Studies relating to the Formation of Semi-met Cu^ICu^{II} *Panulirus interruptus* Haemocyanin†

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Reduction of *Panulirus interruptus* haemocyanin in the methaemocyanin Cu^{II}_2 state with N_2H_4 and $S_2O_4^{2-}$ occurs in two stages. The product of the first stage is semi-met Cu^ICu^{II} which has a UV/VIS spectrum approximately midway between those of met- and deoxy-haemocyanin peaks λ/nm (ε/M^{-1} cm^{-1} per subunit) at 680 ($\approx$120) and 337 ($\approx$2900). At 25 °C (pH 8.7) with N_2H_4 as reductant rate constants k_1 = 0.0268 M^{-1} s^{-1} and k_2 < 5 × 10^{-4} M^{-1} s^{-1} have been obtained. Formation of semi-met (k_1) and deoxy-haemocyanin (k_2) at the intermediate and final stages is confirmed by reaction with O_2 to yield UV/VIS spectra of met- and oxy-haemocyanin respectively. Reduction of methaemocyanin with the strong reductants e_{aq}^- and $CO_2^{\bullet -}$ generated by pulse radiolysis occurs at some other sites on the protein, and no reduction of the Cu^{II}_2 site was observed. With the less strong reductant $MV^{\bullet +}$ derived from methyl viologen (1,1′-dimethyl-4,4′-bipyridinium dichloride) and with methaemocyanin in excess, formation of semi-methaemocyanin was observed presumably by electron transfer from the protein surface. The same behaviour is noted with the photochemically generated 5-deazaflavin radical $dfl^{\bullet -}$ which does not further reduce the protein to the deoxyhaemocyanin state. With pulse-radiolysis-generated oxidising radicals $N_3^{\bullet}$ and $(NCS)_2^{\bullet -}$ deoxyhaemocyanin gives spectroscopically identifiable protein Tyr$^{\bullet}$ (410 nm) and Trp$^{\bullet}$ (510 nm) radicals, but no evidence for oxidation of the Cu^I_2 site. Mechanistic features are discussed including a consideration of the extent to which a redox partner can penetrate the protein to access the active site.

This work is concerned with the chemistry of the binuclear copper active site of the biological O_2-carrier haemocyanin. X-Ray crystal structure information has been reported for the deoxyhaemocyanin protein from *Panulirus interruptus*,[1] and for the O_2-carrying oxyhaemocyanin form from *Limulus polyphemus*.[2] The latter confirms the presence of a bridged $\eta^2:\eta^2$-peroxodicopper(II) active site, as previously identified by Kitajima[3] in model complexes. The binuclear type 3 active site of haemocyanin allows three possible oxidation states: Cu^I_2 (deoxyhaemocyanin), Cu^{II}_2 (oxy- and met-haemocyanin) and Cu^ICu^{II} (semi-methaemocyanin). If the two metal centres are not identical then two different semi-met forms can result. At present there is little information regarding the existence of semi-methaemocyanin. This contrasts with the binuclear iron containing proteins haemerythrin,[4] ribonucleotide reductase,[5] methane monooxygenase[6] and purple acid phosphatase,[7] all of which have semi-met $Fe^{II}Fe^{III}$ forms. Relevant reviews containing information on the latter have appeared.[8-11] Interestingly, a Cu^ICu^{II} state is implicated in the mechanism of the type 3 copper enzyme tyrosinase,[12] which in other respects has properties similar to haemocyanin.

For a full understanding of the chemistry of the haemocyanin active site it is important to explore the properties of the semi-met state. In theory at least, the presence of a single Cu^{II} renders the active site amenable to a wider range of spectroscopic techniques than the spectroscopically silent deoxy and anti-ferromagnetically coupled oxy- and met-haemocyanin forms. Previous work, notably by the Solomon group,[13] has resulted in the identification of semi-methaemocyanin derivatives (referred to as half-methaemocyanin) from the mollusc *Busycon canaliculatum*. These are obtained in the reaction of deoxy-haemocyanin with a nitrite–ascorbic acid mixture to give semi-met products containing NO_2^- or NO_2^--related ligands. Replacement of the latter has been carried out by dialysis with a range of ligating groups *e.g.* CN^-, SCN^-, N_3^-, $CH_3CO_2^-$, I^-, Br^-, Cl^-. Semi-met species have also been prepared from the arthropod *Limulus polyphemus* and from *Neurospora crassa* tyrosine.[14] In addition, Lontie and co-workers[15-17] have reported a mononuclear copper(II) EPR signal upon treating nitrite with *Helix promatia* (mollusc) deoxy- and met-haemocyanin forms, and from the reaction of NO with *Astacus leptodactylus* (arthropod) and *Helix promatia* methaemocyanin. The product has been described as a nitrosyl haemocyanin derivative $Cu^INO^+Cu^{II}$. Differences in the properties of arthropod and mullusc haemocyanin have been observed, but it is not certain how extensive these might be.

In the present work we are concerned with demonstrating the existence of what is referred to as the semi-met (aqua) state of haemocyanin from the arthropod *P. interruptus*. The aqua ion terminology is used to specify the condition in which no anion complexation occurs with the possible exception of OH^-. It has been shown previously that haemocyanin does not undergo electron transfer with inorganic complexes $[Co(dipic)_2]^-$, $[Fe(CN)_6]^{3-}$ (oxidants) or $[Co(sep)]^{2+}$ (reductant) as redox partners: dipic = pyridine-2,6-dicarboxylate (dipicolinate), sep = sepulchrate (1,3,6,8,10,13,16,19-octaazabicyclo[6.6.6]-eicosane).[18,19] Redox interconversion can however be achieved by small (generally neutral) molecules that are able to access the active site. Thus, O_2 and H_2O_2 bring about a two-electron inner-sphere oxidation of Cu^I_2 to Cu^{II}_2.

