Rehorek, M., & Heyn, M. P. (1979) *Biochemistry 18*, 4977–4983.
Shichida, Y., Matuoka, S., Hidaka, Y., & Yoshizawa, T. (1983) *Biochim. Biophys. Acta 723*, 240–246.
Smith, S. O., & Mathies, R. A. (1985) *Biophys. J. 47*, 251–254.
Sperling, W., Carl, P., Rafferty, C. N., & Dencher, N. A. (1977) *Biophys. Struct. Mech. 3*, 79–94.
Stoeckenius, S., Lozier, R. H., & Bogomolni, R. A. (1979) *Biochim. Biophys. Acta 505*, 215–278.
Vincett, P. S., Voigt, E. M., & Reickhoff, K. E. (1971) *J. Chem. Phys. 55*, 4131–4140.

Transient Kinetics of Reduction of Blue Copper Proteins by Free Flavin and Flavodoxin Semiquinones†

G. Tollin,*,‡ T. E. Meyer,‡ G. Cheddar,‡ E. D. Getzoff,§ and M. A. Cusanovich‡

Department of Biochemistry, University of Arizona, Tucson, Arizona 85721, and Department of Molecular Biology, Scripps Clinic and Research Foundation, La Jolla, California 92037

Received October 25, 1985; Revised Manuscript Received February 5, 1986

ABSTRACT: Rate constants have been determined for the electron-transfer reactions between reduced free flavins and flavodoxin semiquinone and several blue copper proteins. Correlations between these values and redox potentials demonstrate that spinach plastocyanin, *Pseudomonas aeruginosa* azurin, *Alcaligenes* sp. azurin, and *Alcaligenes* sp. nitrite reductase have the same intrinsic reactivities toward free flavins, whereas stellacyanin is more reactive (3.3 times) and laccase considerably less reactive (~12 times). Electrostatic interactions between the negatively charged flavin mononucleotide (FMN) and the copper proteins show that the interaction site charges for laccase and nitrite reductase are opposite in sign to the net protein charge and that the signs and magnitudes of the charges are consistent with the known three-dimensional structures for plastocyanin and the azurins and with amino acid sequence homologies for stellacyanin. The results demonstrate that the apparent interaction site charge with flavodoxin is larger than that with FMN for plastocyanin, nitrite reductase, and stellacyanin but smaller for *Pseudomonas* azurin. This is interpreted in terms of a larger interaction domain for the flavodoxin reaction, which allows charged groups more distant from the actual electron-transfer site to become involved. The intrinsic reactivities of plastocyanin and azurin toward flavodoxin are the same, as was the case with FMN, but both stellacyanin and nitrite reductase are considerably less reactive than expected (approximately 2 orders of magnitude). This result suggests the involvement of steric factors with these latter two proteins which discriminate against large reactants such as flavodoxin.

The electron-transfer reactions and spectroscopic properties of the intensely blue copper proteins have been the focus of considerable research [for reviews, see Holwerda et al. (1976), Solomon et al. (1983), and Adman (1985)]. Most kinetic studies have utilized reactions with inorganic and small organic reductants and oxidants, although in some cases protein–protein interactions have been examined (Farver et al., 1982; Augustin et al., 1984; Takabe et al., 1984; Beoku-Betts et al., 1985). Both temperature (Sailasuta et al., 1979) and pH (Rosenberg et al., 1976) effects have been investigated. Ionic strength effects, which are known to be large in other systems, have also been studied to some extent [cf. Sisley et al. (1983)]. This previous work has led to the conclusions that electron-transfer rate constants are relatively insensitive to pH [cf. also Ugurbil and Mitra (1985)] and that the apparent charge at the electron-transfer site has the same sign as the overall protein charge at pH 7 [cf. Holwerda et al. (1976) and Sisley et al. (1983)], although one exception to this has been reported with stellacyanin (Cummins & Gray, 1977).

