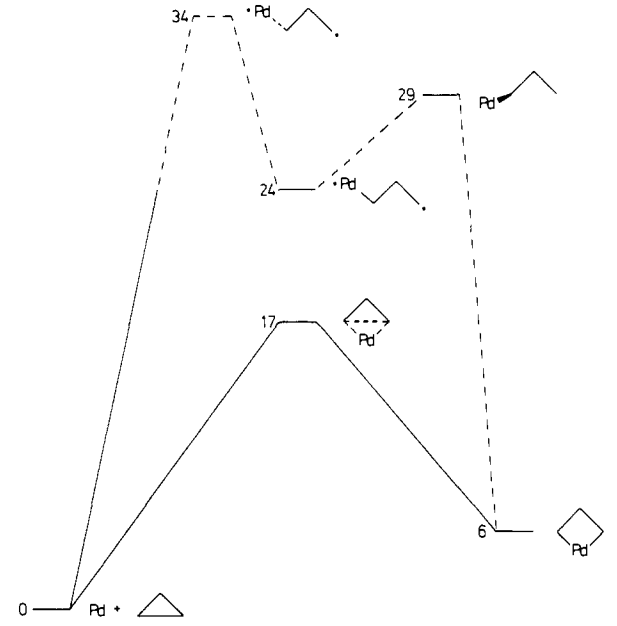

Figure 6. Schematic energy diagram for the different reaction paths followed after a palladium attack on cyclopropane. The energy values are in kcal/mol. The transition states for the corner palladation and for the formation of the metallacycle from the open radical structure are only estimated. The other energies are from the CCI(+Dav.) calculations.

in the CCI calculation leaving out a few reference configurations with coefficients slightly larger than 0.05.

Discussion

The calculated barrier for formation of a metallacyclobutane from palladium and cyclopropane is 17 kcal/mol. Considering the approximations involved in the basis set and the geometry optimization a more realistic barrier is probably around 10 kcal/mol, which according to chemical experience is a reasonable value for this type of reaction. The calculations further show that the edge palladation is a lower energy pathway than the corresponding corner palladation. Thus, the energy minimum of the radical structure formed after a corner attack is 8 kcal/mol higher than the saddle point energy for the edge attack. The saddle point for the corner palladation should be an additional 10 kcal/mol higher in energy. It is thus clear that the effect of ligands on palladium, which will be investigated in a forthcoming study, has to be rather large in order to change the preference for the edge attack compared to the corner attack. The energies for the different structures on the potential energy surface of PdC_3H_6 are shown schematically in Figure 6.

It is clear that in principle a metallacyclobutane could also be formed from the open high energy radical structure by rotation around the C_1–C_2 bond and formation of a bond between palladium and C_3. This would require another energy barrier of 4–5 kcal/mol on going from the radical to the metallacyclobutane, and the barrier for metallocyclobutane formation via a corner attack would then be around 30 kal/mol. Formation of metallacyclobutane is therefore much favored via an edge activation. This is in agreement with experimental results, which have shown that the formation of a platinacyclobutane occurs with retention of configuration at both carbon atoms attacked by the metal.[3f]

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Registry No. Palladium, 7440-05-3; cyclopropane, 75-19-4; palladacyclobutane, 98875-35-5.

Temperature and Electrolyte Effects on the Electron-Transfer Reactions of Cytochrome *c*

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Abstract: The electron-transfer reaction partners of cytochrome *c* are both membrane-bound, imparting heterogeneous character to their reactions. The heterogeneous electron-transfer kinetics of cytochrome *c* reacting at indium oxide electrodes have been evaluated by cyclic voltammetry as a function of temperature in binding (phosphate) and nonbinding (Tris/cacodylic acid) neutral buffer media. The formal heterogeneous electron-transfer rate constant, $k^{o\prime}$, exhibited a biphasic dependence on temperature with a maximum value obtained at 41 °C in phosphate buffer and at 55 °C in Tris/cacodylic acid buffer at pH 7.0. The temperature dependence of the formal potential of cytochrome *c* was also determined under the same experimental conditions from reversible cyclic voltammograms acquired at a potential scan rate of 20 mV/s. The change in reaction center entropy, ΔS_{rc}^{o}, and the formal potential, $E^{o\prime}$ (at 25 °C), for cytochrome *c* were determined to be −13.4 eu and 0.264 V vs. NHE, respectively, in Tris/cacodylic acid buffer and −12.7 eu and 0.256 V vs. NHE in phosphate buffer. While the maximum $k^{o\prime}$ occurred at different temperatures in the two buffer systems, it occurred at the same formal potential, ca. 0.247 V vs. NHE. These results suggest that there is an optimum conformation of ferricytochrome *c* for facile heterogeneous electron transfer that occurs at different temperatures in binding and nonbinding buffer media.

