Positive Activation Volume for a Cytochrome *C* Electrode Process: Evidence for a "Protein Friction" Mechanism from High-Pressure Studies

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The electron exchange kinetics of horse heart cytochrome *c* (Cyt *c*) at 4,4′-bipyridyl- and 4,4′-bipyridyl-disulfide-modified Au electrodes has been studied for the first time under variable pressure conditions (up to 150 MPa), by using fast scanning cyclic voltammetry. A positive activation volume of $+6.1 \pm 0.5$ cm^3 mol^{-1} was determined from the pressure dependence of the heterogeneous standard rate constant in both cases. This value is similar to that for the homogeneous Cyt *c* self-exchange process, predicted from the cross-reaction treatment. A careful analysis based on an extended version of the contemporary charge-transfer theory indicates that the process most probably takes place through an adiabatic ("protein friction") charge-transfer mechanism in which the positive volume of activation results from the pressure-induced increase of the protein's intrinsic viscosity (decrease of the characteristic relaxation mobility), which is also in remarkable agreement with earlier results from studies in which the viscosity was varied directly. This approach allows for variation of the internal protein viscosity without significant alteration of the properties (viscosity, diffusion coefficients) of the aqueous medium.

1. Introduction

Among alternative experimental approaches facilitating new insights into molecular charge-transfer mechanisms, high-pressure kinetic studies are of exceptional importance.[1,2] They provide unique information about the equilibrium and activation volumes of various processes, adding a new and important dimension to the development of fundamental understanding. Let us consider the electron self-exchange (homogeneous) and electrode-exchange (heterogeneous) electron-transfer reactions proceeding in the extreme adiabatic regime known also as the solvent controlled (or solvent friction) mechanism.[3,4] An adequate theoretical model for the corresponding intrinsic (unimolecular) rate constant is given by (see refs 3b,c,e for a present update):

$$k_{\mathrm{et(SF)}} = \nu_{\mathrm{eff}}\left(\frac{\Delta G_{\mathrm{r}}^{*}}{\pi^{3}RT}\right)^{1/2} \exp\left(-\frac{\Delta G_{\mathrm{a}}^{*}}{RT}\right) \qquad (1)$$

where ν_{eff} is a characteristic frequency for the polarization relaxation of the medium, which is coupled to the electron transfer, $\Delta G_{\mathrm{r}}^{*}$ and $\Delta G_{\mathrm{a}}^{*}$ are the reorganization and activation free energy parameters, respectively (vide infra), R is the gas constant, and T is the absolute temperature.

A dielectric continuum approximation is commonly used to express ν_{eff}. For a Debye-type solvent one finds that[3,4]

$$\nu_{\mathrm{eff}} = \nu_{\mathrm{L}} = \tau_{\mathrm{L}}^{-1} = \left(\frac{\epsilon_{\mathrm{s}}}{\epsilon_{\infty}}\right)\frac{RT}{3\eta V_{\mathrm{m}}} \qquad (2)$$

where τ_{L} is the longitudinal relaxation time of the solvent polarization, which is proportional to the Debye relaxation time and, thus, to the solvent (solution) viscosity, η; ϵ_{s} and ϵ_{∞} are static and high-frequency dielectric constants, respectively; and V_{m} is the molar volume. The model predicts the inverted power-law dependence of the intrinsic rate constant on the longitudinal relaxation time of the solution, $k_{\mathrm{et}} \propto \tau_{\mathrm{L}}^{-1}$, and hence, on the solution viscosity, $k_{\mathrm{et}} \propto \eta^{-1}$, in the high friction limit. In the case of non-Debye solvents with several quasidiffusional relaxation times, the effective frequency, ν_{eff}, can be connected with either of these (intrinsically coupled with electron transfer), or with a combination of these.[3d,e] In both cases this leads to viscosity control. As the quasidiffusional motion in the protein interior is similar to one in a viscous liquid, the same is true for the protein-implicated processes.[10,20a] A dependence of the type $k_{\mathrm{et}} \propto \eta^{-\delta}$ (in which δ varies within the range of $0 < \delta \leq 1$), is observed in an intermediate friction regime, or in some other complicated cases[5−7a−c,8] (vide infra). Experimentally, the solvent friction mechanism has been strictly verified for the outer-sphere electrode processes involving redox-active metal complexes and metallo-enzymes.[5−9] In these experiments the

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medium (solution) viscosity was varied by changing the solvent,[5,6] addition of viscous substances,[7,8] or variation of the hydrostatic pressure.[9] The latter approach, especially when applied in combination with one of the others, is most valuable, because it allows for a discrimination between charge-transfer mechanisms through the sign and magnitude of the activation volume without any change in the solution composition, or even the working solution[1,9] (vide infra). However, the high-pressure approach could seem less promising for the case of aqueous systems since the viscosity of water is little sensitive to the applied hydrostatic pressure[9] (vide infra).

