Electron-Transfer Reactions between Flavodoxin Semiquinone and *c*-Type Cytochromes: Comparisons between Various Flavodoxins†

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ABSTRACT: As an extension of previous work from this laboratory using *Clostridium pasteurianum* flavodoxin [Tollin, G., Cheddar, G., Watkins, J. A., Meyer, T. E., & Cusanovich, M. A. (1984) *Biochemistry 23*, 6345–6349], we have measured the rate constants as a function of ionic strength for electron transfer from the semiquinones of *Clostridium* MP, *Anacystis nidulans*, and *Azotobacter vinelandii* flavodoxins to the following oxidants: cytochrome *c* from tuna and horse, *Paracoccus denitrificans* cytochrome c_2, *Pseudomonas aeruginosa* cytochrome *c*-551, and ferricyanide. The rate constants extrapolated to infinite ionic strength (k_∞) for the *C.* MP flavodoxin are all slightly smaller than for the *C. pasteurianum* flavodoxin, as would be predicted on the basis of the higher redox potential of the *C.* MP protein. This indicates that there is a close similarity between the surface topographies of the two proteins in the vicinity of the coenzyme binding site. Moreover, the electrostatic interactions between the two flavodoxins and the various oxidants are also approximately the same. These studies justify our previous use of the crystallographic structure of the *C.* MP flavodoxin to interpret kinetic results obtained with the structurally uncharacterized *C. pasteurianum* flavodoxin. Despite their lower redox potentials, both *Anacystis* and *Azotobacter* flavodoxins are appreciably less reactive toward all of these oxidants (as much as 2 orders of magnitude in some cases) than are the *Clostridium* flavodoxins. This is consistent with crystallographic evidence, which indicates that the accessibility of the flavin mononucleotide (FMN) prosthetic group of *Anacystis* flavodoxin is significantly smaller than that of *C.* MP flavodoxin and suggests that a similar situation exists for *Azotobacter* flavodoxin as well, for which the crystal structure is not known. Whereas tuna and horse cytochromes are less reactive than are *Paracoccus* and *Pseudomonas* cytochromes toward three of the flavodoxin semiquinones, they are more reactive with *Azotobacter* flavodoxin. This indicates that there are appreciable differences in the surface topographies between the *Anacystis* and the *Azotobacter* flavodoxins. The directions and approximate magnitudes of the ionic strength dependencies of the *Anacystis* and *Azotobacter* flavodoxin reaction rate constants are the same as for those of the *Clostridium* flavodoxins for all of the oxidants except *Pseudomonas* cytochrome *c*-551, for which the directions are opposite in sign. This demonstrates that the *Pseudomonas* cytochrome, which has a relatively weak positive electrostatic potential in the vicinity of the heme prosthetic group, interacts differently with these two groups of flavodoxins, most likely as a consequence of the differences in the orientations of the FMN cofactor and in the steric properties of the flavodoxin surfaces. Computer modeling experiments are in agreement with this. These studies support our previous contention that the difference in redox potential between reacting electron-transfer proteins, the location and magnitude of electrostatic potential on the protein surfaces, the relative exposure of the prosthethic groups, and surface topography are all involved in controlling the specificity of a particular protein for its reaction partner. Optimum reactivity is achieved when the reactant proteins have regions of opposite electrostatic potential adjacent to the site of electron transfer. Furthermore, this work provides evidence that relatively small differences in surface topography can produce large effects on the kinetics of electron transfer by altering the mutual orientations of the two proteins within an intermediate complex.

Previous work from this laboratory (Simondsen et al., 1982; Tollin et al., 1984, 1986b; Przysiecki et al., 1985; Meyer et al., 1986) has utilized the semiquinone form of the flavodoxin from *Clostridium pasteurianum* as a reductant for a variety of redox protein classes (cytochromes *c* and *c'*, iron–sulfur proteins, blue copper proteins) and several inorganic oxidants in an effort to elucidate the factors that determine reaction rate constants and biological specificity for electron-transfer proteins. Interpretations of these studies utilizing computer graphic techniques (Simondsen et al., 1982; Weber & Tollin, 1985) have been based upon the X-ray crystallographic structural work on the closely related flavodoxin from *Clostridium* MP (Burnett et al., 1974). In order to determine whether or not this is a suitable model, we have carried out a series of kinetic comparisons between the two flavodoxins. As we will demonstrate below, their properties are closely analogous in all respects. Furthermore, as an extension of this comparison, we have determined the kinetic parameters for two other flavodoxins, from *Anacystis nidulans* [for which a partial crystallographic structure is available (Smith et al., 1983)] and from *Azotobacter vinelandii*. We have found that both similarities and differences exist between these flavodoxins and those from the *Clostridium* species, which support and extend our previous observations (Meyer et al., 1983, 1984, 1986; Tollin et al., 1984, 1986a,b; Przysiecki et al., 1985) that

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redox potential differences, electrostatic interactions, and relatively small differences in surface topography among structurally homologous proteins can produce large effects on the kinetics of their electron-transfer reactions with both small molecules and other redox proteins.

