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Ionic Strength Dependence of the Kinetics of Electron Transfer from Bovine Mitochondrial Cytochrome *c* to Bovine Cytochrome *c* Oxidase†

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ABSTRACT: The effect of ionic strength on the one-electron reduction of oxidized bovine cytochrome *c* oxidase by reduced bovine cytochrome *c* has been studied by using flavin semiquinone reductants generated in situ by laser flash photolysis. In the absence of cytochrome *c*, direct reduction of the heme *a* prosthetic group of the oxidase by the one-electron reductant 5-deazariboflavin semiquinone occurred slowly, despite a driving force of approximately +1 V. This is consistent with a sterically inaccessible heme *a* center. This reduction process was independent of ionic strength from 10 to 100 mM. Addition of cytochrome *c* resulted in a marked increase in the amount of reduced oxidase generated per laser flash. Reduction of the oxidase at the heme *a* site was monophasic, whereas oxidation of cytochrome *c* was multiphasic, the fastest phase corresponding in rate constant to the reduction of the heme *a*. During the fast kinetic phase, 2 equiv of cytochrome *c* was oxidized per heme *a* reduced. We presume that the second equivalent was used to reduce the Cu_a center, although this was not directly measured. The first-order rate-limiting process which controls electron transfer to the heme *a* showed a marked ionic strength effect, with a maximum rate constant occurring at μ = 110 mM (1470 s^{-1}), whereas the rate constant obtained at μ = 10 mM was 630 s^{-1} and at μ = 510 mM was 45 s^{-1}. There was no effect of "pulsing" the enzyme on this rate-limiting one-electron transfer process. These results suggest that there are structural differences in the complex(es) formed between mitochondrial cytochrome *c* and cytochrome *c* oxidase at very low and more physiologically relevant ionic strengths, which lead to differences in electron-transfer rate constants.

As a continuation of ongoing studies of the kinetics of electron-transfer processes involving biological redox proteins, we have investigated the effect of ionic strength on the reaction between bovine ferrous cytochrome *c* (cyt c^{2+})[1] and fully oxidized bovine cytochrome *c* oxidase (CcO). A large body of information has accumulated on the kinetics of CcO reduction by both cyt *c* and nonphysiological reductants. Included in the latter category are ferrocyanide and ferrous sulfate (Krab

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[1] Abbreviations: cyt c^{2+} and cyt c^{3+}, ferrous and ferric cytochrome *c*, respectively; CcO, cytochrome *c* oxidase; 5-DRF and 5-DRFH•, oxidized and semiquinone forms of 5-deazariboflavin, respectively; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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& Slater, 1979), fully reduced and semiquinone species of free flavins (Ahmad et al., 1982), dithionite (Lambeth & Palmer, 1973; Jones et al., 1983; Petersen & Cox, 1980), methyl and benzyl viologens (Petersen & Cox, 1980), reduced phenazine methosulfate (Krab & Slater, 1979; Halaka et al., 1984), ruthenium(II) hexaammine (Scott & Gray, 1980; Sarti et al., 1990), and radical species generated by pulse radiolysis (Van Buuren et al., 1974; Veerman et al., 1982; Kobayashi et al., 1989). Common characteristics of the reactions of CcO with these nonphysiological reductants are that the rate constants tend to be small and that the heme *a* component is reduced much more rapidly than the heme a_3 component or the Cu_a component [except in the pulse radiolysis study of Kobayashi et al. (1989), in which it was reported that Cu_a was the preferred site of reduction of the enzyme by the 1-methylnicotinamide radical].

In order to characterize the reaction of cyt c^{2+} with CcO, the rapid-mixing (stopped-flow) technique has frequently been employed. Although this literature has been thoroughly reviewed (Nicholls, 1974; Antalis & Palmer, 1982; Hill, 1988; Malmström, 1989; Moser & Dutton, 1988), a few points should be mentioned. From the early investigations of Gibson et al. (1965), Andréasson and co-workers (Andréasson et al., 1972; Andréasson, 1975), and Wilson et al. (1975), to the more recent stopped-flow studies of Veerman et al. (1980) or Antalis and Palmer (1982), the kinetics of cyt c^{2+} oxidation have been consistently observed to be multiphasic and ionic strength dependent. The origin of this has been attributed either to ionic strength dependent interactions between cyt *c* and CcO at a single binding site (Antalis & Palmer, 1982) or to the presence of multiple cyt *c* binding sites which have their own intrinsic ionic strength dependencies (Veerman et al., 1980). In the model of Antalis and Palmer (1982), at higher ionic strengths the initial kinetic phase was ascribed to heme *a* reduction, and the second and slower phase to Cu_a reduction. However, these workers found that at low ionic strengths, when cyt *c* was in excess, the reduction of CcO was essentially monophasic, with initial reduction occurring at heme *a*. This was followed by a rapid exchange of reducing equivalents with the Cu_a site, such that an equal number of electrons became localized on the two components. As the ionic strength was increased in these investigations, the ratio of reduced heme *a* to Cu_a was not affected; however, the kinetics became more biphasic, and there was a systematic decrease in the reaction rate. Veerman et al. (1980) ascribed the oxidation of cyt *c* to the reduction of an *a*-type heme in CcO, and the kinetic complexity to the presence of two high- and two low-affinity binding sites for ferrous cyt *c*. This is analogous to the proposal made earlier by Ferguson-Miller et al. (1976) for the contribution of high- and low-affinity binding sites to the biphasic nature of the steady-state kinetics of oxidation of cyt *c* by CcO at low ionic strengths [see Garber and Margoliash, (1990) for a more recent clarification of this model]. A difficulty in the rapid-mix experiments, as was explicitly emphasized by both Veerman et al. (1980) and Antalis and Palmer (1982), was that the ability to monitor the rapid kinetic processes which occurred during the initial reduction of the enzyme by cyt *c* was severely limited by the mixing time of the stopped-flow instrument. As will be demonstrated below, this limitation does not exist in the present laser flash experiments.

While it is generally agreed that the rate constants for reduction of CcO by cyt *c* decrease with increasing ionic strength at $\mu > 100$ mM, the observed behavior of the rate constants at ionic strengths less than 100 mM has been more varied. Davies and co-workers (Davies et al., 1964; Bolgiano, 1988), as well as Smith et al. (1981), have observed either a decrease or no change in the steady-state rate of cyt *c* oxidation by bovine or *Paracoccus denitrificans* CcO for ionic strengths smaller than 100 mM. This unusual kinetic behavior has largely been ignored, and extrapolations to zero ionic strength (Smith et al., 1981), using the rate constants obtained at ionic strengths greater than 100 mM, have been made in order to obtain a theoretical rate constant at infinitely low ionic strength. The justification for this extrapolation is based upon the idea that an electrostatically stabilized complex should be, a priori, optimized for electron transfer, due to the distribution of the appropriate charged residues and/or dipole moments around or near the respective prosthetic groups (Smith et al., 1981; Koppenol & Margoliash, 1982; Margoliash & Bosshard, 1983). In contrast, Antalis and Palmer (1982) observed a rather uniform decrease in the oxidation rate constant from 40 to 540 mM ionic strength, using horse cyt *c* and bovine CcO, with cyt c^{2+} in an 8-fold excess over CcO. This is more consistent with a model involving the formation of the most efficient electron-transfer complex via electrostatic stabilization. However, the validity of the hypothesis that 1:1 complexes formed at low ionic strength are *the* physiologically relevant intermediates in electron transfer has recently been questioned, based upon the observation that rate constants for the processes controlling intramolecular electron transfer within complexes of a variety of mitochondrial cytochromes *c* and yeast cytochrome *c* peroxidase *increase* with increasing ionic strengths, demonstrating that the complex formed at very low ionic strengths is not optimal for electron transfer (Hazzard et al., 1988a,b). As will be shown below, the present results are in agreement with this.

