Charge Transfer

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High-Pressure Probing of a Changeover in the Charge-Transfer Mechanism for Intact Cytochrome *c* at Gold/Self-Assembled Monolayer Junctions**

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High-pressure (HP) kinetic studies provide unique information about the activation volumes for various processes and add a new dimension to the development of fundamental mechanistic understanding.[1–3] Application of this technique along (or in combination) with other kinetic approaches also seems to be promising for biological processes, including charge-transfer (CT) reactions.[4–6] However, even for small and well-characterized proteins such as cytochrome *c* (CytC),[7,8] an intrinsic CT mechanism in "homogeneous" systems is difficult to recognize conclusively as a consequence of the extra structural and dynamic environmental complexity introduced by the participating redox partner.[4,5,9–11] The covalent attachment of "small" complex ions as the redox counterparts at different external sites of CytC[12,13] does not warrant sufficiently smooth variation of intrinsic parameters, such as an electronic coupling (correlated with the CT distance; see below), because of the highly inhomogeneous nature of the protein interior. In this context, artificial bioelectrochemical devices made of electrode-deposited self-assembled monolayer (SAM) films of variable composition and thickness, and CytC or other redox proteins attached or freely diffusing to the SAM terminal groups (also subject to wide variations), were proven to be systems with well-controlled variable parameters, and hence suitable for fundamental studies[8,14–18] and some technological applications.[7,8]

Previous work that exploited SAM-implicated bioelectrochemical systems revealed kinetic behavior and a distinct change in mechanism with the CT distance for cases with CytC irreversibly attached (probably heavily altered in the native structure) to SAM terminal groups.[15–18] Only fragmentary work has been performed for totally intact CytC freely diffusing to electrode/SAM junctions,[8,19,20] which includes our pioneering HP kinetic study of CytC at "primitively" modified Au electrodes.[6] In the latter work, however, it was demonstrated for the first time that the application of hydrostatic pressure allows a variation of the internal viscosity (conformational mobility) of the protein without significant variation of the properties of the aqueous medium.

Herein, we report a changeover in the intrinsic mechanism for freely diffusing, unbound CytC as clearly observed in HP kinetic experiments, which reveal an evident trend in the activation volume from essentially positive (for short-range CT) to essentially negative (for long-range CT) values through an intermediate zero magnitude. By exploiting bioelectrochemical devices that comprise CytC weakly interacting with hydroxy-terminated alkanethiol SAMs of variable thickness ($[-S-(CH_2)_n-OH]$, where $n = 2$, 3, 4, 6, 11) deposited on Au disk electrodes, we took advantage of independent and gradual variation of the electron-transfer (ET) distance (directly affecting electronic coupling[12,13,21–23]), hydrostatic pressure (directly affecting the intrinsic friction of the protein[6,24]), and solution viscosity (externally affecting the intrinsic friction of the protein,[25–27] and applied here for the purposes of cross-testing). Fast-scan cyclic voltammetry (CV)[28,29] was applied in most cases (for $n = 2$, 3, 4, 6) to determine the heterogeneous standard rate constant. This method has been proven to be fully adequate for both the determination of the physical condition (structural accomplishment) of composite systems and the accurate determi-

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nation of dynamic characteristics, such as heterogeneous kinetic constants and diffusion coefficients, under variable experimental conditions.[6,8,30,31] The methods for preparation of the SAM-modified electrodes,[19,20] the instrumentation,[6] and the HP setup[2,6] were similar to those reported elsewhere, and are also briefly described in the Supporting Information.

