anion radicals of benzaldehyde and its nitro derivatives, the odd electron is delocalized on an extensive conjugated system. The barrier heights increase accordingly with the double-bond character of the C_1–C_α bond,[50] but the increment is greater for benzaldehyde than for 4-NBA and 3-NBA, owing to the electron-withdrawing power of the nitro group. As a consequence, a nice correlation is observed (Figure 9) between the experimental barriers and the hfs constants of the formyl proton. Accordingly, the experimental barriers decrease as the nitro group σ^- values increase ($\sigma_p^-(NO_2)$ = 0.828; $\sigma_m^-(NO_2)$ = 0.975).[9]

Solvent interactions and ionic associations make the cis isomer of 3-NBA$^-$· more stable than the trans isomer through noticeable entropy contributions to the free energy of the isomerization process. The free energy of activation of the internal rotation decreases as the solvating power of the solvent or the strength of ionic association increases. Analysis of the diffusional process of the anion radicals in solution has provided direct evidence of specific interactions between the anion radicals and the surrounding solvent molecules.

(50) The Mulliken reduced overlap populations (Mulliken, R. S. *J. Chem. Phys.* **1955**, *23*, 1833, 1841, 2338, 2345) for the C_1–C_α bond, calculated from the MO ab initio STO-3G wave functions, are 0.380, 0.379, 0.378 for benzaldehyde, 3-NBA, and 4-NBA, and 0.441, 0.393, and 0.399 for the corresponding anion radicals, respectively.

Registry No. 3-NBA radical anion, 40951-85-7; 4-NBA radical anion, 34512-33-9; 2-NBA radical anion, 57643-03-5; benzaldehyde, 100-52-7; 4-nitrobenzaldehyde, 555-16-8; 3-nitrobenzaldehyde, 99-61-6; benzaldehyde radical anion, 34473-57-9.

Investigation of Some Intermolecular Electron Transfer Reactions of Cytochrome *c* by Electrochemical Methods

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Abstract: An electrochemical investigation of the reaction of horse heart cyclochrome *c* with the redox proteins from *Pseudomonas aeruginosa*, cytochrome c_{551} and azurin, in the presence of *P. aeruginosa* nitrite reductase/cytochrome oxidase and dioxygen, is described. The electrochemical reduction of horse heart cytochrome *c* is coupled to the reduction of dioxygen via the redox proteins from *P. aeruginosa*; 3.5–3.8 faradays mol^{-1} of dioxygen is consumed.

Electron transfer between proteins is a crucial and essential process in all living organisms. The factors which govern and control these intermolecular reactions and allow rapid electron transfer concomitantly with selectivity and specificity in choice of partners deserve to be elucidated. There have been a number of valuable studies[1-8] which have served to identify some features of protein structure that appear to be important such as the nature and disposition of charged groups on the surface of the protein and the location of the redox center with respect to the surface.

Most electrochemical investigations of redox proteins have made use of small-molecule electrode-active mediators, either bound[9] to the electrode surface or free in solution[10-18] which enhance the rate of electron transfer between electrode and protein. To date, there have been only a few reported[19] cases of *direct* electron transfer to redox proteins, e.g., eukaryotic cytochromes[20,21] *c*, cytochromes[22-25] c_3, and ferredoxin.[26,27] Our own contribution[21] has mainly centered on the use of electrodes upon which 4,4′-bipyridyl or related materials are absorbed. The electrochemistry of horse heart cytochrome *c* is well-behaved at such electrodes and we have proposed[21] that the cytochrome adsorbs onto and desorbs from the electrode surface rapidly, the favorable binding interaction being responsible for the enhancement of the rate of the electrode reaction. In this paper we describe the electrochemical investigation of electron transfer reactions of horse heat