Materials and Methods

Cultures of *C. pasteurianum* (strain W5, ATCC 6013) and *An. nidulans* (ATCC 27344) were purchased from the American Type Culture Collection. *C.* MP was a gift from Dr. Stephen G. Mayhew. These were grown in iron-depleted media in 10-L batches. Procedures for growing the *Clostridium* species and for isolating and purifying their flavodoxins have already been described (Mayhew, 1971). The procedure for growing the cyanobacterium (*An. nidulans*) and for isolating and purifying its flavodoxin has also been described (Smillie & Entsch, 1971). The flavodoxin from *Az. vinelandii* was prepared by the procedure of Hinkson and Bulen (1967). The UV–vis absorption spectra of the various flavodoxins were similar to published results and were used as the criteria for purity. Horse cytochrome *c* (type VI) and tuna cytochrome *c* (type VI) were purchased from Sigma Chemical Co. and used without further purification, although in a few experiments material was used that had been subjected to ion-exchange chromatography, without any difference in results being observed. *Pseudomonas aeruginosa* cytochrome *c*-551 and *Paracoccus denitrificans* cytochrome c_2 were obtained as described in Meyer et al. (1983). An extinction coefficient of 30 mM^{-1} cm^{-1} at the reduced α maximum was assumed in order to calculate the concentrations of the cytochromes. Potassium ferricyanide was AR grade purchased from Mallinckrodt Chemical Co. and used without further purification. Deazariboflavin was a gift from Dr. C. C. Cheng.

All of the experiments were carried out in 5.0 mM potassium phosphate buffer, pH 7.2 at 23.5 °C, at various ionic strengths (12–500 mM) obtained by the addition of appropriate amounts of NaCl. Reactions were studied by using stopped-flow spectrophotometry under pseudo-first-order conditions (oxidant in excess) and were monitored at 580 nm where the disappearance of flavodoxin semiquinone could be observed. Experimental procedures were as described previously (Simondsen et al., 1982; Tollin et al., 1984).

All of the kinetic traces, except when ferricyanide was the oxidant, were biphasic, as previously observed (Tollin et al., 1984). The kinetic data were corrected for this biphasicity as described in Simondsen et al. (1982). Thus, rate constants were obtained from only the fast portion of the kinetics traces, which typically represented 80% of the total. The time-dependent absorbance changes were exponential over at least 3 half-lives.

Pseudo-first-order rate constants (k_{obsd}) were obtained for at least three different oxidant concentrations at each ionic strength. Over the ionic strength range and oxidant concentration range (5–30 μM) used, all of the reactions studied gave linear plots of k_{obsd} vs. oxidant concentration, except for the horse cytochrome *c* reactions at the lowest ionic strengths, which displayed nonlinear effects at higher cytochrome concentrations [cf. Simondsen et al. (1982)]. The apparent second-order rate constants for electron transfer from flavodoxin semiquinone to the oxidized electron acceptors were calculated from the slopes of the linear plots. We estimate the error in these values to be ±15%. For the horse cytochrome *c* reactions, the second-order rate constants and the limiting first-order rate constants were evaluated by a computer-fitting procedure as described previously (Simondsen et al., 1982). In most cases, second-order rate constants were