In the case of haemerythrin two semi-met forms have been identified and these are defined as semi-met$_r$ from the reduction of methaemerythrin, and semi-met$_o$ from the oxidation of deoxyhaemerythrin.[4] Both these approaches were adopted therefore in the present work, and the products are likewise examined by UV/VIS and EPR spectroscopy. The selection of reagents was according to one-electron redox properties as well as size of the reagent. In addition to dithionite and hydrazine, the aquated electron e_{aq}^- and radicals produced by pulse

† *Non-SI units employed:* M = mol dm^{-3}, eV ≈ 1.6 × 10^{-19} J.

radiolysis of aqueous solutions containing formate, methyl viologen (1,1′-dimethyl-4,4′-bipyridinium dichloride), azide, thiocyanate and the photochemically generated 5-deazaflavin radical, have been studied. In the latter procedures the concentration of radicals and hence the extent of redox change can be accurately controlled. A particular difficulty is the relatively weak UV/VIS absorption of both deoxy- and met-haemocyanin, and the need to use protein concentrations in the range 10^{-4}–10^{-3} M.

Experimental

Protein.—Samples of oxyhaemocyanin from *Panulirus interruptus* were prepared and purified as described previously.[20] We commenced these studies using separated subunit a, but as the work progressed found it equally informative to use the native b/c subunit mix. The deoxyhaemocyanin form was obtained as previously described.[20] To prepare methaemocyanin a 50-fold excess of hydrogen peroxide (BDH, AnalaR) was added to a solution of oxyhaemocyanin in 0.050 M Tris–HCl buffer at pH 8.7 [Tris = tris(hydroxymethyl)aminomethane], with 0.005 M ethylenediaminetetraacetate (edta) present, and left for 20 h at room temperature. The H_2O_2 reacts with the deoxyhaemocyanin component of such solutions. Excess of oxidant was then removed by dialysis against a 300-fold excess v/v of the desired buffer with three changes. The protein concentration was determined by absorbance measurements at 337 nm (shoulder), $\varepsilon = 3700\ M^{-1}\ cm^{-1}$ per active site. This value was obtained by quantitative conversion of oxyhaemocyanin, which has $\varepsilon = 2.0 \times 10^4\ M^{-1}\ cm^{-1}$ at 337 nm. Solutions of methaemocyanin denature upon freezing–thawing as judged from changes in the UV/VIS spectrum, and were freshly prepared for each experiment and used within 2 d of preparation. Protein solutions were dialysed into the appropriate air-free buffer prior to reaction. In the absence of M^{2+} (*e.g.* Ca^{2+}), subunit a is present as a monomer at pH $\geqslant 8.4$ and as a hexamer for pH $\leqslant 7.8$, whereas the native b/c mix is hexameric under all conditions. Concentrations of oxy- and deoxy-haemocyanin were determined by UV/VIS absorbance measurements using peak positions λ/nm ($\varepsilon/M^{-1}\ cm^{-1}$ per subunit) of 337 (2.0×10^4)[21] for oxyhaemocyanin and 280 (1.04×10^5)[22] for deoxyhaemocyanin. Concentrations of methaemocyanin were determined using the peak position 680 (200) and the shoulder 337 (3700);[20] all ε values are per binuclear copper site.

Buffers.—Buffer solutions of 0.045 M phosphate K_2HPO_4–KH_2PO_4 (BDH, AnalaR), pH 7.0 and $I = 0.100$ M, were used in pulse-radiolysis experiments. Solutions of 0.050 M Tris (Sigma), pH 7.0–9.0 were used in all other studies. The pH was adjusted by addition of an HCl solution (BDH, Convol), and the ionic strength to $I = 0.100$ M with NaCl (BDH, AnalaR) in all experiments not involving pulse radiolysis.

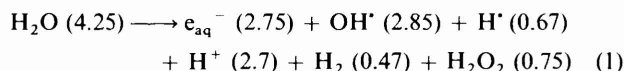
Standard Procedures.—The reductants hydrazine hydrate, $N_2H_4{\cdot}H_2O$ (BDH, AnalaR), and sodium dithionite, $Na_2S_2O_4$ (Fluka), were dissolved in air-free (N_2-saturated) solutions of Tris buffer (0.05 M), containing 0.005 M disodium dihydrogenethylenediaminetetracetate, Na_2H_2edta (BDH, AnalaR). Hydrazine solutions were made up by weight, and dithionite concentrations determined by titration against a solution of potassium hexacyanoferrate(III), $K_3[Fe(CN)_6]$ (BDH, AnalaR), peak 420 nm ($\varepsilon = 1010\ M^{-1}\ cm^{-1}$).

Reactions were studied at 25.0 ± 0.1 °C under strict air-free conditions, and monitored by UV/VIS spectrophotometry using Perkin Elmer Lambda 9, Philips Analytical 8700 and Shimadzu UV-2101PC instruments. Solutions of methaemocyanin were made up with the appropriate reductant inside a Miller–Howe glove-box (N_2 atmosphere), and transferred (≈2 min) to the spectrophotometer. Reactions were monitored in repetitive scan mode in the range 300–850 nm. The reductant was maintained in at least ten-fold excess of protein. First-order rate constants k_{obs} were calculated from slopes of $\ln(A_t - A_\infty)$ against time (t) graphs using absorbance (A) values at 680 nm.

Pulse Radiolysis Studies.—Experiments were carried out using a beam of 2.5 MeV electrons produced by a Van de Graaff generator (KS-3000, High Voltage Engineering Europa) at the Cookridge Radiation Research Centre. Pulse lengths were typically in the range 0.2–1.0 μs. Individual pulse sizes were recorded using a secondary emission chamber (SEC) which was calibrated daily using thiocyanate dosimetry.[23] Analysing light either from a tungsten quartz–iodine filament lamp (Wotan HLX 64640) or from a 250 W xenon lamp (Wotan XBO 250 W/4) was transmitted through the sample cell (2.5 cm path length) and monochromator to either an EMI 9781B or a Hamamatsu R 446 photomultiplier. Short-wavelength cut-off filters of either 385 or 500 nm, as appropriate, were placed before the cell so as to minimise scattered light effects and photolysis of the sample. For observations on time-scales less than 200 μs, the xenon lamp was flashed to enhance its brightness. The tungsten lamp was used on time-scales > 1 ms since on these time-scales the xenon lamp is subject to greater instabilities due to movement of the arc. The light level was sampled and recorded before an electron pulse was triggered. The detection system permits the recording of signals on time-scales from nanoseconds to seconds. Experiments were performed at room temperature (20 ± 1 °C).