Mammalian cytochrome *c* is a water-soluble heme protein that functions as an electron carrier between cytochrome *c* reductase and cytochrome *c* oxidase, components of oxidative phosphorylation. The physiological redox partners of cytochrome *c* are membrane-bound (inner membrane of mitochondria), whereas cytochrome *c* resides in the cytosol between the inner and outer membranes and is believed to be associated with the outer surface of the inner membrane.[1] Cytochrome *c* electron transfer is believed to occur at the solvent-exposed edge of the heme group by an outer-sphere mechanism.[2] The dipole moment of cyto-

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chrome *c* orients the enzyme within the electrostatic field of its physiological redox partners for effective electron transfer.[3,4]

The effect of temperature on the conformation of ferri- and ferrocytochrome *c* has been widely investigated.[1,5-19] Ferrocytochrome *c* is very stable and except for minor conformational changes maintaines its native structure between the extremes of 4 and 97 °C at pH 7.[14] Ferricytochrome *c* is less stable than ferrocytochrome *c* due to the net +1 charge on the heme group that is largely embedded within the hydrophobic interior of the enzyme's protein sheath.[2,15] The heme group is neutral in ferrocytochrome *c*. Ferricytochrome *c* retains its native coordination of the heme group from 20 to 77 °C at pH 5.25[15] while undergoing mild conformational changes throughout this temperature range that involve a gradual opening of the crevice surrounding the solvent-exposed heme edge with increasing temperature.[19] The reduction of ferricytochrome *c* has widely been reported to be accompanied by a small conformational change[19] that includes closing of the crevice about the solvent-exposed heme edge.[20] Since the conformations of the two redox states of cytochrome *c* are affected differently by temperature changes, the conformational changes attendent to electron transfer vary with temperature.

The temperature dependencies of both the formal potential and the rate of heterogeneous electron transfer for cytochrome *c* at tin-doped indium oxide electrodes are reported in this paper. Cytochrome *c* has been shown to behave quasi-reversibly at this electrode.[21,22] The change of formal potential with temperature has been previously determined by spectroelectrochemical experiments that employed an electrochemical mediator for the homogeneous reduction of cytochrome *c*.[9-11,13,17,18] The homogeneous electron-transfer rates for cytochrome *c* and many redox partners have been studied by a variety of methods.[3,4,23-34] However, the effect of temperature on the direct, heterogeneous electron transfer between cytochrome *c* and an electrode has not been reported. The present study of heterogeneous electrochemical electron-transfer behavior is of interest due to the interfacial character of electron transfer between cytochrome *c* and its physiological redox partners.

Two different buffer/electrolyte media were selected for use in this study to probe effects on both the formal potential and the formal heterogeneous electron-transfer rate constant as a function of temperature arising from anion binding to cationic lysine residues on the surface of cytochrome *c*. Tris/cacodylic acid buffer provides a nonbinding buffer medium for cytochrome *c*,[8,35] whereas phosphate buffer has been clearly shown to exhibit anion binding to cytochrome *c*, electrostatically altering the stabilization of the heme group in the hydrophobic pocket of the molecule.[36] While other anions have also been shown to bind to cytochrome *c*,[36] the systems selected for this study provide extremes for nonbinding and binding media.

Experimental Section

Horse heart cytochrome *c*, type VI, Sigma Chemical Co., was purified by chromatography on carboxymethylcellulose (CM-52, Whatman) according to a published procedure.[37] The purified cytochrome *c* was then lyophilized and stored at −4 °C. Due to the long times required for a temperature study (several hours), very pure cytochrome *c* was required. The process of lyophilizing denatures some ferricytochrome *c*, and not all of the sample returns to its native form when dissolved in water. This was determined from the observation that purified, lyophilized cytochrome *c* would show band separation when repurified whereas purified, nonlyophilized cytochrome *c* passed through the column as a single band. Therefore, a small portion of the lyophilized, purified cytochrome *c* was repurified and stored in solvent prior to experimental use. The cytochrome *c* was used as received from the column in the 0.2 M ionic strength phosphate buffer eluent for the experiments in phosphate buffer. To obtain cytochrome *c* in Tris/cacodylic acid buffer, the phosphate ions were first removed by repeated dilutions followed by concentrations using a stirred ultrafiltration cell (Amicon Model 52) with a YM5 filter. Tris(hydroxymethyl)aminomethane was used as received from Sigma Chemical Co. (Trizma Base, reagent grade). Cacodylic acid, hydroxydimethylarsine oxide (Sigma Chemical Co., 98% pure), was recrystallized twice from 2-propanol. Water used in this work was purified with a Milli RO-4/Milli-Q system (Millipore Corp.) and exhibited a resistivity of 18 MΩ on delivery. All solutions were pH 7.0. Cytochrome *c* concentrations were determined by the reduced minus oxidized difference molar absorptivity $\Delta\epsilon = 21\,100\ M^{-1}\ cm^{-1}$ at 550 nm[38] on a Beckman Acta MVII spectrophotometer. Ferricytochrome *c* was reduced by dithionite. All other chemicals used in this work were ACS reagent grade.