The experiments described herein involve the very rapid (<1 μs) in situ generation of reductants in the form of flavin semiquinones using the laser flash photolysis technique [cf. Tollin et al. (1986)]. When such photochemistry is carried out in the presence of a given redox protein (P_1) and its physiological electron acceptor (P_2), under the appropriate conditions one can selectively reduce the lower redox potential partner, P_1, and follow the subsequent electron transfer to the acceptor, P_2, according to the equations:

$$Fl_{ox} \xrightarrow{h\nu,\ \text{EDTA}\ k_1} FlH^{\bullet} \qquad (1)$$

$$FlH^{\bullet} + (P_1)_{ox} \xrightarrow{k_{2a}} Fl_{ox} + (P_1)_{red} + H^+ \qquad (2a)$$

$$FlH^{\bullet}\ (P_2)_{ox} \xrightarrow{k_{2b}} Fl_{ox} + (P_2)_{red} + H^+ \qquad (2b)$$

$$(P_1)_{red} + (P_2)_{ox} \xrightarrow{k_3} (P_1)_{ox} + (P_2)_{red} \qquad (3)$$

where Fl_{ox} and $FlH^{\bullet}$ correspond to the oxidized and semiquinone flavin species, respectively. Equations 2a and 2b are depicted as parallel second-order one-electron-transfer reactions, the relative concentrations of $(P_1)_{red}$ and $(P_2)_{red}$ produced being dependent upon the ratio of the respective rate constants and the relative concentrations of $(P_1)_{ox}$ and $(P_2)_{ox}$. Thus, if $k_{2a} \gg k_{2b}$ and/or $[(P_1)_{ox}] \gg [(P_2)_{ox}]$, then $[(P_1)_{red}] \gg [(P_2)_{red}]$, and the contribution of eq 2b to the overall reaction sequence will be minimal. Furthermore, under typical experimental conditions, the concentration of $FlH^{\bullet}$ generated by the laser flash via reaction 1 is relatively small (≤ 1 μM in the absence of added protein). Hence, for protein concentrations ≥ 10 μM, pseudo-first-order conditions exist for reactions 2a, 2b, and 3. It is also important to note that reactions 2a and 2b are always in competition with flavin semiquinone disproportionation (not shown), and thus must be more rapid than this process in order to result in effective protein reduction.

The transfer of an electron from P_1 to P_2 is depicted as a one-step process in eq 3. In most cases, however, this can be expected to be a multistep process, involving the formation of a collisional electron-transfer complex, and perhaps other intermediates as well. Assuming that the reactions in eq 1 and 2a are sufficiently rapid so that they do not become rate limiting (see below), plots of k_{obs} vs $[(P_2)_{ox}]$ should be linear if reaction 3 is a true second-order process. In several previous studies, however, and in the present experiments with cyt *c* and CcO, hyperbolic plots were obtained for the concentration dependence of k_{obs}. This suggests that reaction 3 consists of at least two steps, one of which is a rate-limiting first-order process which controls the electron-transfer reaction. Specifically, this type of behavior has been observed during reactions of several mitochondrial cyt *c* with native and site-directed mutants of cyt *c* peroxidase (Hazzard et al., 1988a,b), and in the reduction of *Anabaena* and spinach ferredoxin $NADP^+$ reductase by their respective ferredoxins (Bhattacharryya et al., 1987; Walker et al., 1990).

It has been previously shown that direct reduction of oxidized resting CcO by several isoalloxazine derivatives (i.e., flavin analogues; E_m values for riboflavin are −230 and −210 mV for the semiquinone and fully reduced couple, respectively; Draper & Ingraham, 1968) occurs very slowly and that the predominant reductant is the fully reduced anionic species, FH^-, formed via FH• disproportionation, rather than the semiquinone (Ahmad et al., 1982). For the purposes of the present investigation, we have chosen to use the lower potential 5-deazariboflavin (5-DRF; E_m = −630 mV; Blankenhorn, 1976) as a reductant for several reasons. First, the fully reduced 5-DRF is very unreactive toward reduction of redox proteins which are able to react readily with the semiquinone species (5-DRFH•; Edmondson et al., 1972). Hence, any electron-transfer reaction which occurs using this flavin analogue most probably involves only one electron donation from the semiquinone species. Second, due to its low reduction potential, one-electron transfer to *c*-type cytochromes by 5-DRFH• has a second-order rate constant which is at the diffusion-controlled limit, $>10^9$ M^{-1} s^{-1} (Meyer et al., 1983). As will be demonstrated below, reduction of CcO by 5-DRFH• is considerably slower than this. Therefore, in a laser flash photolysis experiment using 5-DRF, under suitable conditions the initial reduction of cyt *c* does not become rate limiting and proceeds at much faster rates than does direct reduction of CcO by 5-DRFH•. Thus, the conditions specified above in eqs 1–3 are met by this system.

Upon addition of cyt *c* to a solution containing 5-DRF and CcO in the course of a laser flash photolysis experiment, several effects were observed; the magnitude of reduced oxidase generated per laser flash was increased significantly over that observed in the absence of cyt *c*. Rapid reduction of the oxidase occurred at the heme *a* site in a monophasic exponential process, whereas oxidation of reduced cyt *c* was multiphasic, the fastest and largest phase corresponding in rate constant to the reduction of the heme *a* component of CcO. The observed rate constant for reduction of CcO depended nonlinearly on the oxidase concentration, and the first-order rate-limiting process which controls electron transfer from cyt *c* to CcO showed a marked ionic strength effect, with a maximum occurring at μ = 110 mM.

