Kinetic Studies on the Electron-Transfer Reaction between Cytochrome c_3 and Flavodoxin from *Desulfovibrio vulgaris* Strain Hildenborough†

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Received April 22, 1994; Revised Manuscript Received June 22, 1994®

ABSTRACT: The kinetic properties of the electron-transfer process between reduced *Desulfovibrio vulgaris* cytochrome c_3 and *D. vulgaris* flavodoxin have been studied by anaerobic stopped-flow techniques. Anaerobic titrations of reduced cytochrome c_3 with oxidized flavodoxin show a stoichiometry of 4 mol of flavodoxin required to oxidize the tetraheme cytochrome. Flavodoxin neutral semiquinone and oxidized cytochrome c_3 are the only observable products of the reaction. At pH 7.5, the four-electron-transfer reaction is biphasic. Both the rapid and the slow phases exhibit limiting rates as the flavodoxin concentration is increased with respective rates of 73.4 and 18.5 s^{-1} and respective K_d values of 65.9 ± 9.4 μM and 54.5 ± 13 μM. A biphasic electron-transfer rate is observed when the ionic strength is increased to 100 mM KCl; however, the observed rate is no longer saturable, and relative second-order rate constants of 5.3×10^5 and 8.5×10^4 M^{-1} s^{-1} are calculated. The magnitude of the rapid phase of electron transfer diminishes with the level of heme reduction when varying reduced levels of the cytochrome are mixed with oxidized flavodoxin. No rapid phase is observed when 0.66e^--reduced cytochrome c_3 reacts with an ~25-fold molar excess of flavodoxin. At pH 6.0, the electron-transfer reaction is monophasic with a limiting rate of 42 ± 1.4 s^{-1} and a K_d value of ~8 μM. Increasing the ionic strength of the pH 6.0 solution to 100 μM KCl results in a biphasic reaction with relative second-order rate constants of 5.3×10^5 and 1.1×10^4 M^{-1} s^{-1}. *Azotobacter vinelandii* flavodoxin reacts with reduced *D. vulgaris* cytochrome c_3 in a slow, monophasic manner with limiting rate of electron transfer of 1.2 ± 0.06 s^{-1} and a K_d value of 80.9 ± 10.7 μM. These results are discussed in terms of two equilibrium conformational states for the cytochrome which are dependent on the pH of the medium and the level of heme reduction [Catarino *et al.* (1991) *Eur. J. Biochem. 207*, 1107–1113].

Cytochrome c_3 is a tetraheme-containing redox protein isolated from sulfate-reducing bacteria that has been extensively studied due to its role in electron transport *in vivo* and as a model to understand cooperative interactions on oxidation–reduction properties. Small electron-transfer proteins, such as flavodoxins or ferredoxins, have been shown to interact with the multiheme protein forming protein–protein complexes which have been useful to probe electron-transfer chains operating between electron donors and respiratory electron acceptors in sulfate reducing bacteria. Insights into this system can be addressed from two aspects: (i) the intramolecular electron transfer occurring within the multiheme protein and (ii) the intermolecular electron transfer to an interacting partner (i.e., flavodoxin).

Intramolecular electron transfer among the four hemes in the protein has been studied by a number of authors using various approaches. The majority of studies has centered on the mechanism of cytochrome c_3 reduction using nonphysiological chemical reductants. Reduction kinetics by reaction with sodium dithionite in different cytochrome c_3 preparations were analyzed (Favaudon *et al.*, 1978; Tabushi *et al.*, 1983; Capeillère-Blandin *et al.*, 1986). Pulsed-radiolysis kinetic studies performed in the presence of methyl viologen (van Leeuwen *et al.*, 1982) and flash photolysis (Akutsu *et al.*, 1992) were used to study the reduction kinetics of the four hemes of cytochrome c_3. A more detailed analysis was recently undertaken correlating kinetic measurements with a thermodynamic model which suggests that the redox properties were modulated by a proton-linked process and giving an overall insight on the multielectronic pathway (Catarino *et al.*, 1991). The data show a biphasic behavior which is interpreted as two of the hemes (hemes 4 and 1) being reduced by sodium dithionite significantly faster than hemes 2 and 3. Heme 4 (the most positive and most rapidly reduced) transfers electrons by equilibration with hemes 2 and 3. Transfer of electrons from heme 1 to hemes 2 and 3 was suggested to occur at a slower rate, therefore, making the process biphasic. Heme cooperativity (a pH–redox-linked process) and associated protein conformational changes are thought to give rise to this complex biphasic kinetic behavior. The general picture emerging from these studies is that the most positive heme (heme 4) is reduced most rapidly and is suggested to be the heme involved in the interaction with the other redox partners.

Several hypothetical complexes have been proposed on the basis of spectroscopic data and molecular modeling approaches since the three-dimensional structures are available or can be made available through homology modeling. Cytochrome c_3 from sulfate-reducing bacteria has been considered to be a model system for the study of protein–protein interactions

† This work was supported by grants from the National Institutes of Health [GM-29433 (D.E.E.), GM 41482 (J.L.G. and J.J.G.M.)] and from Junta Nacional de Investigacão Cientîficae Tecnlógica and NATO (J.J.G.M.). Partial funding for the stopped-flow apparatus used in this work was provided by the Emory University Research Committee.