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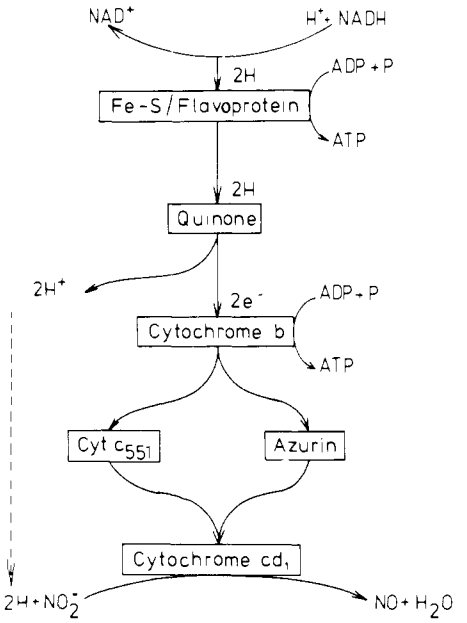

Figure 1. Respiratory chain of the bacterium *Pseudomonas aeruginosa.*

cytochrome *c* subsequent to the initial electron transfer at the electrode. The system chosen for study comprises components from the respiratory chain (Figure 1) of *Pseudomonas aeruginosa*: the "blue" copper protein azurin, the cytochrome c_{551}, and the cytochrome oxidase/nitrite reductase (*Pseudomonas* ferrocytochrome c_{551}–O_2 oxidoreductase, EC 1.9.3.2) which contains two heme groups and is referred to herein as oxidase cd_1. A preliminary account of one such reaction has been published.[28]

Experimental Section

Protein Purification. Horse heart cytochrome *c*, type III, obtained from the Sigma Chemical Co. was purified[29] to remove all polymeric and deamidated forms by ion-exchange chromatography. The respiratory chain (Figure 1) proteins from *Pseudomonas aeruginosa*—azurin, cytochrome c_{551}, and cytochrome oxidase/nitrite reductase—were purified according to the method of Parr et al.[30]

Supporting Electrolyte. The electrolyte used in all electrochemical experiments was 0.1 M $NaClO_4$, 0.02 M phosphate buffer, pH 7, 1 mM 1,2-bis(4-pyridyl)ethene, the surface modifier being obtained from the Aldrich Chemical Co. and recrystallized from ethanol/water. All other reagents were of Aristar grade.

Electrochemistry. Dc cyclic voltammograms were obtained by using an Oxford Electrodes potentiostat and recorded on a Bryans X–Y recorder 26000 A3. The cell was of all-glass construction and a capacity of 1 cm^3. The working electrode, a 4-mm diameter gold disk, supplied by Oxford Electrodes, was polished before each experiment by using an alumina (particle size 1 μm)/water slurry on cotton wool and then washed with distilled water. The reference electrode was a Radiometer type K401 saturated calomel electrode (SCE) and the counterelectrode a platinum gauze.

The bulk reduction experiment was carried out in a closed Perspex cell of volume 7.0 cm^3 as previously described.[28]

Results and Discussion

Under the experimental conditions employed in this work and over the entire range of rates of potential scan used (1–200 mV s^{-1}) horse heart cytochrome *c* gives cyclic voltammograms consistent[31] with a heterogeneous rate constant[21] of 1.5×10^{-2} cm s^{-1} and diffusion coefficients[21] for the oxidized and reduced forms of the protein of $\sim 10^{-6}$ cm^2 s^{-1}.

However, under *the same solution conditions* of pH, ionic strength, and concentration of surface modifier, and when the same