Materials and Methods

Bovine cytochrome *c* (Sigma, type V) was prepared by ion-exchange chromatography on CM-cellulose. Conversion of all the protein to the oxidized species was performed by addition of a slight excess of ferricyanide followed by passage through an anion-exchange column (Bio-Rad AG 1×8). Protein concentration was determined by using $\Delta\epsilon_{550}$ = 21 mM^{-1} cm^{-1} for the reduced minus oxidized difference spectrum. Typically, stock cyt *c* solutions were prepared and stored frozen at concentrations >1 mM. Bovine cytochrome *c* oxidase was isolated by a modification of the Yonetani procedure (1960, 1961, 1967). During the last stages of the ammonium sulfate fractionations, the enzyme was brought up into buffer containing 0.1% lauryl maltoside (Calbiochem) and finally equilibrated on a Sephadex G-50 column which had been preequilibrated with 10 mM Tris containing 0.1% lauryl maltoside. Following concentration, the enzyme was stored in liquid nitrogen. Aliquots of frozen enzyme were thawed only 1 time. Concentration of the enzyme, expressed in terms of enzyme concentration (i.e., cytochrome aa_3), was determined by using $\Delta\epsilon_{604-630}$ = 20.5 mM^{-1} cm^{-1} (Yonetani, 1960) for the reduced minus oxidized difference spectrum obtained by photochemical reduction using either lumiflavin or 5-deazariboflavin in the presence of EDTA. The molecular activity of the enzyme was determined spectrophotometrically by the procedure of Smith (1955). A turnover number of 175 s^{-1} was determined for the aerobic oxidation of cyt c^{2+}, which compares well with literature values (Yonetani, 1960, 1967). The sample of enzyme provided by Prof. T. E. King had been prepared by the procedure of Kuboyama et al. (1972).

5-Deazariboflavin was synthesized by Dr. M. C. Walker in this laboratory by the procedure of Smit et al. (1986). The basic reaction buffer for the kinetic experiments was 10 mM Tris, pH 7.4, containing 0.5 mM EDTA and 100 μM 5-deazariboflavin. Ionic strength was adjusted by addition of KCl. The laser flash photolysis system has been discussed previously (Simondsen & Tollin, 1983). The methodology of the laser flash experiment is comparable to that used in earlier investigations involving cyt *c* and cytochrome *c* peroxidase, and is described fully elsewhere (Hazzard et al., 1988a,b; Mauro et al., 1988; Miller et al., 1988; also see the introduction). A minimum of four transient decay curves, collected at the appropriate wavelengths (see Results), were averaged for rate constant determinations at each oxidase concentration. At 604 nm, pseudo-first-order rate constants (k_{obs}) were obtained either by manual plotting of log (Δ signal) vs time or by computer fitting to a single-exponential rate equation using the program SI-FIT (OLIS, Inc.). The plots were linear for at least 4 half-lives. Agreement between the rate constants derived by the two methods was usually quite good. Analysis of biphasic decays at 550 nm was performed by using the program SI-FIT and fitting the data with a two-exponential rate equation. Since the predominant change in absorbance at 550 nm coincided with the monophasic absorbance change at 604 nm (see below), for the sake of simplicity pseudo-first-order rate constants were obtained from data measured at this latter wavelength. For the nonlinear plots of k_{obs} vs oxidase concentration, data were analyzed based on the minimal reaction scheme (eqs 5–6) given in the text according to the procedure of Simondsen et al. (1982), as more fully described in Miller et al. (1988). From the analysis of these hyperbolic plots, we can directly determine the apparent equilibrium constant for complex formation, K_{app}, and the limiting rate constant, k_6.

Results and Discussion

Difference Spectra for Photoreduction of Cytochrome c Oxidase and Cytochrome c. Figure 1a shows reduced minus oxidized difference spectra in the visible and Soret regions for 20 μM CcO and 20 μM cyt *c*, generated by steady-state illumination of solutions containing the individual proteins, 5-DRF, and EDTA. Photoirradiation was performed until

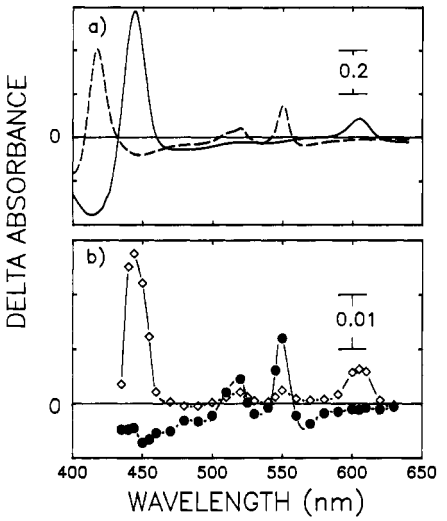

FIGURE 1: Redox difference spectra of cyt *c* and CcO. (a) Steady-state redox difference spectra of solutions containing (---) cytochrome *c* (20 μM) or (—) cytochrome *c* oxidase (10 μM), 5-DRF (100 μM), and EDTA (0.5 mM) in 10 mM Tris buffer at pH 7.4. (b) Transient redox difference spectra obtained following laser flash photolysis of a solution containing cyt *c* (20 μM), CcO (10 μM), 5-DRF (100 μM), and EDTA (0.5 mM) in 10 mM Tris buffer at pH 7.4. (●) Immediately after the laser flash; (○) 20 ms after the laser flash.

there was no further change in absorbance at 550 nm (cyt *c* reduction) or 605 nm (CcO reduction). Figure 1b shows the reduced minus oxidized difference spectrum from 430 to 630 nm obtained by laser flash photolysis of solutions containing equimolar concentrations of CcO and cyt *c* in the presence of 5-DRF and EDTA at low ionic strength. (At wavelengths shorter than 430 nm, too much reduction of the 5-DRF occurred due to the monitoring beam; therefore, this wavelength was the lower limit that we could achieve in the flash photolysis experiment.) The difference spectrum obtained immediately after the laser flash (solid circles) is essentially that due to cyt *c* reduction, with positive absorption bands occurring at 550 and 520 nm and a marked trough at 450 nm. After 20 ms, strong positive bands were observed at 605 and 445 nm (open circles). There was also some residual absorbance due to the incomplete oxidation of reduced cyt *c* (see below), as indicated by the smaller peaks at 520 and 550 nm. Aside from these latter wavelengths, however, the 20-ms difference spectrum is essentially the same as that obtained for the steady-state reduction of free CcO, shown in Figure 1a, with one very important difference. The ratio $\Delta A_{445}/\Delta A_{605}$ calculated from the steady-state difference spectrum is 6.9, which is consistent with an equal distribution of electrons between the heme *a* and a_3 centers of CcO (Vanneste, 1966; Blair et al., 1986; Antalis & Palmer, 1982). In contrast, this ratio in the 20-ms time-resolved spectrum is 3.7. If we assume that in the flash experiment the degree of attenuation of both the 445- and 605-nm bands due to the larger monochromator slit widths (band-pass = 8 nm) is the same (which is a reasonable approximation considering the similar broadness of both bands), then the difference between the steady-state and time-resolved difference spectra indicates that an equal distribution of electrons between the *a* and a_3 heme centers is not achieved on a 20-ms time scale. Calculation of the relative amounts of reduction of the two hemes in the time-resolved difference spectrum depends upon the weighting factor used for the contributions of the two hemes to the total extinction coefficient at these two wavelengths. When the values given by Vanneste (1966) and Antalis and Palmer (1982) are used, a $\Delta A_{445}/\Delta A_{605}$ ratio of 2.8 is expected if only the heme *a* component underwent reduction, whereas a ratio of 3.3 is expected with the values given in Blair et al. (1986). Thus, within the limits of the experiment, the transient difference spectrum is consistent with the direct reduction of predominantly the heme *a* component by cyt *c*.