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® Abstract published in *Advance ACS Abstracts,* August 1, 1994.

0006-2960/94/0433-10386$04.50/0

with other small electron carrier proteins (Cambillau *et al.*, 1988; Stewart *et al.*, 1988, 1989). The interaction between *Dm. baculatum* Norway 4[1] cytochrome c_3 and ferredoxin I from the same organism was studied in detail by NMR and cross-linking studies (Guerlesquin *et al.*, 1985; Capeillère-Blandin *et al.*, 1986, Dolla & Bruschi, 1988; Dolla *et al.*, 1991). Also, polyanions have been proposed as a model for studying the binding process (Mus-Veteau *et al.*, 1992). *Desulfovibrio vulgaris* Hildenborough cytochrome c_3 also interacts with rubredoxin and flavodoxin (Moura *et al.*, 1980; Stewart *et al.*, 1988, 1989). These complexes were initially interpreted to be consistent with a 1:1 stoichiometry. A recent detailed analysis of the alterations in heme ^{1}H NMR chemical shifts upon protein–protein titrations (*Desulfovibrio salexigens* flavodoxin with three homologous cytochrome c_3 preparations, which exhibit a wide range of isoelectric points) was interpreted to show a 2:1 stoichiometry, involving the binding of 2 mol of cytochrome c_3/mol of flavodoxin with relative K_d values of 5.3 μM and 550 μM (Palma *et al.*, 1994). Ongoing work on the interaction of *D. vulgaris* Hildenborough flavodoxin with cytochrome c_3 also suggests that the same model applies (J. J. G. Moura, unpublished data). Park *et al.* (1991) studied the complex formed between *D. vulgaris* Miyasaki F and ferredoxin I from the same organism and, on the basis of 2D NMR assignments and NMR profiles obtained on protein–protein titrations, also claim a stoichiometry for the complex involving 2 cytochromes c_3 molecules per monomer of ferredoxin.

The experimental support provided by kinetic measurements, as well as our well-documented work on complex formation between *D. vulgaris* Hildenborough cytochrome c_3 and flavodoxin (Stewart *et al.*, 1988; Palma *et al.*, 1994) and on the complex cytochrome c_3–rubredoxin (Stewart *et al.*, 1989), led us to investigate the mechanism of electron transfer occurring between these two proteins. In particular, it is established that the interaction has an electrostatic nature being driven by the charge complementarity of acidic surface residues in flavodoxin and a patch of positive charge (lysine residues) identified in the close vicinity of heme 4.

A further interest in the study of the flavodoxin–cytochrome c_3 complex comes from the fact that recently we could reconstitute a soluble electron-transfer chain from aldehydes to molecular hydrogen involving four proteins (aldehyde oxidoreductase, flavodoxin, cytochrome c_3, and hydrogenase) where the electron transfer as well as the modulation of redox potentials is crucial for the overall scheme (Barata *et al.*, 1993).

EXPERIMENTAL PROCEDURES

Materials

Azotobacter (A.) vinelandii and *D. vulgaris* flavodoxins were purified according to published procedures (Hinkson & Bulen, 1967; Dubourdieu & LeGall, 1970). The published extinction coefficients were used in order to calculate the concentration of the two flavodoxins and to judge their purities. *D. vulgaris* cytochrome c_3 was purified as described by Der Vartanian and LeGall (1974), and its concentration was determined using an extinction coefficient of 120 000 M^{-1} cm^{-1} at the reduced α-band maximum (553 nm). The buffers used in this study were 10 mM potassium phosphate, pH 7.5, and 10 mM MES[2] /KOH, pH 6.0. Where stated, the ionic strength was adjusted with KCl to 100 mM. EDTA (1 mM) was included in all of the buffers used.

Methods

Samples were made anaerobic by several cycles of alternate degassing under vacuum and flushing with argon that had been previously passed through a column of heated BASF catalyst (Williams *et al.*, 1979).

Cytochrome c_3 was reduced by photoreduction in the presence of 5-deazariboflavin and EDTA (Massey & Hemmerich, 1978). Samples were then transferred to the stopped-flow spectrophotometer by means of tonometers equipped with three-way stopcocks. The stopped-flow apparatus used was purchased from Kinetic Instrument, Inc., Ann Arbor, MI. Data were collected with a Nicolet Model 4094 digital oscilloscope, and the data files were tranferred to an IBM XT computer with an IEEE interface and analysed using the MEDAS kinetics decay analysis program (EMF Software Inc., Knoxville, TN) by fitting the experimental traces to either a one- or a two-exponential curve. The program was run and reinitialized with different initial estimates several times for each data set, in order to avoid convergence to a local minimum. Typically, between 200 and 500 iterations were sufficient for a satisfactory fitting of the experimental data. Cytochrome c_3 reoxidation was monitored at 553 nm and flavodoxin semiquinone formation at 620 nm. The traces recorded at the two different wavelengths were always found to be kinetically equivalent and consistent with the generation of 4 mol of flavodoxin semiquinone/mol of reduced cytochrome c_3. In all stopped-flow experiments, the flavodoxin concentration was at least 20-fold higher than the cytochrome c_3 concentration, in order to satisfy pseudo-first-order kinetic conditions.