A nonisothermal electrochemical cell was used for this work. The half-cell containing the working electrode and the liquid junction of the reference electrode was encased in a water jacket. The Ag/AgCl (1.00 M KCl) reference electrode was physically isolated from the thermostated region of the cell by small diameter glass tubing and remained at

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room temperature. Solution volume was approximately 1 mL, and a platinum auxiliary electrode was used. Tin-doped indium oxide OTE materials were obtained from PPG Industries. The OTE electrodes were cleaned by successive 5-min sonications in Alconox solution, in 95% ethanol, and twice in purified water.[39] The working electrode was attached to the bottom of the cell by a retainer plate and an O-ring seal. Each working electrode was only used for one experiment.

Potential-step chronocoulometry experiments were performed by stepping 400 mV negative from an initial potential of 522 mV vs. NHE. The experiments were conducted in 0.2 M ionic strength, pH 7.0, Tris/cacodylic acid buffer. Data were acquired at 10 points/s by an integrating analog/digital converter interfaced to a UNC microcomputer.[40] Base-line data were acquired for 1 s prior to the potential step, and their average was subtracted from all subsequent points. Background electrolyte data were acquired for each experiment with the working electrode used for that experiment and subtracted from the total coulometric response. The computer-recorded current was then digitally summed to yield charge. An in-house constructed potentiostat of conventional design was used for all electrochemical work. The triangle wave generator that was used to perform cyclic voltammetric experiments was triggered by the computer. Data were acquired at a rate calculated to obtain 2 data points/mV. A 20-μs conversion time, 12 bit, fast Datel ADC-HX12B analog/digital converter based interface card was used to acquire cyclic voltammetric data. Data showed 60-Hz noise and were digitally smoothed by a 12-point polynomial smoothing routine.[41] Formal potentials were determined from the average of the peak potential values from reversible cyclic voltammograms acquired at low potential scan rates. The difference in the computer acquired background (buffer electrolyte alone) and total (electroactive species present) cyclic voltammograms was used in these determinations. A program then determined the potential at which the maximum cathodic and anodic currents occurred from each digitally stored cyclic voltammogram providing precise values for formal potentials. The standard deviations for cathodic peak potentials, anodic peak potentials, and formal potentials for replicate experiments were typically less than 1 mV (see figure legends for specific values).

Heterogeneous electron-transfer rate constants were determined from cyclic voltammetric peak separation by the method of Nicholson.[42]

Results

A series of control experiments were performed to confirm that the cell being used in this work behaved in a nonisothermal manner.[43] In a nonisothermal cell, the temperature of the reference electrode is held constant, in this case at ambient room temperature (22 ± 1 °C), while the temperature of the redox couple of interest is varied. Under such conditions, the reaction center entropy change, $\Delta S_{rc}°$, is given by[43]

$$\Delta S_{rc}° = nF(dE°'/dT) = S_{red}° - S_{ox}° \quad (1)$$

where n is the number of electrons transferred, F is the Faraday constant, $E°'$ is the formal potential, and T is the temperature of the redox couple of interest. The liquid junction potential difference between the two electrolyte media used in this work, Tris/cacodylic acid and phosphate buffer in the presence and absence of NaCl, was determined to be less than 1 mV by measuring the potential of the silver/silver chloride reference electrode against a saturated calomel electrode in each medium. The uncertainty in the determination of $\Delta S_{rc}°$ by this procedure is ±1 eu.[43]

The model system chosen for the evaluation of the nonisothermal behavior of the cell used in this work was $K_3Fe(CN)_6$. The temperature dependence of both the formal potential and the formal heterogeneous electron-transfer rate constant, $k°'$, for this redox couple is shown in Figure 1. A reaction center entropy change of −37 eu was obtained by using eq 1 from the $E°'$ data shown in this figure. Lin and Breck[44] extrapolated to zero ionic strength and reported a reaction center entropy change of −43 eu. Evaluation of their data at 0.2 M ionic strength yielded a