The most commonly used copper proteins for kinetic analysis have been plant plastocyanins, bacterial azurins, tree (*Rhus vernicifera*) laccase, and stellacyanin. All but laccase are simple, low molecular weight proteins containing a single copper atom. Amino acid sequences of azurin, plastocyanin, and stellacyanin indicate that they are homologous, although the sequences are difficult to align because of insertions and deletions (Boulter et al., 1977; Norris et al., 1983). The three-dimensional (3-D) structures of azurins (Adman et al., 1978; Norris et al., 1983) and plastocyanin (Colman et al., 1978; Guss & Freeman 1983) show that both proteins have very similar copper binding sites and that there is considerable conservation of β-type secondary structure and indicate where insertions and deletions are likely to have occurred. Laccase is a large oxidase that contains, in addition to the intensely blue or type 1 copper, a paramagnetic but otherwise spectrally invisible type 2 copper and a diamagnetic, UV-absorbing type 3 binuclear copper center. Previous kinetic studies have indicated that stellacyanin is significantly more reactive, and the type 1 copper center in laccase is considerably less reactive, than plastocyanin and azurin [cf. Holwerda et al. (1976)]. On

† Supported in part by grants from the National Institutes of Health (AM15057 to G.T. and GM21277 to M.A.C.).
* Author to whom correspondence should be addressed.
‡ University of Arizona.
§ Scripps Clinic and Research Foundation.

0006-2960/86/0425-3363$01.50/0

the basis of relative Marcus theory calculations of self-exchange rate constants, Rosenberg et al. (1976) have concluded that the copper center in plastocyanin is inherently more reactive than that in azurin.

In our approach to the investigation of the mechanisms of electron-transfer reactions of redox proteins, laser flash photolysis is used to generate either neutral or charged free flavin semiquinones, and the transient kinetics of protein reduction are followed spectrophotometrically. Alternatively, flavodoxin semiquinone is produced by steady-state photolysis, and protein reduction is followed by rapid-mixing techniques. Using these methods, we have established relationships between rate constants for reduction by reduced free flavins and flavodoxin semiquinone and the redox potentials of the protein oxidant for homologous series of *c*-type cytochromes, high redox potential ferredoxins (HiPIP's), and cytochromes *c'* (Meyer et al., 1983, 1984, 1986; Tollin et al., 1984; Przysiecki et al., 1985). We were also able to show that deviant behavior could be related to the relative degree of exposure of the redox center or to steric hindrance to approach of the reductant. Electrostatic effects on the rate constants for reaction of flavin mononucleotide (FMN) semiquinone or flavodoxin semiquinone with electron-transfer proteins could be analyzed by fitting the kinetic data and extrapolating to infinite ionic strength. We have found that the signs and the magnitudes of the electrostatic interactions generally did not correlate with net protein charge but rather were consistent with local and long-range interaction at the specific site of electron transfer. Steric, electrostatic, and redox potential effects were all greatly magnified in flavodoxin semiquinone reactions as compared to FMN reactions, providing a quantitative basis for understanding protein–protein specificity. As will be demonstrated below, our results are generally consistent with earlier work with regard to the relative reactivities of the copper proteins, although we find azurin and plastocyanin to have closely similar reactivities. Further, our knowledge of the roles of steric and electrostatic factors in determining reaction rate constants for this class of electron-transfer protein has been appreciably extended.

