Formation of an Iron–Sulfur Cluster by the Reduction of Sulfate with the Blood Pigment of an Ascidian in the Presence of Iron

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Received August 12, 1982

The morula blood cells of ascidians concentrate a colored organic reducing agent [1–4], sulfate [5], and various metals [2, 3, 6]. In some species of the sub-order Phlebobranchia where the predominant metal is vanadium [2, 6], these components are present in molar concentrations [3]. Although present in much lower concentrations iron has been found in the blood cells of the three sub-orders of the Ascidiacea, Aplousobranchia, Phlebobranchia, and Stolidobranchia [2, 6]. In species from the last of these, no vanadium generally is present in the blood cells. The compounds formed by the metals in the blood cells are presently unknown. However, there is evidence that the vanadium in Phlebobranchs is present largely as hydrated vanadium(III) [7, 8], and there is conflicting evidence that the vanadium is largely membrane bound with some vanadium in th granules present in the intracellular vacuoles [9]. In the Stolidobranch, *Botryllus schlosseri*, iron has also been found to be localized in electron dense granules within the vacuoles [10].

The metal-free pigment from the ferrocytes of the Stolidobranch, *Pyura stolonifera*, has been isolated by precipitation at pH 9 in the absence of oxygen following lysis of the cells in dilute acid (HCl at pH 3). The compound, which has been given the name, apo-ferreascid, is redissolved in dilute acid, and reprecipitated as its acetate salt by the addition of acetic acid: *Anal.* $(C_{20}H_{31}N_5O_{13})_n$ C, H, N. Sedimentation velocity experiments of the salt in acetate buffer (pH 3.9 I 1.0) yield a molecular weight of about 5200 daltons corresponding to n being 9. Field desorption mass spectroscopic studies of the salt give a peak at about 5000 daltons. The complete molecular structure of the compound is presently unknown. However, ^{13}C and ^{1}H n.m.r. spectra of the compound, and its acid hydrolysis product confirm colorimetric tests that the compound contains a derivative of glucose. In addition GC–MS studies of the acetyl/propionate derivatives of the acid hydrolysate show the amino acids, alanine, valine, leucine, isoleucine, phenylalanine, tyrosine, serine, glutamic acid and aspartic acid, in total accounting for approximately 20% of the compound, although the compound does not give a positive biuret test. The ^{13}C spectrum suggests the presence of a nitrogen heterocycle, possibly related to isoxanthopterin because this compound as well as other pteridines have been isolated previously from a number of ascidians [11]. Absorption and circular dichroism studies show the presence of the isolated compound in the whole cells. The details of the isolation and structural investigations will be published elsewhere.

When apo-ferreascid acetate is reacted anaerobically with a solution of iron(II) sulfate, a yellow solid immediately precipitates. Any gases liberated during the reaction were collected and examined by mass spectroscopy. There were no traces of sulfur compounds, but carbon dioxide was formed. Using [U-^{14}C]-acetate it was shown that this was not formed by oxidation of the acetate, and, therefore, it must be formed during the oxidation of the apo-ferreascid. The yellow precipitate is very sensitive to oxygen, and in the presence of trace amounts of oxygen turns blue (yellow in transmitted light when viewed on a microscope). The blue precipitate, formed when the reaction was carried out aerobically, gave analyses (C, H, N, S, Fe) consistent with $(C_{168}H_{280}N_{54}O_{115}S_4)Fe_2S_2$. It possesses labile sulfide which is released as hydrogen sulfide when the compound is reacted anaerobically with concentrated hydrochloric acid. The liberated hydrogen sulfide was measured quantitatively by mass spectroscopy, and from this measurement it was determined that a mass of about 5000 daltons possesses two labile sulfides. Aerobic treatment with concentrated hydrochloric acid gives sulfur dioxide with smaller amounts of hydrogen sulfide. The visible absorption spectrum of the solid dispersed in a polyester disk has a shoulder at 540 nm, in a similar position to a shoulder in the spectrum of 2-Fe ferredoxins [12]. An anaerobic core extrusion reaction with thiophenol [13] gives a solution possessing an absorption spectrum with A_{540}/A_{440} of 0.9, a value obtained for 2-Fe ferredoxins [13]. The laser Raman spectrum has peaks at 255, 300, 435 and 590 cm^{-1}. The 2-Fe protein, Adrenodoxin, has bands at 297, 350, and 397 cm^{-1}, with the first and last being assigned to vibrations involving the labile sulfide [14]. These

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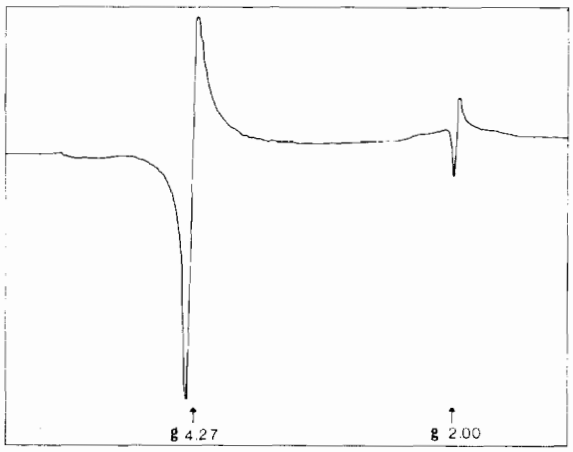

Fig. 1. E.S.R. spectrum (X-band) of ferreascid at 4.2 K.