For freely diffusing reactant species such as CytC, the experimentally determined standard heterogeneous rate constant is obtained at zero overvoltage (zero driving force) within the framework of the conventional encounter pre-equilibrium model with the accuracy of a scaling factor, that is, the pre-equilibrium term that could be assumed to be constant within the series of SAMs with identical terminal groups.[6,8,31–34] This heterogeneous rate constant can be associated with the intrinsic unimolecular rate constant representative of either nonadiabatic (long-range) or adiabatic (short-range) CT processes.[6,31] Theoretical work that accounts for both mechanisms and the smooth changeover between them is available.[35–40] The recently updated[18] expression is shown in Equation (1), where H_{if} is the

$$k_{ET}^{0} = \frac{(H_{if})^2}{\hbar}\frac{\rho_m}{1+g}\left(\frac{\pi^3 RT}{\lambda}\right)^{1/2}\exp\left(-\frac{\Delta G_a}{RT}\right) \quad (1)$$

electronic coupling matrix element, λ is the reorganization free energy, and ρ_m is the density of electronic states in the metal (electrode). The activation free energy under the experimental conditions of the present work can be defined as $\Delta G_a \approx \lambda/4$.[6,8,22,30,31]

The adiabaticity criterion g, which acts as a control parameter, is given by Equation (2),[18,34,36,39,40] where the

$$g = \frac{\pi^3 RT(H_{if})^2\rho_m}{\hbar\nu_{eff}\lambda} \quad (2)$$

effective frequency ν_{eff} is related to a single or several relaxation process(es) in the vicinity of the reaction zone, which are intrinsically coupled to electron transfer (actually, $\nu_{eff} \approx \eta$, where η is the effective viscosity of the medium[35–40]). With either $g \ll 1$ or $g \gg 1$, which depends on the values of intrinsic parameters from Equation (1) (especially of H_{if}, the value of which can be varied greatly in our experiments), one arrives at two different expressions for the intrinsic rate constant. These equations include phenomenological extensions for the long-range CT [Eq. (3)], where R_e is the CT

$$k_{ET} \propto \exp[-\beta(R_e - R_0)] \quad (3)$$

distance and β is the decay parameter, which is normally of the order of about 1 $Å^{-1}$,[12–18,23,31] and for the short-range CT [Eq. (4)], where δ is an "empirical" solvent–protein coupling

$$k_{ET} \propto \eta^{-\delta} \quad (4)$$

parameter with values between 0 and 1, and $\delta \approx 1$ represents full solvent–protein coupling.[25–27,32,35–40]

A general expression for the activation volume of any kind of microscopic barrier-crossing process, including ET, can be defined as shown in Equation (5).[1–3] By substitution of

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

Equations (1) and (3), and omitting minor terms, one obtains Equation (6).[41–43] This equation indicates that the experimentally measurable volume of activation [Eq. (5)] may originate from the effects of pressure on the ET distance as a result of shrinking of the reactive (SAM/protein) system and/or a change in the medium (SAM/protein/solvent) reorganization energy (Franck–Condon factor).

$$\Delta V_{a(NA)} = \beta RT\left(\frac{\partial R_e}{\partial P}\right)_T + \frac{1}{4}\left(\frac{\partial\lambda}{\partial P}\right)_T \quad (6)$$

On considering the effect of pressure on the adiabatic, viscosity-dependent ET process [Eqs. (1) and (4)], and again omitting the minor terms, one arrives at Equation (7),[3,44]

$$\Delta V_{a(AD)} = \beta RT\left(\frac{\partial\ln\eta}{\partial P}\right)_T + \frac{1}{4}\left(\frac{\partial\lambda}{\partial P}\right)_T \quad (7)$$

from which it follows that in the case of full dynamic control [Eq. (4); $\delta \approx 1$], viscosity changes arising from increasing pressure may result in a large positive contribution provided that viscosity is affected by pressure. This is the case for all known liquids except water.[3,6,44] Actually, for most solvents, η increases exponentially with pressure and yields a maximum net viscosity-related contribution as high as $+20\ cm^3\ mol^{-1}$ (as the upper limit[3,44]).

In addition, we assume that for composite multicomponent systems, as in the present case, the overall medium reorganization energy can be roughly reproduced by summation over individual components [Eq. (8)],[45–47] where the

$$\lambda = \lambda_{solv} + \lambda_{prot} + \lambda_{SAM} \quad (8)$$

three contributions to λ represent the solvent, protein, and SAM interior, respectively (see below).