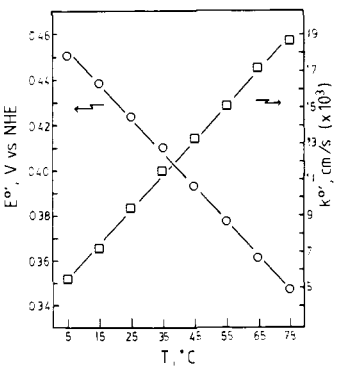

Figure 1. Temperature dependence of the formal potential and the formal heterogeneous electron-transfer rate constant for potassium ferricyanide. Results shown are the averages of three separate experiments at three different indium oxide electrodes. All solutions contained 1.0 mM $K_3Fe(CN)_6$ and were pH 7.0 and 0.20 M ionic strength. Two solutions only contained Tris/cacodylate buffer, and the third solution also contained 0.10 M NaCl. Temperatures (°C), formal potentials (mV vs. NHE), and formal heterogeneous electron-transfer rate constants ($\times 10^3$, cm/s): 5, 451.4 (±0.9), 5.5 (±1.4); 15, 438.7 (±0.2), 7.2 (±1.6); 25, 423.8 (±0.6), 9.4 (±2.0); 35, 410.3 (±1.0), 11.5 (±2.5); 45, 393.2 (±0.6), 13.3 (±2.6); 55, 377.4 (±0.4), 15.1 (±2.6); 65, 361.2 (±0.4), 17.2 (±3.4); and 75, 347.1 (±2.1), 18.7 (±4.2). Parentheses contain standard deviations.

matching value of −37 eu for this same system. Moreover, a formal potential for $K_3Fe(CN)_6$ at 25 °C determined from potentiometric redox titrations has been reported that agrees with the value determined in the present work from cyclic voltammetric results, namely, 424 mV vs. NHE.[45] These results establish that the cell configuration used in this work is nonisothermal. The temperature dependence of the $k°'$ value for $K_3Fe(CN)_6$ reacting at indium oxide electrodes has also been determined by cyclic voltammetry at scan rates of 500 mV/s.[42] In order to evaluate $k°'$ as a function of temperature, the diffusion coefficient of $Fe(CN)_6^{3-}$ was determined for each temperature. As is evident from the results shown in Figure 1, the $k°'$ value for this redox system is linearly dependent upon temperature.

The temperature dependence of the pH of the two buffer solutions used in this work was determined from 5 to 55 °C. Both phosphate and Tris/cacodylate buffers were prepared to be pH 7.00 at 22 °C and 0.20 M in ionic strength. The pH of the phosphate buffer varied with temperature in a manner that was similar to that previously reported for 0.025 M phosphate buffer,[46] with pH values of 7.09 at 5 °C and 6.88 at 55 °C. The pH of Tris/cacodylate acid buffer was 7.22 at 5 °C and 6.55 at 55 °C, exhibiting a larger temperature dependence than the phosphate buffer but not as large as for Tris/HCl buffers.[46] Both ferricytochrome c[47] and ferrocytochrome c[14] remain conformationally stable between the pH values given above.

Experiments performed with cytochrome c followed the same procedure as described above and in the Experimental Section. The temperature dependence of the diffusion coefficient of cytochrome c was determined by potential-step chronocoulometry, assuming a value of 1.16×10^{-6} cm^2/s at 25 °C.[48] This diffusion coefficient was used in conjunction with the slopes of the Q vs. $t^{1/2}$ plots to obtain corresponding diffusion coefficients at the respective temperatures (see Figure 2). Diffusion coefficients are directly proportional to temperature divided by the viscosity of the medium. Various equations differ by the parameters used to predict the proportionality constant. Theoretical D values reported in this paper are based on a proportionality constant calculated by using the literature D value for cytochrome c and the viscosity of pure water at 25 °C. Deviations between experimental and theoretical values are less than 10% over the

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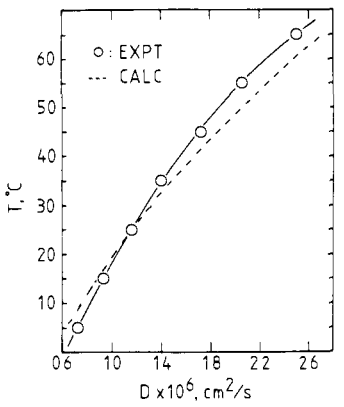

Figure 2. Temperature dependence of the diffusion coefficient of cytochrome *c*. Experimental results obtained from the average of six experiments (open circles). Calculated values (dashed line). Temperatures and values of experimental diffusion coefficients ($\times 10^6$, cm^2/s): 5 °C, 0.72 (±0.04); 15 °C, 0.93 (±0.03); 25 °C, 1.16 (literature value); 35 °C, 1.40 (±0.03); 45 °C, 1.72 (±0.05); 55 °C, 2.06 (±0.05); 65 °C, 2.5 (±0.1). Solution conditions: Tris/cacodylic acid buffer, pH 7.0, cytochrome *c* concentrations from 51 to 78 μM.