The intrinsic mechanisms of biochemical redox processes are of special interest, especially with respect to the applicability of the contemporary adiabatic electron-transfer model (eqs 1 and 2, refs 10 and 11c,d). In particular, in the case of cytochrome *c* (Cyt *c*) as a representative small redox protein with a well-known molecular structure,[12] numerous studies on both its homogeneous[13−15] and heterogeneous[11,16,17] electron-transfer reactions have been performed. However, up to now the high-pressure kinetic studies were limited to homogeneous processes.[15] Previous high-pressure electrochemical experiments on Cyt *c* were performed at equilibrium only, to determine the redox reaction volume.[18] At the same time, a number of kinetic studies at ambient pressure, in which solution viscosity has been varied by adding viscous substances (usually sugars), have revealed a viscosity-dependent behavior for the observed electron-transfer rate constants.[11d,14a,d,e,g,16d] Most authors interpreted these results as a manifestation of a "conformationally gated" mechanism in which some large-scale (usually intermolecular, e.g., docking) conformational step (not coupled directly to the electron hopping) is rate determining.[14d,e,g,16d,f] On the contrary, some of us some time ago,[10a] on the grounds of the adiabatic charge-transfer model[3,4] (eqs 1 and 2), have argued that the high intrinsic viscosity of proteins (i.e. slow relaxational mobility, vide infra) may facilitate a manifestation of the adiabatic ("protein friction") mechanism at much larger electron-transfer distances (for weaker electronic interaction strengths) than is typically found for simple liquid-phase reactions. By way of illustration, we refer to bioelectrochemical systems that consist of a metal (Au) electrode, the mixed self-assembled monolayer films of variable thickness including inert diluent (CH_3-terminated) and "active" (pyridinal-terminated) groups, in which the latter specifically binds to the Cyt *c* heme.[11] Results of these and related studies[16] are in accord with the adiabatic ("protein friction") mechanism, implying direct coupling of the protein's fluctuational conformational motion with the electron-hopping process, rather than the "gated" mechanism (vide infra).

Redox proteins such as Cyt *c* differ from most redox-active metal complexes in that they are normally insoluble in solvents other than water. On the other hand, numerous experimental studies (including measurements of various structural-dynamic and physicochemical parameters,[10c,19a−d] and specific kinetic investigations[10,20a]), as well as molecular dynamics studies,[19e−g,20b,c] convincingly demonstrated that the protein interior, especially its peripheral vicinity around active sites, can be viewed as a liquidlike "droplet" of much higher viscosity than the surrounding aqueous medium.[19,20] The liquidlike nature of the protein's interior becomes quite obvious solely from the fact that the viscosity of the protein exterior may affect its internal viscosity (measurable as such[19d]).[10,20a] Of course, the characteristic relaxation time(s) of the protein interior is much larger than that of neat water.[20] It is well established that the viscosity of most liquid substances, including the polar, nonpolar, low and highly viscous liquids and their mixtures, except water, increase monotonically and roughly exponentially with increasing pressure, often doubling between 0.1 and 100 MPa.[9,21] For the tris(bipyridine)Co(III/II) [$Co(BP)_3^{3+/2+}$] electrode reaction in three different organic solvents, Swaddle et al.[9] reported from high-pressure kinetic studies positive volumes of activation ranging from +9 to +12 $cm^3\ mol^{-1}$, attributable mostly to the pressure-induced viscosity effect operating through eqs 1 and 2.[3,4] One may propose that for the aqueous protein systems, the applied hydrostatic pressure does not appreciably alter the viscosity of the solvent water, but should alter the intrinsic viscosity of the protein globule, properties which are essentially different from water, and should be more similar to any other viscous liquid or mixture of liquids different from water. Hence, in an appropriate bioelectrochemical kinetic experiment involving redox proteins under high pressure, one may expect the manifestation of the adiabatic ("protein friction") mechanism, even for an aqueous system, through an increase in the protein's intrinsic viscosity with pressure. The expected manifestation should occur via a positive and significant volume of activation. In the opposite case of the uncomplicated nonadiabatic (tunneling) mechanism, a negative volume of activation would be expected on the basis of the extended nonadiabatic charge-transfer model[9] (vide infra).

It should also be mentioned that the application of the high-pressure electrochemical methodology to biochemical redox processes seems more advantageous compared to the analogous homogeneous studies, since it does not require the participation of a redox partner other than the electrode. For cross-exchange processes, the intrinsic activation volumes are often masked by the large reaction volumes that result from the participation of a low molecular weight redox partner or other protein.[15] It is usually difficult from a methodological standpoint to directly determine the rate constant of the homogeneous electron self-exchange process with participation of the redox protein.[15] Presumably this is even more difficult to do at elevated pressures. On the contrary, biochemical electrode-exchange kinetics at high pressures can be readily studied by using a simple homemade high-pressure vessel and conventional electrochemical techniques as demonstrated below.

In the present study, we report for the first time high-pressure kinetic studies of Cyt *c* electrode reactions aimed at the elucidation of the electron exchange mechanism (via the activation volume) and the role of the coupled conformational fluctuations (relaxations) of the protein globule.

2. Experimental Section

Materials. Horse heart Cyt *c* was purchased from Sigma Chemical Co (100% purity based on H_2O content of 4%) and used either as received, by direct dissolving in 0.02 M Tris buffer (pH 7.0) containing 0.1 M $NaClO_4$ (Fluka), or after exhaustive ultra-filtration against the same buffer solution. The working concentration of Cyt *c* was 2.5−5 mg mL^{-1}, that is $(2-4) \times 10^{-4}$ M, throughout all the experiments. Both kinds of solution preparations gave essentially the same results. The 4,4′-bipyridyl (BP) and 4,4′-bipyridyl-disulfide (BPDS) compounds were purchased from Fluka and Acros, respectively. Other chemicals were from Fluka and were used as received. Millipore water, degassed with argon, was used throughout.