Figure 1a shows the dependence of the experimental standard heterogeneous rate constant, k_{ET}^{0}, on the number of SAM methylene units. The matching numerical values, the data obtained at different concentrations of glucose (0, 100, 200, 300, and 400 $g\,L^{-1}$), and the corresponding viscosity values are available in the Supporting Information. The upper curve, clearly distinguishable for SAMs with $n = 2$, 3, and 4, represents kinetic data obtained in the absence of a viscous additive, and the two other curves that merge with the former one at $n = 6$ and 11 represent kinetic data obtained at a higher solution viscosity in the presence of 200 and 400 $g\,L^{-1}$ glucose, respectively. Kinetic data obtained at $n = 6$ and 11, which give larger electrode–reactant separations, demonstrate an exponential decay of the rate constant with increasing distance [Eq. (3)] in all three cases, with a slope of about 1 per CH_2 unit. This finding is in excellent agreement with previous results obtained for various composite systems.[12–18,23,31] Meanwhile, SAMs with $n = 2$ and 3 clearly demonstrate a

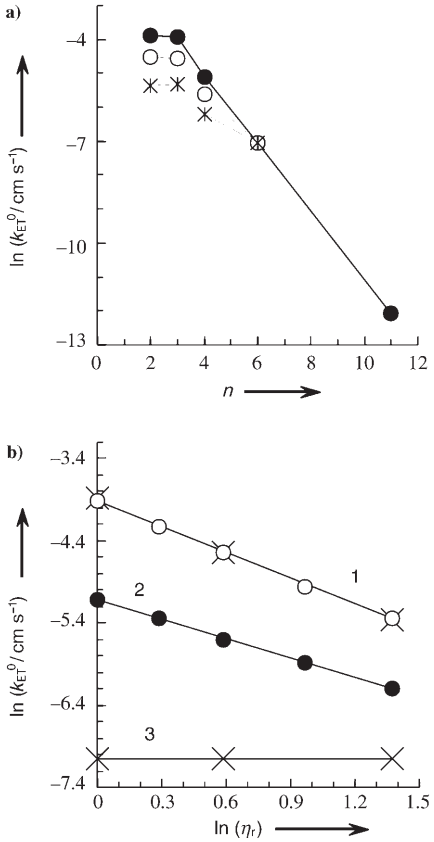

Figure 1. a) Natural logarithm of the heterogeneous standard rate constant for the CytC electron exchange at Au electrodes modified by hydroxy-terminated *n*-alkanethiol SAMs ($n=2$, 3, 4, 6, 11) versus number of methylene units *n* at different solution viscosities. Upper curve (●): no viscous additive, $\eta_r=1$; middle curve (○), $\eta_r=1.80$; lower curve (*), $\eta_r=3.96$. Note that all three curves merge at $n=6$ and 11, and form a single sloped line. b) Natural logarithm of the heterogeneous standard rate constant for the CytC electron exchange at Au electrodes modified by hydroxy-terminated *n*-alkanethiol SAMs versus logarithm of solution relative viscosity (η_r). Plot 1: two coinciding plots for $n=2$ (×) and $n=3$ (○); plot 2: $n=4$ (●); plot 3: $n=6$ (×). Note that the numerical values are averaged over three to five independent experiments. The symbols are larger than actual error bars throughout.