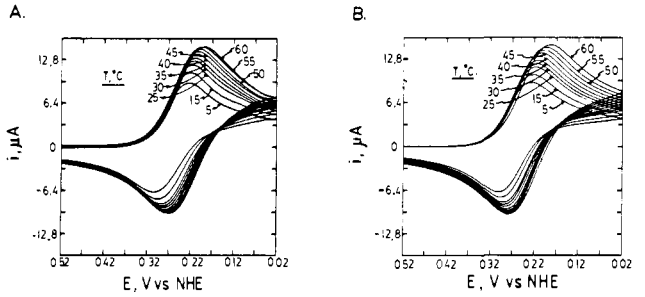

Figure 3. Temperature dependence of cyclic voltammograms of Cytochrome *c*. (A) Experimental results: Solution conditions: pH 7.0 phosphate buffer, 0.20 M ionic strength, 74 μM cytochrome *c*. Scan rate = 0.203 V/s, electrode area = 1.25 cm^2 (determined by potential-step chronocoulometry of cytochrome *c* at 25 °C assuming $D^{25\,°C}$ is 1.16×10^{-6} cm^2/s). (B) Simulated results. Conditions as given in A. Simulation parameters: $E^{\circ\prime}$ (see Figure 3), D (see Figure 1), and $k^{\circ\prime}$ (see Figure 4). The electrochemical-transfer coefficient was 0.5.

temperature range studied and may be explained by experimental results being obtained in 0.2 M ionic strength buffer whereas theoretical values are based on the viscosity of pure water.

A series of cyclic voltammograms obtained for cytochrome *c* in phosphate buffer for the temperature range of 5–60 °C is shown in Figure 3. The negative shift of both the cathodic and anodic peaks indicates the shift of the formal reduction potential with increasing temperature. Figure 4 shows the dependence of the formal potential upon temperature for cytochrome *c* in phosphate buffer. Linearity is observed from 5 to 55 °C, and $dE^{\circ\prime}/dT = -5.5 \times 10^{-4}$ V/deg. This slope yields a ΔS_{rc}° of −12.7 eu, which compares well with the value of −12.9 eu reported by Taniguchi et al.[18] for the mediated reduction of cytochrome *c* in phosphate buffer, pH 7.0. The formal potential at 25 °C is ca. 256 mV vs. NHE.

While the reduction potential for cytochrome *c* varied linearly with temperature from 5 to 55 °C, the heterogeneous electron-transfer rate constant showed a sharp break in behavior at approximately 41 °C in phosphate buffer. The corresponding reduction potential for the maximum rate constant is 247 mV vs. NHE. Figure 5 shows the results from one experiment. This behavior was observed in each of the experiments; however, the values of the heterogeneous electron-transfer rate constant at any temperature varied by approximately ±50% between experiments.

The electrochemical response for cytochrome *c* in Tris/cacodylic acid buffer, a nonbinding buffer, at the same pH and ionic strength was evaluated. Figure 5 shows the effect of temperature on the heterogeneous rate constant for cytochrome *c* in Tris/cacodylic acid buffer over the range 5–65 °C. Two differences in behavior

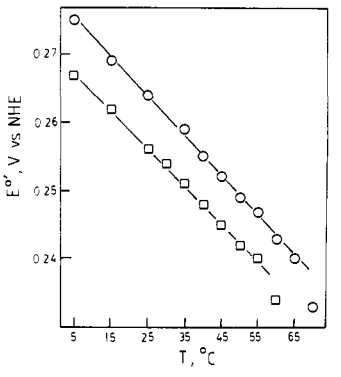

Figure 4. Temperature dependence of the formal potential of cytochrome *c*. (□) Phosphate buffer, pH 7.0, 0.20 M ionic strength. $dE^{\circ\prime}/dT = -5.5 \times 10^{-4}$ v/°C (5–55 °C), $\Delta S_{rc}^{\circ} = -12.7$ eu, $r = -0.9993$. Results shown are the averages of six separate experiments. Three experiments were in phosphate buffer only and three experiments were in phosphate buffer that also contained 0.1 M NaCl. Temperatures (°C) and formal potentials (mV vs. NHE): 5, 267 (±2.0); 15, 262 (±1.2); 25, 256 (±1.0); 30, 254 (±0.6); 35, 251 (±1.0); 40, 248 (±1.0); 45, 245 (±1.3); 50, 242 (±1.1); 55, 240 (±0.5). (○) Tris/cacodylic acid buffer, pH 7.0, 0.20 M ionic strength. $dE^{\circ\prime}/dT = -5.8 \times 10^{-4}$ V/°C (5–65 °C), $\Delta S_{rc}^{\circ} = -13.4$ eu, $r = -0.9989$. Each value is the average of three separate experiments. Temperature (°C) and formal potential (mV vs. NHE): 5, 275 (±0.6); 15, 269 (±0.8); 25, 264 (±0.8); 35, 259 (±1.0); 40, 255 (±1.2); 45, 252 (±0.8); 50, 249 (±0.5); 55, 247 (±0.9); 60, 244 (±0.5); 65, 240 (±0.8).