Instrumentation. The pressure vessel and electrochemical cell were similar to those described by Swaddle et al.[2b,22a] with the difference that the working electrode was a 2 mm ϕ gold disk (Metrohm, Type 6.1204.140), sealed in a Teflon cylinder. The working electrode together with the auxiliary electrode

(platinum wire of area much larger than that of the working electrode) and the reference electrode (Ag/AgCl) were sealed into the cell cap by two O-rings. To isolate the inner fluid (4 M KCl) from the working solution, the reference electrode was placed in a flexible plastic tube with a Vycor tip at the end, providing the electrical contact between these liquids. Pure *n*-hexane was used throughout as a pressurizing liquid to minimize parasitic currents outside the cell. The working volume of the high-pressure electrochemical cell was 5 mL. The assembled pressure vessel containing the cell was placed in a thermostated water jacket equilibrated at 25.0 ± 0.1 °C. The working disk electrode was cleaned before each pressure cycle experiment by polishing with alumina slurry (3 and 0.5 μm granule sizes) on a Buehler polishing pad, rinsed with Millipore water, and modified by immediate dipping in solutions containing 10^{-2} M BP or 10^{-3} M BPDS for ca. 10 min.[17a,18b]

The fast scanning cyclic voltammetry (CV) measurements were performed with a PAR 273 instrument controlled by Mode 270 software, with pressure steps of 25 MPa (in total, under 8 different pressure conditions), and a maximum hydrostatic pressure of 150 MPa was applied. Care was taken that the results obtained at the lowest pressure at the start and the end of the pressure cycle were in good agreement.[2b,9,18b] In the present investigations, preference was given to the fast scanning CV (Nicholson) method for the following reasons: A crucial point to obtain reproducible electrochemical kinetic data is a condition (cleanness) of the working electrode surface that usually becomes worse (leading to the degradation of kinetic characteristics) during the electrochemical experiments. Since the high-pressure studies do not allow cleaning of the electrode before each series of CV scans (at a given pressure), minimization of the overall pressure cycle time was essential. The fast-scanning CV method[22] allows the simultaneous determination of the electrode surface condition, as well as almost all necessary parameters from the same experiment by a variation of the CV scan rate only, which reduces the overall experiment time (pressure cycle with 8 different pressure conditions) to ca. 4 h only (including a period of 20 min required for temperature equilibration between each newly adjusted pressure). We did not perform any corrections for a parasitic peak shift due to the uncompensated resistance of the electrochemical cell, since recent impedance experiments[11b] indicated that under experimental conditions similar to ours (but at an even lower ionic strength), this kind of resistance may amount to 300−500 Ω only, which leads to a negligible maximum potential shift of ≤2 mV (at the highest currents exploited). In our case such a shift should be much smaller. The essential similarity of our results obtained at different Cyt *c* concentrations is in good agreement with this conclusion. In any case, the uncontrolled potential shift due to the uncompensated resistance could not lead to the pressure-induced changes of the heterogeneous rate constant observed in this work (section 3), since this would require a change of I_p with pressure, which can be excluded due to the essential pressure independence of *D* (vide infra).

3. Results

Several series of kinetic experiments for the Cyt *c* electrode reaction under different pressure conditions were performed with both BP and BPDS as electrode modifiers. The cyclic voltammograms were recorded at potential scan rates between 0.01 and 8.0 V s^{-1}. Within the range from 0.01 to 0.5−1.0 V s^{-1} (depending on the applied pressure) the position of the peak maximum and the peak-to-peak separation were unchanged. The latter was close to 60 mV (actually 64 ± 4 mV), indicating that the process was nearly reversible according to theory.[23] Typical CV curves for the case of the BPDS-modified Au working electrode, at $P = 150$ MPa, and lower potential scan rates are shown in Figure 1. The slight effective deviations of the peak-to-peak separation from the theoretical value, 59 mV,[23] when using the high-pressure electrochemical cell (mounted into the pressure vessel), can be explained on the basis of somewhat more noisy electrochemical signals (due to the considerable complexity of the high-pressure cell environment) compared to the conventional electrochemical cell. With the latter as a reference system, the peak-to-peak separation normally was 61 ± 2 mV for both modifiers, in agreement with the "best" published values (vide infra).[17]

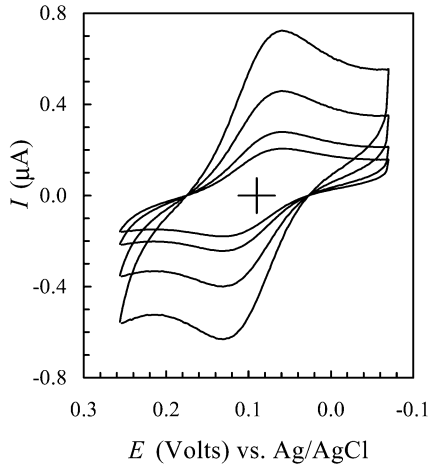

Figure 1. Cyclic voltammograms for the Cyt *c* (3 mg mL^{-1}) redox process at a BPDS-modified Au electrode in 0.1 M $NaClO_4$ + 0.02 M Tris buffer (pH 7.0) + 10^{-3} M BPDS, $P = 150$ MPa. Scan rates: 0.01, 0.02, 0.05, and 0.1 V s^{-1}. Potential vs Ag/AgCl reference electrode.