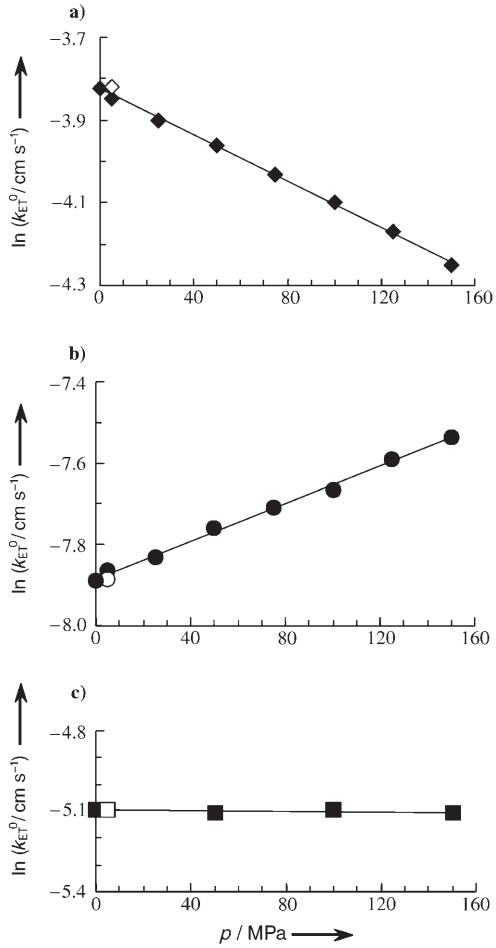

Figure 2. Natural logarithm of the heterogeneous standard rate constant for the CytC electron exchange at Au electrodes modified by hydroxy-terminated *n*-alkanethiol SAMs ($n=3$,4,6) versus hydrostatic pressure *p*: a) $n=3$: ◇, ◆; b) $n=6$: ○, ●; c) $n=4$: □, ■. The symbols are larger than actual error bars for each pressure-cycle experiment. The reported numerical values for the activation volumes are averaged over three to five independent pressure cycles.

plateau region. The data for the SAM with $n=4$ fall in the intermediate region.

Figure 1b shows the standard heterogeneous rate constant, k_{ET}^0, versus the relative solution viscosity for SAMs with $n=2$, 3, 4, and 6. Phenomenologically, these dependencies can be approximated by Equation (4) with power indexes (the actual slopes in Figure 2b) of $\delta=1$ ($n=2, 3$), 0.7 ($n=4$), and 0 ($n=6$, 11; the latter case is not shown in Figure 2b; see Supporting Information). The "limiting" value of about 0.6 was detected in the case of specific adsorption on the pyridine-terminated thin SAMs (plateau region).[18] Somewhat larger slopes were found for an ET within the "homogeneous" system involving zinc-substituted CytC, and wild-type and mutant cupriplastocyanin, which ranged from 0.7 to 0.9.[9–11] Interestingly, full viscosity control ($\delta\approx1$) has been observed for photoinduced CT from the artificial Ru-coordinated polypeptide electron donor to ferri-CytC,[48] which occurs through the loosely bound (encounter) complex as opposed to the "preformed" (tight) complex involving the same reactants ($\delta\approx0.6$); both patterns occur simultaneously. The latter findings closely match the whole pattern of CytC bioelectrochemical CT in both the tightly bound and freely diffusing (to Au-deposited SAMs comprising ω-COOH and ω-OH) regimes, respectively.

In addition, recent experimental and computational results obtained for a related system—a "charge-modified" complex of a photosynthetic reaction center (PRC) and cytochrome c_2[49,50]—together with our detailed analysis[51] for the case of CytC freely diffusing to hydroxy-terminated SAMs, strongly suggest the formation of a solvent-separated, softly stabilized complex as an encounter-reactive associate (see below).

The dependence of $\ln(k_{ET}^0)$ on the hydrostatic pressure up to 150 MPa for SAMs with $n=3$, 4, and 6 is shown in Figure 2a–c, respectively. It can be seen that for a SAM with $n=3$, the value of $\ln(k_{ET}^0)$ decreases linearly with pressure and yields a positive volume of activation of $+6.7\pm0.5$ cm^3 mol^{-1}

(Figure 3a, and the Supporting Information). This value is very close to $+6.1 \pm 0.5\ cm^3 mol^{-1}$ which is found in the case of Au electrodes modified by 4,4′-bipyridyl and 4,4′-bipyridyl disulfide (the case of "primitive" thin SAMs).[6] On the contrary, for a much thicker SAM with $n = 6$, we found that the value of $\ln(k_{ET}^0)$ increases linearly with increasing pressure to yield a negative volume of activation of $-5.5 \pm 0.5\ cm^3 mol^{-1}$ (Figure 2b). In the case of a SAM with $n = 4$, an intermediate behavior is displayed with no effect of pressure on k_{ET}^0, to give $\Delta V_a \approx 0$ (Figure 2c and Supporting Information). To our knowledge, this is the first observation of a changeover of the HP kinetic response for a given bio(electro)chemical system. Hence, it is demonstrated that the HP studies provide direct, adequate, and fully complementary kinetic information suitable for the mechanistic analysis, as compared to more conventional experimental approaches.