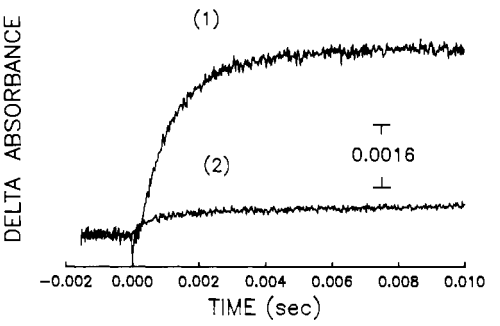

FIGURE 2: Spectral changes observed at 605 nm for the reduction of CcO by 5-DRFH• in the presence (curve 1) and absence (curve 2) of cyt *c*. Protein concentrations were 11 μM oxidized CcO and 20 μM ferric cyt *c*. Buffer conditions were as in Figure 1.

Kinetics of Reduction of CcO by Free Flavins in the Absence and Presence of Cytochrome c. Figure 2 shows transient kinetic traces obtained at 605 nm following laser flash photolysis of a solution containing fully oxidized CcO, 5-DRF, and EDTA in the absence and presence of equimolar cyt *c*. In the absence of cyt *c*, a relatively rapid reduction of the heme *a* component was observed, although the magnitude of the increase in absorbance at 605 nm was small. Addition of cyt *c* to the reaction mixture resulted in a 7-fold increase in the intensity of the 605-nm signal, which is in direct proportion to the increase in the amount of heme *a* reduced per laser flash.

The second-order rate constant determined for the direct reduction of fully oxidized CcO by 5-DRFH• is 1.8×10^7 M^{-1} s^{-1} (data not shown) at both 10 and 110 μM ionic strength. This value is on the borderline of being measurable by this technique, and thus probably represents an upper limit, due to the competition from semiquinone disproportionation ($k = 4.3 \times 10^9$ M^{-1} s^{-1} under conditions similar to those employed in the present studies; M. C. Walker, unpublished results). For a protein which has a reported midpoint reduction potential which ranges from approximately +220 to +340 mV [see Antalis and Palmer (1982) or Kojima and Palmer (1983) for a more complete discussion of the literature values] for the heme *a* component, this second-order rate constant is quite small. For example, in the case of mitochondrial and bacterial *c*-type cytochromes which have redox potentials from +50 to +370 mV, the second-order reduction rate constants obtained with 5-DRFH• are all close to the diffusion-controlled limit, $(2–3) \times 10^9$ M^{-1} s^{-1} (Meyer et al., 1983). The present data with CcO are in agreement with the results of Ahmad et al. (1982), in which it was shown that the reduction of CcO by the higher potential flavin derivatives, lumi- and riboflavin, gave second-order rate constants that were consistent with a reaction involving the fully reduced flavin species, which is produced by disproportionation of the semiquinone. Thus, the rate constant for reduction by the semiquinone was too small to compete with disproportionation ($<10^7$ M^{-1} s^{-1}). Unlike these higher potential flavins, however, Edmondson et al. (1972) have shown that the fully reduced 5-DRF is an unreactive species. Thus, the most logical explanation for the small magnitude of CcO reduction observed in Figure 2 for the reaction in the absence of cyt *c* is that there is a significant loss of the reactant species resulting from the disproportionation reaction due to the sluggish reaction between 5-DRFH• and CcO. As will be important to considerations presented

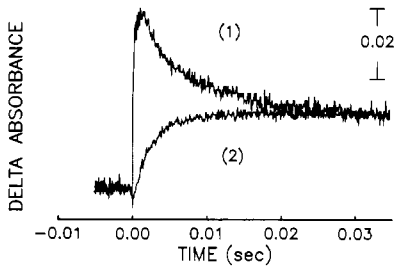

FIGURE 3: Spectral changes occurring at 550 (curve 1) and 604 nm (curve 2) for the reduction of CcO on a 40-ms time scale in the presence of cyt c at μ = 10 mM. Protein concentrations were 10 μM oxidized CcO and 20 μM ferric cyt c. Buffer conditions were as in Figure 1.

below, the lack of an ionic strength effect on the kinetics of reduction of CcO by the electrostatically neutral 5-DRFH$^{\bullet}$ also suggests that there is no ionic strength dependent change in the conformation of the oxidase between 10 and 100 mM that results in a change in the accessibility of heme a to reduction. From these experiments, we can also conclude that there exists a kinetic barrier to the direct reduction of CcO by 5-DRFH$^{\bullet}$, since there is such a large thermodynamic driving force (ΔE_m = ~930 mV) for the reaction (see below for further discussion).

Figure 3 shows 40-ms kinetic traces obtained at 550 and 605 nm for a solution containing equimolar cyt c and fully oxidized CcO following flash-induced reduction by 5-DRFH$^{\bullet}$ at an ionic strength of 10 mM. At 605 nm (curve 2), there was a very rapid initial small decrease in absorbance, relative to the preflash base line, followed by a larger monophasic increase. The initial decrease can be ascribed to cyt c reduction and the subsequent increase to heme a reduction (cf. Figure 1). At 550 nm, there was a very rapid rise in absorbance, followed by a decay to a value which is positive relative to the preflash base line. Consistent with the conclusions drawn from the time-resolved redox difference spectra shown in Figure 1b, the kinetic traces indicate a very rapid reduction of cyt c by 5-DRFH$^{\bullet}$, producing the increase in absorbance at 550 nm, followed by its oxidation [note that there is very little contribution of CcO to the total absorbance change at 550 nm (cf. Figure 1)]. By comparing the rate of cyt c reduction in the presence of CcO at low ionic strength with that of cyt c alone, we have determined that complexation of ferric cyt c with fully oxidized CcO does not impede the reduction of the cyt c by 5-DRFH$^{\bullet}$ (data not shown). This is in agreement with earlier results with other flavin semiquinones (Ahmad et al., 1982). It should also be pointed out that the absolute magnitude of the 550-nm signal in the laser flash experiment has been attenuated due to the relatively narrow bandwidth of the cyt c α peak (half-bandwidth = 8.6 nm) and the wide-slit width of the monochromator used to monitor the flash reaction (band-pass = 8 nm). The peak is also attenuated due to cyt c reoxidation by CcO. The degree of attenuation of the 550-nm α band for cyt c was calculated by extrapolating the decay curve to zero time and by comparing $|\Delta A_{550}|/|\Delta A_{570}|$ in the spectra obtained from the spectrophotometer and that from the laser flash apparatus. For the spectrum shown in Figure 1a, the ratio was 4.7, whereas in the flash photolysis experiment (Figure 1b) the ratio was 2.4 for the transient difference spectrum obtained at zero time. Thus, under these experimental conditions, there was an approximately 50% attenuation of the 550-nm band of cyt c. For CcO, the attenuation of the 605-nm band was much smaller, due to the larger half-bandwidth (20 nm). Using $|\Delta A_{605}|/|\Delta A_{630}|$, we determined that there was an attenuation of only 26% for the