Materials and Methods

The laser flash photolysis and stopped-flow techniques and methods of data collection and analysis were as described previously (Ahmad et al., 1981; Simondsen & Tollin, 1983; Meyer et al., 1983, 1984; Tollin et al., 1984; Przysiecki et al., 1985). The pseudo-first-order decay of the flavin semiquinone and appearance of the reduced protein were monitored in the 575–600-nm region under anaerobic conditions over three to four half-lives. The protein purification procedures were as follows. *Pseudomonas aeruginosa* NCTC 10332 azurin was purified by using a modification of the method of Ambler (1963). *Alcaligenes* sp. NCIB 11015 azurin was purified by using the general procedure developed by Suzuki and Iwasaki (1962). *Alcaligenes* sp. NCIB 11015 nitrite reductase was purified by using the method of Masuko et al (1984) as a guideline. Plastocyanin was obtained from spinach by using the general procedure of Ellefson et al. (1980). Laccase and stellacyanin were purified from 100 g of acetone powder of the September harvest of Japanese lacquer tree (*R. vernicifera*) sap supplied by Saito and Co., Tokyo, by using the general procedure of Reinhammer (1970). Purity was established from the ratio of the protein absorbance (~280 nm) to that of the blue copper band (~600 nm), which in all cases was consistent with literature values. Protein concentrations for the kinetic experiments were varied over a 5–10-fold range (the extinction coefficient values used are given in Table I). Depending upon the reaction studied, concentrations were 0.5–7 or 14–300 μM. Typically, three to five concentrations were used to determine the second-order rate constants. No saturation effects were observed at the higher protein concentrations. FMN was purified by passage through a Bio-Gel P-2 gel filtration column equilibrated with double-distilled water (Nagy et al., 1982) and lyophilized. Solutions were made up just prior to use. Standard buffers contained 40 μM flavin, 10 mM ethylenediaminetetraacetic acid (EDTA), and 20 mM sodium phosphate, pH 7.0, for lumiflavin and riboflavin experiments. The 16 mM ionic strength buffer for the FMN experiments contained 1 mM EDTA and 5 mM potassium phosphate; the 60 mM ionic strength buffer contained 5 mM EDTA and 16 mM potassium phosphate. Higher ionic strengths were made up with 10 mM EDTA and 20, 52, 100, and 222 mM potassium phosphate, pH 7.0, giving ionic strengths of 96, 160, 256, and 500 mM, respectively.

For most of the proteins, we determined the second-order rate constants for reduction by both flavin semiquinone and fully reduced flavin (the latter is a product of semiquinone disporportionation, which is always in competition with the electron transfer to the redox protein). In general, for the copper proteins the rate constants for the reaction with fully reduced flavins were more difficult to measure (and are thus less accurately determined) than for other redox proteins because the values are very similar to those for the semiquinone reaction (see below). This made deconvolution of the two processes rather difficult.

For computer graphics analysis, atomic coordinates were taken from the Brookhaven Protein Data Bank (Bernstein et al., 1977). Exposed molecular surfaces were calculated by using the program MS (Connolly, 1983). The molecular surface calculation mathematically positions a water-sized probe sphere (1.4-Å radius) representing a solvent water molecule to touch the van der Waals surface of the protein. Molecular surface calculations used individual van der Waals radii including implicit hydrogen atoms (Getzoff et al., 1986). Computer graphics analysis of the molecular surfaces and their underlying stereochemistry was done by using the interactive graphics language GRAMPS (O'Donnell & Olson, 1981) and the molecular modeling program Granny (Connolly & Olson, 1985).

Results and Discussion

Our previous work (Meyer et al., 1983, 1984, 1986; Tollin et al., 1984; Przysiecki et al., 1985) has shown that the second-order rate constants for the reduction of homologous series of cytochromes *c*, cytochromes *c'*, and HiPIP's by free flavin semiquinones can be correlated with the differences in redox potential (ΔE) between the reactants, as expected on the basis of Marcus electron-transfer theory (Marcus, 1964). By use of an equation developed by Marcus, two parameters are required to fit the data: ν_{ET} (which measures the limiting rate constant as ΔE approaches infinity and thus defines an intrinsic reactivity) and $\Delta G^{*}(0)$ (which is called the intrinsic barrier and which reflects the structural rearrangement that must occur prior to electron transfer). The ν_{ET} values for the reduction of the above series of proteins by lumiflavin semiquinone have been found to vary from 9.6×10^7 M^{-1} s^{-1} (for the HiPIP's) to 2.8×10^8 M^{-1} s^{-1} (for the cytochromes *c'*) and the $\Delta G^{*}(0)$ values from 2.75 (for the cytochromes *c*) to 3.7 kcal mol^{-1} (for the HiPIP's). In the present series of experiments, we do not have enough copper proteins with a wide enough range of redox potentials to adequately determine the parameter values for this electron-transfer group. However, we can make use of our previous results to ask the following