Anaerobic titrations of reduced cytochrome c_3 with oxidized flavodoxins were carried out in an gas-tight cuvette under an argon atmosphere. The cytochrome c_3 solution was made anaerobic and reduced as described above, and a gas-tight Hamilton 500-μL syringe equipped with a Chaney adapter, containing an argon-saturated flavodoxin solution, was attached to the cuvette under an argon atmosphere. Aliquots of the flavodoxin stock solution (10 μL) were added to the cytochrome c_3 solution, and the absorption spectrum was recorded on a CARY 14 spectrophotometer after each addition.

RESULTS

Stoichiometry of the Electron Transfer from Reduced D. vulgaris Cytochrome c_3 to Oxidized D. vulgaris and A. vinelandii Flavodoxins. In order to interpret the kinetics of electron transfer from reduced cytochrome c_3 to flavodoxin, measurement of the stoichiometry of the electron-transfer reaction was required. Three hemes of *D. vulgaris* cytochrome c_3 have midpoint potentials in the range of –320 to –355 mV, and the most positive heme (heme 4) has a midpoint potential around –190 mV (Moreno *et al.*, 1992). *D. vulgaris* flavodoxin has two redox couples with oxidized/semiquinone midpoint potential at pH = 7.78, determined to be –149 mV and the semiquinone/hydroquinine couple at –438 mV (Dubourdieu *et al.*, 1975). *A. vinelandii* flavodoxin has respective potentials of –200 and –500 mV at pH 8.0 (Watt, 1979). From

[1] *Desulfovibrio (D.) desulfuricans* Norway 4, renamed *D. baculatus* Norway 4, was recently reclassified as *Desulfomicrobium (Dm.) baculatum* Norway 4 (Devereux, *et al.*, 1990).

[2] Abbreviations: Cyt c_3, cytochrome c_3; EDTA, ethylenediaminetetraacetic acid; 5-deazariboflavin, 5-carba-5-deazariboflavin; MES, 2-(*N*-morpholino)ethanesulfonic acid.

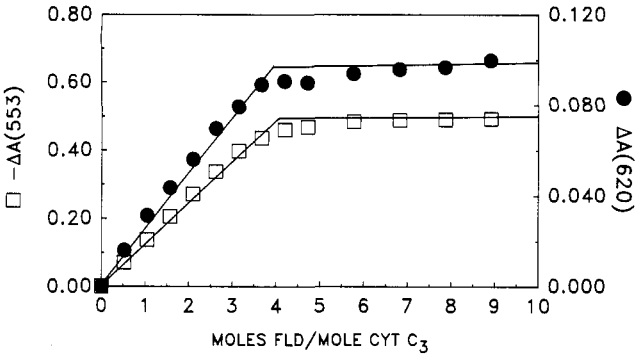


FIGURE 1: Anaerobic titration of reduced *D. vulgaris* cytochrome c_3 with oxidized *D. vulgaris* flavodoxin. *D. vulgaris* cytochrome c_3 (5.3 μM in 10 mM potassium phosphate buffer at pH 7.5, 1 mM EDTA, and 0.5 μM deazariboflavin) was photoreduced in an anaerobic cuvette, and absorption changes were monitored on the addition of aliquots of deaerated oxidized *D. vulgaris* flavodoxin (550 μM). All data are corrected for dilution.

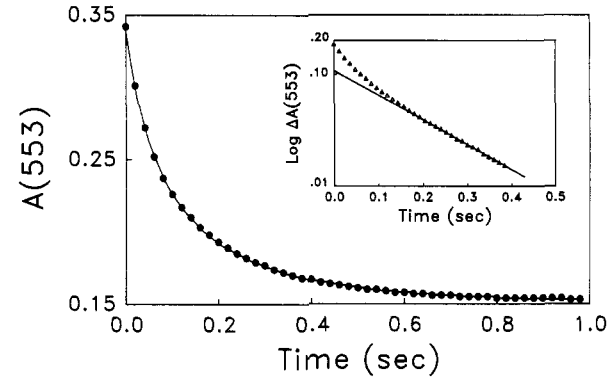


FIGURE 2: Anaerobic reaction between fully-reduced *D. vulgaris* cytochrome c_3 (1.1 μM) and oxidized *D. vulgaris* flavodoxin (25 μM) in 10 mM potassium phosphate and 1 mM EDTA, pH 7.5. The kinetic trace was recorded at 553 nm. The solid line is the experimental trace, whereas the filled circles are generated by computer fitting according to the equation:

$$A(t) = 0.0763 \exp(-25.6t) + 0.133 \exp(-5.19t) + 0.1527$$

Inset: Semilogarithmic plot of the experimental data points (open triangles).

thermodynamical considerations, the electron transfer from the cytochrome to flavodoxin should occur in one-electron steps with flavodoxin semiquinone formation as the major product with little or no flavodoxin hydroquinone formation.