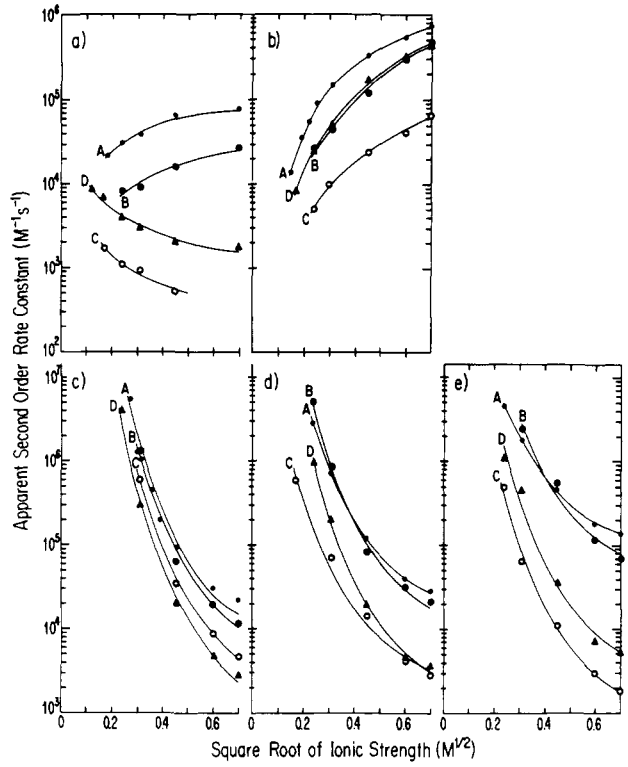

FIGURE 1: Ionic strength dependence of the apparent second-order rate constants for the oxidation of various flavodoxin semiquinones by several oxidants. All reactions were carried out in 5 mM phosphate buffer, pH 7.0, with the addition of varying amounts of NaCl. Flavodoxins are (A) *C. pasteurianum*, (B) *C.* MP, (C) *Az. vinelandii*, and (D) *An. nidulans*. Oxidants are (a) *Pseudomonas* cytochrome *c*-551, (b) ferricyanide, (c) horse cytochrome *c*, (d) tuna cytochrome *c*, and (e) *Paracoccus* cytochrome c_2.

determined at five different ionic strengths. These values were fit with a theoretical model for electrostatic interactions developed by Watkins (1986), and described in more detail below [cf. also Tollin et al. (1984)], to obtain an electrostatically corrected rate constant (k_∞). Although other theoretical models can be used [cf. Tollin et al. (1984)], we have generally found that the relative values of k_∞ are model-independent. Since our purpose in this work was to compare various flavodoxins, we have not attempted to evaluate other theoretical approaches. We estimate that the error in the extrapolation to obtain k_∞ is no greater than the intrinsic error in the rate constant values themselves (i.e., ±15%).

Results and Discussion

When flavodoxin semiquinone and an appropriate oxidized electron acceptor were mixed in the stopped-flow apparatus, a net decrease in absorbance was observed at 580 nm. This absorbance change was mainly due to the formation of oxidized flavodoxin, as a consequence of electron transfer from the flavodoxin semiquinone to the electron acceptor. In Figure 1 is shown a plot of the dependencies of the calculated second-order rate constants for these reactions on ionic strength (I). The solid lines through the experimentally determined points were obtained by utilizing the equation (eq 1) of Watkins (1986) to obtain a least-squares fit to the data points [see Meyer et al. (1984, 1986) and Tollin et al. (1984) for discussion]. This equation was derived by treating the interaction domains of the two reacting molecules as parallel plates with radius ρ, carrying electrostatic charges of Z_1 and Z_2, and separated by a distance of R_{12}, with an effective dielectric constant between the plates of D_e.

$$\ln k(I) = \ln k_\infty - V_{it}X(I) \qquad (1)$$

Table I: Electrostatics of Flavodoxin Semiquinone Oxidation by Various Oxidants

oxidant/ flavodoxin	$E_{m,7}$ (mV)	Z_1[a]	Z_2[a]	V_{ii}[b] (kcal/ mol)	k_∞ (M^{-1} s^{-1})
horse cytochrome *c*	+260	+6.7			
C. pasteurianum	−132		−4.0	−23.1	2.7×10^3
C. MP	−92		(−4)	−23.0	2.1×10^3
Azotobacter	−165[c]		−4.2	−23.8	0.83×10^3
Anacystis	−221		−4.4	−25.0	0.4×10^3
tuna cytochrome *c*	+260	+5.6			
C. pasteurianum			−3.4	−16.1	8.7×10^3
C. MP			(−4)	−19.3	4.6×10^3
Azotobacter			−2.7	−13.4	1.3×10^3
Anacystis			−4.2	−20.2	0.74×10^3
Paracoccus cytochrome c_2	+250	+5.1			
C. pasteurianum			−2.8	−12.3	5.9×10^4
C. MP			(−4)	−17.5	2.3×10^4
Azotobacter			−4.4	−19.1	0.46×10^3
Anacystis			−4.5	−19.8	1.3×10^3
Pseudomonas cytochrome *c*-551	+270	−1.3			
C. pasteurianum			−2.8	+3.3	1.0×10^5
C. MP			(−4)	+4.3	0.35×10^5
Azotobacter		(+1)	−4.4	−3.6	0.31×10^3
Anacystis		(+1)	−4.0	−3.3	1.2×10^3
ferricyanide	+430	−3			
C. pasteurianum			−5.4	+7.2	2.9×10^6
C. MP			−6.3	+8.4	1.9×10^6
Azotobacter			−5.4	+7.3	0.22×10^6
Anacystis			−6.3	+8.4	2.0×10^6