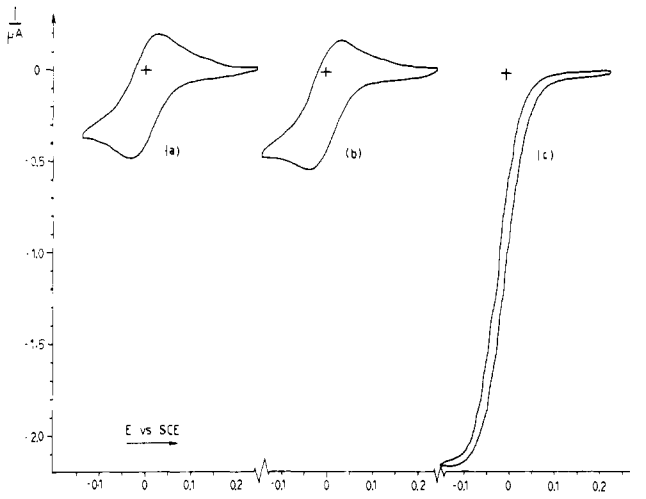

Figure 2. Dc cyclic voltammograms of 0.75 cm^3 supporting electrolyte with successive additions of (a) horse heart cytochrome (III) *c* (0.44 mM), (b) *Pseudomonas* cytochrome oxidase (6 μM), and (c) *Pseudomonas* azurin (0.25 mM). Potential scan rate 1 mV s^{-1} in the range +0.25 to −0.15 V vs. SCE.

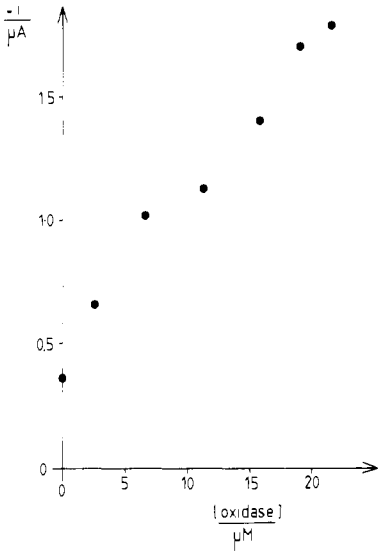

Figure 3. Catalytic current, *i*, as a function of *Pseudomonas* cytochrome oxidase concentration, measured from voltammograms recorded at 1 mV s^{-1}. Horse heart cytochrome *c*, 0.44 mM; and azurin, 0.10 mM.

electrochemical technique is used, all the proteins obtained from *Pseudomonas aeruginosa*—the copper-containing protein azurin (0.38 mM), cytochrome c_{551} (1.2 mM), and the oxidase cd_1 (10 μM)—fail to exhibit any electrochemistry. Thus, addition of any of these respiratory proteins from *P. aeruginosa* to a solution of horse heart cytochrome *c* does not give rise to any additional Faradaic current due to *direct* electron transfer to these proteins.

Figure 2a shows a single sweep cyclic voltammogram of a solution containing only horse heart cytochrome *c*. Addition of the oxidase cd_1 in the presence of dioxygen from *P. aeruginosa* to this solution produces (Figure 2b) only a small increase in current consistent with a slow coupled catalytic reaction, proposed in Scheme I. This observation is consistent with the slow rate[32]

Scheme I

$$2\text{HHCyt}^{III}c + 2e^- \rightleftharpoons 2\text{HHCyt}^{II}c \quad (1)$$

$$2\text{HHCyt}^{II}c + \text{oxidase } c(\text{III})d_1(\text{III}) \rightleftharpoons 2\text{HHCyt}^{III}c + \text{oxidase } c(\text{II})d_1(\text{II}) \quad (2)$$

$$\text{oxidase } c(\text{II})d_1(\text{II}) + {}^1/_2O_2 + 2H^+ \rightarrow \text{oxidase } c(\text{III})d_1(\text{III}) + H_2O \quad (3)$$

(28) Hill, H. A. O.; Walton, N. J.; Higgins, I. J. *FEBS Lett.* **1981**, *126*, 282–4.
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Scheme II

$$2HHCyt^{III}c + 2e^- \rightleftharpoons 2HHCyt^{II}c \quad (4)$$

$$2HHCyt^{II}c + \left\{\begin{matrix} 2Az(II) \\ 2Cyt^{III}c_{551} \end{matrix}\right. \rightleftharpoons \left.\begin{matrix} 2Az(I) \\ 2Cyt^{II}c_{551} \end{matrix}\right\} + 2HHCyt^{III}c \quad (5)$$

$$\text{oxidase } c(III)d_1(III) + \left\{\begin{matrix} 2Az(I) \\ 2Cyt^{II}c_{551} \end{matrix}\right. \rightleftharpoons \left.\begin{matrix} 2Az(II) \\ 2Cyt^{III}c_{551} \end{matrix}\right\} + \text{oxidase } c(II)d_1(II) \quad (6)$$

$$\text{oxidase } c(II)d_1(II) + {}^1/_2O_2 + 2H^+ \rightarrow \text{oxidase } c(III)d_1(III) + H_2O \quad (7)$$