Anaerobic titrations of four-electron-reduced cytochrome c_3 with oxidized flavodoxin, both from *D. vulgaris* (Figure 1) and from *A. vinelandii* (data not shown), were monitored spectrophotometrically under strict anaerobic conditions. In each case 4 mol of flavodoxin are required to completely oxidize cytochrome c_3, suggesting that the FMN in flavodoxin is reduced to its neutral semiquinone state and no flavin–hydroquinone accumulates during the titration. Furthermore, it is shown in Figure 1 that the spectral changes observed at 620 nm (a wavelength at which only the flavodoxin semiquinone absorbs) closely parallel the absorbance changes at 553 nm, determined by the disappearance of the α-band of reduced cytochrome c_3. The magnitude of the absorbance change measured at 620 nm at the end of the titration is also consistent with the formation of 4 mol of flavodoxin semiquinone. Altogether, these data show that flavodoxin FMN semiquinone is the only product generated upon electron transfer from reduced cytochrome c_3 to oxidized flavodoxin. These data are consistent with the expectation that the macroscopic redox potentials of the *D. vulgaris* cytochrome c_3 hemes (Moreno *et al.*, 1992) are lower than the redox potentials of the flavodoxin oxidized/semiquinone and above that of the flavodoxin semiquinone/hydroquinone couples (Dubourdieu *et al.*, 1975; Watt, 1979).

Electron Transfer from Reduced D. vulgaris Cytochrome c_3 to Oxidized D. vulgaris Flavodoxin. The rate of electron transfer from reduced *D. vulgaris* cytochrome c_3 to oxidized flavodoxin was measured in the stopped-flow apparatus under anaerobic conditions and at two different ionic strengths. It is necessary that the samples be kept under strictly anaerobic conditions, since reduced cytochrome c_3 reacts with oxygen at a rapid rate (the $t_{1/2}$ for the oxidation of reduced *D. vulgaris* cytochrome c_3 by air-saturated buffer is less than 1 s). The oxidation of cytochrome c_3, monitored by the decrease of absorbance at 553 nm, occurs simultaneously with the reduction of flavodoxin FMN to semiquinone as measured by the increase of the absorbance at 620 nm.

Initially, the rate of electron transfer from reduced *D. vulgaris* cytochrome c_3 to oxidized *D. vulgaris* flavodoxin was studied at low ionic strength. When 1.1 μM of fully-reduced cytochrome c_3 was mixed with 25 μM oxidized flavodoxin (pseudo-first-order conditions), biphasic absorbance changes were observed at both 553 and 620 nm (Figure 2 and data not shown). The time-dependent absorbance changes were fit to the sum of two exponentials and the respective rates and amplitudes of the two phases determined. Analysis of the traces recorded either at 553 nm or at 620 nm gave identical results. The amplitude of the initial rapid phase generally comprised about 40% of the total absorbance change.

Table 1: Influence of Level of Heme Reduction on Kinetic Behavior of Electron Transfer from Cytochrome c_3 (1.1 μM) to Flavodoxin (25 μM) at pH 7.5

hemes reduced	kinetic behavior	amplitude of phases	k_{obs} (s^{-1})
0.66	monophasic	(slow only)	6.9
1.38	biphasic	0.010 (fast)	25.9
		0.060 (slow)	5.4
2.5	biphasic	0.034 (fast)	25.6
		0.073 (slow)	6.4
2.9	biphasic	0.043 (fast)	23.3
		0.098 (slow)	5.2
3.9	biphasic	0.079 (fast)	24.4
		0.110 (slow)	5.0

In order to understand the origin of the two observed kinetic phases, we performed similar experiments, by varying the degree of reduction of the cytochrome c_3 molecule. We found that the ratio between the amplitude of the fast with that of the slow phase decreases when the level of heme reduction decreases, but that the values of the rate constants for the two phases remain unaffected. The experimental data are summarized in Table 1. Interestingly, the faster initial phase is no longer detectable when 0.66 electron-reduced cytochrome c_3 is mixed with oxidized flavodoxin as documented in Figure 3. The trace recorded at 553 nm, shown in Figure 3, shows a monophasic decay, and the semilogarithmic plot in the inset is linear over several half-lives. When fully-reduced cytochrome c_3 was mixed with oxidized flavodoxin in the presence of 100 mM KCl, two distinguishable kinetic phases are also observed (see below).

Effect of D. vulgaris Flavodoxin Concentration on Kinetics of D. vulgaris Cytochrome c_3 Oxidation. Pseudo-first-order rate constants for both the slow and the fast phases of the oxidation of cytochrome c_3 vs the concentration of *D. vulgaris* flavodoxin were determined at pH 7.5 at two ionic strengths and are shown in Figure 4. Several conclusions can be reached from these data. First of all, nonlinear dependencies of k_{obs}

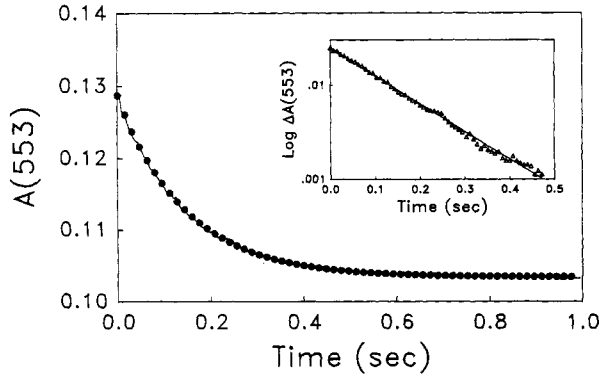


FIGURE 3: Anaerobic reaction of 0.66-electron-reduced *D. vulgaris* cytochrome c_3 with oxidized *D. vulgaris* flavodoxin. All other experimental conditions are as described in Figure 2. The solid line is the experimental trace. Filled circles are generated by computer fitting according to the equation:

$$A(t) = 0.0253 \exp(-6.872t) + 0.1034$$

Inset: Semilogarithmic plot of the experimental data points (open triangles).