[a] With $D_e = 10$ and $Z_2 = -4$ for *C.* MP flavodoxin [cf. Tollin et al. (1984)], Z_1 values for the cytochromes (except for *Pseudomonas c*-551) were calculated. With this Z_1 value, Z_2 values for the other flavodoxins were calculated. For *Pseudomonas c*-551, this same procedure was used to obtain a Z_2 value for *C. pasteurianum* flavodoxin. However, for the *Anacystis* and *Azotobacter* flavodoxins, Z_1 was arbitrarily set at +1 to obtain a Z_2 value. For ferricyanide, $D_e = 50$ and $Z_1 = -3$ were used to calculate Z_2. [b] The parameters used in the analysis to obtain active-site charges were as follows: $\rho = 7.25$ Å for the cytochromes, $\rho = 4.5$ Å for ferricyanide, and $R_{12} = 3.5$ Å. These are similar to those used previously (Tollin et al., 1984). [c] This value for $E_{m,7}$ was determined by ourselves in 100 mM phosphate buffer at pH 7.0 and is estimated to be accurate to ±20 mV.

k_∞ is the rate constant extrapolated to infinite ionic strength where all of the electrostatic factors are screened out, V_{ii} ($V_{ii} = \alpha Z_1 Z_2 R_{12} D_e^{-1} \rho^{-2}$, where $\alpha = 128.47$) is the electrostatic interaction energy, and $X(I)$ [$X(I) = (1 + \kappa\rho)^{-1} \exp(-\kappa\rho)$, where $\kappa = 0.3295 I^{1/2}$] is an ionic strength dependent term. The k_∞ values were obtained by applying eq 1 for a particular value of ρ and R_{12} (the latter was taken to be the van der Waals radius 3.5 Å). Typically, a series of fits at different ρ values were obtained, and the value (±0.5 Å) that yielded the minimum least-squares error was used. Of the parameters which define V_{ii}, all but the values of Z_1 and Z_2 are fixed, allowing a determination of the effective interaction site charge of one of the reactants by assuming a charge for the other reactant (cf. Table I). Thus, the values of Z_1 or Z_2 (but not the fits to yield V_{ii} and k_∞) are directly proportional to D_e and ρ^2 and inversely proportional to R_{12} and the assumed Z value. As can be seen in Figure 1, the fits to the data points are generally excellent. The k_∞, V_{ii}, and Z_1 and Z_2 values obtained from these analyses are given in Table I.

When ferricyanide was used as the oxidant for any of the four flavodoxin semiquinones, the rate constant was found to increase with increasing ionic strength, indicating a repulsive electrostatic interaction between the reactants (Figure 1b). This is consistent with the fact that ferricyanide is negatively charged (−3) and that all of the flavodoxins that were studied are net negatively charged protein molecules at pH 7. The calculated electrostatic potential surface for *C.* MP flavodoxin shows that approximately three-fourths of the molecular surface, including the entire exposed flavin mononucleotide (FMN) region, is negatively charged (Weber & Tollin, 1985). Although the four ferricyanide curves are parallel to each other, the magnitudes of the k_∞ values are different (cf. Table I and Figure 1b). We will discuss these differences below.

Pseudomonas c-551 was the only protein oxidant that showed repulsive (minus–minus) electrostatic interactions (Figure 1a). With this cytochrome, the electrostatic interaction was repulsive with the flavodoxin semiquinones from *C. pasteurianum* and *C.* MP and was attractive for the flavodoxin semiquinones from both *An. nidulans* and *Az. vinelandii*. This rather surprising result will be discussed further below. Both horse and tuna cytochromes *c* are net positively charged proteins and showed attractive interactions with all four flavodoxins (Figure 1c,d). The calculated electrostatic potential surface for the tuna cytochrome is almost totally positive, with only small patches of negative charge located on the side of the protein furthest from the heme; furthermore, the area immediately surrounding the solvent-exposed heme region is completely covered by positive potential (Weber & Tollin, 1985). *Paracoccus* c_2 is a net negatively charged protein; however, it has been previously shown to behave as a cation when it oxidizes *C. pasteurianum* flavodoxin semiquinone (Tollin et al., 1984). This establishes that localized positive charges adjacent to the site of electron transfer (heme edge) have more influence on the electrostatics of intermediate complex formation than either the net protein charge or charged amino acid side chains more removed from the active site. These experiments demonstrate that all of the flavodoxins studied behave similarly with *Paracoccus* c_2 (Figure 1e).