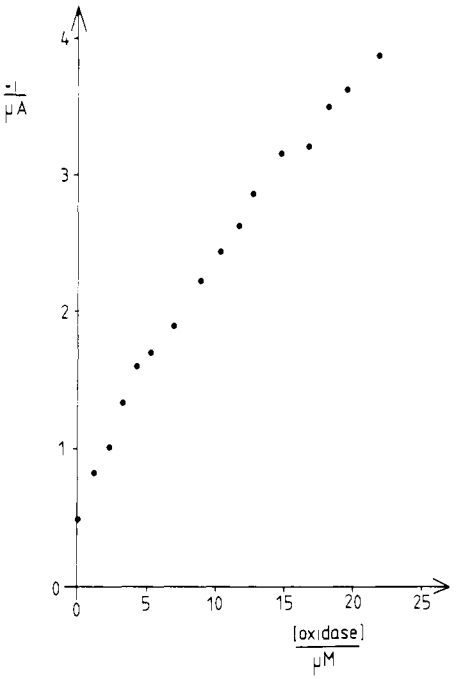

Figure 4. As in Figure 3, but for 0.44 mM horse heat cytochrome *c* and 0.29 mM cytochrome c_{551}.

of reaction of horse heart cytochrome (II) *c* with oxidase cd_1. In contrast, addition of either azurin or cytochrome c_{551} to the same solution containing horse heart cytochrome *c*, the oxidase cd_1, and dioxygen has a dramatic effect on voltammograms recorded at slow rates of potential scan (Figure 3c). No peak is observed and a large catalytic current, i_k, flows at reducing potentials. Such observations imply rapid catalytic regeneration of horse heart cytochrome (III) *c* by both azurin and cytochrome c_{551}, *which are themselves both recycled by the oxidase* cd_1, in conjunction with the reduction of dioxygen to water. We propose Scheme II to describe these reactions. These reactions are known to be fast.[33-36] Thus, the electrons are being introduced into the respiratory chain of *P. aeruginosa* (Figure 1) at a point *corresponding* to cytochrome *b* using, in this case, horse heart cytochrome *c* as a *biochemical* mediator. The electrons continue on down the chain to the terminal electron acceptor, which can be, and is, dioxygen. Bulk reduction experiments[28] in a closed system showed that 3.5–3.8 faraday was consumed per mole of dioxygen reduced, consistent with the four-electron reduction of dioxygen to water at pH 7.

In an attempt to simplify the analysis of Scheme II, we measured the kinetic current, at a slow scan rate, as a function of concentration of the oxidase cd_1, using a fixed concentration of horse heart cytochrome *c* and *either* azurin *or* cytochrome c_{551}. These results, shown in Figure 3 and 4, suggest that the catalytic current is limited by the rate of recycling of azurin or cytochrome

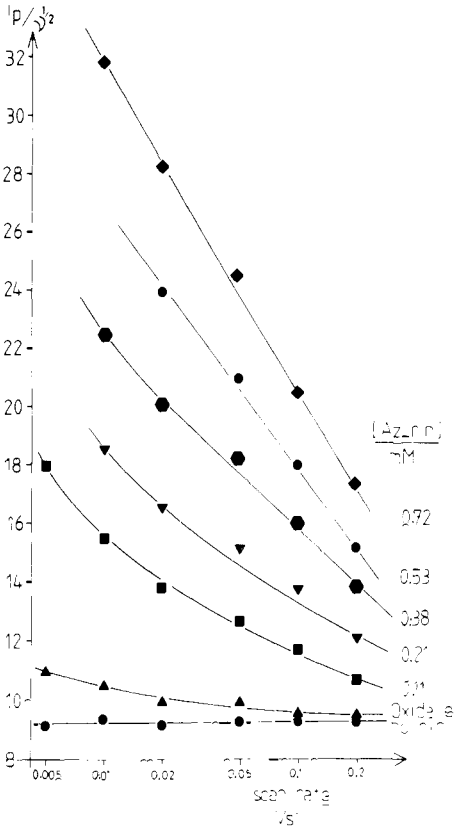

Figure 5. Diagnostic plots of current function $i_k/\nu^{1/2}$ vs. log ν, at increasing concentrations of *Pseudomonas* azurin.