In summary, three different strategies, namely, variation of the SAM thickness, solution viscosity, and hydrostatic pressure, all clearly indicate a definite switch in the intrinsic mechanism behind the change in specific performance with the change in CT distance. Importantly, this general conclusion is model-free, that is, independent of the theoretical interpretation. Consecutively, it is reasonable to attribute the observed kinetic effects that result from the alkane chain length, viscosity, and pressure variations to respective changes of the intrinsic unimolecular rate constant. Indeed, it is very unlikely that some variation of the internal order of the SAMs with the alkane chain length[52,53] would cause significant changes in the pre-equilibrium term, to exactly mimic three kinds of effects theoretically predictable *specifically* for the intrinsic CT constant. This argument, above all, is valid in the case of a "bulky" reactant such as CytC reacting in the free regime at the solvent-separated distance from the SAM "surface", as it is presumably almost insensitive to the extent of the SAM disorder (which is actually unimportant in many practical cases[8,15,16,31]). Furthermore, this natural assumption allows one to rationalize the whole variety of experimental results in terms of the self-consistent unified (extended) CT theory [Eqs. (1), (2), (6), and (7)].[54]

To this extent, within the framework of the generalized theoretical model adopted here, the HP kinetic results fully conform with two types of intrinsic mechanisms of CT, namely, nonadiabatic (long-range) and adiabatic (short-range) processes, in good accordance with two other experimental manifestations fully exhibited by our experiments. The individual components that contribute to the experimental volumes of activation (see Supporting Information) can be estimated on the basis of Equations (6) and (7) by assuming that the overall value of $\lambda = 0.6$–$0.8\ eV$[8,17–20] (representing the Franck–Condon term) is roughly composed of about 50% "outer-sphere" (solution) component, which can contribute about half of the activation volume, and about 50% "inner-sphere" protein reorganization component that is negligibly altered by pressure (resulting in a zero activation volume contribution).[42] Moreover, the reorganizational component due to the SAM interior seems to be negligible ($\lambda_{SAM} \approx 0$),[47] which also yields the zero activation volume increment. Further bioelectrochemical HP kinetic experiments with the participation of other, less well-studied redox proteins are planned to extend profound CT mechanistic studies.

Finally, this is the first time that the CT mechanism changeover has been clearly detected by a HP kinetic strategy. The changeover in CT mechanism has been cross-tested and proven by three independent experimental approaches. Furthermore, two distinct CT mechanisms and their changeover have been detected for freely diffusing, intact CytC molecules. The exponential decrease in the standard rate constant with increasing CT distance at larger CytC–electrode separations ($n = 6$, 11) has been shown to be in full accord with the well-established signature of nonadiabatic CT. Full viscosity control has been detected for freely diffusing CytC, presumably operating in the extreme adiabatic, "friction-controlled" regime in the case of thinner SAMs ($n = 2$, 3). The "intermediate" regime for the CT of CytC ($n = 4$) has been detected by using all three approaches. In addition, all the experimental results can be nicely described within the unified theoretical framework (extended CT theory).

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[54] An attempt to ascribe the observed effects (or one of the effects) to the variation of the pre-equilibrium term unavoidably gives rise to a cascade of rather strained and controversial assumptions; for example, that the kinetic viscosity effects for CytC CT in the cases of "homogeneous"[48] and electrochemical, namely, tightly bound[15,18] and freely diffusing (this work), regimes are of totally different origin. Similarly, the designation of the onset of the plateau region in Figure 1a to some disorder in short SAMs ($n = 2, 3, 4$) makes the explanation of analogous occurrences for the cases of ω-Py[17,18] and ω-COOH[15,18] SAMs (which occur at $n \approx 8$ and 12, respectively) very confusing, as the alkanethiol SAMs with $n = 6$–12 are known to be the most stable within the sequence[53]. Further extended analysis of these and related mechanistic aspects will be presented elsewhere.[51]