The peak midpoint (formal) potential for the Cyt *c* redox reaction was ca. 50 mV (versus the Ag/AgCl reference electrode), and increased gradually to ca. 90 mV at $P = 150$ MPa, in excellent agreement with earlier results.[18b] This shift results in a reaction equilibrium volume for the oxidation of Cyt *c* amounting to +5.0 cm^3 mol^{-1} (vide infra).[18c]

The values of background-corrected reductive peak currents, $I_{p(corr)}$, plotted versus the square root of the potential scan rate, υ (at the lower scan rates as indicated above), in all cases displayed straight lines intercepting the origin, Figure 2. Together with the above-mentioned behavior, this is a direct indication that the process is diffusion limited and electrochemically reversible at low scan rates.[23] Thus, from the slopes of these dependencies (or the representative values) the diffusion coefficient of Cyt *c* can be calculated by using eq 3:[23c,d]

$$I_{p(corr)} = -0.446nF\left(\frac{nF}{RT}\right)^{1/2} C_o D^{1/2} \upsilon^{1/2} \qquad (3)$$

where *n* is the number of transferred electrons (here $n = 1$), *F* is the Faraday constant, C_o is the reactant (Cyt *c*) concentration in the bulk, and *D* is the diffusion coefficient of the reactant. The calculated values of *D* for Cyt *c* were similar in all cases, (8.0 ± 0.5) × 10^{-7} cm^2 s^{-1}, and in good agreement with the "best" values from the literature.[17a,e,f] Note the independence of *D* on the applied pressure within the range 0.1−150 MPa, which is also in agreement with the approximate independence of the solvent viscosity on *P* (according to the Stokes−Einstein equation, see ref 10c, e.g.). This becomes quite visible from an inspection of Figure 2. An essentially similar result was obtained

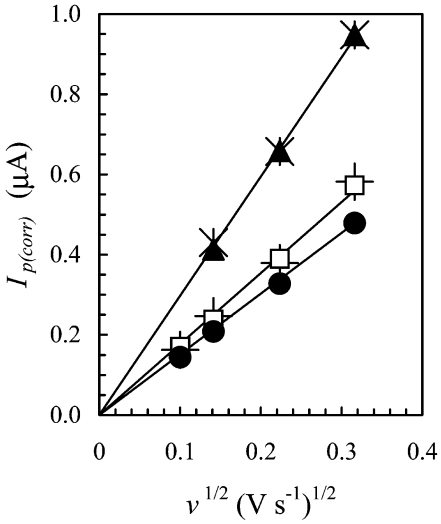

Figure 2. Typical dependencies for the background-corrected peak current versus the square root of the scan rate (within 0.01−0.1 V s^{-1}) obtained at different Cyt *c* concentrations under ambient and the highest pressure conditions: (circles) P = 0.1 MPa, C = 2.5 mg mL^{-1}; (squares) P = 0.1 MPa, C = 3 mg mL^{-1}; (crosses) P = 150 MPa, C = 3 mg mL^{-1}; (triangles) P = 0.1 MPa, C = 5 mg mL^{-1}; (asterisks) P = 150 MPa, C = 5 mg mL^{-1}.

TABLE 1: Typical CV Experimental Series Providing the Determination of k^o_{el} According to Eq 4,[23] at P = 150 MPa[a]

v (V s^{-1})	ΔE_p (mV)	Ψ	k^o_{el} (10^{-3} cm s^{-1})
3	105	0.50	8.53
4	110	0.42	8.27
5	114	0.38	8.37
6	118	0.36	8.69
7	126	0.32	8.34
average			**8.44**

[a] Protein concentration 3 mg/mL, BP-modified Au electrode in 0.1 M $NaClO_4$ + 0.02 M Tris buffer (pH 7.0) + 10^{-3} M BP.

by Swaddle et al.[9a,b] for the $Co(BP)_3^{3+/2+}$ diffusion coefficient in aqueous media while studying the corresponding electrode process.

At higher scan rates, $v \geq 1.0$ V s^{-1}, at ambient pressure (and $v \geq 0.5$ V s^{-1}, at the highest pressure applied), the CV peak-to-peak separation increased gradually, indicating the onset of a kinetic factor.[23] In this regime, according to the method of Nicholson,[23] the heterogeneous standard rate constant, k^o_{el}, can be determined from eq 4:

$$\Psi = \frac{(D_O/D_R)^{\alpha/2}(RT)^{1/2}k^o_{el}}{(\pi nFD_O v)^{1/2}} \quad (4)$$

in which, for the diffusion coefficients of the oxidized and reduced forms, it was assumed that $D_O = D_R = D$, and for the charge-transfer coefficient, $\alpha = 0.5$,[17d,f] by directly using the numerically evaluated theoretical relationship between the peak-to-peak separation, ΔE_p, and the Ψ function. Table 1 illustrates the procedure for the determination of k^o_{el} through the values of ΔE_p and Ψ. For a given P, several scans at different v were performed and the average value at each P was determined to improve the statistics. At high scan rates, the signal noise normally increases due to instrumental factors (Figure 3a).[11,17] To achieve higher accuracy in the determination of k^o_{el}, the curve smoothening procedure available in the Mode 270 software was usually applied (Figure 3b), enabling a peak potential determination with an accuracy of ±1 to 2 mV. However, the CV curve