Table I: Electron-Transfer Rate Constants for Copper Proteins

	redox potential (mV)	lumiflavin semiquinone, $k \times 10^{-7}$ (M^{-1} s^{-1})	lumiflavin, fully reduced, $k \times 10^{-7}$ (M^{-1} s^{-1})	riboflavin semiquinone, $k \times 10^{-7}$ (M^{-1} s^{-1})	ϵ_{600nm}[c] (mM^{-1})
(A) laccase	434	[a]	0.21 ± 0.02	[b]	5.7
(B) spinach plastocyanin	370	2.4 ± 0.2	2.5 ± 0.4	2.0 ± 0.2	4.9
(C) *Pseudomonas* azurin	327	2.2 ± 0.1	2.2 ± 1.0	1.8 ± 0.2	4.8
(D) *Alcaligenes* azurin	299	2.2 ± 0.3	2.2 ± 0.2	1.8 ± 0.2	4.8
(E) *Alcaligenes* nitrite reductase	260	1.9 ± 0.2	2.1 ± 0.5	0.9 ± 0.1	3.7
(F) stellacyanin	180	4.1 ± 0.3	2.0 ± 0.5	3.8 ± 0.3	4.1

[a] Too slow to measure. [b] A value of (0.23 ± 0.02) × 10^7 M^{-1} s^{-1} was obtained with fully reduced riboflavin for this protein. [c] These values were chosen from among those that have been reported in the literature. There is some uncertainty as to whether the differences are in fact real or represent experimental error. The values for the azurins and plastocyanin are probably the most reliable. If we were to use these same extinction coefficients for all of the proteins, the conclusions based upon this table and Figure 1 (see text) would not be changed.

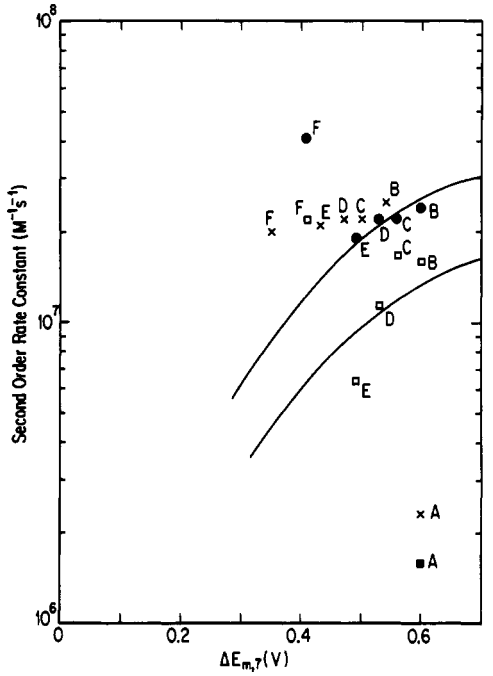

FIGURE 1: Semilog plot of second-order rate constants for reduction of various copper proteins by free flavins vs. the difference in redox potential between reactants ($\Delta E_{m,7}$). Solid lines correspond to plots of the Marcus exponential equation using parameters as given below. Letter designation of proteins is as given in Table I. Symbols: (●) LFH·; ν_{ET} = 4.4 × 10^7 M^{-1} s^{-1}; $\Delta G^*(0)$ = 3.5 kcal/mol (these parameters were determined by a least-squares fit to the data points; however, because of the limited number of points, they should be considered as approximations); (×) fully reduced lumiflavin; (□) FMNH· (k_∞ values); ν_{ET} = 2.3 × 10^7 M^{-1} s^{-1}; $\Delta G^*(0)$ = 3.5 kcal/mol (these parameters are merely estimates); (■) fully reduced FMN (k_∞ value).