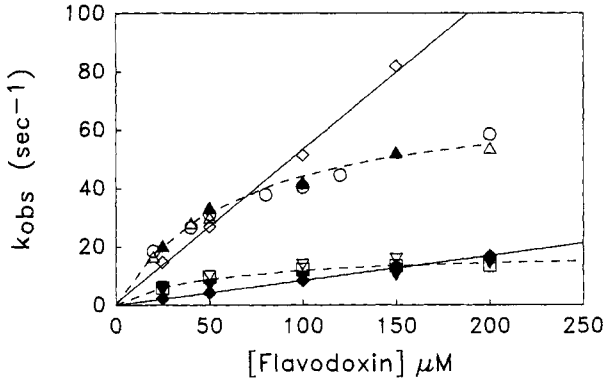


FIGURE 4: Dependence of the observed rates of oxidation of reduced or partially reduced *D. vulgaris* cytochrome c_3 on *D. vulgaris* flavodoxin concentration. The experiments were carried out anaerobically at pH 7.5 in 10 mM sodium phosphate buffer with 1.1 μM cytochrome c_3. *Open circles and triangles*: k_{obs} obtained from the faster component of biphasic traces recorded respectively at 553 and 620 nm using fully reduced *D. vulgaris* cytochrome c_3 and oxidized *D. vulgaris* flavodoxin. *Filled triangles*: Data obtained from the faster component of biphasic traces recorded at 553 nm using 2-electron-reduced *D. vulgaris* cytochrome c_3 and oxidized *D. vulgaris* flavodoxin in the absence of KCl. *Open squares*: k_{obs} obtained from monophasic traces recorded at 620 nm using 0.8-electron-reduced *D. vulgaris* cytochrome c_3 and oxidized *D. vulgaris* flavodoxin. *Open inverted triangles*: k_{obs} obtained from the slower component of biphasic traces recorded at 553 nm using 2-electron-reduced *D. vulgaris* cytochrome c_3 and oxidized *D. vulgaris* flavodoxin. *Filled inverted triangles*: k_{obs} obtained from the slower component of biphasic traces recorded at 553 nm using fully-reduced *D. vulgaris* cytochrome c_3 and oxidized *D. vulgaris* flavodoxin. *Dashed lines*: computer best fits to eq 1 with the following parameters: *Fast phase*: $K_d = 65.91 \pm 9.42$ μM; $k_{el} = 73.41 \pm 4.44$ s^{-1}; *Slow phase*: $K_d = 54.45 \pm 12.95$ μM; $k_{el} = 18.53 \pm 1.6$ s^{-1}. *Open diamonds*: k_{obs} obtained from the faster component of biphasic traces recorded at 553 nm using fully-reduced *D. vulgaris* cytochrome c_3 and oxidized *D. vulgaris* flavodoxin in the presence of 100 mM KCl. The line is the least-squares regression through the experimental points. The calculated second-order rate constant is 5.3×10^5 M^{-1}s^{-1}. *Filled diamonds*: k_{obs} obtained from the slower component of biphasic traces recorded at 553 nm using fully reduced *D. vulgaris* cytochrome c_3 and oxidized *D. vulgaris* flavodoxin in the presence of 100 mM KCl. The line is the least-squares regression through the experimental points. The calculated second-order rate constant is 8.5×10^4 M^{-1}s^{-1}.

vs flavodoxin concentration are observed for both the slow and the fast phases observed at low ionic strength. Such behavior can be attributed to intermediate complex formation between the reduced cytochrome c_3 and the oxidized flavodoxin, according to the equation:

$$\text{Cyt } c_3\{n\} + \text{Fld ox} \xrightarrow{K_d\{n\}} [\text{complex}] \xrightarrow{k_{el}\{n\}} \text{Cyt } c_3\{(n-1)\} + \text{FldH} \quad (1)$$

where K_d is the dissociation constant of the complex, k_{el} is the rate constant for electron transfer within the complex, and $\{n\}$ indicates the number of reduced hemes in the cytochrome c_3 molecules. The formation of an electrostatic complex between *D. vulgaris* cytochrome c_3 and *D. vulgaris* flavodoxin has been suggested on the basis of a previous molecular modeling study (Stewart *et al.*, 1989).

It was possible, by computer analysis of the experimental data in terms of eq 1, to estimate the values for K_d and k_{el} for the fast and the slow components, respectively. The results of such analysis are listed in the legend to Figure 4. It is noteworthy that similar K_d values are obtained, within experimental error, for the slow and the fast kinetic phases. These data suggest the presence of a unique binding site for flavodoxin on the cytochrome c_3 molecule.

When similar experiments were performed in the presence of 100 mM KCl, a linear dependence of the rate of cytochrome c_3 oxidation on the concentration of flavodoxin is observed, suggesting that electrostatic forces play a major role in the stabilization of the intermediate complex. It is of interest, however, to point out that, at the higher flavodoxin concentrations, the observed rate of electron transfer is actually faster than the limiting rate (k_{el}) observed at low ionic strength. Therefore, when complex formation between the two proteins is disrupted, the bimolecular process of electron transfer occurs at a faster rate. In contrast, the rate of bimolecular electron transfer between flavodoxin semiquinone and oxidized cytochrome *c* is slower than the rate of electron tranfer in the complex (De Francesco *et al.*, 1987).