All the solutions for pulse radiolysis were prepared using water from a BDH deioniser (conductivity < 0.05 μS cm^{-1}). Samples of met- and deoxy-haemocyanin (≈2 cm^3) were dialysed against 3 × 500 cm^3 deaerated buffer (45 mM phosphate, pH 7). The dialysed protein samples were stored under N_2 and transported in solution with ice/water cooling. Buffer solutions ≈20 cm^3 of 45 mM phosphate, pH 7, containing the relevant reagents for radical generation were saturated with N_2O. Calculated amounts of concentrated deaerated protein solution where then added to the buffer solution under argon to give the required concentration. No gas was bubbled through the protein solution thereby avoiding denaturation.

The radiolysis of water 10^{-7} s after the initial ionisation event is represented by equation (1). The figures in equation (1) refer

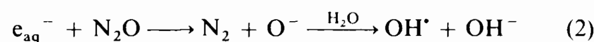


$$H_2O\ (4.25) \longrightarrow e_{aq}^-\ (2.75) + OH^{\bullet}\ (2.85) + H^{\bullet}\ (0.67) + H^+\ (2.7) + H_2\ (0.47) + H_2O_2\ (0.75) \quad (1)$$

to G values, which are the number of moles ($\times 10^7$) of product per joule of energy absorbed. Primary radicals e_{aq}^- and $H^{\bullet}$ are reducing agents, and $OH^{\bullet}$ is an oxidant. In order to obtain totally reducing or totally oxidising solutions of the different radicals the following procedures were used.

(*i*) Hydrated electrons, e_{aq}^-: argon (BOC, 99.998%) saturated solutions were used containing 0.05 M 2-methylpropan-2-ol (BDH) to convert $HO^{\bullet}$ and $H^{\bullet}$ to the relatively unreactive $^{\bullet}CH_2(CH_3)_2COH$ radicals. The hydrated electron which remains is a very powerful reducing agent ($E^\circ = -2.9$ V) with a broad absorption peak at 715 nm.

(*ii*) Formate radicals, $CO_2^{\bullet -}$: these radicals were generated by pulse radiolysis of N_2O-saturated solutions containing 0.01 M sodium formate (BDH). In this procedure the e_{aq}^- are converted to $OH^{\bullet}$ according to equation (2), and $OH^{\bullet}$ and $H^{\bullet}$



$$e_{aq}^- + N_2O \longrightarrow N_2 + O^- \xrightarrow{H_2O} OH^{\bullet} + OH^- \quad (2)$$

$$OH^{\bullet}(H^{\bullet}) + HCO_2^- \longrightarrow H_2O(H_2) + CO_2^{\bullet -} \quad (3)$$

generate $CO_2^{\bullet -}$ ($E^\circ = -1.85$ V) as in equation (3).

(*iii*) Methyl viologen radicals, $MV^{\bullet +}$: conditions were the same as for formate radical generation, with the addition of

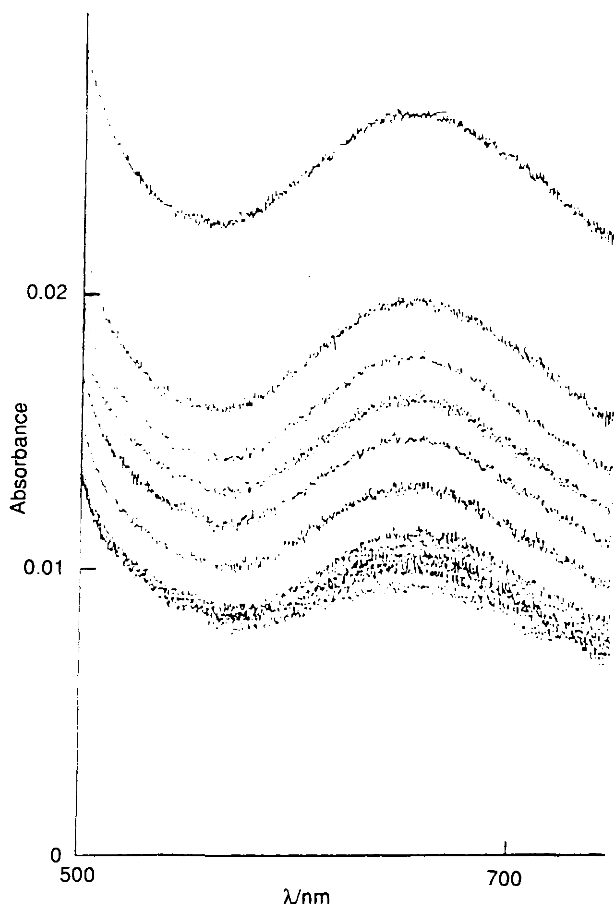


Fig. 1 The decrease in absorbance of *Panulirus interruptus* subunit a monomer methaemocyanin (1.3×10^{-4} M) on reduction (25 °C) with dithionite (1.4 mM) in the presence of edta (5 mM) at pH 8.7, $I = 0.100$ M (NaCl). Spectra were recorded at 90 min intervals and show the end of the first stage (second spectrum) and the onset of the second stage