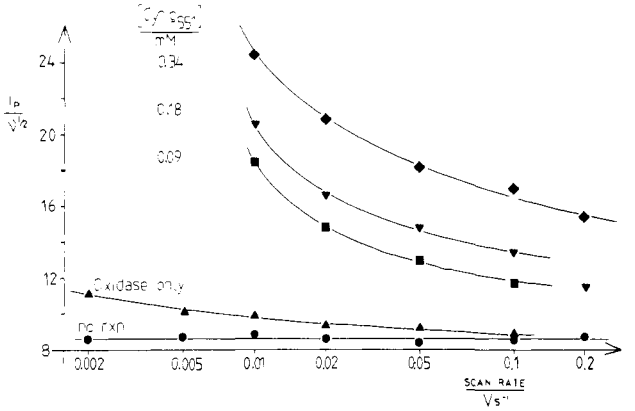

Figure 6. Diagnostic plots of current function $i_k/\nu^{1/2}$ vs. log ν at increasing concentrations of *Pseudomonas* cytochrome c_{551}.

c_{551} by the oxidase cd_1. Because reaction 7 is irreversible, increasing the concentration of oxidase cd_1 will tend to drive reactions 5 and 6 in a forward direction, i.e., left-to-right as written. Since it is reaction 5 which is the electrochemically coupled reaction, we might approximate Scheme II by the simplified Scheme III *provided all experiments are carried out in the presence of*

Scheme III

$$O + e^- \rightleftharpoons R$$

$$R + Z \xrightarrow{k_f} O$$

oxidase and dioxygen: where $k_f = k[Z]$, the pseudo-first-order rate constant. We did not find it practicable to use the 10-fold molar excess of azurin or cytochrome c_{551} over horse heart cytochrome *c* which would be required to achieve first-order conditions. Under the conditions used we should expect to observe, at slower scan rates when the extent of reaction in the diffusion layer is considerable, rather less current than expected from the first-order case. The theory[37] applicable to Scheme III yields a

(33) Morton, R. A.; Overnell, J.; Harbury, H. A. *J. Biol. Chem.* **1970**, *245*, 4653–7.
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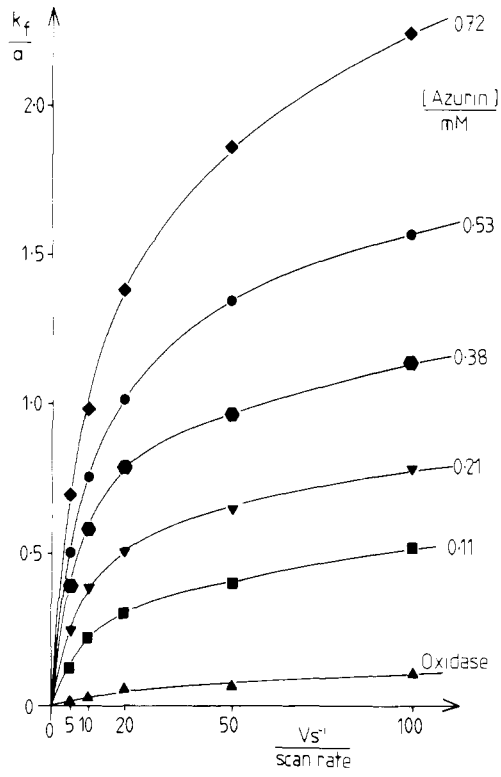

Figure 7. Plots of the kinetic parameter k_f/a against $1/\nu$ for each experiment with *Pseudomonas* azurin.