Effect of pH on the Rate of Electron Transfer. On the basis of previous NMR data, it has been suggested that pH could modulate the heme-interacting potentials of *D. vulgaris* cytochrome c_3 (Moura *et al.*, 1982). It was therefore of interest to investigate whether lowering the pH of the buffer had any influence on the electron transfer reaction between reduced cytochrome c_3 and flavodoxin. It should be pointed out here that the potential for the semiquinone/hydroquinone flavodoxin couple is independent of pH between 6.0 and 7.5; however, the oxidized/semiquinone couple is *increased* by 90 mV. The data in Figure 5 show the kinetic results obtained when reduced cytochrome c_3 and flavodoxin were mixed in 10 mM MOPS/KOH at pH 6.0 under conditions of either low or high ionic strength.

Under conditions of low ionic strength, only a single kinetic phase is observed and the data are satisfactorily fit by a single-term exponential in contrast to the kinetic behavior observed at pH 7.5 (Figure 2). This observation suggests a loss of interaction among the reduced hemes in the cytochrome c_3 molecule when the pH is decreased from 7.5 to 6.0. The intermediate electrostatic complex between the two proteins is more stable at pH 6.0 than at pH 7.5, as judged by a more pronounced saturation behavior at the lower pH value. Since we did not collect data at flavodoxin concentrations lower than 25 μM, the calculated K_d value (approximately 8 μM) should be regarded as an estimated upper limit. The estimated value of k_{el}, the rate of electron transfer within the complex, is 42 s^{-1}, a value intermediate between the limiting rates calculated for the slow and the fast kinetic phases observed at pH 7.5 (Figure 4).

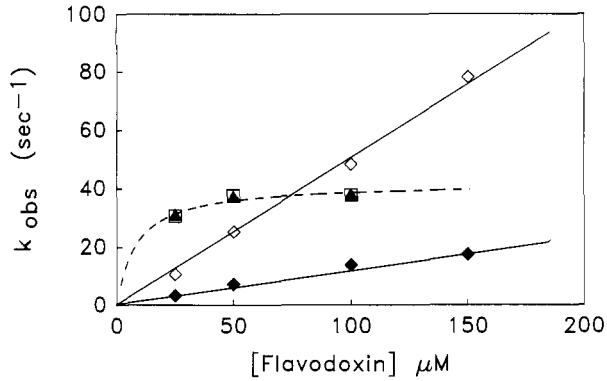


FIGURE 5: Dependence of the observed rates of oxidation of fully reduced *D. vulgaris* cytochrome c_3 on *D. vulgaris* flavodoxin concentration. The experiments were carried out anaerobically at pH 6.0 in 10 mM MES/KOH buffer. *Filled triangles and open squares*: k_{obs} obtained from monophasic traces recorded at 553 and 620 nm, respectively, using fully-reduced *D. vulgaris* cytochrome c_3 and oxidized *D. vulgaris* flavodoxin. *Dashed lines*: Computer best fits to eq 1 with the following parameters: K_d = 8.48 ± 1.8 μM; k_3 = 42.0 ± 1.4 s^{-1}. *Open diamonds*: k_{obs} obtained from the faster component of biphasic traces recorded respectively at 553 nm using fully-reduced *D. vulgaris* cytochrome c_3 and oxidized *D. vulgaris* flavodoxin in the presence of 100 mM KCl. *Filled diamonds*: k_{obs} obtained from the slower component of biphasic traces recorded respectively at 553 nm using fully-reduced *D. vulgaris* cytochrome c_3 and oxidized *D. vulgaris* flavodoxin in the presence of 100 mM KCl. The second-order rate constants are 5.3 × 10^5 and 1.1 × 10^4 M^{-1} s^{-1} for the fast and the slow phase, respectively.

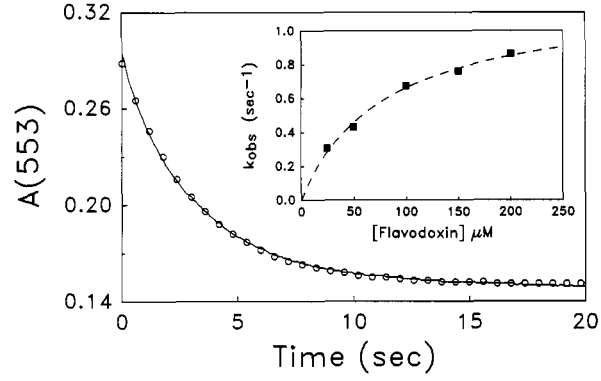


FIGURE 6: Anaerobic reaction between fully-reduced *D. vulgaris* cytochrome c_3 (1 μM) and *A. vinelandii* oxidized flavodoxin (25 μM) in 10 mM potassium phosphate and 1 mM EDTA, pH 7.5. The kinetic trace was recorded at 553 nm. The solid line is the experimental trace; the open circles are generated by computer fitting according to the equation:

$$A(t) = 0.138 \exp(-0.308t) + 0.1504$$

Inset: Dependence of the k_{obs} of oxidation of reduced *D. vulgaris* cytochrome c_3 on *A. vinelandii* flavodoxin concentration. *Dashed lines*: Computer best fits to eq 1 with the following parameters: K_d = 80.93 ± 10.7 μM; k_{el} = 1.2 ● 0.06 s^{-1}.