In our previous experiments with *C. pasteurianum* flavodoxin (Simondsen et al., 1982), nonlinear concentration dependencies were oberved with horse cytochrome *c* at ionic strengths of 0.075 M and below. This was attributed to a reaction mechanism involving intermediate complex formation followed by rate-limiting intracomplex electron transfer. In this work, we have observed similar effects with all of the other flavodoxins studied. Furthermore, the limiting first-order rate constants obtained by computer-fitting procedures were all within a factor of 2 of one another under comparable conditions. Thus, the same mechanistic pathway was presumbaly being followed by all of the flavodoxins in their reactions with horse cytochrome *c*.

The magnitudes of the ionic strength effects for the cytochromes are not the same for each flavodoxin as were the ferricyanide effects (cf. V_{ii} values in Table I). This is probably due to the larger flavodoxin surface areas encountered by the protein oxidants, which result in different apparent active-site charges (and/or different ρ and D_e values) for each flavodoxin. The calculated charges using the same ρ and D_e values for all of the flavodoxins are shown in Table I.

The net charge on the *Pseudomonas c*-551 molecule is negative, and the calculated electrostatic potential surface has relatively small and somewhat scattered areas of both negative and positive potential (Weber & Tollin, 1985), with the positive potential located predominantly in the region closest to the exposed heme edge and the negative potential localized principally on the back surface of the protein. The experiments shown in Figure 1 demonstrate that *Pseudomonas c*-551 behaves as a cation when it accepts electrons from the semiquinones of both *Anacystis* and *Azotobacter* flavodoxins but