question: does this group of proteins form a single reactivity series (i.e., do they fall on a single Marcus curve)? The answer to this question is clearly shown by the data in Figure 1 and Table I. We conclude, from the results obtained with lumiflavin and riboflavin semiquinones and with fully reduced lumiflavin,[1] that plastocyanin and the two azurins are homologous in their reactivities toward these reagents (within experimental error), whereas stellacyanin has a significantly higher reactivity (3.3-fold for lumiflavin semiquinone) and laccase an appreciably lower reactivity (~12-fold for lumiflavin on the basis of the rate constants for the fully reduced form).[2] This is in partial agreement with the earlier work in this field (see above) and represents the first instance for which we can compare the relative reactivity results obtained by using our approach with those obtained in other laboratories. The main discrepancy involves the relative reactivities of azurin and plastocyanin. However, it should be noted that the previous assessment is based upon calculations of protein self-exchange rate constants (Rosenberg et al., 1976) and that the values for these constants can vary over many orders of magnitude, depending upon the reaction used as the source of data for their calculation, and thus can be very different from the directly measured value (Augustin et al., 1984; Ugurbil & Mitra, 1985). Furthermore, many of the studies were done with ionic reagents, and stoichiometric binding to the copper protein (Goldberg & Pecht, 1976; Cho et al., 1984) as well as multiple interaction sites [cf. Adman (1985)] could have complicated the results. We have also extended the previous work to include nitrite reductase, which is seen to have an intrinsic reactivity identical with plastocyanin and azurin toward lumiflavin semiquinone. We can conclude from this that the structural and stereochemical features of the copper binding site in this protein should be similar to those of these other two copper proteins, even though it is much larger in size (M_r 70K for the dimer) (Masuko et al., 1984). This is consistent with recent X-ray absorption spectroscopic measurements (Sano & Matsubara, 1984). The differences in reactivity for stellacyanin and laccase could reflect either steric (i.e., accessibility) effects [cf. Holwerda et al. (1976)] or differences in copper ligation, or both. The copper binding sites for the azurins and plastocyanins are quite similar, on the basis of the 3-D structure work (cf. Figure 3, below), whereas stellacyanin lacks methionine (one of the possible ligating groups)[3] and thus must have at least a partially different copper site.

Riboflavin semiquinone is somewhat less reactive toward all of the copper proteins than is lumiflavin semiquinone, a pattern that we have consistently observed with other classes of redox proteins (Meyer et al., 1984, 1986; Przysiecki et al., 1985) and that we have attributed to a steric effect of the ribityl side chain.

[1] The relatively small differences between the rate constant values for the reduction by lumiflavin semiquinone and fully reduced lumiflavin of plastocyanin, the two azurins, and nitrite reductase are probably not significant. However, the difference for stellacyanin is clearly larger than our experimental uncertainty. The reason for the relative slowness of the fully reduced lumiflavin reaction with stellacyanin is not apparent.

[2] Laccase reacts too slowly with lumiflavin semiquinone to compete with disproportionation, and thus we cannot determine a value for k_2. In this case, however, the k_2 value for the reaction with fully reduced flavin can be measured quite accurately. Thus, a quantitative comparison using this rate constant is meaningful.

[3] Norris et al. (1983) have concluded that the copper is either tridentate or pentacoordinate in *Alcaligenes denitrificans* azurin. There are three definite or strong ligands, His-46, Cys-112, and His-117, and two possible, but weak, ligands, Met-121 and the carbonyl oxygen of Gly-45.