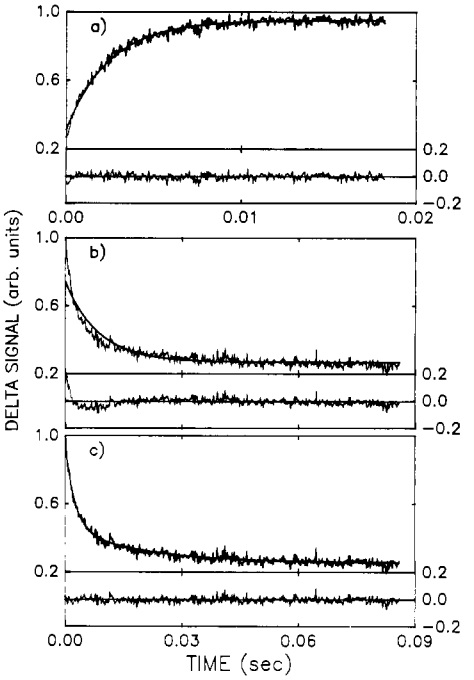

FIGURE 4: Deconvolution of kinetic traces obtained at 550 and 604 nm using single- and double-exponential curves. (a) Single-exponential fit to data at 604 nm. (b) Single-exponential fit to data at 550 nm. (c) Double-exponential fit to data at 550 nm.

605-nm band resulting from the wider slit width of the laser apparatus. When corrections in the magnitude of the absorbance changes at 550 and 604 nm were made taking the band attenuation into account, and using values for $\Delta\epsilon$ of 22 and 20 mM^{-1} cm^{-1} for cyt c and heme a of CcO (Vanneste, 1966; Antalis & Palmer, 1982), respectively, the concentration of cyt c which was reoxidized at t = 40 ms was 0.44 μM, and the concentration of CcO which was reduced at this time was 0.25 μM. Hence, the ratio of cyt c oxidized to heme a reduced at t = 40 ms was approximately 1:0.5. These calculations also assumed no spectral contribution of cyt c or CcO to the transients at 604 and 550 nm, respectively (cf. Figure 1). Attempts to measure absorbance changes at wavelengths greater than 800 nm so as to monitor possible redox state changes in the copper centers were unsuccessful.

It is evident from Figure 3 that the signal at 550 nm did not return to the preflash base line on a time scale on which there was no more change observed at 605 nm. The decay at 550 nm which occurred at t = 40 ms was calculated to be 67% of the total amount of reduced cyt c generated in the laser flash. As will be discussed in more detail below, on longer time scales (i.e., 100 ms) we calculate that an additional 12% of the reduced cyt c was reoxidized and that this second oxidation phase occurred via a distinct kinetic process.

These results are generally in agreement with the experiments of Antalis and Palmer (1982), in that cyt c oxidation by CcO occurs via a biphasic process at low ionic strength, and for every one electron donated by cyt c half a reducing equivalent residues on heme a. Presumably, the other half reducing equivalent resides on Cu_a (Antalis & Palmer, 1982), although in our studies direct monitoring of the Cu_a signal was not possible.

Figure 4 shows a kinetic analysis of the transients observed at 605 and 550 nm. In Figure 4a, the smooth curve represents a single-exponential fit to the data at 605 nm. The agreement is clearly quite good, as is indicated by the calculated residuals. A small deviation occurs at the very beginning of the trace, which is probably due (at least in part) to contributions from

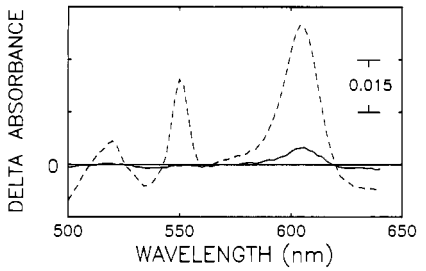

FIGURE 5: Steady-state irradiation of a mixture of cyt *c* (8 μM) and CcO (4 μM) in the presence of lumiflavin (7 μM) in Tris buffer (pH 7.4) containing EDTA (0.5 mM) at μ = 10 mM. (—) 20-s and (---) 50-s irradiation.

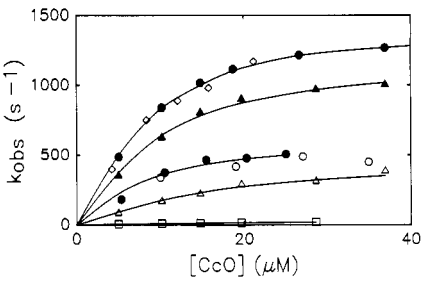

FIGURE 6: Plots of k_{obs} vs [CcO] for the reduction of CcO at various ionic strengths. (●) Simultaneous addition of increasing amounts of cyt *c* and CcO in a 2:1 molar ratio at μ = 10 mM. For all other reactions, cyt *c* was held constant at 20 μM and titrated with the indicated concentration of CcO. Reactions were performed at ionic strengths of (○) 10, (▲) 60, (●) 110, (△) 210, and (□) 510 mM. (◇) corresponds to data collected using the oxidase preparation provided by Prof. T. E. King at μ = 110 mM. The buffer for all reactions was 10 mM Tris at pH 7.4 (μ = 10 mM) containing 0.5 mM EDTA, 100 μM 5-DRF, and the appropriate amount of KCl for the changes in ionic strength.

cyt *c* reduction. The pseudo-first-order rate constant derived from this fit is 409 ± 7 s^{-1}. Figure 4b shows a single-exponential fit to the data obtained at 550 nm for the same reaction solution. Note that there is a systematic deviation over most of the trace observed in the residuals, indicating that a second slower kinetic process is clearly detectable at 550 nm. Figure 4c shows the 550-nm kinetic trace fitted with a two-exponential equation. The agreement between the observed and theoretical curve is clearly considerably better. The values for the two rate constants are 416 ± 24 and 51 ± 5 s^{-1}, with amplitudes of 70 and 30% of the total signal change, respectively. Thus, the first-order rate constant for the increase in absorbance at 605 nm and the rate constant for the fast phase of the signal decay at 550 nm are, within experimental error, the same. On this basis, we can conclude that the fast component of the kinetic process corresponds to direct reduction of the heme *a* component of CcO by reduced cyt *c*. At the present time, we cannot specify the identity of the oxidant in the slower second electron-transfer process. However, in view of the absence of this kinetic phase at 604 nm, we can say that the second site is certainly not the heme *a* center. As noted above, attempts to measure absorbance changes at wavelengths greater than 800 nm, so as to monitor possible redox state changes in the Cu_a center, were unsuccessful.

Similar slow cyt *c* oxidation phases have been observed by others, in particular by Antalis and Palmer (1982). Interestingly, they too could not measure any spectral change at 604 nm at low ionic strength for this slow kinetic process. On the basis of changes in the Soret region and the loss of the slow phase upon incubation of the enzyme with CN^-, it was concluded that this slow phase was due to reduction of the heme a_3 species. The fact that the rate constant for the slow phase in our experiments is larger than that reported by these workers (3 s^{-1}) is not surprising, considering the fact that the experimental conditions were not equivalent, especially with respect to buffer, detergent, and temperature, and that these workers used horse cyt *c* rather than bovine cyt *c* as in the present experiments. As pointed out by Antalis and Palmer (1982), the rate of this slow kinetic process is significantly smaller than the catalytic turnover rate, which is also the case in our experiments. Because of this fact, we did not investigate this process any further.