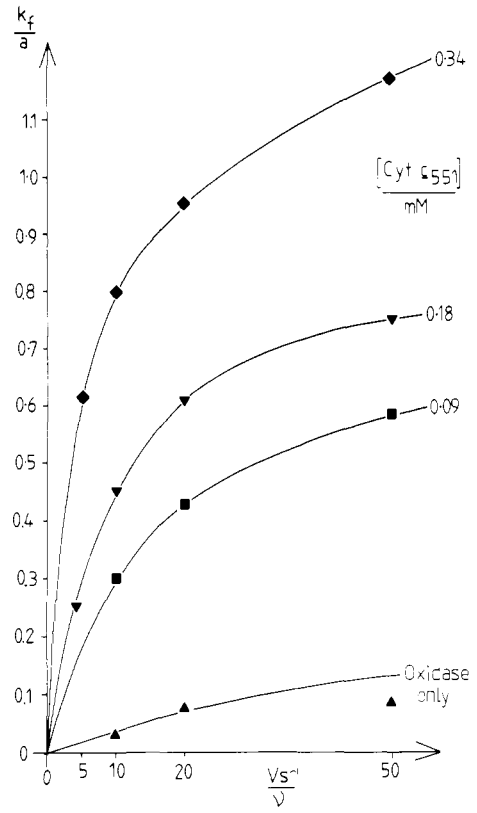

Figure 8. Plots of the kinetic parameter k_f/a against $1/\nu$ for each experiment with *Pseudomonas* cytochrome c_{551}.

"diagnostic" plot of $i_k/\nu^{1/2} \equiv i_k/i_d$ vs. ν, where ν is the scan rate, to confirm that the experimental results conform to the theoretical model. Figures 5 and 6 show results obtained at various concentrations of azurin and cytochrome c_{551}, respectively. Comparison with the theoretical plots (see Figure 17 of ref 37) indicates that we are indeed dealing with a coupled catalytic reaction that can be approximated by Scheme III. Thus, this simple electrochemical method can be used to detect and, to some extent, characterize quite complicated inter-protein electron transfer reactions.

Despite the simplifying assumptions made, we considered it worthwhile to attempt to derive kinetic information from the data available. Using a working curve (see Figure 14 of ref 37) of i_k/i_d vs. kinetic parameter, $(k_f/a)^{1/2}$, we have replotted the data as k_f/a, where $a = Fv/RT$, against $1/\nu$ which is linear under first-order conditions. However, the results for azurin (Figure 7) and cytochrome c_{551} (Figure 8) show a marked deviation from the predicted linearity, the more so at slower scan rates consistent with the idea that first-order conditions cannot be maintained at slow rates of potential scan. The reactant, Z, is not in sufficiently large excess and so is consumed to a considerable extent in the diffusion layer during the time course of a slower scan rate experiment. Second-order kinetics are being observed for which the theory is much more complex. The initial slope of each curve at infinite scan rate (when, of course, there would be no reaction at all) is, however, proportional to the true pseudo-first-order rate constant, k_f^0:

$$k_f^0 = (nF/RT)(\text{initial slope})$$

Drawing tangents to the curves at $1/\nu = 0$ yields values of k_f^0 for each experiment which are plotted in Figure 11 and 12 against the concentration of azurin and cytochrome c_{551}, respectively. An alternative method of finding k_f^0 is to calculate the effective pseudo-first-order rate constant, k_f', from each k_f/a value at a given scan rate, where

$$k_f' = (F/RT)\nu(k_f/a)$$

Plotting k_f' vs. $1/\nu$ and extrapolating to $1/\nu = 0$ will give the pseudo-first-order rate constant at infinite scan rate—k_f^0. The intercepts from Figure 9 and 10 for azurin and cytochrome c_{551}, respectively, are again plotted in Figures 11 and 12. These figures show that there is good agreement between the two methods. The