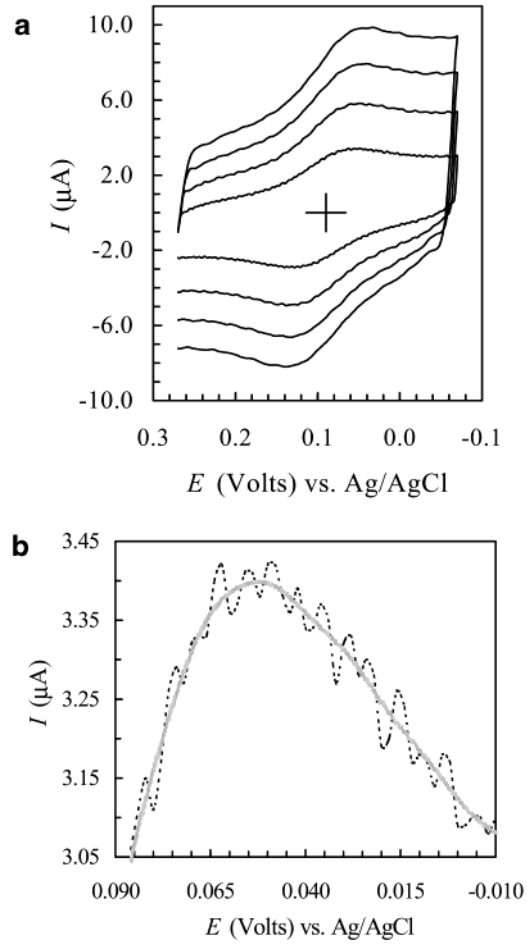

Figure 3. (a) Cyclic voltammograms (raw data) for the Cyt *c* (3 mg mL^{-1}) redox process at a BPDS-modified Au electrode in 0.1 M $NaClO_4$ + 0.02 M Tris buffer (pH 7.0) + 10^{-3} M BPDS, P = 150 MPa. Scan rates: 1, 2, 3, and 4 V s^{-1}. Potential vs Ag/AgCl reference electrode. (b) Strongly enlarged fragment of the inner curve of part a (taken at a scan rate of 1 V s^{-1}) before and after the curve smoothening procedure (illustration, see text for details).

refinement procedure does not affect the accuracy of the calculated kinetic constants notably. Certainly, the main result does not change whether this procedure is applied or not. It should also be mentioned that even if some systematic inclination occurs that will lead to an error in the determination of the absolute values of k^o_{el} (which is obviously not large in our case), the method still allows for the accurate determination of the relative changes caused by the variation in pressure, as has also been demonstrated earlier in ref 22c. Different series of experiments on the pressure dependence of the heterogeneous standard rate constant for the Cyt *c* electrode reaction yielded essentially similar results, all indicating the gradual decrease in k^o_{et} with increasing P. The values of k^o_{et} at ambient pressure for both BP and BPDS as electrode modifiers were the same within the experimental uncertainty. The average value of this standard rate constant from all the experimental measurements under these conditions was $k^o_{et} = (1.4 \pm 0.2) \times 10^{-2}$ cm s^{-1}, which is also in good agreement with the "best" values quoted in the literature.[17b,c] This value decreased in each high-pressure cycle by about 1.4−1.5 times at the highest applied pressure (150 MPa), yielding the average volume of activation of +6.1 ± 0.5 cm^3 mol^{-1} calculated for each cycle according to eq 5:[1,2]

$$\Delta V_a = -RT\left[\frac{\partial(\ln k^o_{el})}{\partial P}\right]_T \quad (5)$$

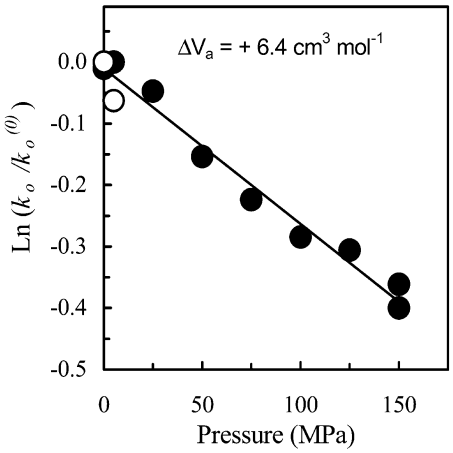

Figure 4. Logarithm of the relative experimental (standard heterogeneous) rate constant versus the applied pressure for the Cyt *c* redox process at the BP-modified Au electrode. Closed symbols (circles) indicate measurements recorded on increasing the pressure. Open symbols indicate control measurements at the end of the pressure cycle.

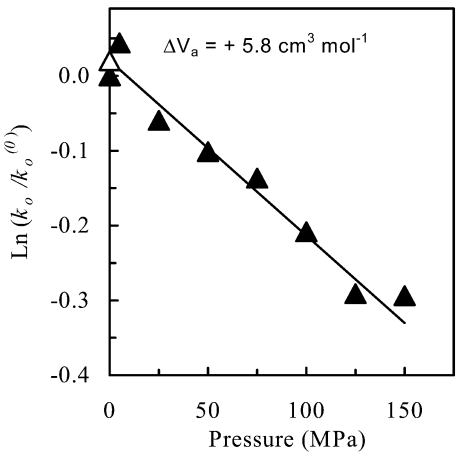

Figure 5. Logarithm of the relative experimental (standard heterogeneous) rate constant versus the applied pressure for the CytC redox process at the BPDS-modified Au electrode. Solid symbols (triangles) indicate measurements recorded on increasing the pressure. The open symbol indicates control measurement at the end of the pressure cycle.

Figures 4 and 5 represent typical dependences of the logarithm of the reduced rate constants, $k^{o}_{el}/k^{o}_{el}{}^{(0)}$, where the denominator indicates the value of k^{o}_{el} at ambient pressure within the same pressure cycle.

In summary, despite some scatter in the kinetic data amounting to ca. 20% for totally independent experiments (including a new preparation of the electrodes and solutions employed), the reproducibility of the determination of k^{o}_{el} within the given cycle was better than 5% (at each pressure value including the control experiments following the release of pressure), indicating a good reliability for the reported activation volume.