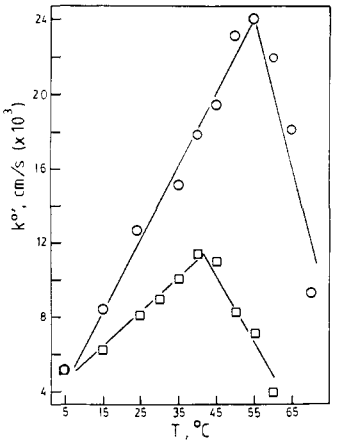

Figure 5. Temperature dependence of the formal heterogeneous electron-transfer rate constant of cytochrome *c* reacting at indium oxide electrodes. (□) Phosphate buffer, pH 7.0, 0.20 M ionic strength. Temperatures (°C) and formal heterogeneous electron-transfer rate constants ($\times 10^3$, cm/s): 5, 5.2; 15, 6.1; 25, 8.0; 30, 8.9; 35, 10.0; 40, 11.3; 45, 10.8; 50, 8.2; 55, 7.1; 60, 3.8. (○) Tris/cacodylic acid buffer, pH 7.0, 0.20 M ionic strength. Temperatures (°C) and formal heterogeneous electron-transfer rate constants ($\times 10^3$, cm/s): 5, 5.1; 15, 8.3; 25, 12.5; 35, 15.1; 40, 17.8; 45, 19.4; 50, 23.1; 55, 23.9; 60, 21.8; 65, 18.0; 70, 9.0. Results shown for each case were obtained on one cytochrome *c* solution.

are noted: the rate constant is larger in Tris/cacodylic acid than in phosphate buffer, and the break in kinetic behavior with temperature occurs at approximately 55 °C, as opposed to 41 °C in phosphate buffer. The reduction potential for cytochrome *c* in Tris/cacodylic acid buffer (Figure 4) is ca. 8 mV anodic of that in phosphate at any particular temperature, with a formal reduction potential of 264 mV vs. NHE at 25 °C. A ΔS_{rc}° of −13.4 eu is experimentally indistinguishable from the −12.7 eu value observed in phosphate buffer.

Experimentally determined values of $E^{\circ\prime}$, D, $k^{\circ\prime}$, cytochrome *c* concentration, and electrode area were used to digitally simulate cyclic voltammograms. The simulation algorithm is based on Butler–Volmer theory. A discrepancy was noted between theoretical and experimental currents which varied with scan rate and temperature (Figure 6). The difference was largest at fast scan rates and between 35 and 40 °C in both buffer systems. The cathodic peak current showed the largest difference. This type

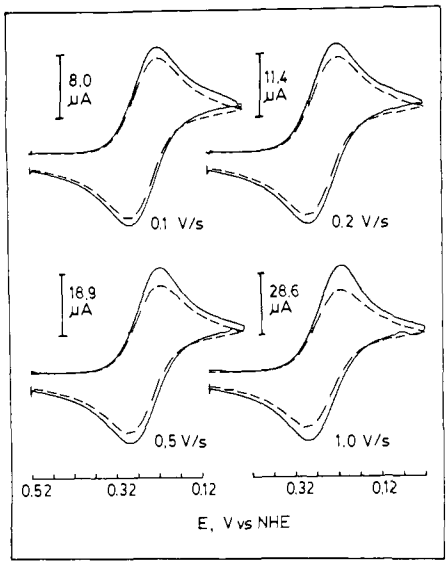

Figure 6. Comparison of experimental and simulated cyclic voltammograms for the reaction of cytochrome *c* at an indium oxide electrode. Experimental conditions: Tris/cacodylic acid buffer, pH 7.0, 0.20 M ionic strength, 96 μM cytochrome *c*, electrode area = 1.25 cm^2, T = 40 °C. Scan rates given on figure. Formal potential = 255 mV vs. NHE, $k^{\circ\prime} = 2.01 \times 10^{-2}$ cm/s, $D = 1.56 \times 10^{-6}$ cm^2/s. Experimental results (solid lines) and simulated results (dashed lines).

of behavior indicates that ferricytochrome *c* is weakly absorbed at the electrode surface.[49] When faster scan rates are used, the contribution of adsorbed species to the total current becomes more important, since the charge consumed by adsorbed reactant remains constant while the charge consumed by solution resident reactant decreases.

Discussion

The differences observed in the formal potential of cytochrome *c* reacting at indium oxide electrodes in the two buffers (phosphate and Tris/cacodylic acid) used in this study are attributed to anion binding in the case of phosphate buffer. Differences in the formal heterogeneous electron-transfer rate constant for this reaction between the two buffer media may be due to a combination of anion binding effects and changes in the character of the electrode/solution interface. Discussion is presented that suggests that kinetic differences may be dominated by the former.