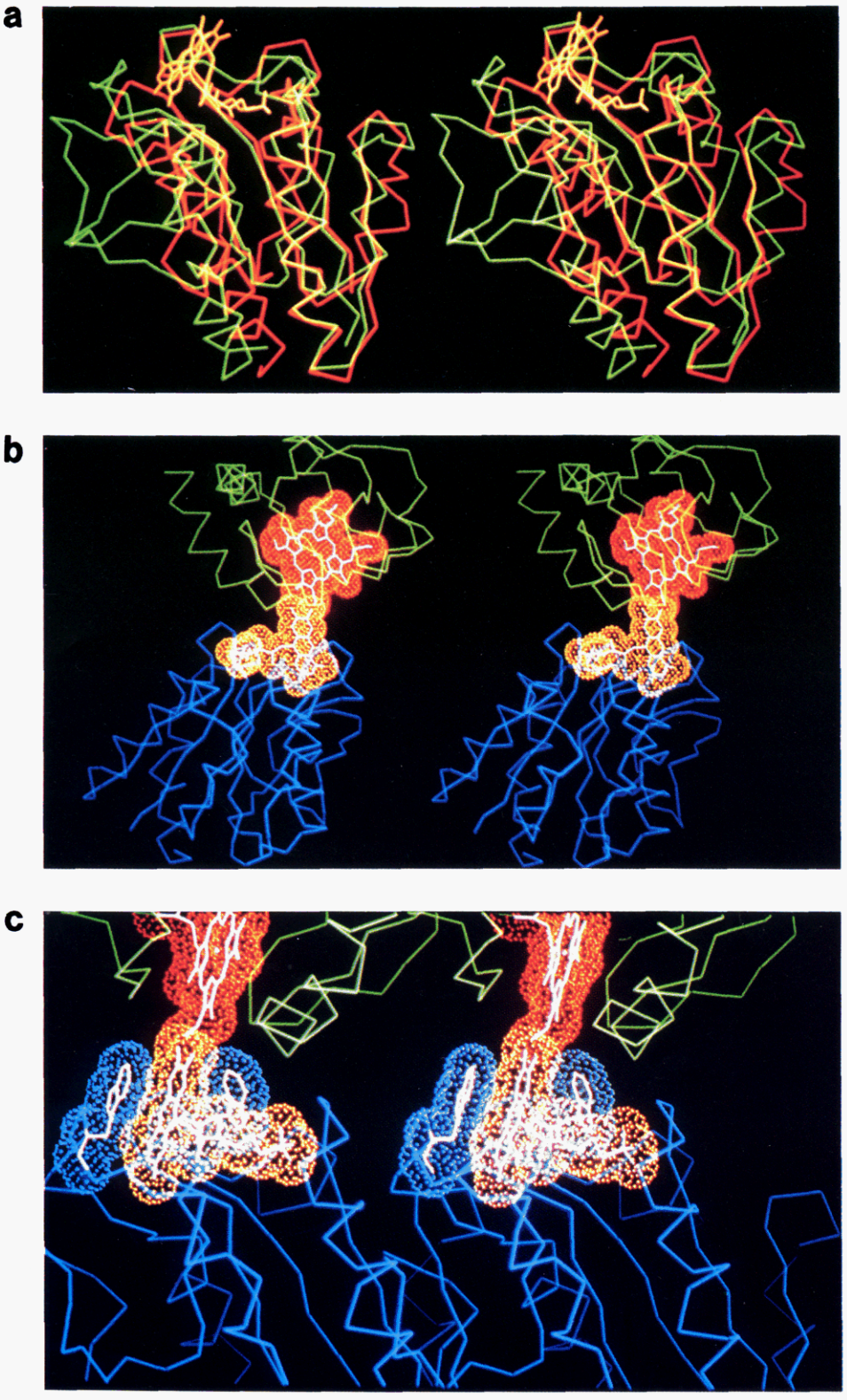

FIGURE 2: Computer graphic stereoviews of *Anacystis* and *C.* MP flavodoxins and the *Anacystis* flavodoxin–tuna *c* complex. (a) Superposition of the backbones and FMN moieties of flavodoxins from *C.* MP (backbone red, FMN orange) and *An. nidulans* (backbone green, FMN yellow). Yellow regions of the backbone correspond to overlap or crossing of the two chains. The molecules were aligned by using the C_α atoms of the parallel sheets. This view shows differences in the orientation of the isoalloxazine ring in the flavodoxins, as well as the extra residues in the longer chain of *Anacystis* flavodoxin. (b) *Anacystis* flavodoxin docked with tuna cytochrome *c* in the same orientation as proposed for *C.* MP flavodoxin. Intolerably close contacts between the FMN and heme are visible in this view. The cytochrome (green backbone) is above and the flavodoxin (blue backbone) below. Dot surfaces have been drawn with a uniform radius of 1.4 Å. (c) Close-up view of the contact region, rotated approximately 90° relative to (b) and including (blue dot surfaces) the Trp residues that adjoin the FMN ring in *Anacystis* flavodoxin (the outer Trp was assigned from electron density only).

behaves as an anion when the reductant is either of the *Clostridium* flavodoxin semiquinones. This indicates that electrons were transferred into the heme of cytochrome *c*-551 from two different regions on the cytochrome surface, depending upon which of the two groups of flavodoxins was used as the reductant.