4. Discussion

The experimentally determined standard heterogeneous rate constant, in the framework of the conventional encounter preequilibrium model, can be written as[4e,f,g,5,24]

$$k^{o}_{el} = K_A k^{o}_{et} \tag{6}$$

where K_A is a statistically averaged equilibrium constant proportional to the probability of finding the reactant species at the active site near the electrode. For the adiabatic mechanism, k_{et} is defined by eq 1, and for the case of the nonadiabatic (tunneling) mechanism, by eq 7:[3c,e,23c,25]

$$k_{et(NA)} = \frac{(H_{if})^2}{\hbar}\rho_m\left(\frac{\pi^3 RT}{\Delta G_r^*}\right)^{1/2}\exp\left(-\frac{\Delta G_a^*}{RT}\right) \tag{7}$$

where H_{if} is the electronic coupling matrix element, and ρ_m is the density of electronic states in the metal (electrode). In the nonadiabatic regime, the intrinsic rate constant, eq 7, depends exponentially on the charge-transfer distance (through the parameter H_{if}), and is independent of the solution viscosity.[8b,11c,d] It is commonly accepted in the literature that the two parameters, ΔG_r^* and ΔG_a^*, appearing in eq 1 or 7, are connected through the simple relationship, $\Delta G_a^* \approx {}^1/_4\Delta G_r^*$. The exact expression[24c,25] behind this formula is

$$\Delta G_a^* = \frac{(\Delta G_r^* - \Delta G_o^*)^2}{4\Delta G_r^*} - H_{if} \tag{8}$$

Equation 8 indicates that when the values of ΔG_r^* and H_{if} are comparable and ΔG_o^* is zero, the simple "one-fourth" rule is valid.[8b] For k^{o}_{el}, and hence for k^{o}_{et}, ΔG_o^* is zero (the present case) and $\Delta G_a^* \approx {}^1/_4\Delta G_r^* - H_{if}$.

The volume of activation, determined from the pressure dependence of the experimental heterogeneous rate constant by eq 5, may in general terms originate from the pressure dependence of the parameters K_A, $\Delta G_r^*(\Delta G_a^*)$, $\tau_L(\eta)$, or H_{if}. The immediate inference that emerges on the grounds of the recent landmark work by Swaddle et al.[9] is that the obtained positive volume of activation should be directly connected with the viscosity control of the Cyt *c* redox reaction at the electrode (as revealed by the experiments employing solutions with viscous additives[11d,16d]). Further detailed analysis confirms this suggestion. It was argued in previous work[9a,b] that the preequilibrium term K_A, which is largely determined by the structure of the electrical double layer near the electrode surface, should not be notably pressure dependent within the applied pressure range, i.e., should not contribute appreciably to the observed volume of activation. Such a contribution, if the case, should be connected with some squeezing (shrinking) of the reactive system, directly or indirectly facilitating the intrinsic charge-transfer event (via the higher value of H_{if}., eqs 7 and 8, see ref 26d), and be manifested as a negative contribution to the overall effective volume of activation.[26d] Meanwhile, the gated mechanism, if considered to be viscosity sensitive through the preequilibrium term, K_A,[16d] can also give rise to the positive activation volume, formally indistinguishable from the "true" kinetic (adiabatic) value. As already mentioned above, for the heterogeneous Cyt *c* systems of interest, the gated mechanism has been excluded on the grounds of detailed analysis given elsewhere.[11c,d] This is quite obvious, however, also from the elementary analysis of solely the nature of the present electrochemical data—the classical shape: no additional peaks on the CV curves indicative of pre- or post-adsorption at the electrode, the zero intercept for the linear dependence of I_p on $\upsilon^{1/2}$, excluding the involvement of kinetic steps other than outer-sphere electron transfer,[23] in Figures 1−3 (see also further discussion below). Hence, most probably, the observed positive value cannot originate from the preequilibrium factors. Therefore, we only consider further the terms originating from the intrinsic charge-transfer mechanism. In addition, we will restrict our analysis to the adiabatic mechanism only. The processes occurring at the metal electrodes all seem to be essentially

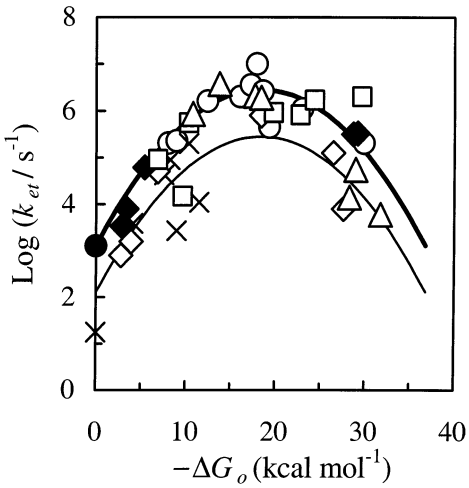

Figure 6. The Marcus-type plot shows the free energy dependence of the Cyt *c* unimolecular electron-transfer rate constant from a number of different studies on both homogeneous and heterogeneous electron transfer. The solid diamonds represent "homogeneous" processes that exhibit a dependence on the external solution viscosity, refs 14a,d−g. The solid circle shows the unimolecular electrochemical electron-transfer rate constant at short distances which also displays a viscosity dependence.[11d,16d] Other data are from refs 13a−c and 14b,c.

adiabatic (except for some specific cases[16f]), operating through eqs 1 and 2, rather than eq 7.[3−10,11c,d] We note, however, that the contribution due to the parameter H_{if}, when originating from the preexponential factor (nonadiabatic mechanism, eq 7), like one due to K_A, should be small and negative, and cannot explain the present result.