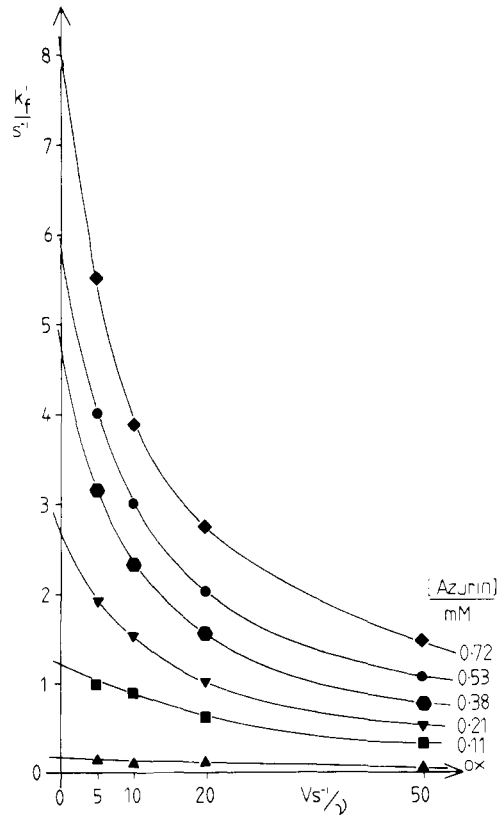

Figure 9. Effective pseudo-first-order rate constant, k_f', as a function of reciprocal scan rate extrapolated to $1/\nu = 0$, for experiments with *Pseudomonas* azurin.

(37) Nicholson, R. S.; Shain, I. *Anal. Chem.* **1964**, *36*, 706–23.

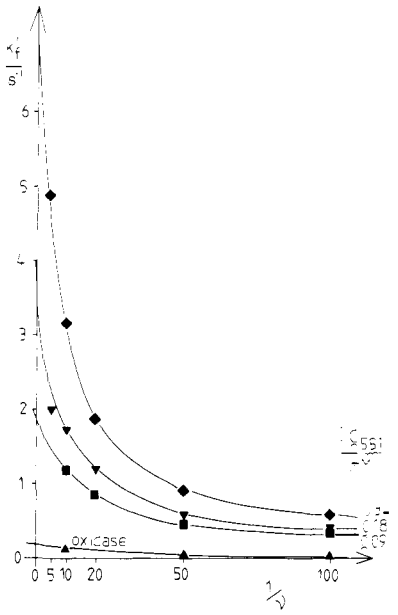

Figure 10. Effective pseudo-first-order rate constant, k_f', as a function of reciprocal scan rate, extrapolated to $1/\nu = 0$, for experiments with *Pseudomonas* cytochrome c_{551}.

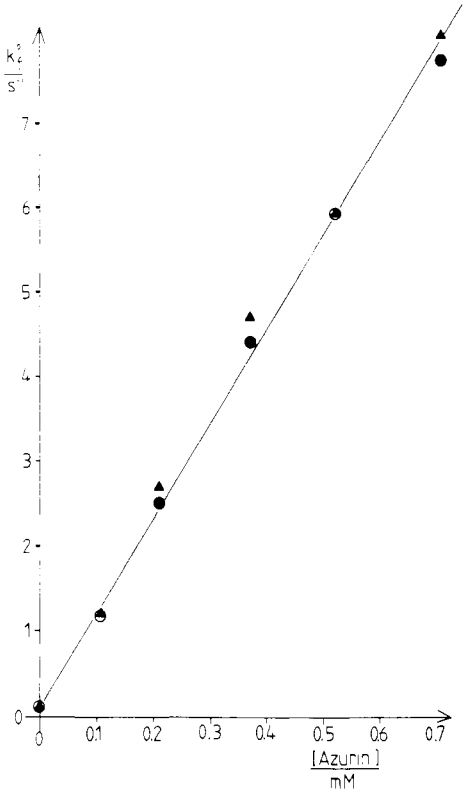

Figure 11. Pseudo-first-order rate constant, k_f^0, as a function of concentration of *Pseudomonas* azurin. Initial slope obtained by drawing tangents (●) and from incercepts of Figure 9 (▲).

slope of each graph is equal to the homogeneous second-order rate constant. For oxidation of horse heart cytochrome (II) *c* by azurin we obtain $k = 1 \times 10^4$ M^{-1} s^{-1}, and, for oxidation by cytochrome c_{551}, $k = 2 \times 10^4$ M^{-1} s^{-1}. Both rate constants were obtained at 293 K and an ionic strength of $I = 0.135$ M.