Table II: Analysis of the Effect of Ionic Strength on the Reduction of Copper Proteins by FMN Semiquinone[a]

	net charge[b]	V_{ii} (kcal/mol)	Z_1	$k_\infty \times 10^{-7}$ (M^{-1} s^{-1})
laccase[c]	+	1.27	−1.2	0.17
spinach plastocyanin	−8	2.51	−3.0	1.6
Pseudomonas azurin	−2	1.72	−2.0	1.7
Alcaligenes azurin	0	0	0	1.1
Alcaligenes nitrite reductase	+	1.14	−1.4	0.6
stellacyanin	5	−0.70	0.8	2.2

[a] The parameters used in the analysis were as follows: ρ (the radius of the interaction domain) = 4.5 Å, D_e (the dielectric constant within the interaction domain) = 50, Z_2 = −1.9 (for FMN semiquinone) and −2.9 (for fully reduced FMN), and r_{12} = 3.5 Å (this assumes that the entry point for electrons is at the protein surface, i.e., the exposed histidine). These are similar to ones that we have used previously (Meyer et al., 1984, 1986; Przysiecki et al., 1985). [b] Net charges are based on sequences, except for nitrite reductase and laccase for which the sequences are unknown. With these proteins, the net charge assigned is based on chromatographic behavior. [c] The values for this protein were obtained with fully reduced FMN (Z_2 = −2.9).

Plots of the apparent second-order rate constants vs. the square root of the ionic strength for the reduction of the copper proteins by FMN semiquinone (or by fully reduced FMN in the case of laccase) are shown in Figure 2. The solid lines were obtained from theoretical fits to the data using the approach that we have described previously (Meyer et al., 1984, 1986; Tollin et al., 1984; Przysiecki et al., 1985) with the parameters as given in Table II. As is evident, the agreement with experiment is quite good. The numerical results of the theoretical calculations are also presented in Table II. The V_{ii} values represent the electrostatic potential energy of the interaction and are comparable in magnitude with those we have obtained for other redox proteins. The Z_1 values, which represent the charge at the site of electron transfer in the copper proteins, have the same sign as the net protein charge[4] except for laccase and nitrite reductase. This result is similar to previous observations (Meyer et al., 1984, 1986; Tollin et al., 1984) for those cytochromes in which localized charge effects were predominant, especially where the charge distribution was not uniform. In contrast, for those redox proteins in which there were no charged side chain residues at the presumed site of electron transfer, or where the charges were more uniformly distributed, the interaction site charges obtained from kinetics were more in accord with net protein charges. Both laccase and nitrite reductase are large proteins, and therefore it is not unexpected that interaction site charges should be different from net charges. Additional structural work is required for further analysis of the kinetic data with these proteins.

The 3-D structures of poplar plastocyanin, *P. aeruginosa* azurin, and *Alcaligenes denitrificans* azurin are similar to one another [cf. Norris et al. (1983)]. Computer graphics representations of the plastocyanin and *Pseudomonas* azurin structures are shown in Figure 3. The copper is nearest to the surface of the protein at a region known as the "hydrophobic patch", which presumably is the site of electron transfer. The copper itself is not exposed to solvent, but the edge of one of the histidine ligands (in plastocyanin, His-87, and in azurin, His-117) is at the surface. In azurin the hydrophobic patch is bounded by residues 11–14, 37–44, 65–68, and 114–120. In plastocyanin, this region is bounded by

[4] Note that our results with stellacyanin are consistent with those of Cummins and Gray (1977).