When considering the most probable adiabatic mechanism, the contributions from the preexponential factor (through τ_L, which is proportional to η) and the exponential factor (through ΔG_a*, which is equivalent to ΔG_r*) should be strictly discriminated. In the adiabatic regime, the preexponential term represents an "effective" low-frequency mode (formally it can be a single mode) that is dynamically coupled to the charge-transfer step. In contrast, the term ΔG_r* is a cumulative sum of all the low- and high-frequency modes that are coupled to charge transfer through the Franck−Condon principle and contribute in a weighted manner.[25] The term ΔG_r* contains the inner- and outer-sphere components, $\Delta G_r^* = \Delta G_{r(in)}^* + \Delta G_{r(out)}^*$.[24] Recent theoretical studies of the reorganization energy in Cyt *c* indicated that the inner-sphere (heme) contribution is less than 2 kcal mol^{-1} [26a] and that the protein's "outer sphere" (interior) contribution is about 12−16 kcal mol^{-1}, with the remainder (4−5 kcal mol^{-1}) associated with the solvent.[26b,c] Figure 6 displays the dependence of log k_{et} on ΔG_o*, including the monomolecular (intrinsic) rate constants for a broad variety of processes with the participation of Cyt *c*, in which Cyt *c* was attached either to other redox-active (partner) proteins,[14a,b] metal complexes,[13,14c−g] or to the SAM-modified metal electrodes.[11,16] One can see that the major part of kinetic data can be represented by a single bell-shaped dependence constructed on the basis of eqs 1 and 8, using a single value for reorganization free energy, ΔG_r = 18 kcal mol^{-1}, and a single value for the "effective" characteristic relaxation time, $\tau_{eff} = 1.9 \times 10^{-7}$ s, a typical value for the interior of globular proteins.[20] This value nicely resembles the one detected for the longest relaxation time of 3.5×10^{-7} s (room temperature, 60% glycerol solution) coupled to the electron transfer from the Cyt *c* subunit to the bacteriochlorophyll dimer in *Rps. sulfoviridis*.[20e] The rate constants for the processes tested for, and found to be viscosity controlled, are indicated by solid symbols (including the value for the unimolecular electrode process at zero overvoltage, $\Delta G_o^* = 0$,[11] see the corresponding figure caption). All other processes, although not directly tested vs the viscosity effects, can be assumed to be adiabatic also or marginally adiabatic, as can be judged on the grounds of a novel charge-transfer distance analysis.[11c,d] In light of this analysis, also a reconsideration of the existing database for the electron-transfer processes in natural complexes of Cyt *c* in favor of the adiabatic mechanism can be expected (in support of this view, e.g., see refs 14a and 20e). Note that the bell-shaped character of this dependence again confirms the "true" charge-transfer feature of the Cyt *c* processes under consideration, rather than the gated mechanism, for which, normally, the rate constant should be independent of ΔG_o*.[29] As one can see, the nature of the Cyt *c* redox partner is of minor importance in determining the value of ΔG_r* (ΔG_a*) for these processes. This generality may arise because the intrinsic reorganization of the protein interior of Cyt *c*, in contrast to earlier expectations, is relatively large compared to the aqueous medium or the partner redox enzymes.[26b,c]

At the same time, theoretical estimates of the volume effect originating from the Franck−Condon factor (reorganization energy appearing in the exponential term) associated with the protein interior indicate a very small negative contribution.[26d] For simple outer-sphere electron-transfer processes, typical volume effects (arising from the pressure dependence of dielectric properties of water) were found to be negative and of the order of −4 to −7 cm^3 mol^{-1}.[22,27] The observed activation volumes for the "simple" outer-sphere electrode charge-transfer processes studied in aqueous solutions (the essential point, vide infra) including eight redox-active metal complexes were all found to be negative, ranging within −1.6 to −9.1 cm^3 mol^{-1},[22] in satisfactory agreement with the theoretically predicted values. A very special point is that in the case of an adiabatic mechanism, the pressure dependence of the parameter τ_L, and its macroscopic equivalent, η, may show up as a large positive volume of activation, provided that the process takes place in a medium different from aqueous solution. The above-mentioned electrode processes in aqueous media all exhibit negative volumes of activation, since the adiabatic nature cannot show up through the change in viscosity.[9] The positive volumes of activation observed for the electrode reactions of cyano metal complexes are due to other specific effects, viz. strong ion-pairing with alkali-metal counterions participating in the activation process.[8a,b,22] Interestingly, the volume of activation changes from +7.3 to −4.4 cm^3 mol^{-1}, when going from the Na^+ to tetraethylammonium containing electrolyte for the $Mo(CN)_8^{3-/4-}$ electrode process,[22] indicating the onset of a "normal" outer-sphere pattern.