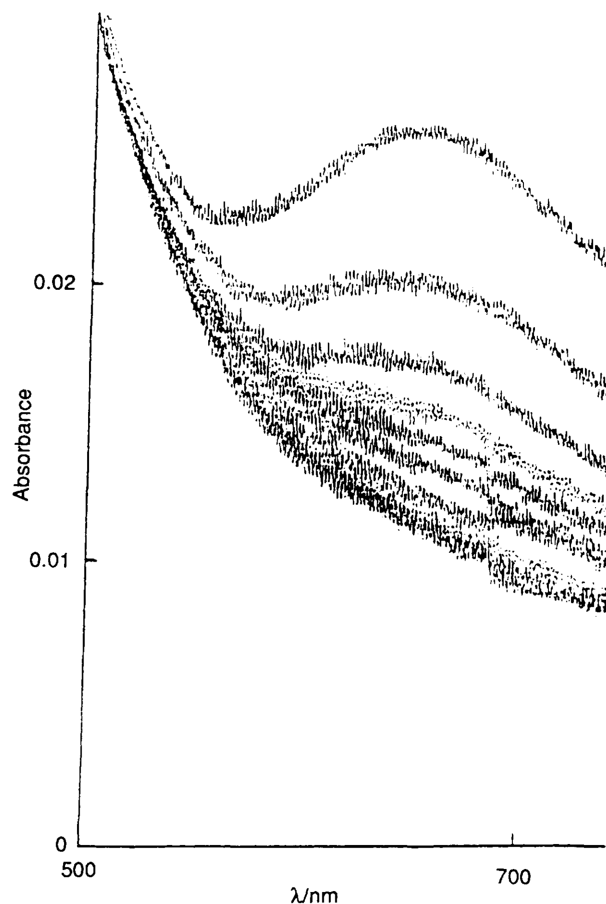


Fig. 2 The decrease in absorbance occurring in the second stage of the reduction (25 °C) of *Panulirus interruptus* subunit a monomer methaemocyanin (2.5×10^{-4} M) with hydrazine (2.9 mM) in the presence of edta (5 mM) at pH 8.7, $I = 0.100$ M (NaCl). Spectra were recorded at 100 min intervals

$\approx 1.0 \times 10^{-4}$ M methyl viologen (Sigma Chemicals), equation (4).

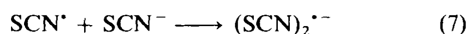
$$CO_2^{\bullet -} + MV^{2+} \longrightarrow CO_2 + MV^{\bullet +} \quad (4)$$

(*iv*) Azide radicals, $N_3^{\bullet}$: pulse radiolysis of N_2O-saturated solutions containing 0.01 M sodium azide (BDH, AnalaR) produces $N_3^{\bullet}$ ($E^{\circ} = 1.33$ V) according to reaction (5).

$$OH^{\bullet} + N_3^- \longrightarrow OH^- + N_3^{\bullet} \quad (5)$$

(*v*) $(SCN)_2^{\bullet -}$ radicals: pulse radiolysis of N_2O-saturated solutions containing 1.0 mM potassium thiocyanate (BDH, AnalaR) produces this radical, equations (6) and (7). The

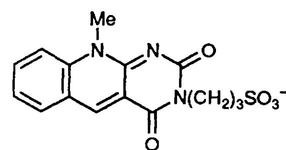
$$OH^{\bullet} + SCN^- \longrightarrow OH^- + SCN^{\bullet} \quad (6)$$



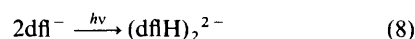
$$SCN^{\bullet} + SCN^- \longrightarrow (SCN)_2^{\bullet -} \quad (7)$$

radical is a powerful oxidant ($E^{\circ} = 1.29$ V), with an absorption peak at 480 nm ($\varepsilon = 7199$ M^{-1} cm^{-1}).

Photochemical Studies.—The 5-deazaflavin reagent, potassium 10-methyl-5-deazaisoalloxazine-3-propanesulfonate K^+ dfl^-, was kindly supplied by Dr. P. M. H. Kroneck, Konstanz, Germany. All reactions were performed in air-free 0.050 M Tris–HCl (pH 7.0–9.0), containing 0.015 M edta, $I = 0.100$ M (NaCl). Concentrations of dfl^- determined by weight gave solutions with UV/VIS peaks at 323 and 392 nm, both with ε = 9300 M^{-1} cm^{-1}. Illumination ($\approx$10 min) of air-free solutions of



dfl^- (0.1–2.0 mM) containing 15 mM edta, at pH 8.7 (50 mM Tris), using a 250 W projector lamp, resulted in the formation of a species with an absorbance peak at 307 nm, $\varepsilon = 7500$ M^{-1} cm^{-1}. The UV/VIS spectrum obtained in similar to that reported by Massey and Hemmerich,[24,25] and corresponds to the dimer $(dflH)_2^{2-}$, equation (8). The latter is the precursor



$$2dfl^- \xrightarrow{h\nu} (dflH)_2^{2-} \quad (8)$$

of the reducing radical, $dflH^{\bullet -}$ ($E^{\circ} = -0.65$ V),[26] which forms upon subsequent illumination, equation (9). Although

$$(dflH)_2^{2-} \xrightarrow{h\nu} 2dflH^{\bullet -} \quad (9)$$

the $(dflH)_2^{2-}$ species is stable in the absence of photochemical excitation, $dflH^{\bullet -}$ exists only during sustained illumination from the projector lamp. The extent of reduction is controlled by the illumination time and distance of the sample cell from the projector lamp (5 cm). To minimise excessive temperature rises due to illumination over long time-scales, a quartz cell containing water was placed between the lamp and cell to act as heat filter. Stock solutions of methaemocyanin ($\approx$0.5 mM) were

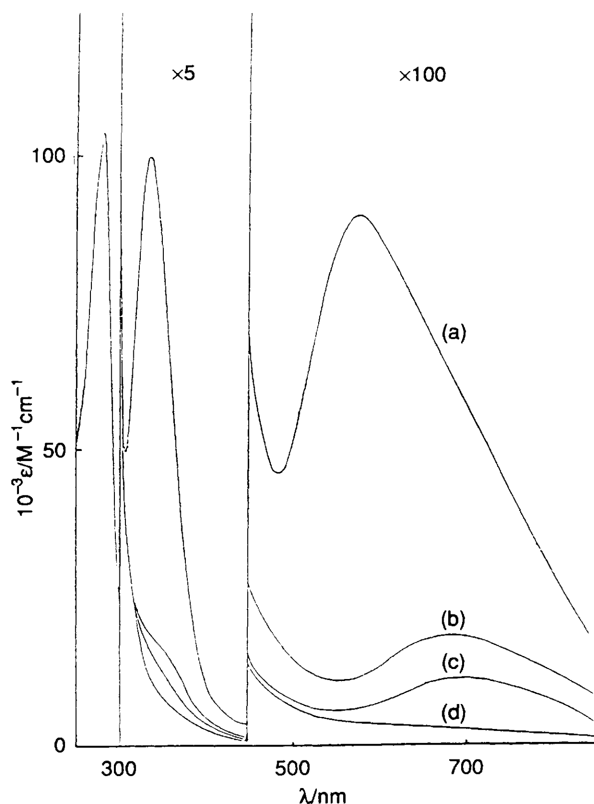


Fig. 3 The UV/VIS spectra of (*a*) oxy-, (*b*) met-, (*c*) semi-met- and (*d*) deoxy-haemocyanin from *Panulirus interruptus* at pH 8.7. A single wavelength scale is used over the range indicated