At pH 6.0 and in the presence of 100 mM KCl, we did observe that electron transfer from cytochrome c_3 to flavodoxin exhibited a biphasic kinetic behavior (Figure 5), similar to that observed at pH 7.5. The dependence of k_{obs} on flavodoxin concentration for both the slow and the fast phases is superimposable with that observed at pH 7.5 under the same conditions of ionic strength. The formation of an intermediate complex is not kinetically detectable, suggesting that electrostatic forces are also responsible for stabilization of the complex at this pH. The calculated second-order rate constants for the slow and the fast kinetic phases observed at pH 6.0 are very similar to those observed at pH 7.5 (at the same ionic strength).

Electron Transfer from Reduced D. vulgaris Cytochrome c_3 to Oxidized A. vinelandii Flavodoxin. *A. vinelandii* flavodoxin has a redox potential for the oxidized/semiquinone couple that is about 50 mV more negative as compared to that of *D. vulgaris* flavodoxin. Since the difference in redox potentials has been demonstrated to affect significantly the rate of electron transfer between partner redox proteins (Cheddar *et al.*, 1986), it was of interest to study the kinetics of electron transfer from reduced *D. vulgaris* cytochrome c_3 to oxidized *A. vinelandii* flavodoxin.

The absorbance changes observed at 553 nm when fully-reduced cytochrome c_3 (1 μM) was mixed with *A. vinelandii* flavodoxin (25 μM) are shown in Figure 6. The kinetic trace is satisfactorily fit by a single exponential (as depicted in the figure) rather than two exponentials as observed with *D. vulgaris* flavodoxin (Figure 2). Flavodoxin semiquinone appeared (followed by the change of the absorbance measured at 620 nm) at a rate identical with the oxidation of the cytochrome (data not shown).

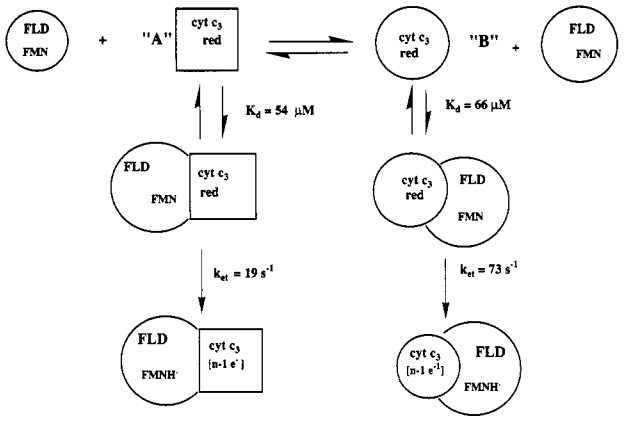
The dependence of the observed pseudo-first-order rate constants on the concentration of *A. vinelandii* flavodoxin was determined and is summarized in the inset of Figure 6. These data show the formation of an intermediate electron-transfer complex between the two proteins which is formed with an affinity very similar to that of the *D. vulgaris* flavodoxin–cytochrome c_3 complex (K_d = 80 μM). However, the intrinsic rate of electron transfer (1.2 s^{-1}) is diminished to a far greater extent than expected solely on the basis of the redox potential difference. These data suggest that the stability of the intermediate complex and the redox potential differences are not *per se* sufficient to drive efficient electron transfer between redox partners. It is likely that a major factor in accounting for the relatively slower rate of electron transfer to *A. vinelandii* flavodoxin is an unfavorable orientation of the flavin and the heme groups within the complex. A similar conclusion was reached on comparison of the rates of electron transfer from the semiquinone forms of *Clostridium pasteurianum* and *A. vinelandii* flavodoxin and oxidized horse heart cytochrome *c* (De Francesco *et al.*, 1987).

DISCUSSION

Cytochrome c_3 is a complex redox protein in that each heme of the tetraheme system has differing oxidation–reduction potentials that are modulated by a conformational A ⇌ B equilibrium. This conformational equilibrium is altered by the level of heme oxidation–reduction and by the pH of the medium. In the case of *Desulfovibrio gigas* cytochrome c_3, the pH where equal conformers of A and B occur is pH 6.3 for the oxidized protein and is 8.5 for the $4e^-$-reduced protein. Each intermediate redox level exhibits a different p*K* value for this conformational equilibrium within the 2.2 Δp*K* range (Catarino *et al.*, 1991). Unfortunately, such detailed p*K*a data are not available for *D. vulgaris* cytochrome c_3 which also exhibits such a Bohr-linked conformational equilibria between two states (Moura *et al.*, 1982). Inasmuch as the *D. vulgaris* cytochrome c_3 exhibits a different isoelectric point than the *D. gigas* protein, the p*K*a values may differ between the two proteins.