Due to the fact that in the flash experiments we observed residual reduced cyt *c* on time scales on which there was no longer any observable change occurring at 604 nm, we carried out a steady-state irradiation of a solution containing cyt *c* and CcO, in a molar ratio of 2:1, in the presence of 5-DRF and EDTA. The redox difference spectra obtained after 20 and 50 s of white light irradiation are shown in Figure 5. In the 20-s spectrum, we observed an increase in absorbance at 605 nm which corresponds to reduction of approximately 0.2 μM CcO, whereas at 550 nm there was virtually no absorbance due to the presence of reduced cyt *c*. Thus, on time scales considerably longer than were accessible with the laser flash technique, we observed essentially complete oxidation of all of the reduced cyt *c* at low levels of reduction. This implies that, based upon the data presented in Figure 4, there is an additional slow kinetic process involving the oxidation of reduced cyt *c*. Again, this process is too slow to be relevant to catalytic turnover, and was not investigated any further. In the 50-s steady-state spectrum, we observed a further increase in absorbance at 604 nm as well as a marked increase at 550 nm. These spectral changes are consistent with the reduction of 1.6 μM heme *a* and 1.11 μM cyt *c*, which corresponds to reduction of 44% of the total oxidase heme ($a + a_3$) and 15% of the cyt *c*. This agrees well with what would be expected based on an estimate of the redox potential difference between cyt *c* and heme *a* (approximately 40 mV), which implies that the system is close to equilibrium. Again, reduction of the Cu_a center was not determined in these spectra.

Figure 6 shows plots of k_{obs} vs [CcO] obtained upon laser photolysis when solutions containing 20 μM oxidized cyt *c* and 100 μM 5-DRF were titrated with CcO at several ionic strengths. In these experiments, the concentration of cyt *c* reduced per laser flash was <1 μM. Thus, with respect to CcO, pseudo-first-order conditions were maintained. The k_{obs} values were calculated from the spectral changes measured at 605 nm. At μ = 10 mM, we have also included data obtained from experiments in which a 2:1 molar ratio of cyt *c* and CcO was increased by adding incremental amounts of both proteins (open circles). At μ = 110 mM, data are presented which were obtained from a preparation of CcO kindly provided by Dr. Tsoo E. King. Note that for this latter preparation the k_{obs} values fall on the same curve as determined with our own preparation. Furthermore, the kinetic data obtained at 550 nm with this material were also indistinguishable from that observed with our preparation (not shown).

We have also determined that when the CcO and cyt *c* solution was fully reduced by prior white light irradiation of the sample, and then exposed to air for a time sufficient to allow both proteins to return to their fully oxidized states, no change occurred in the kinetics of heme *a* reduction by cyt *c* during a subsequent laser flash photolysis experiment (not shown). Thus, "pulsing" or activating the enzyme had no apparent effect on the reduction kinetics (Colosimo et al., 1981).

At all ionic strengths, k_{obs} became independent of the change in CcO concentration, and approached a limiting value which

was dependent on the ionic strength of the reaction solution. At the higher ionic strengths ($\mu \geq 60$ μM) and at concentrations less than 20 μM, where there is a smaller concentration of preformed complex, the data suggest that CcO reduction proceeds via at least a two-step mechanism, involving transient complex formation between reduced cyt *c* and oxidized CcO. The effect of complexation of oxidized cyt *c* by CcO will be discussed further below. These data have been analyzed by nonlinear least-squares fitting according to the following minimal kinetic mechanism [cf. Simondsen et al., (1982) for details]:

$$FH^{\bullet} + \text{cyt } c^{3+} \xrightarrow{k_4} F_{ox} + \text{cyt } c^{2+} \quad (4)$$

$$\text{cyt } c^{2+} + \text{cyt } a^{3+} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \text{cyt } c^{2+}\cdot\text{cyt } a^{3+} \quad (5)$$

$$\text{cyt } c^{2+}\cdot\text{cyt } a^{3+} \xrightarrow{k_6} \text{cyt } c^{3+} + \text{cyt } a^{2+} \quad (6)$$

The pseudo-first-order rate constant for the reaction in eq 4 at 20 μM cyt *c* is 4×10^4 s^{-1}, which is significantly larger than the maximum value for the limiting rate constant for CcO reduction at any ionic strength (cf. Figure 6). Thus, cyt *c* reduction was not rate-determining. The data obtained at 10 mM ionic strength upon increasing the concentrations of *both* cyt *c* and CcO provide further support for this contention, inasmuch as k_{obs} was approximately independent of protein concentration. These results also imply that at this low ionic strength not only CcO reduction but also the initial cyt *c* reduction by the flavin semiquinone occurred within an electrostatically stabilized complex between cyt *c* and CcO. The close agreement between the data at 10 mM ionic strength for the titration experiment in which only the CcO concentration was increased, and the experiment in which additions of both proteins were made, supports the idea that the limiting value for k_{obs} does not reflect the rate of dissociation of oxidized cyt *c* from a complex between the oxidized proteins, a point that will be discussed below.

The apparent equilibrium constant for the formation of a competent electron-transfer complex between reduced cyt *c* and oxidized CcO is equal to the ratio of the rate constants in eq 5, $K_{app} = k_5/k_{-5}$. A value for this quantity can in principle be obtained from the concentration dependence of k_{obs}. This, of course, ignores any contribution from complexation of CcO by cyt c^{3+}; this will be considered more fully below. Equation 6 represents a rate-limiting first-order intracomplex electron transfer from reduced cyt *c* to oxidized heme *a*. The solid lines in Figure 6 correspond to the best fits according to the above mechanism as derived from nonlinear least-squares regression analysis. Values for K_{app} and the limiting first-order rate constant k_6 (which reflects the processes that control intracomplex electron transfer) are given in Table I. Also given in Table I are the values obtained when lumiflavin ($E_m = -230$ mV for the semiquinone/oxidized couple) was used as a reductant at an ionic strength of 110 mM. From these latter values, it is apparent that neither K_{app} nor the limiting rate constant were dependent on the nature of the flavin reductant, and thus represent properties of the proteins alone.

As the ionic strength was increased from 10 to 110 mM, there was a 2.3-fold increase in the value of k_6, which was then followed by a sharp drop in this rate constant as the ionic strength was further increased to 210 and 510 mM. In the range of 10–110 mM, there was a much smaller effect of ionic strength on K_{app}, while for $\mu > 110$ mM there was a decrease in K_{app} paralleling that of k_6. Thus, the data indicate that the first-order process(es) which control(s) electron transfer from cyt *c* to CcO is optimized at an ionic strength of approximately 110 mM.