Phosphate anions bind to the positively charged lysine residues surrounding the solvent-exposed heme edge.[35,36] This reduces charge repulsion between the positively charged heme group of ferricytochrome *c* and the positively charged lysine residues, thus stabilizing the oxidized form of cytochrome *c*. This is reflected by the reduction potentials for cytochrome *c* in phosphate buffer being negative of those in Tris/cacodylic acid buffer. As mentioned previously, the reduced form of cytochrome *c* is very stable and undergoes mild conformational changes throughout the temperature range used for this study.[14,19] The oxidized form is less stable and undergoes larger conformational changes. The formal potential indicates the relative stability of the oxidized and reduced forms. As the temperature is increased, the crevice around the solvent-exposed heme edge opens and allows greater exposure of the heme to solvent, thus stabilizing the positively charged heme group of ferricytochrome *c*. Previous studies have indicated that in ferricytochrome the Fe–S (the sulfur of the Met-80 residue) bond gradually and continuously weakens with the increase in temperature,[2,5,15] as evidenced by the increasing bond length due to the increasing vibrational energy. The residues adjacent to the Met-80 group are also flexible.[12,15] Moore and Williams[15] have reported that the conformational difference between the two oxidation states of cytochrome *c* is centered in the region about Ile-57. The conformational change on reduction is primarily a closing of the crevice about the solvent-exposed edge.[20] The difference in conformation is associated with a change in the Fe–S bond length which affects conformational changes by a "pull–push" mechanism.[15] Similarly, the temperature-dependent Fe–S bond length may have a role in controlling the crevice about the heme within each redox state as a function of temperature. The reaction center entropy changes for cytochrome *c* determined in both buffer media used in this work are small in magnitude, negative in sign, and, within experimental error, the same (i.e., −13 (±1) eu). The sign and magnitude of this reaction center entropy change are consistent with a small conformational change to a more compact structure in the environment about the heme upon reduction.[18] Charge-induced outer-sphere solvent reorganization can only be a small factor in this entropy change since the heme is largely shielded from the solvent. Moreover, a positive contribution to ΔS_{rc}° would be expected for a +1 to a neutral-charge change.[43]

The negative entropy change for the reduction of cytochrome *c* has also been attributed to the decrease in size of cytochrome *c* when reduced, followed by water filling the void, with subsequent structure making through hydrogen bonding.[9,10,11] This model is based upon the biphasic behvior of temperature dependence of the formal potential of cytochrome *c* in the presence of chloride anions. The break occurred at 42 °C. Below 42 °C a ΔS_{rc}° of −10.2 eu was observed, while above 42 °C a value of −75.0 eu was obtained. Three of the experiments in phosphate buffer in this work were conducted in the presence of 0.1 M NaCl (see Figure 4). No chloride effect was observed. This difference in results for temperatures above 42 °C may be due to several factors. The previous work employed spectropotentiostatic experiments using optically transparent thin-layer electrode (OTTLE) cells and a mediator to couple the redox state of cytochrome *c* to the potential applied to the gold minigrid working electrode. Thus the OTTLE results are based on optical measurements that probe redox states in the bulk of solution. The results presented here are based on cyclic voltammetric responses and are therefore dependent on the flux of electroactive species at the electrode/solution interface and not bulk solution conditions. The dimension of the diffuse double layer under the present experimental conditions (i.e., 0.20 M ionic strength) is less than 5 Å,[50] and cytochrome *c* has a diameter of ca. 25–30 Å not including the solvation sheath.[51] These factors suggest that cytochrome *c* molecules undergoing electron-transfer reactions in these experiments have characteristics that are largely due to the bulk solvent properties. However, if the physiological binding domain of cytochrome *c* is indeed facing the electrode surface during electron transfer, chloride anions may be excluded from this region, preventing the observation of the chloride-dependent behavior observed by the OTTLE technique above 42 °C.

The maximum $k^{\circ\prime}$ for the reaction of cytochrome *c* occurs at the same formal potential in these two buffer systems. This suggests that there is the same relative conformational difference between ferri- and ferrocytochrome *c* at the peak $k^{\circ\prime}$ in both the buffers even though the temperature at this point is 14 °C lower in phosphate buffer than in Tris/cacodylic acid buffer. In both buffer systems an increase in temperature allows greater solvent exposure to the positively charged heme group in ferricytochrome *c*, thus stabilizing the oxidized form. In phosphate buffer there is the further stabilization of ferricytochrome *c* due to the binding of phosphate anions to the positively charged residues about the solvent-exposed heme edge.[36] Thus the conformation at which a maximum $k^{\circ\prime}$ is observed occurs at a lower temperature in phosphate buffer than in the noninteractive Tris/cacodylic acid buffer. A reasonable assumption is that the controlling factor which accounts for the break in kinetic behavior with change in temperature is the conformation of ferricytochrome *c* with respect to the conformation of the transition state. Thus, ease of electron transfer may increase with temperature, due to the above reasons,

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Table I. Surface Excess of Cytochrome *c* vs. Temperature