Table 1 Rate constants (25 °C) for the first-stage reduction of *Panulirus interruptus* subunit a ($\approx 1 \times 10^{-4}$ M) with hydrazine; Tris (50 mM), edta (5 mM), pH 8.7, I = 0.100 M (NaCl), λ = 680 nm

$[N_2H_4]$/M	$10^3k_{1obs}/s^{-1}$
0.011	0.55
0.026	1.03
0.038	1.07
0.054	1.52
0.085	2.33
0.103	2.57

used. A small volume ($\approx$0.02 cm^3) of photochemically prepared $(dflH)_2^{2-}$ was mixed with methaemocyanin ($\approx$1.2 cm^3) inside a UV/VIS cell under anaerobic conditions, to give typically a 20-fold excess of protein over $dflH^{\bullet-}$ on illumination. Protein reduction was carried out at 20 ± 1 °C and absorbance changes were monitored over the range 300–500 or 500–850 nm, depending on the concentration of protein used, by periodically transferring the optical cell to a UV/VIS spectrophotometer.

Electron Paramagnetic Resonance Measurements.—The EPR studies on Bruker ER 200D spectrometers fitted with Oxford instrument cryostats were made with the help of Dr. P. F. Knowles in the Department of Biochemistry and Molecular Biology at the University of Leeds (using a blow-off liquid-nitrogen cooling facility), and with Dr. W. J. Ingledew in the Department of Biochemistry at St. Andrews University (temperatures down to liquid helium). Samples obtained by dithionite or $dflH^{\bullet-}$ reduction of methaemocyanin were generated inside a UV/VIS spectrophotometric cell in order to confirm the identity of the product prior to EPR measurements. In the case of dithionite the reductant was removed by dialysis of the protein solution (24 h, 4 °C). The protein

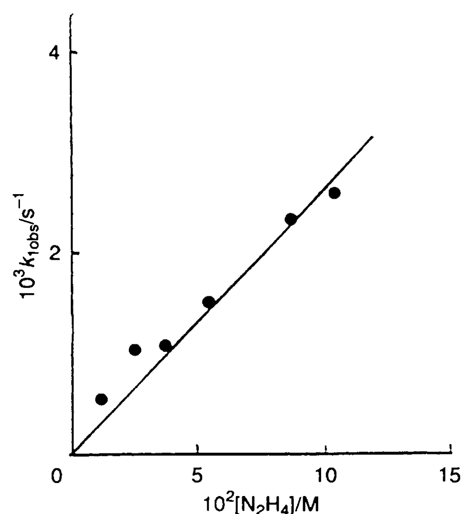


Fig. 4 The dependence of rate constants (25 °C) for the first-stage reduction of *Panulirus interruptus* subunit a ($\approx 1 \times 10^{-4}$ M) with hydrazine on reductant concentration; Tris (50 mM), edta (5 mM), pH 8.7, I = 0.100 M (NaCl)

remained reduced as judged by the regeneration of methaemocyanin upon exposure to air.

Samples were transferred into a N_2-filled EPR tube using a Hamilton gas-tight syringe under a constant stream of N_2 by means of a two-way tap. In addition, some experiments were designed to avoid the need for external transfer of protein sample solutions. Thus, $dflH^{\bullet-}$ reduced protein samples were generated directly inside the EPR tube. Protein from pulse-radiolysis experiments was introduced internally into an EPR tube attached at an angle of 90° to a specially adapted pulse radiolysis cell. Samples were frozen inside the EPR tube by careful immersion in liquid nitrogen before transferring to the spectrometer, a process requiring up to $\approx$2 h. Typically X-band EPR spectra of protein samples ($\approx$0.5 mM haemocyanin) were run at 100 K or 3.5–100 K. No sharp bands were observed for g values between 1.5 and 1.6 at a frequency of 9.45 GHz.

Results

Reactions of Methaemocyanin with Hydrazine and Dithionite.—Two stages were observed in the reductions of monomer methaemocyanin (0.1–0.5 mM) with N_2H_4 and $S_2O_4^{2-}$ under anaerobic conditions, Figs. 1 and 2. The product of the first stage gives a UV/VIS spectrum with absorption coefficients intermediate between met- and deoxy-haemocyanin, spectrum (*c*) in Fig. 3, consistent with the formation of semi-methaemocyanin. The decrease in absorption coefficients (Δε) recorded were $\approx$800 M^{-1} cm^{-1} at 337 nm and $\approx$80 M^{-1} cm^{-1} at 680 nm. After dialysis to remove excess of reductant, exposure to air resulted in the regeneration of the initial methaemocyanin spectrum, although some flocculence was observed upon prolonged exposure to air. Typically $\approx$80% of the initial concentration of methaemocyanin was re-formed in the case of hydrazine, and $\approx$60% with dithionite. With hydrazine (0.011–0.103 M) first-order rate constants k_{1obs}, Table 1, show a linear dependence on the reductant concentration, Fig. 4, as in equation (10).

$$k_{1obs} = k_1[N_2H_4] \quad (10)$$

From a least-squares treatment (with weighting to pass through the origin) k_1 (25 °C) = 0.0268 ± 0.004 M^{-1} s^{-1}, at I = 0.100 M (NaCl). Kinetic analyses were not as precise in the case of dithionite with a second-order rate constant of order of magnitude 0.5 M^{-1} s^{-1}. The second stages are very much slower and given a further decrease in absorbance to the UV/VIS