The rate constant obtained for the oxidation of horse heart cytochrome *c* by *Pseudomonas* cytochrome c_{551} is in good agreement with the literature value[33] of 1.57×10^4 M^{-1} s^{-1} at 277.5 K and $I = 0.2$ M. That for oxidation by *Pseudomonas* azurin

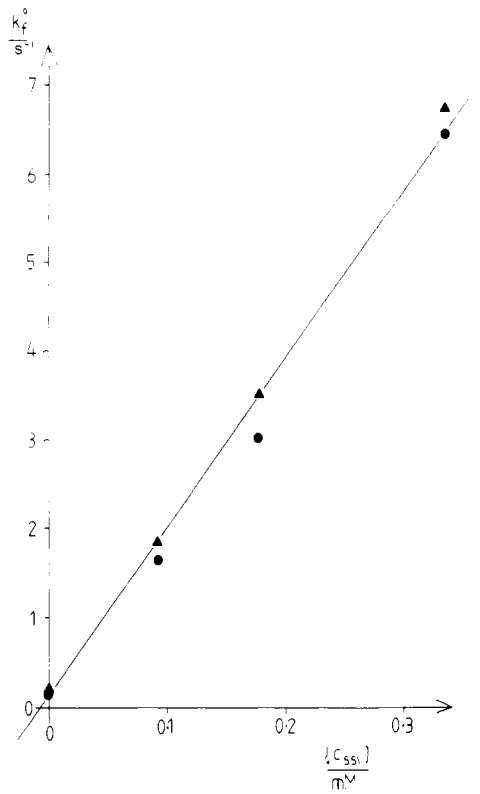

Figure 12. Pseudo-first-order rate constant, k_f^0, as a function of concentration of *Pseudomonas* cytochrome c_{551}. Initial slope obtained by drawing tangents (●) and from intercepts of Figure 10 (▲).

is not available, but the literature gives[36] a value of the rate constant of the *reverse* reaction

$$\text{Az(I)} + \text{HHCyt}^{\text{III}}c \xrightarrow{k} \text{Az(II)} + \text{HHCyt}^{\text{II}}c$$

of $k = 1.1 \times 10^3$ M^{-1} s^{-1} at 298 K and $I = 0.1$ M. The redox potential of *Pseudomonas* azurin ($E^{\circ\prime} = 0.304$ V vs. NHE) is some 40 mV more positive than that of horse heart cytochrome *c* ($E^{\circ\prime} = 0.260$V vs. NHE) so that the calculated rate constant for the *reduction* of azurin by cytochrome *c* is 0.63×10^4 M^{-1} s^{-1}, in good agreement with the value of 1×10^4 M^{-1} s^{-1} derived from our analysis of the cyclic voltammetry.

Conclusions

The collection of proteins in the working electrode compartment constitutes a terminal oxidase together with its attendant electron-transport proteins. The cytochrome cd_1 protein is not a very effective enzyme for the reduction of dioxygen to water. Nevertheless, the system corresponds to an "oxygen electrode" in which the *four*-electron reduction of dioxygen is achieved relatively rapidly at pH 7. Effective coupling of a terminal oxidase which was an efficient catalyst for the reduction of dioxygen might have considerable value.

Although it has been necessary to make various approximations to simplify the inherently complicated set of reactions of Scheme II, it has been possible to use electrochemical methods to study biologically relevant inter-protein electron transfer reactions.

Acknowledgment. We thank the Science and Engineering Research Council for support and a studentship to N.J.W. We are grateful to our colleagues, Professor I. J. Higgins and Drs. A. E. G. Cass, M. J. Eddowes, and A. P. F. Turner for advice and help.

Registry No. Cytochrome c_{551}, 9048-77-5; cytochromeoxidaselnitrite reductase, 9027-00-3; cytochrome *c*, 9007-43-6.