in a product can also be determined. Any V^{III} in a product was converted to V^{IV} by bubbling O_2 through the sample;[14] the subsequent increase in the V^{IV} signal indicates the amount of V^{III}. An important consideration was that the V^{IV} signal strength was dependent on pH with a total loss of signal at pH 7. This pH effect is due to the formation of EPR-silent, spin-coupled V^{IV} dimers and oligomers at pH 3-11 (predominant at neutral pH).[3b,15] The signal was strongest at pH < 3 where V^{IV} is monomeric; therefore, samples were buffered at pH 2 before measurement (after reaction at pH 7). Redox reactions between tunichrome and V^{V} or V^{IV} go to completion at pH 7 as confirmed by similar results when EDTA (pH 7), instead of pH 2 buffer, was added in excess to allow EPR measurements of V^{IV} at neutral pH.

The mole equivalents of V^{V} or V^{IV} were varied as follows: V:Mm-1 = 1:1, 2:1, and 4:1. After complete reaction at pH 7, the signal was measured at pH 2 before and after oxygenation of V^{III} (e.g., Figure 1). A parallel control, having V^{IV} but no Mm-1, was also measured to calibrate total vanadium. Using the measurements of V^{IV}, V^{III}, and total vanadium, the $\%V^{IV}$ and $\%V^{III}$ in a product mixture were estimated.[16] (Due to ca. 20% error in comparing a product mixture to its control, the percentages are not shown.) The percentage of V^{V} was estimated by subtracting the sum of $\%V^{IV}$ and $\%V^{III}$ from 100% total vanadium. The percentages showed that in each case essentially all (within 100 ± 20%) of the product vanadium was present as V^{IV} and V^{III}, except from 4 mol equiv of V^{V} in which remaining V^{V} was found (5-20%). The most reliable calculations are made using EPR measurements taken from an individual sample. Therefore, for each product mixture, the measurements of V^{IV} and V^{III} were compared to calculate a ratio (Table I). These ratios are qualitatively reproducible, but their quantitative aspect should not be overemphasized.

The EPR studies reveal that tunichrome can reduce V^{V} or V^{IV} to appreciable levels of V^{III}. Mm-1 can donate up to four electrons to a suitably strong oxidant, such as V^{V} (entry 6). In this example, all four available electrons from Mm-1 were necessary to reduce all but 5-20% of the V^{V} reactant (4 mol equiv) to V^{IV} and V^{III}.

Interestingly, the generation of V^{III} is more facile from V^{V} than from V^{IV}. Comparing 1 mol equiv of each (entries 1 and 4), one finds that V^{IV} reactant was mostly not reduced (85%), whereas V^{V} reactant was fully reduced to V^{III}. Hence, V^{V} must be reduced to a transient V^{IV} intermediate which is reduced further. It is perhaps surprising that this intermediate V^{IV} is reduced, in contrast to V^{IV} reactant (1 mol equiv). However, the intermediate V^{IV} could interact with its co-intermediate—a tunichrome semiquinone—which is a powerful reductant.[17] This situation could facilitate the formation of V^{III}. We know that the reduction and/or complexation of V^{V} is rapid,[4,8,9] which may prevent it from equilibrating with newly formed V^{III}. In contrast to a semiquinone, the Mm-1 starting material is presumably a much weaker reductant such that it does not readily reduce V^{IV} reactant.[17]

Although Mm-1 reduced V^{V} at all mole equivalents tested, the reduction of V^{IV} occurred according to a particular pattern. The first 2 mol equiv of V^{IV} with Mm-1 were mostly not reduced (entry 1, 2); however, 2 additional mol equiv were significantly reduced to V^{III} (entry 3). The results suggest that both catecholic groups are preferentially complexed by V^{IV} prior to the activation of redox reactivity. The activation may be due to complete complexation of tunichrome and/or to formation of V^{IV} pairs. However, no spectroscopic evidence of tunichrome–vanadium complexes was found in these studies, including EPR analyses at pH 7 (no EDTA). The addition of pH 2 buffer to product mixtures—dark green solutions containing dark suspensions—gave clear, pale green solutions. At pH 2, the spectra of product mixtures and V^{IV} controls were superimposable, indicating that the V^{IV} species are predominantly free pentaaquovanadyl ions, $VO(OH_2)_5^{2+}$ (peak to peak line separation ~110 G), rather than catecholate–V^{IV} complexes which typically have smaller line separations.[18] Similarly, for the experiments with EDTA added at pH 7, the spectra of product mixtures and EDTA–V^{IV} controls were superimposable. It is possible that tunichrome–vanadium complexes exist at pH 7 but are EPR-silent due to spin-coupled interactions between metal centers.