Figure 2a shows the X-ray structure of *C.* MP flavodoxin superimposed on that of *An. nidulans* flavodoxin (Ludwig et al., 1982; M. L. Ludwig, personal communication). As was previously concluded (Ludwig et al., 1982), alignment of the parallel β sheets of these two flavodoxins demonstrates that the isoalloxazine rings of the FMN prosthetic groups are not superimposable. The flavin rings are inclined at an angle of about 30°, and the C_α and C_β atoms of the outer aromatic residues (Trp-90 for *C.* MP and Trp-95 for *Anacystis*) are not spatially equivalent (not shown). Furthermore, the dimethylbenzene end of the flavin ring (presumably the site of electron transfer) is appreciably more solvent accessible in *C.* MP flavodoxin than in *An. nidulans* flavodoxin (Ludwig et al., 1982). Parts b and c of Figure 2 show a computer-generated hypothetical electron-transfer complex (M. L. Ludwig, personal communication) between *An. nidulans* flavodoxin and tuna cytochrome *c*, using as a model the *C.* MP flavodoxin–tuna cytochrome *c* complex proposed by Simondsen et al. (1982). In the *Anacystis* complex, the cytochrome and the flavodoxin were constrained to have the same mutual orientation as in the *Clostridium* complex. In the *Anacystis* complex, however, this orientation causes the heme and the flavin prosthetic groups to overlap each other. These intolerably close contacts require that a sterically acceptable complex must have a different relative positioning of the two proteins. Inasmuch as the complete structure of the *Anacystis* flavodoxin is not yet available, a more detailed analysis of possible complexes cannot be made. However, due to the structural homologies among the *c*-type cytochromes [cf. Tollin et al. (1984) and Weber and Tollin (1985)] this same steric interference would be expected to occur with an *Anacystis* flavodoxin–*Pseudomonas* cytochrome *c*-551 complex. In this case, however, because of the small and scattered electrostatic potential at the surface of the cytochrome *c*-551, the difference in protein orientation apparently results in a change in the sign of the electrostatic interaction. A further contribution to this effect is as follows. In constructing computer graphic models of the electron-transfer complexes between *C.* MP flavodoxin and several *c*-type cytochromes, Weber and Tollin (1985) observed that there was a complementary interaction between the molecular surfaces formed by residues 7–10 in flavodoxin and residues 14–17 in horse (or tuna) cytochrome *c*, which restricted molecular rotations at the intermolecular interface and thereby greatly contributed to the uniqueness of the proposed complex. This was also the case for *Rhodospirillus rubrum* cytochrome c_2, but *not* for *Pseudomonas* cytochrome *c*-551. With this latter cytochrome, the substitution of Ala for Gln at position 16 results in less correspondence with the surface topography of flavodoxin. This feature, plus the location of fewer lysine residues near the exposed heme edge, makes the *Pseudomonas* complex with flavodoxin considerably less constrained than the others and would thus more easily permit the variation in relative orientations between the two protein molecules, which is required by the steric interference between the FMN and the heme.

In Figure 3 is shown a semilogarithmic plot of the second-order rate constants extrapolated to infinite ionic strength (k_∞) vs. the difference in redox potential between the semiquinone oxidation state of the flavodoxins and the oxidized state of the various cytochromes and ferricyanide ($\Delta E_{m,7}$). The broken line in the figure, which is included to facilitate comparisons among the various flavodoxins, is a plot of the Marcus exponential equation using the parameters ($\nu_{ET} = 5 \times 10^9$ M^{-1} s^{-1} and $\Delta G^\ddagger(0) = 10.7$ kcal/mol) that were used in our previous work with *C. pasteurianum* flavodoxin (Tollin et al., 1984)

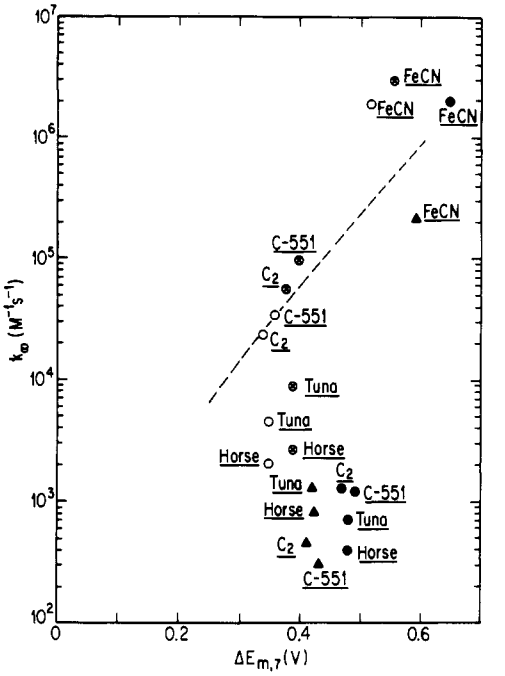

FIGURE 3: Semilogarithmic plot of electrostatically corrected second-order rate constants (k_∞) for oxidation of various flavodoxin semiquinones by oxidants vs. differences in redox potential. The dashed line is the theoretical curve from Tollin et al. (1984). ⊗, *C. pasteurianum*; ○, *C.* MP; ●, *An. nidulans*; ▲, *Az. vinelandii*.