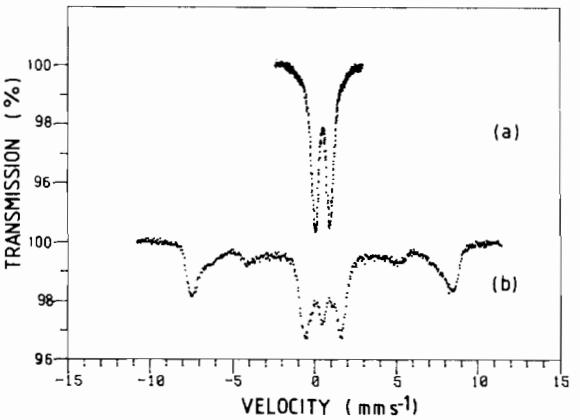

Fig. 2. Mössbauer spectrum at 4.2 K of ferreascid enriched with ^{57}Fe and dispersed in boron nitride: (a) the zero-field spectrum; and (b) with an applied longitudinal field of 5.0 T.

results suggest that ferreascid has an Fe_2S_2 core, which is found in the 2-Fe ferredoxins. However, in the ferredoxins the coordination of the metal is completed by mercaptan groups from cysteine residues, whereas apo-ferreascid does not possess these groups. This difference in the coordination could explain the fact that ferreascid does not give the characteristic ESR spectrum of a 2-Fe ferredoxin that has a signal with g about 1.94 [12].

The ESR spectrum of ferreascid at 4.2 K is given in Fig. 1. The signal at g 4.27 in the 77 K spectrum was integrated and its area compared to that for the analogous signal of K[Fe(EDTA)] [15]. With equal percentages of iron, the ferreascid signal had approximately the same area as that for K[Fe(EDTA)].

The Mössbauer spectrum of the blue form of ferreascid enriched with ^{57}Fe and dispersed in boron nitride is shown in Fig. 2. The zero-field spectrum at 4.2 K (Fig. 2a) has a single, slightly asymmetric quadrupole doublet with $\delta = 0.53 \pm 0.01$ and $\Delta E_Q = 0.91 \pm 0.02$ mm s^{-1} (*vs.* metallic Fe at room temperature). The asymmetry could be due to two superimposed virtually identical quadrupole doublets coming from two inequivalent iron sites, has been found previously for example, for the oxidized 2-Fe *Scenedesmus* ferredoxin [16]. The isomer shift and quadrupolar splitting are the same as the values found for iron(III) semiquinone complexes [17]. With an applied longitudinal field of 5.0 T, the complex spectrum (Fig. 2b) can be resolved into two components of approximately the same area. A paramagnetic component has a typical six line spectrum with the low intensity of lines 2 and 5 showing that the majority of electron spins are aligned along the applied field direction, but that some are at an angle to the field causing the 'trailing' on the inside edges of the outer lines 1 and 6. This compound has $\delta = 0.5 \pm 0.1$ mm s^{-1} and $H_{int} = 54.2 \pm 0.2$ T. The internal field is consistent with high spin Fe(III). The second component which is diamagnetic, weakly paramagnetic, or more likely, antiferromagnetically coupled, accounts for most of the centre of the spectrum with $\delta = +0.98 \pm 0.2$ mm s^{-1} and $H_{in} = 1.8 \pm 0.2$ T. The antiferromagnetic coupling was confirmed by a variable temperature magnetic susceptibility study.

The ferreascid described above was prepared *in vitro*. Although ferreascid has not been isolated intact from the blood cells there is evidence to show the insoluble compound is present in the cells. Since the cells contain sulfate [18], and high concentrations of apo-ferreascid, when iron enters the globules the reaction to form ferreascid should proceed. Electron microscopy studies of the morula blood cells of the Stolidobranch, *Botryllus schlosseri,* has shown that iron is localized in electron dense granules within the globules [10]. For *Pyura stolonifera,* a major proportion of the cellular iron is found in the cell residue after lysis. For example, freeze-dried whole blood cells that contain 175 ± 20 ppm Fe on lysis give a cell residue with 1160 ± 20 ppm Fe and a lysate that on freeze drying has 170 ± 110 ppm Fe. The ratio of the weight of iron in the residue to that in the lysate is 2.0 ± 1.2. Bearing in mind that the iron from some ferreascid would be liberated on cell lysis in dilute acid, this result is consistent with the majority of the iron being in the insoluble granule within the cell. Further evidence of the presence of the iron sulfide complex within the cells is that frequently on lysis of the cells in dilute hydrochloric acid hydrogen sulfide is detected.

Similar strong reducing agents analogous to apoferreascid have been isolated by the above procedure from the Aplousobranch, *Podoclavella moluccensis*

and the Phlebobranchs, *Ascidia ceratodes, Phallusia julinüa,* and *Ecteinascidia hataii.*

Acknowledgements

The authors gratefully acknowledge financial support from the Australian Research Grants Committee. Technical assistance with the ESR and Mössbauer measurements by W. Garrett and B. Maguire are also gratefully acknowledged. The field desorption mass spectroscopy study was carried out by Professor P. Derrick, the magnetic susceptibility study by Professor E. Sinn, and the sedimentation velocity experiment by Dr. D. J. Winzor. The authors are grateful for this assistance.

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