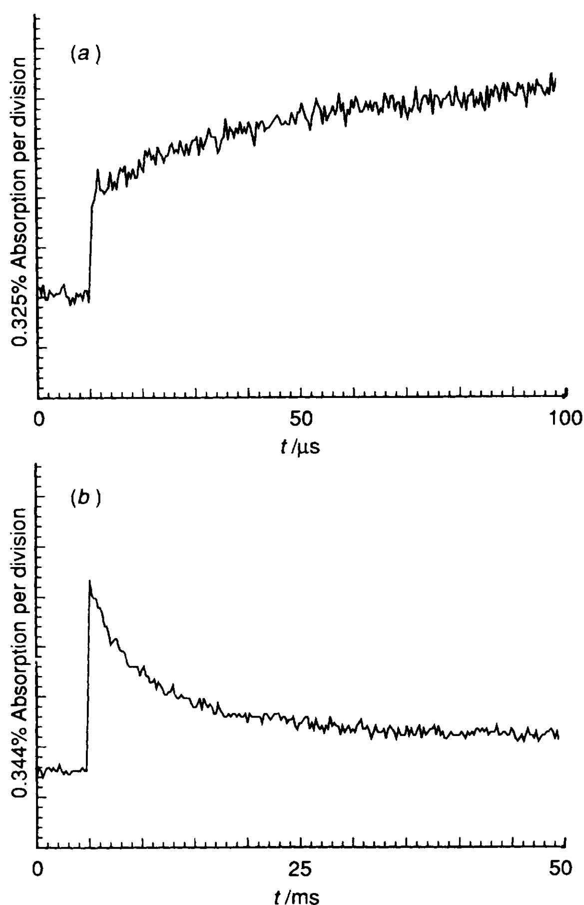


Fig. 5 Voltage *vs.* time traces at 325 nm on pulse radiolysis of *Panulirus interruptus* native subunit b/c hexamer mix of methaemocyanin (3×10^{-5} M) in N_2O-saturated solution in the presence of formate (0.010 M), V_0 = 1537 mV (*a*) or 1455 mV (*b*); pH 7 (45 mM phosphate), I = 0.100 M

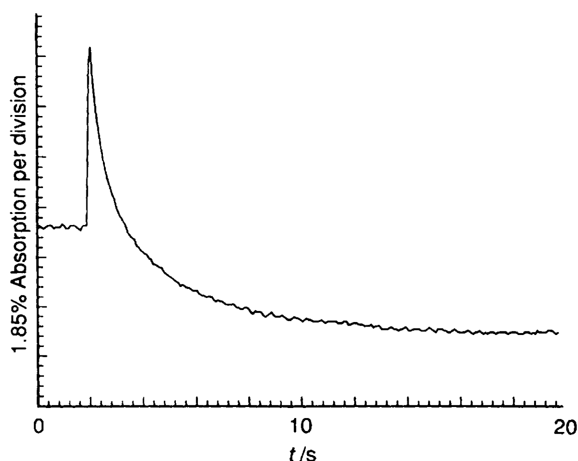


Fig. 6 Voltage *vs.* time traces at 350 nm (V_0 = 1079 mV) on pulse radiolysis of *Panulirus interruptus* native subunit b/c hexamer mix as methaemocyanin (3.0×10^{-5} M) in N_2O-saturated solution in the presence of formate (0.005 M) and methyl viologen (0.78 mM); pH 7 (45 mM phosphate), I = 0.100 M

spectrum of deoxyhaemocyanin. After dialysis to remove excess of reductant, exposure to air gives the characteristic oxyhaemocyanin peak at 337 nm. Kinetic studies on the second stage were limited due to the slowness of the reaction and doubts over protein stability. With hydrazine 0.04–0.32 M, second-order rate constants $k_2 < 5 \times 10^{-4}$ M^{-1} s^{-1} were obtained. In the case of dithionite (2.4–11.9 mM) absorbance decreases required > 48 h, and there is an additional problem of dithionite stability over such long periods.

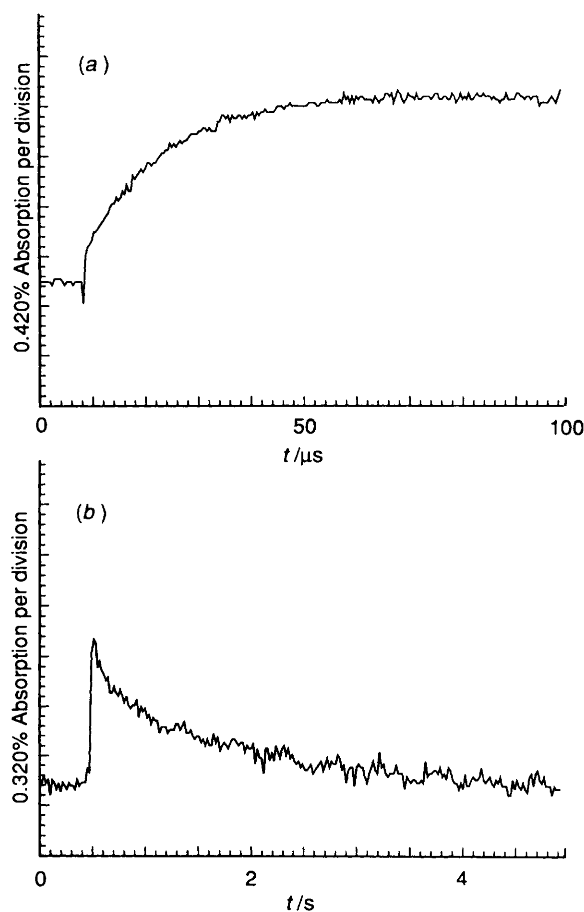


Fig. 7 Voltage *vs.* time traces at 325 nm on pulse radiolysis of *Panulirus interruptus* native subunit b/c hexamer mix deoxyhaemocyanin (3.0×10^{-5} M) in N_2O-saturated solution in the presence of azide (0.010 M), V_0 = 1189 mV (*a*) or 1566 mV (*b*); pH 7 (45 mM phosphate), I = 0.100 M

Pulse-radiolysis Reduction of Methaemocyanin.—The reaction of the native subunit b/c hexamer mix methaemocyanin (3×10^{-5} M) with $CO_2^{\cdot -}$ and e_{aq}^- gave an increase in absorbance at wavelengths in the range 325–350 nm. In the case of $CO_2^{\cdot -}$, formation of a transient occurred within ≈ 100 μs, Fig. 5(*a*), and was followed by a decay over ≈ 50 ms to give a small positive absorbance, Fig. 5(*b*). In the case of e_{aq}^- the absorbance increase is faster and decay to the original baseline is complete within ≈ 10 ms. The transients formed were not identified.