Table I: Kinetic Parameters for the Reduction of Bovine Cytochrome *c* Oxidase by Bovine Cytochrome *c*[a]

flavin reductant	ionic strength (mM)	$K_{app} \times 10^{-4}$ (M^{-1})	k_6 (s^{-1})
5-DRFH$^{\bullet}$	10	9.8	628
5-DRFH$^{\bullet}$	60	9.5	1200
5-DRFH$^{\bullet}$	110	10.7	1470
LFH$^{\bullet}$	110	10.0	1400
5-DRFH$^{\bullet}$	210	5.5	470
5-DRFH$^{\bullet}$	510	2.2	45

[a] Experimental conditions: 5 mM Tris buffer at pH 7.4 containing 1 mM EDTA, 0.1% lauryl maltoside, and either 100 or 80 μM flavin for 5-DRFH$^{\bullet}$ and LFH$^{\bullet}$, respectively. Ionic strength was varied by the addition of KCl. Values obtained by nonlinear least-squares fitting to k_{obs} vs concentration data shown in Figure 6, according to the mechanism given in the text (eqs 5–6) [cf. Simondsen et al. (1982) for details].

Conclusions

A possible explanation for the kinetic barrier found for CcO reduction by free flavin semiquinones is that the heme *a* is highly buried within the protein matrix, so that accessibility of the reductant to this center is severely limited. Raman spectra (Sassaroli et al., 1989), which indicate that heme *a* is far removed from the bulk solvent and that there are only a limited number (2) of water molecules which can interact with this group, are consistent with this. Similar kinetic sluggishness toward the reaction of flavin semiquinones has also been observed in the reduction of the ferryl species of yeast cyt *c* peroxidase (Hazzard et al., 1987; Miller et al., 1988), which is believed to have a midpoint potential of approximately +1 V (Purcell & Erman, 1976). It is interesting that both enzymes, the oxidase and the peroxidase, which not only are the physiological oxidants of mitochondrial cyt *c* but also are involved in multiple electron reduction reactions involving dioxygen or its two-electron reduction product, H_2O_2, behave in a similar manner toward reduction by nonprotein electron donors. This may be a consequence of the need to protect highly reactive intermediates, which is achieved by sequestering the catalytic site deeply within the polypeptide matrix (Poulos & Finzel, 1984).

Inasmuch as cyt *c* has an approximately 900-mV higher redox potential than does deazariboflavin and is a much larger molecule, the effectiveness of this electron-transfer reaction raises the possibility that there is a pathway that is inaccessible to the free flavin semiquinones, which permits rapid electron transfer upon binding of the physiological donor. The ability to access such a pathway could be due to the induction of a conformational change in the oxidase, which does not occur with the free flavins. This pathway is also apparently not inducible by charge neutralization effects alone, inasmuch as a 10-fold increase in ionic strength did not alter the rate constant for reduction of free CcO by 5-DRFH$^{\bullet}$ (see above).

Considerable evidence exists for spectral perturbations induced by complex formation between cyt *c* and CcO at low ionic strength. Falk and Ångström (1983) observed changes in the ferric cyt *c* proton NMR spectrum in a solution containing 3% CcO. Bosshard and co-workers (Michel & Bosshard, 1984; Weber et al., 1987; Michel & Bosshard, 1989) have demonstrated differences in the circular dichroism spectra of both ferric cyt *c* and fully oxidized oxidase upon mixing. Interestingly, the redox state of the oxidase is apparently related to its ability to undergo a conformational change upon

complexation, inasmuch as in a 1:1 complex between the reduced proteins, it was suggested that only a change in the CD spectrum of cyt *c* could be observed. More recently, Hildebrandt et al. (1990) have reported differences between the resonance Raman spectrum for an electrostatically stabilized horse heart cyt *c* and bovine CcO complex and the sum of the two spectra for the individual proteins. Again, it was concluded that complex formation perturbed the cyt *c* heme environment more than that of the oxidase. It is interesting to note that the reduction potential of cyt *c* can be decreased by approximately 20–50 mV in the presence of liposomes, submitochondrial particles, or proteins with which it can form electrostatically stabilized complexes such as CcO [reviewed in Nicholls (1974)] or *Clostridium pasteurianum* flavodoxin (Hazzard et al., 1986).

The above-mentioned data indicate that in the electrostatically stabilized complex between cyt *c* and CcO, the majority of observed effects are related to the cyt *c*, with only minimal, if any, effects observed in the oxidase. In contrast, our kinetic results indicate that if conformational changes occur within the oxidase upon cyt *c* binding, they must be coupled to electron transfer to the heme *a*. However, if this involves some form of electron-transfer gating, there is no a priori requirement that such a conformational event must be accompanied by a change in the spectral properties of the enzyme.

Previous steady-state kinetic results have suggested that the electrostatically stabilized complex at low ionic strength may not be the most efficient at electron transfer (Davies et al., 1964; Smith et al., 1981; Boliagno et al., 1988). Furthermore, the ionic strength of the mitochondrial inner membrane space is ca. 100–200 mM (Cortese et al., 1990). The present data on the ionic strength dependence of the first-order rate constant for electron transfer are relevant to these considerations. Thus, the optimal ionic strength for electron transfer was found to be 110 mM, not the ionic strength where the electrostatic stabilization should be strongest (μ = 10 mM). Thus, we can conclude from a direct measurement that the low ionic strength complex between cyt *c* and CcO is not optimized with respect to the rate of electron transfer. Above an ionic strength of 110 mM, a decrease in this first-order rate constant was also observed, suggesting that the higher ionic strength transient complexes are also less efficient for electron transfer. The simplest structural interpretation of these results is that, as the ionic strength increases from 10 to 110 mM, the proteins are able to assume a more favorable orientation or geometric relationship relative to one another, which permits more rapid electron transfer. It is possible that an electrostatic environment within CcO, which is present at the lower ionic strength and for which cyt *c* has a high affinity, becomes masked, and another region on the protein surface becomes the preferred binding site, causing an alternative orientation of the proteins within the complex. If this latter orientation leads to better contact between the redox centers, a larger intracomplex electron-transfer rate constant would result. The precise structural basis for this rate of optimization is uncertain, but based on the fact that the rate constant for direct reduction of CcO by 5-DRFH$^{\bullet}$ is independent of ionic strength, we can conclude that there is probably no gross structural change in the oxidase, due solely to electrostatic effects, that makes heme *a* more accessible to reductants. The fact that k_6 decreases at ionic strengths above 110 mM implies that electrostatic forces are important in providing an approximately correct orientation between the two proteins within a transient complex.

An alternative, but less likely, explanation for the increase in k_6 upon going from 10 to 110 mM ionic strength is that this rate constant corresponds to a rate-limiting dissociation of ferric cyt *c* from the complex with oxidized CcO. This is possible, since in our experiments the concentration of cyt c^{3+} is always much greater than that of cyt c^{2+}. The strongest argument against this mechanism is that the rate of cyt *c* reduction is not significantly affected by complex formation. Thus, dissociation is not required to get initial reduction of the cyt *c*, and the k_{obs} values for reduction of CcO by cyt *c* in a preformed complex at low ionic strength should represent the intracomplex electron-transfer rate constant [or the first-order process(es) controlling that transfer]. Consistent with this is the fact that a similar value for k_6 was obtained when cyt *c* was titrated with excess CcO at low ionic strength. Furthermore, if k_6 does represent the first-order dissociation of cyt c^{3+}, this rate constant should be ionic strength independent, which is clearly not the case.