Since previous kinetic data on this class of proteins have employed reduction by nonphysiological reductants, it was important to see if similar kinetic behavior is exhibited by a cytochrome c_3 on *oxidation* by a redox protein that forms a binary complex. Flavodoxin has been used extensively in electron-transfer studies with cytochrome systems [cf. De Francesco *et al.* (1987)], forms an electrostatic complex with

Scheme 1: Model for *D. vulgaris* Cytochrome c_3 Complexation and Electron Transfer to *D. vulgaris* Flavodoxin at pH 7.5 and at Low Ionic Strength



cytochrome c_3 as monitored by NMR (Santos *et al.*, 1984) and by molecular modeling (Stewart *et al.*, 1989), and has a physiological relevance in that it can participate in electron transfer from an aldehyde oxidoreductase to cytochrome c_3/hydrogenase in sulfate-reducing bacteria (Barata *et al.*, 1993).

The data presented in this study demonstrate that *D. vulgaris* flavodoxin is reduced by cytochrome c_3 to the semiquinone form in a stoichiometry of 4:1. Kinetic data at low and at high ionic strength demonstrate the formation of a kinetically-detectable electrostatic complex between the two proteins as found with cytochrome *c*–flavodoxin electron-transfer studies (Simondsen *et al.*, 1982). The biphasic rate of electron transfer observed at both high and low ionic strengths suggests factors influencing the differential rate to be independent of protein complex formation. Although recent NMR studies have demonstrated a binding of 2 mol of cytochrome c_3/mol of flavodoxin (Palma *et al.*, 1994), we feel this situation is not responsible for the observed biphasic kinetic behavior since (a) the flavodoxin concentration was always in excess of cytochrome c_3 (>20-fold) and (b) the K_d values observed for the binding of the second mole of cytochrome c_3 to flavodoxin is approximately 10-fold weaker than the kinetically determined K_d values for either kinetic phase of the reaction reported here. Finally, the observation of biphasic kinetic behavior at ionic strength values where a kinetically viable complex of cytochrome c_3 and flavodoxin is not formed provides the most persuasive argument against the possibility of electron transfer from cytochrome c_3 to flavodoxin occurring at two separate sites as being responsible for the biphasic kinetic behavior.

The data in Table 1 demonstrate that the "fast" phase of electron transfer occurs when the cytochrome has appreciable populations of 4 reduced hemes or 3 reduced hemes. When the population of cytochrome c_3 is predominantly 2 or 1 reduced hemes, the "slow" phase predominates. It should also be noted that the electron-transfer rate becomes monophasic at pH 6 at a rate intermediate between the "slow" and "fast" phases. These observations suggest that the origin of the biphasic rate of electron transfer is in the conformational equilibria between the A and B conformers of the cytochrome c_3 (see Scheme 1).

By analogy with the *D. gigas* protein, *D. vulgaris* cytochrome c_3 is proposed to be largely in the "B" conformer when reduced at pH 7.5. During the oxidation by flavodoxin at this pH value, the protein undergoes a B to A transformation where the conformer population is largely A after a 2e$^-$ oxidation. The A conformer is still capable of complexation with flavodoxin with a K_d value of equal magnitude to that of the B conformer; however, the rate of electron transfer decreases by a factor of 4. On increasing the ionic strength of the solution, the two proteins do not form a kinetically-detectable electrostatic complex; however, electron transfer still occurs in a biphasic manner with rates that reflect the relative populations of the B and A conformers.

On lowering the pH of the system to 6, the electron-transfer rate from cytochrome c_3 to flavodoxin is monophasic, which suggests the cytochrome to be in a single A conformational state. At this pH value, the K_d for complex formation is lower than at pH 7.5 and the value for k_{et} is faster than the "slow" phase attributable to the reaction of conformer "A" with flavodoxin at pH 7.5. These alterations in binding constant and in rate may reflect a different geometry of the complex of flavodoxin with reduced cytochrome c_3.

Analogous kinetic experiments with *Azotobacter* flavodoxin exhibited no biphasic kinetic behavior under any of the conditions tested. While the K_d for binding of *Azotobacter* flavodoxin to the cytochrome was not much different than that of *D. vulgaris* flavodoxin, the value for k_{et} was much slower (~60-fold slower than the limiting "fast" phase and 15-fold slower than the "slow phase"). Thus, the relatively slow rate of electron transfer occurs whether the cytochrome is in either the A or the B conformational state. We interpret the lack of biphasic electron-transfer kinetics between cytochrome c_3 and *Azotobacter* flavodoxin to an unfavorable heme–flavin orientation in the complex of the two proteins which is not influenced by the conformational state (A or B) of the reduced cytochrome. The three dimensional structure of *Azotobacter* flavodoxin is not known, and therefore, detailed molecular possibilities to account for the much slower rate of electron transfer between the two proteins are unknown at this time. Previous work on flavodoxin–cytochrome *c* electron-transfer reactions has suggested the rate of electron transfer to be correlated with the level of heme perturbation on complex formation between the two proteins (De Francesco *et al.*, 1987; Tollin *et al.*, 1987). Efforts to detect a similar differential heme perturbation on complexation of *D. vulgaris* or *Azotobacter* flavodoxins with cytochrome c_3 by circular dichroism spectroscopy were precluded by the strong heme absorption by the tetraheme cytochrome at the concentrations required for complex formation.

In conclusion, this study further demonstrates the utility of cytochrome c_3 as a good model to study conformer-linked electron-transfer reactions with partner proteins involving either oxidative or reductive reactions.

ACKNOWLEDGMENT

The authors thank the staff of the UGA Fermentation Plant for growing the cells and Mr. M.-Y. Liu for his help in the purification of the proteins used in this study.

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