	$\Gamma^\circ \times 10^{12}$, mol/cm^2	
temp, °C	Tris/cacodylic acid	phosphate
5	5.8[a]	4.4[b]
15	7.3	6.3
25	8.4	6.8
30		7.6
35	9.5	7.6
40	9.9	8.1
45	9.8	8.0
50	9.3	7.9
55	7.8	7.7
60	5.9	6.2
65	1.7	

[a] Surface excesses calculated from the difference in the calculated and experimental cathodic peak currents at a scan rate of 0.5 V/s.[44] [b] As in (*a*) but at a scan rate of 0.2 V/s.

to a point where the fluctuation of the nuclear coordinates to the transition state becomes difficult. This may occur at a point where high spin character begins to develop. Moore and Williams[15] have reported that ferricytochrome *c* is 12% high spin at 67 °C in pH 5.25 buffer. Also, the position of the aromatic ring on the Phe-82 relative to the heme may pass through an optimum for effective orbital overlap, resulting in an increase in electron-transfer probability. This argument would require that the electron-transfer process be nonadiabatic. Moore and Williams also reported[15] that the temperature-dependent NMR shifts of cytochrome *c* residues which are caused by conformation changes were biphasic with the break occurring at ca. 65 °C. Their work was conducted at pH 5.25. Previous work[2] has indicated that the Fe–S bond is less perturbed by temperature at pH 5.25 than at pH 7. Thus, the biphasic behavior of the NMR shifts with change in temperature may be related to the biphasic kinetic behavior reported in this paper. Electrolyte and pH affect the change of the Fe–S bond length with temperature. Optimum electron-transfer kinetics are then observed for a specific Fe–S bond length and position of adjacent residues, which is reflected by a corresponding formal potential. The solvent environment of the ferricytochrome *c* affects the temperature at which this optimum conformation occurs.

Another potential contribution to the temperature-dependent break in kinetics is the variation in the surface excess (the moles of adsorbed reactant per unit electrode area) of ferricytochrome *c* that was observed in this work. Differences between calculated and experimental peak currents were used to determine the surface excess of adsorbed ferricytochrome *c* on the electrode.[52] At faster cyclic voltammetric scan rates, although the measured peak current is larger, the total number of cytochrome *c* molecules reduced is smaller than at slower scan rates. The current consumed by a fixed number of adsorbed cytochrome *c* molecules therefore becomes a larger fraction of the total current observed at faster scan rates. This trend is indeed observed, as shown in Figure 6. It should be recognized that at slow scan rates, where formal potential values were determined, the effect of adsorption on the recorded cyclic voltammograms is not discernable. A maximum surface excess was observed around 40 °C for both buffer systems. A surface coverage of 8.3×10^{-12} mol/cm^2 corresponds to a monolayer of adsorbed cytochrome *c* when a value of 2000 Å^2/adsorbed cytochrome *c* molecule is used[53] (see Table I for surface coverage values at various temperatures). Eddowes and Hill[54] have suggested that reversible adsorption is necessary for facile electron transfer between cytochrome *c* and electrodes. Other groups have indicated that irreversible adsorption of cytochrome *c* at many electrode surfaces results in irreversible electrochemical behavior.[55] Eddowes and Hill describe the adsorption process of their system as hydrogen bonding between the lysines adjacent to the solvent-exposed edges and the 4,4′-bipyridyl molecules of the chemically modified gold electrode used in their work. It could be that for the indium oxide electrode used in this study, the lysine residues hydrogen-bond to the oxide groups of the electrode surface.

It is interesting to note that the variation in intramolecular electron-transfer rate constant with temperature has been reported for ruthenium complex modified cytochrome *c*. Isied et al. noted an increase in rate constant with temperature from 3 to 44 °C.[56] This was observed in phosphate buffer, pH 7.0, for which we report a maximum rate of heterogeneous electron transfer at 41 °C. However, no results were reported for temperatures above 44 °C in the work that is cited above.[56] Gray and co-workers[57] studied the same Ru modified cytochrome *c* system and reported no significant change in kinetic behavior over the temperature region 0–80 °C. Yet a graph showing intramolecular electron-transfer rate constant for this complex vs. temperature in phosphate buffer, pH 7.0, shows evidence of a peak at approximately 40 °C.

Very recent studies by Taniguchi and co-workers have described the temperature dependence of the formal potential of cytochrome *c* as a function of solution pH[58] and the presence of chloride.[59] The results of these studies are consistent with those that are presented here regarding chloride effects.

Conclusions

This study describes the temperature dependence of the rate of heterogeneous electron transfer for cytochrome *c* reacting at indium oxide electrodes. The condition of the Fe–S bond of ferricytochrome *c* is postulated to be the controlling factor for the rate of electron transfer. Solvent conditions mitigate the effect of temperature on the Fe–S bond. Also, experimental evidence indicates that ferricytochrome *c* is weakly adsorbed on the electrode surface prior to electron transfer.

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