As a reference redox system for Cyt *c*, let us consider in more detail the $Co(BP)_3^{3+/2+}$ couple extensively studied by different authors.[6a,b,9a,b] This system, studied in aqueous solution under high pressure, exhibited a negative volume of activation, $\Delta V_a = -8.6$ cm^3 mol^{-1}, in satisfactory agreement with the theoretical values (vide supra). In contrast, in organic solvents (acetonitrile, acetone and propylene carbonate) the activation volumes were essentially positive, 9.1, 10.2, and 12.2 cm^3 mol^{-1}, respectively (Table 2).[9a,b] The latter values can be readily attributed to the manifestation of the pressure dependence of η, contributing through the adiabatic mechanism, eqs 1 and 2. This conclusion nicely agrees with the observation of viscosity control for this process, through the variation of η by the change in solvent composition.[6a,b]

TABLE 2: Comparison of High-Pressure Kinetic Results for the $Co(BP)_3^{3+/2+}$ and Cyt *c* Electrode Reactions in Different Media

redox system	medium	ΔV_a, $cm^3\ mol^{-1}$	ref
$Co(BP)_3^{3+/2+}$	organic solvents (3)	$+10.7 \pm 1.5$	9a
$Co(BP)_3^{3+/2+}$	H_2O	-8.6	9a
Cyt *c* (electrode)	protein + H_2O	$+6.1 \pm 0.5$	this work
Cyt *c* (self-exchange)	protein + H_2O	+7.0 (calcd)	28a

Returning now to the Cyt *c* system, the fundamental details revealed by this work and elsewhere can be summarized as follows:

(a) The volume of activation for the electrode process from the present study is positive and significant, viz. $\Delta V_a = +6.1$ $cm^3\ mol^{-1}$.

(b) This positive volume of activation cannot be caused by the counterion reorganization effects as were specifically found for the cyano metal redox processes.[2b,22] For the latter it is due to a very specific charge distribution and redistribution on the periphery of cyanocomplexes,[7d,8c] and has not been mentioned for any other electrochemical redox processes.[2b,9a,b] In addition, most recent experiments revealed a very weak effect of ionic strength on heterogeneous Cyt *c* charge transfer, indicating a minor dynamic role of the ionic medium in this process.[11d]

(c) The electrode processes involving Cyt *c* attached to Au-coated SAMs (the case of thin SAMs) with two different modes of binding were shown to be viscosity controlled when viscosity was varied by adding sucrose to the aqueous electrolyte.[11d,16d]

(d) The above-mentioned electrode processes involving Cyt *c* are not conformationally gated, but rather the "true" charge-transfer processes occurring in the adiabatic regime. This is quite evident from the specific nature of the electrochemical response (present work and ref 11) and the detailed analysis of experimental results on the viscosity, solvent isotope, and SAM composition effects analyzed in detail in ref 11d.

(e) The observed volume of activation of $+6.1$ $cm^3\ mol^{-1}$ cannot be influenced by the reaction equilibrium volume for the oxidation of Cyt *c* amounting to $+5.0$ $cm^3\ mol^{-1}$ (which trivially corresponds to -5.0 $cm^3\ mol^{-1}$ for the Cyt *c* reduction). This activation volume is related to the standard intrinsic rate constant (determined at the standard equilibrium potential at which the oxidation and reduction rate constants are essentially the same). Under these conditions the equilibrium volumes of these partial (half) reactions do not show up in the activation volume (they eliminate each other whatever their values are). This fundamental conclusion is true as in the case of any other redox reaction involving complex ions, e.g., thoroughly listed elsewhere.[2b,22]

From a consideration of all these points, it follows that the observed volume of activation of $+6.1$ $cm^3\ mol^{-1}$ is caused by the adiabatic mechanism of electron transfer, and originates from the pressure-dependent change of internal viscosity (relaxational characteristics) of Cyt *c*, which is coupled to electron transfer via eqs 1 and 2. Such a conclusion seems logical in light of the well-known fact that the viscosity of all the solvents including protic, aprotic, low and highly viscous liquids, except water, and their mixtures, is essentially pressure-dependent. The calculated (high-side) pressure-induced viscosity-related activation volume is $+20$ $cm^3\ mol^{-1}$.[9c] Obviously, a negative contribution of up to $-(6\ to\ 10)$ $cm^3\ mol^{-1}$ (by absolute value) may counterbalance this value.[2b,22,27] The resulting volumes of activation for a $Co(BP)_3^{3+/2+}$ couple in nonaqueous media are of the order of $+10$ $cm^3\ mol^{-1}$ (Table 2), as already mentioned.[9a,b] Approximately the same (or similar) should be true for the case of Cyt *c* if it is considered as a small droplet of a viscous liquid, essentially different from water (vide supra).[19,20] Corrections for the minor terms could be applied, but seem to be of little purpose since they are small and tend to counterbalance each other (hence, do not change the essential conclusion).[9a,b]

Interestingly, for a homogeneous self-exchange reaction of Cyt *c*, the activation volume extracted from the cross-reaction volumes by using the Marcus−Stranks approach was found to be $\Delta V_a^{(hom)} = +7$ $cm^3\ mol^{-1}$.[28a] The similarity with the value found in the present study is striking,[28b] taking into account that this value mostly originates from the preexponential term, being negligible for the Franck−Condon factor due to the protein interior (the activation volume contribution from the Franck−Condon factor of an aqueous medium should be negative, vide supra, but its contribution is small within the total ΔG_r); hence the so-called "fifty-percent rule" based on a consideration of activation volumes related to the Franck−Condon (exponential) factors of homogeneous and electrode processes[2b,22] is not directly applicable here.

In conclusion we note that the activation volume for the electron exchange process of the redox protein, Cyt *c*, at the modified metal electrode has been studied for the first time. An extended comparative analysis revealed both the similarities and fine differences between the elementary charge-transfer processes involving low molecular weight redox-active metal complexes and this redox protein. In the latter case, by applying hydrostatic pressure, tuning of the protein's internal viscosity is possible without a notable altering of the external viscosity of solvent water, and hence the protein's diffusive properties therein.

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