The present results are in contrast to those of Antalis and Palmer (1982), who observed a monotonic decrease in the rate constant for the reaction between ferrous cyt *c* and bovine CcO as the ionic strength was increased above 40 mM. However, it should be noted that these workers used horse cyt *c*, as opposed to the bovine cyt *c* used in our work. Furthermore, the reduced cytochrome was present in an 8-fold excess over CcO, and at low ionic strength, much of the reaction occurred within the dead time of the stopped-flow apparatus.

Studies on the intracomplex electron-transfer reaction from horse or yeast iso-1 cytochromes *c*, as well as several site-specific mutants of the latter species, to the compound I species of cytochrome *c* peroxidase (Hazzard et al., 1988a,b) have shown that in these reactions, the electrostatically stabilized complex formed at low ionic strength is also not optimal for electron transfer. In agreement with conclusions drawn from the kinetic experiments, theoretical docking simulations for this system by Northrup et al. (1988) indicate that, whereas a relatively small region for electron transfer probably exists for cyt *c*, multiple interaction sites might exist on the electron acceptor. Thus, there is no well-documented evidence to suggest that a single complex, electrostatically stabilized or not, exists between cyt *c* and its redox partner(s). In the only reported case of an attempted co-crystallization of cyt *c* with a redox partner, cytochrome *c* peroxidase, it was found that whereas the structure of the peroxidase could be resolved, the cyt *c* structure could not be clearly defined (Poulos et al., 1987). Thus, even within a highly ordered crystal environment, there exists the possibility of multiple orientations of cyt *c* with its redox partner.

The behavior of K_{app} with ionic strength in the present experiments is perplexing. The fact that it remains essentially unchanged over the ionic strength range of 10–110 mM is not consistent with the notion that this equilibrium constant, derived from the kinetic analysis, represents a true association constant for the one-electron reduced complex (k_5/k_{-5}). Typically, the dissociation constant for the cyt *c*·CcO complex at low ionic strengths has been determined to be approximately 1–4 μM [$K_a \sim (1–6) \times 10^6$ M^{-1}; Mochan & Nicholls, 1972]. On the basis of measurements of fluorescence energy transfer induced by binding porphyrin cyt *c* to bovine oxidase over a range of ionic strengths, Kornblatt et al. (1984) have indicated that the dissociation constant decreases uniformly from a value of 0.1 μM at 5 mM ionic strength to 10 μM at 95 mM ionic strength, as would be expected for an electrostatically stabilized complex. Thus, the behavior of K_{app} derived from the kinetics of reduction by cyt *c* can be interpreted as reflecting a con-

tribution from the decrease in the concentration of free CcO caused by complexation between fully oxidized CcO and ferric cyt *c*. A second possibility is that there exists more than one electrostatically stabilized complex which influences K_{app}. At ionic strengths greater than 110 mM, K_{app} decreases in a manner which would be expected of a predominantly electrostatically stabilized complex.

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Registry No. 5-DRFH, 78548-68-2; CcO, 9001-16-5; cyt *c*, 9007-43-6; LFH, 34533-61-4; heme *a*, 57560-10-8.

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Transcription by Eucaryotic and Procaryotic RNA Polymerases of DNA Modified at a d(GG) or a d(AG) Site by the Antitumor Drug *cis*-Diamminedichloroplatinum(II)†

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ABSTRACT: We have investigated whether DNA modified at a d(GG) or a d(AG) site by the chemotherapeutic drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP) can be used as template by wheat germ RNA polymerase II. The templates used in the present study were obtained by ligation of double-helical oligodeoxyribonucleotides, containing 18 pyrimidine bases and 2 central dG, or dA and dG, bases on one strand and 18 purine bases and 2 central dC, or dT and dC, bases on the complementary strand. Therefore, the *cis*-DDP adducts are only present on one strand of each of the two templates and are regularly spaced by 18 pyrimidine bases. These constructs allowed us to investigate the effect of *cis*-DDP on transcription of the platinated strand and of the complementary unplatinated sequence. Transcription experiments were carried out in the presence of dinucleotide primers and either a single triphosphate substrate (abortive elongation) or the full set of triphosphate substrates dictated by the template sequence (productive elongation). The results show that the eucaryotic RNA polymerase can catalyze dinucleotide-primed reactions on platinated DNA. However, the eucaryotic enzyme behaved very differently depending on which strand was transcribed. Thus, transcription elongation was completely blocked on the strand carrying the metal complex, whereas transcription elongation was not blocked on the complementary template strand. However, on this latter strand and with the platinated polymers, productive elongation was slightly inhibited. Furthermore, abortive elongation leading to dinucleotide-primed trinucleotide formation was enhanced on the template strand complementary to that carrying the *cis*-DDP adducts. Similar results were obtained in transcription of the platinated templates by *Escherichia coli* RNA polymerase, suggesting that the *cis*-DDP-induced effect is associated with modifications of the basic catalytic properties of the transcriptases.

cis-Diamminedichloroplatinum(II) (*cis*-DDP)[1] has been introduced by Rosenberg et al. (1969) as a powerful chemotherapeutic drug for the treatment of certain human cancers (Loehrer & Einhorn, 1984). Numerous studies suggest that the curative effect of the metal complex can be attributed to the attack of cellular DNA and to the formation of several types of adducts with DNA bases. In vivo and in vitro, *cis*-DDP reacts preferentially with adjacent purine residues, yielding two major adducts, *cis*-[$Pt(NH_3)_2${d(GpG)}](N7,N7) and *cis*-[$Pt(NH_3)_2${d(ApG)}](N7,N7), representing 65% and 25% of the bound platinum, respectively. Several results also suggest that the antitumor activity of *cis*-DDP is related to the intrastrand adducts [reviewed by Eastman (1987), Lippard (1987), and Reedijk (1987)]. These findings have stimulated a number of research studies, with the aims of deciphering whether platinated DNA may be biochemically active and of gaining a better understanding of the mechanism of action of the drug at the level of gene expression. For example, the

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[1] Abbreviations: *cis*-DDP, *cis*-diamminedichloroplatinum(II); bp, base pair(s); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)-aminomethane. An asterisk denotes a deoxyribonucleotide modified by *cis*-diamminedichloroplatinum(II). d(GG/CC) is a double-stranded oligodeoxyribonucleotide of 20 bp, containing 18 pyrimidine bases and 2 central dG bases on one strand and 18 purine bases and 2 central dC bases on the complementary strand. d(G*G*/CC) is the corresponding double-stranded sequence in which the dG-dG bases are modified by *cis*-DDP. $d(GG/CC)_m$ and $d(G*G*/CC)_m$ are multimers of the ligated d(GG/CC) and d(G*G*/CC) motifs, respectively. d(AG/TC), d-(A*G*/TC), $d(AG/TC)_m$, and $d(A*G*/TC)_m$ correspond to d(GG/CC), d(G*G*/CC), $d(GG/CC)_m$, and $d(G*G*/CC)_m$ in which one of the central dG·dC bp is replaced by a dA·dT bp, respectively. Dinucleoside monophosphate primers and trinucleoside diphosphate products are referred to as dinucleotides and trinucleotides, respectively.

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