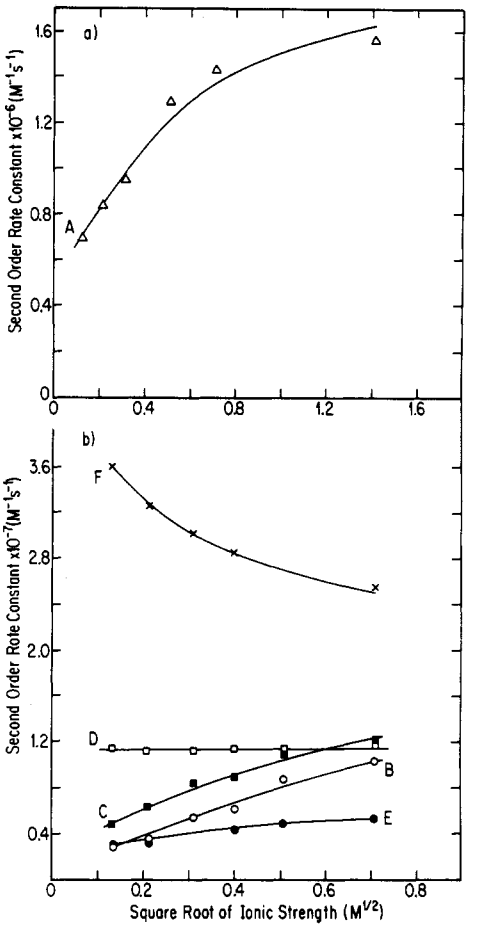

FIGURE 2: Ionic strength dependence of rate constants for reduction of various copper proteins by FMN semiquinone and fully reduced FMN (only for curve A). Letter designation of proteins is as given in Table I.

residues 10–12, 33–36, 62–64, and 86–90. In spinach plastocyanin, the negative charge observed in FMN kinetics may be due to interaction with the negative charges at Glu-59, Glu-60, Asp-61, and possibly Glu-9, which are all about 14 Å away from His-87 (Farver et al., 1982; Guss & Freeman, 1983). In *Pseudomonas* azurin, the charges are more uniformly distributed (Adman et al., 1978), and the smaller negative charge observed in FMN kinetics is probably due to the net effects of Asp-11, Lys-41, Asp-62, Asp-69, Lys-70, and Asp-71, which are 9–17 Å from His-117. Asp-11 should be dominant on the basis of its proximity to the copper site (9 Å). In *Alcaligenes* azurin, opposing charges at Asp-11, Lys-38, Lys-41, and Asp-69 cancel one another, consistent with the kinetics which indicate no ionic strength effect and thus zero interaction site charge.

The 3-D structure of stellacyanin is unknown, but the amino acid sequence is homologous to those of azurin and plastocyanin (Bergman et al., 1977). Alignment is a serious problem except near the copper ligands, but the small positive charge observed in the kinetic experiments can be rationalized by contributions of Arg-43, Arg-44, Lys-91, and Asp-94, at a minimum. On the basis of the amino acid sequence, the carbohydrate binding sites (Asn-X-Thr) are likely to be at the opposite end of the protein from the copper. Since stellacyanin is intrinsically more reactive with small flavins than either plastocyanin or azurin, the carbohydrate apparently does not cause steric hindrance. It is not obvious from the pattern of possible insertions and deletions why stellacyanin should be more reactive, but one can predict that there will be more substantial differences in the 3-D structure of the hydrophobic