In similar experiments with methyl viologen, concentrations of methaemocyanin in the range $(0.3–3.0) \times 10^{-4}$ M, $MV^{\cdot +}$ decay at wavelengths 330–390 nm over ≈ 20 s was observed, Fig. 6. Calculation of $\Delta\varepsilon$ at 330–390 nm showed the product to be similar to the first-stage product in the reduction of methaemocyanin with hydrazine and dithionite, *i.e.* spectrum (*c*) in Fig. 3, with absorption coefficients approximately intermediate between deoxy- and met-haemocyanin; Methaemocyanin is reformed on exposure to air consistent with semi-methaemocyanin formation. No further reduction to deoxyhaemocyanin is observed. Note however that the protein is in excess of $MV^{\cdot +}$ in these experiments.

Pulse-radiolysis Oxidation of Deoxyhaemocyanin.—Solutions of the native subunit b/c hexamer mix as deoxyhaemocyanin (≈ 3×10^{-5} M) reacted with $N_3^{\cdot}$ within ≈ 100 μs, Fig. 7(*a*), to give a transient spectrum with absorbance peaks at 410 and 510

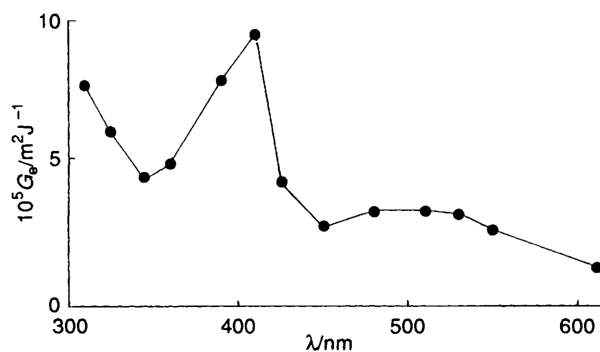


Fig. 8 The transient spectrum on pulse radiolysis of *Panulirus interruptus* native subunit b/c hexamer mix of deoxyhaemocyanin (3.0×10^{-5} M) in N_2O-saturated solution in the presence of azide (0.010 M); pH 7 (45 mM phosphate), I = 0.100 M

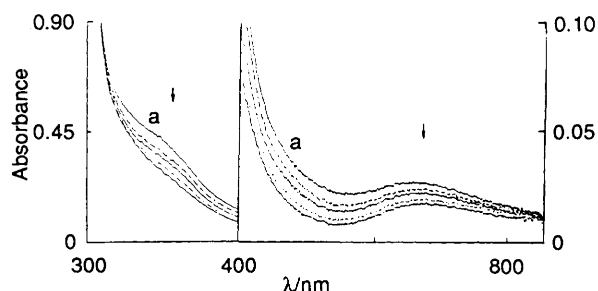


Fig. 9 The effect of projector lamp (250 W) irradiation on the UV/VIS spectrum of an air-free solution of *Panulirus interruptus* subunit a monomer methaemocyanin (1.38×10^{-4} M), containing dfl^- (4.2×10^{-6} M), at pH 8.7 (50 mM Tris) in the presence of edta (15 mM), I = 0.100 M (NaCl). The initial spectrum (a) is indicated. Spectra were recorded after 1, 10, 60 and 250 min of irradiation. No further absorbance decrease was observed

nm characteristic of the tyrosine and tryptophan radicals respectively, Fig. 8.[27,28] Subsequently absorbance decay to the baseline occurred over ≈5 s, Fig. 7(*b*), with no evidence for any active-site oxidation. Similar absorbance changes were observed with the $(SCN)_2^{\bullet -}$ radical.

Reduction of Methaemocyanin with the 5-Deazaflavin Radical, $dflH^{\bullet -}$.—Solutions of subunit a monomer methaemocyanin $(0.8–1.7) \times 10^{-4}$ M, at pH 8.7 (50 mM Tris–HCl) in the presence of 0.015 mM edta, react with $dflH^{\bullet -}$ (protein in 10 to 30-fold excess), to give a decrease in absorbance in the range 300–850 nm. The $dflH^{\bullet -}$ is regenerated and its role is that of a catalyst. With a 30-fold excess of protein over $dflH^{\bullet -}$, the decrease was complete in ≈2 h illumination from a 250 W projector lamp, Fig. 9. The reaction time decreased to ≈40 min with a 10-fold excess of protein. The UV/VIS spectrum of the product is intermediate between those of met- and deoxyhaemocyanin, and is similar to that of the hydrazine, dithionite and $MV^{\bullet +}$ reactions. No further reaction occurred on illumination for a further 24 h. In the presence of air the initial methaemocyanin spectrum is regenerated, although as in the case of dithionite and hydrazine, flocculence is sometimes observed on prolonged exposure to air.

EPR Studies.—The possibility of identifying Cu^ICu^{II} by EPR spectroscopy was explored. No characteristic signal due to Cu^{II} was observed for methaemocyanin solutions (≈0.5 mM), except for a weak feature centred at g ≈ 2 as observed in previous studies.[29] The two copper(II) ions of the met form are believed to be antiferromagnetically coupled.[3] More relevant, no copper(II) signal was observed from the reaction of subunit b/c hexamer mix with $MV^{\bullet +}$, or from the first stage of the reaction of subunit a monomer with dithionite. In the case of the product from the reaction of $dflH^{\bullet -}$ with subunit a monomer methaemocyanin, a weak signal characteristic of Cu^{II} was observed, but could not be reproduced in subsequent experiments.