A fortuitous finding is that V^{V} is almost completely reduced to V^{III} by using 1 mol equiv of Mm-1 (entry 4; Figure 1). In our ongoing studies, these conditions will be useful for generating tunichrome–V^{III} products for structural characterization and comparison to the native V^{III} complex.

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Protein Electrochemistry at High Pressure

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Various techniques coupled with high-pressure methods have proven to be useful for the characterization of proteins.[1-3] For the first time we have combined protein electrochemistry with high pressures. In this paper, we discuss the implications of the effects of compression on the reduction potential of horse heart cytochrome *c*. We derive the corresponding volumes of reaction and determine the difference in the compressibility between ferri- and ferrocytochrome *c*. By measuring the change in the standard potential of a reaction ($E°$) as a function of pressure, one can obtain the volume of reaction ($\Delta V°$): $(\partial E°/\partial P)_T = -(1/nF)\Delta V°$, where $\Delta V°$ equals the sum of the standard molar volumes of the products minus the sum of the standard molar volumes of the reactants, $\Delta V° = \sum v°_p - \sum v°_r$.

Thus, a process that involves a net decrease in volume ($\Delta V° < 0$) will be favored by compression. If we consider a reduction process, such an effect will cause a shift in the standard potential of the couple in the positive direction.

Previous investigations of electrode processes at high pressure[4,5] dealt mainly with pressures below 3 kbar using experimental details

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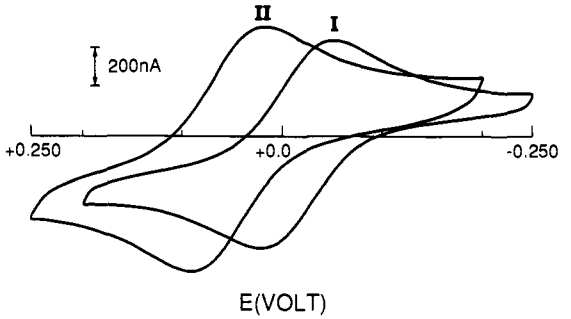

Figure 1. Cyclic voltammograms of cytochrome *c* vs Ag/AgCl/NaCl (0.1 M) at a cysteine-modified gold electrode at ambient pressure (I) and at 4 kbar (II). Pretreatment of the electrode included manual polishing with diamond paste (1 and 0.25 μm) and sonication, followed by electrochemical cleaning by cycling in sulfuric acid (0.1 M). The electrode was finally dipped in a 1 mM cysteine solution for 10 min. The electrolyte solution contained 0.1 M NaCl, 10 mM bis-tris buffer, 1 mM cysteine, pH 7.0. Measurements were made with a BAS 100B potentiostat at a scan rate of 50 mV/s.

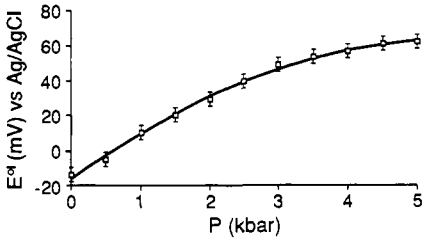

Figure 2. Pressure dependence of the formal potential of cytochrome *c*. At ambient pressure, $E^{\circ\prime} = -14$ mV vs Ag/AgCl/NaCl (0.1 M) or +257 vs NHE.

not suitable for the study of biological macromolecules. With a newly developed electrochemical cell,[6] functional at hydrostatic pressures as high as 10 kbar, we have measured the formal potential of the ferri–ferrocytochrome *c* couple vs a Ag/AgCl/NaCl (0.1 M) reference electrode. Cyclic and square wave voltammetry were obtained using solid gold electrodes derivatized with L-cysteine.[7,8] The cyclic voltammograms exhibit quasireversible behavior with peak currents linear with the square root of the scan rate up to 200 mV/s at ambient pressure. We found the peak splittings of the voltammetric response to be constant and lower than 75 mV up to 5 kbar (Figure 1). From the average of the anodic and cathodic cyclic voltammetric peaks we derived formal potential ($E^{\circ\prime}$) values for cytochrome *c*. The pressure dependence of the potential is shown in Figure 2. The observed trend indicates that the reduction of the protein is favored by the application of pressure and hence must involve a net decrease in volume. From the derivative of the fitting polynomial we can extract the volumes of reaction (ΔV°) associated with reaction 1 (see Table I).