to compare the intrinsic reactivities of a larger group of oxidants. The ν_{ET} value corresponds to the limiting rate constant as the activation energy approaches zero and is a measure of intrinsic reactivity, and the $\Delta G^\ddagger(0)$ value represents the energy required to reach the transition state, when $\Delta E_{m,7} = 0$, and measures the extent of structural and solvent reorganization that is occurring [cf. Tollin et al. (1984)]. This type of data presentation has the advantage of allowing redox potential effects to be easily detected, thus facilitating establishment of the role of other factors (e.g., sterics in determining kinetic reactivity. The k_∞ values for the two *Clostridium* flavodoxins, although similar to one another for each of the oxidants, reflect the more positive redox potential of the *C.* MP flavodoxin semiquinone (i.e., the k_∞ values are uniformly smaller for the *C.* MP flavodoxin). This would argue that both of these proteins have similar flavin exposure and similar surface topography adjacent to the prosthetic group, and thus have similar steric hindrance toward oxidants. The relatively small ferricyanide ion is less sterically hindered than any of the much larger protein molecules, and for this reason the k_∞ values fall above the theoretical curve for the proteins. The large difference in the k_∞ values for the tuna and horse cytochromes relative to other *c*-type cytochromes (for example, with *C. pasteurianum* flavodoxin, the value of k_∞ for *Pseudomonas* *c*-551 is approximately 37 times larger than for horse cytochrome *c*) has previously been attributed to a larger degree of steric hindrance experienced by these cytochromes in forming a productive electron-transfer complex with the *C. pasteurianum* flavodoxin semiquinone [cf. Meyer et al. (1984) and Tollin et al. (1984)]. This work demonstrates that an analogous situation exists for *C.* MP flavodoxin (k_∞ for cytochrome *c*-551 is 16.7 times that of horse cytochrome *c*).

The k_∞ values for electron transfer from the semiquinone oxidation state of both *Anacystis* and *Azotobacter* flavodoxins to the various cytochromes are uniformly smaller than for the *Clostridium* flavodoxins (as much as 2 orders of magnitude in some cases), in spite of the fact that the difference in redox

potential is larger for these proteins (cf. Table I). This suggests that substantial steric effects are operative. As mentioned above, the X-ray crystal structure of *Anacystis* flavodoxin (Ludwig et al., 1982) when compared with that of *C.* MP flavodoxin shows that the dimethylbenzene edge of the flavin is clearly less solvent exposed in the *Anacystis* flavodoxin. Presumably, this increases the distance over which electron transfer must proceed and is the cause of the lowered reactivity [cf. Tollin et al. (1986a) for a correlation between reactivity and solvent exposure]. An additional feature of the *Anacystis* flavodoxin reactions is that the range of k_∞ values for the various cytochromes is considerably smaller than for the *Clostridium* flavodoxins (3.3 vs. 37 for *C. pasteurianum* flavodoxin). Differences in ρ and D_e could contribute to this effect, as well as differences in protein–protein surface contacts caused by the change in mutual orientation within the intermediate complex. Although a crystal structure for *Azotobacter* flavodoxin has not been obtained, the kinetic results suggest that this protein, like the *Anacystis* flavodoxin, is more sterically hindered than *C.* MP flavodoxin. However, the steric properties of the surface regions of the *Azotobacter* and the *Anacystis* flavodoxins are not identical, inasmuch as the relative reactivities of the various cytochromes are appreciably different (Figure 3). For example, although tuna and horse cytochromes are less reactive toward *Anacystis* flavodoxin than are the two bacterial cytochromes, the inverse is true for *Azotobacter* flavodoxin (although tuna and horse retain the same *relative* reactivities toward both flavodoxins).

The k_∞ values for the ferricyanide reactions with *Anacystis* and *Azotobacter* flavodoxin semiquinones are both significantly larger than for the cytrochrome reactions and are closer to the values for the *Clostridium* flavodoxins (Figure 3). This is consistent with ferricyanide being less sterically hindered than the cytochromes and thus being less influenced by the steric constraints on the FMN exposure.

Conclusions

The results of the above experiments are consistent with our previous work in this series (Meyer et al., 1984, 1986; Tollin et al., 1984, 1986b; Przysiecki et al., 1985) and clearly demonstrate the large effects that electrostatic interactions, differences in redox potentials between oxidant and reductant, and relative accessibilities of the prosthetic groups exert on the rate constants for electron transfer between redox proteins. In addition, we have shown that *C. pasteurianum* flavodoxin, which we used in our earlier kinetics studies, behaves very similarly to *C.* MP flavodoxin for which the crystallographic structure is known and for which models of electron-transfer complexes have been constructed. Finally, we have been able to demonstrate a clear-cut example of a fourth major factor that controls reaction rate constants, namely, mutual orientations of reaction partners within an intermediate complex [cf. also Makinen et al. (1983)].

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Registry No. Cytochrome *c*, 9007-43-6; cytochrome c_2, 9035-43-2; cytochrome *c*-551, 9048-77-5; ferricyanide, 13408-62-3.

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