Discussion

The reductants hydrazine, dithionite, $MV^{\bullet +}$ and $dlfH^{\bullet -}$ reduce methaemocyanin under anaerobic conditions to a product with ε values in the range 300–850 nm intermediate between deoxy- and met-haemocyanin. Such absorbance changes are consistent with the formation of semi-methaemocyanin, here written as Cu^ICu^{II}. Exposure of this product to air results in the regeneration of the methaemocyanin spectrum with no evidence for the more intense oxyhaemocyanin spectrum, Fig. 3, thus ruling out formation of a mixture of deoxy- and met-haemocyanin. Detailed studies have been carried out in the case of hydrazine which gives a second-order rate constant k_1 = 0.0268 M^{-1} s^{-1}. Hydrazine and dithionite, but not $dflH^{\bullet -}$, further reduce the haemocyanin to the deoxy form in a second much slower step. We have no information as to whether this reaction can occur with $MV^{\bullet +}$ because pulse-radiolysis experiments require that the protein is in excess. On exposure to air the deoxyhaemocyanin product gives oxyhaemocyanin (peak at 337 nm). In the case of hydrazine, the rate constant for k_2 is $< 5 \times 10^{-4}$ M^{-1} s^{-1}. The instability of methaemocyanin and dithionite over long reaction times makes the quantitative study of this reaction more difficult.

Pulse-radiolysis experiments in which the more powerful reducing and oxidising radicals are generated result in side reactions with the polypeptide and no active-site redox changes are observed. Thus the reactions of $CO_2^{\bullet -}$ (E° = −1.85 V)[30] or e_{aq}^- (E° = −2.9 V)[31] with methaemocyanin give a rapid absorbance increase and then decay. One possibility is that there is an initial reduction of a disulfide bridge on *P. interruptus* haemocyanin (subunits a and b have three each) to the disulfide radical, as has been observed previously in the case of other proteins.[32-34] Similarly no evidence was obtained for active-site oxidation of deoxyhaemocyanin with $N_3^\bullet$ (E° = 1.33 V)[22] or $(SCN)_2^{\bullet -}$ (E° = 1.29 V),[35] which instead give formation of $Tyr^\bullet$ and $Trp^\bullet$ radicals as transients.[27,28] In these four cases reaction occurs preferably at or near the protein surface rather than at the ≈20 Å buried active site.

The abilities of different reducing agents to react at the active site of methaemocyanin is dependent on their size as well as E° values. The two reagents which are able to convert met- to deoxy-haemocyanin in two 1 e^- steps, hydrazine (E° in the range −1.74 to −2.42 V, depending on pH),[36] and dithionite which generally reacts as $SO_2^{\bullet -}$ (E° = −0.66 V, pH 7),[36] are small molecules which could well penetrate the protein matrix and react at the active site. If penetration does occur it is perhaps surprising that hydrazine at least does not bring about a direct 2 e^- reduction to deoxyhaemocyanin. The importance of size is further illustrated by the failure of the larger $dflH^{\bullet -}$ (E° −0.65 V)[25] to reduce haemocyanin beyond the semi-met state despite an E° which is comparable to that of dithionite. Unfortunately we have no information as the whether $MV^{\bullet +}$ can bring about the second stage of reduction of methaemocyanin.

An important feature in the reduction of methaemocyanin is the different reactivities of the two copper ions. Thus the large organic radical $dflH^{\bullet -}$, which is unlikely to penetrate the protein, presumably reacts by long-distance electron transfer from the protein surface. There is however no reduction of the second copper(II) to give deoxyhaemocyanin. Also in the case of hydrazine and dithionite, which might access the active site, the reduction of the second copper occurs much less readily than the first. This suggests that there is a barrier to reduction of the second copper, which may relate to changes in co-ordination number at the active site from a bridged, most likely μ-hydroxo semi-met form to non-bridged deoxyhaemocyanin Cu^I_2.

Accessibility of (or distance to) the second copper may also be less favourable. In this context we note that CO binds to only one copper(I) site in deoxyhaemocyanin,[38–40] and access may be a relevant factor. Although the copper active site is 20 Å buried,[1,2] recent X-ray crystal structure information has indicated a path of well ordered water molecules to within ≈5 Å of the closest copper, with a partial obstruction at ≈10 Å.[41] It is possible that small molecules use this route to access the active site.

The binuclear iron(III) and Tyr˙ containing enzyme ribonucleotide reductase (subunit R2) is another protein with a buried active site ≈10 Å from the protein surface.[42] Here little or no reaction with dithionite is observed, but reaction occurs with $MV^{•+}$ suggesting that aromatic/hydrophobic interactions are important.[43] Moreover reduction of both iron(III) sites is observed, with reduction of the second rapid and non-rate determining.

In spite of the compelling UV/VIS spectrophotometric evidence provided for semi-met(aqua)haemocyanin formed by reduction of methaemocyanin, no unequivocal EPR evidence in support has been obtained. Previous approaches to semi-met generation have all involved the use of NO or NO_2^- to give ligated species displaying a characteristic copper(II) signal.[13–17] Most of this work has been on mollusc as opposed to arthropod haemocyanin which may behave differently. Solomon and co-workers[13–17] have reported substitution of the exogenous ligand with a variety of species including H_2O to form semi-met(aqua)haemocyanin, again with observation of a copper(II) EPR spectrum. This different behaviour is surprising. Symons and Peterson[44] have reported an EPR-active species formed from γ-radiolysis of frozen oxyhaemocyanin solutions at 77 K which is stable at < 270 K.[44] The latter species has been tentatively assigned as $Cu^{II}–O_2^{2-} – Cu^{I}$. A similar mixed-valence form of oxyhaemocyanin has been postulated as an intermediate in the superoxide dismutase-like activity of haemocyanin.[45]

To summarise, UV/VIS absorbance spectra observed for one-electron reduced *Panulirus interruptus* methaemocyanin with hydrazine, dithionite, $dmbipy^{•+}$ and $dflH^{•-}$ indicate formation of semi-met(aqua)haemocyanin. Confirmation stems from its re-oxidation to methaemocyanin in air. Further reduction to deoxyhaemocyanin (which with O_2 gives oxyhaemocyanin) is observed in the case of hydrazine and dithionite. Confirmation by EPR spectroscopy has not been observed and broadening of the copper(II) signal, or quenching by some extrinsic effect is believed to be occurring. Observations on monomer and hexamer *P. interruptus* haemocyanin are identical.

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