ferricytochrome *c* (aq) + Ag(s) + Cl^-(aq) ↔ ferrocytochrome *c* (aq) + AgCl(s) (1)

$$\Delta V^{\circ} = \bar{v}_{\text{ferrocyt}} + v^{\circ}_{\text{AgCl}} - \bar{v}_{\text{ferricyt}} - v^{\circ}_{\text{Ag}} - v_{Cl^-} \quad (2)$$

From eq 2, with the experimentally determined $\Delta V^{\circ}_{P=0\text{kbar}}$ for reaction 1, and standard molar volumes for Ag, AgCl, and Cl^- ion,[6] we calculate the volume difference between the oxidized and reduced forms of cytochrome *c* at ambient pressure, $\bar{v}_{\text{ferrocyt}} - \bar{v}_{\text{ferricyt}} = -24$ mL/mol. This is a significant volume change between two redox states. Our result is consistent with those of structural investigations[9-11] and physical and chemical studies[12-17] that indicate that ferrocytochrome *c* has a more compact structure than ferricytochrome *c*.

Table I. Electrochemical Reaction Volumes

pressure (kbar)	ΔV° (mL/mol)	pressure (kbar)	ΔV° (mL/mol)
0	−27	3	−13
0.5	−25	3.5	−10
1	−23	4	−7.9
1.5	−20	4.5	−5.5
2	−18	5	−3.0
2.5	−15		

As the pressure is increased, the changes in potential and the volume changes that we observe include those of the equilibrium related to the reference electrode. However, the volume change of the reference electrode is negligible compared to that of the overall reaction and has been estimated[6] to be less than 1 mL/mol at 0 kbar and the pressure dependence to be less than 1 mV/kbar. The potential shift that we observe, +76 mV at 5 kbar, is much larger than this. Therefore, it reflects mainly the effect of compression on the cytochrome *c* electron-transfer process. This is certain in the low-pressure end of our present working range, where the ΔV° values for the overall reaction are greater than the ΔV° value of the reference electrode. At the upper end, the volumes involved become smaller and of the order estimated for the reference electrode, and we cannot discriminate one from the other. Nevertheless, near 5 kbar the difference in volume between ferri- and ferrocytochrome *c* becomes of the order of the experimental error and hence insignificant.

With the rate of change of ΔV° with pressure ($\partial \Delta V^{\circ}/\partial P = 4.9$ mL mol^{-1} kbar^{-1}), we also calculated the difference in the isothermal compressibility ($\Delta\beta$) between the reduced and oxidized forms of cytochrome *c*: $\Delta\beta \approx (-1/v')(\partial \Delta V^{\circ}/\partial P)_T = -5.1 \times 10^{-13}$ cm^2/dyne (-5.1×10^{-4} kbar^{-1}), where $v' = (1/2)(\bar{v}_{\text{ferrocyt}} + \bar{v}_{\text{ferricyt}}) = 9370$ mL/mol.[21]

Typical isothermal compressibilities calculated for globular proteins range between 5 and 15×10^{-12} cm^2/dyne.[18,19] Thus, the differential compressibility that we found amounts to less than 10% of the overall compressibility. This result is in reasonable agreement with estimates by Kharakoz and Mkhtaryan,[20] but is in contrast to the results of Eden et al.,[21] who report it to be as high as 40%. This apparent discrepancy may be explained in terms of the effect of the ionic strength on the structure of the protein.[17] Our $\Delta\beta$ and ΔV° values provide evidence that, at the high ionic concentration that we used, 0.1 M NaCl (pH 7), the solution structure of horse heart cytochrome *c* is for the most part preserved upon electron transfer. In addition, these results demonstrate that high-pressure electrochemical measurements of metalloproteins provide valuable thermodynamic information as well as insights into the structural (through ΔV) and dynamic (through compressibilities) differences between their oxidized and reduced forms.

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