper(II) charge-transfer transitions and the lack of an EPR signal being due to antiferromagnetic coupling between the coppers via an endogenous protein bridge. While the optical spectra of the oxy forms of the arthropods and molluscs are generally similar, specific differences have been stressed between the two phyla in terms of absorption band energies and, in particular, their CD spectra.[5] A second difference between the oxy forms of the two phyla which has been reported is that only the molluscs undergo ligand displacement of the peroxide, based on negative results for *Limulus polyphemus* (an arthropod).[6] However, we have recently shown that *Limulus*, which is in a different subphylum from other arthropods, was unique in this regard and, in fact, the rest of the arthropods tested underwent the most facile ligand-displacement reactions.[7] The most emphasized general difference between the arthropods (A) and molluscs (M) is related to their catalase activity.

$$2H_2O_2 \xrightarrow{Hc_M} 2H_2O + O_2 \quad (1)$$

Only the molluscs exhibit high catalase activity,[8] being regenerable

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