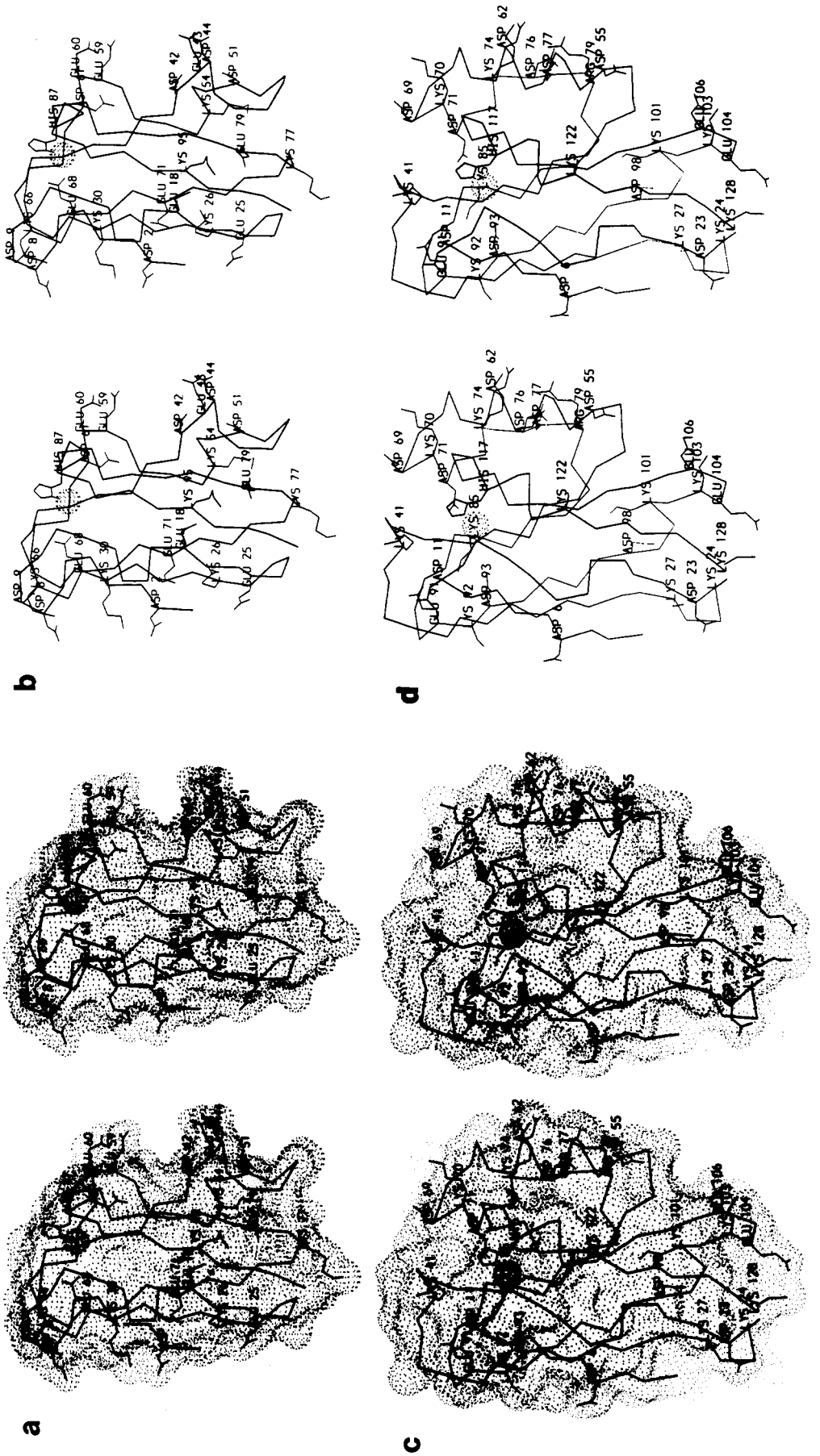

FIGURE 3: Computer graphics stereo views of (a, b) plastocyanin (poplar) and (c, d) azurin (*P. aeruginosa*). The copper ion (sphere, upper center), which has one exposed His-liganding side chain (His-87 in plastocyanin and His-117 in azurin), is located at one end of the β barrel forming the major structural element of both molecules. In panels b and d the molecules are displayed as α-carbon chain with side chains shown for the exposed His-liganding side chain, negative residues, and positive residues. In panels a and c the α-carbon chains with ligand and charged side chains are orientated as in panels b and d, respectively, but with the exposed molecular surface shown as dots. Note that, in spinach plastocyanin, Asp-9 is replaced by Glu, Asp-8 is replaced by Gly, Ser-45 is replaced by Glu, and Lys-66 is replaced by Pro.

Table III: Analysis of the Effect of Ionic Strength on the Reduction of Copper Proteins by Flavodoxin Semiquinone[a]

	V_{ii} (kcal/mol)	Z_1	$k_\infty \times 10^{-5}$ (M^{-1} s^{-1})
spinach plastocyanin	8.90	−3.9	1.3
Pseudomonas azurin	0.92	−0.4	0.27
Alcaligenes nitrite reductase	5.35	−2.4	0.004
stellacyanin	−6.85	3.0	0.013

[a] The parameters used in the analyses were as follows ρ = 7.25 Å, D_e = 15, Z_2 = −4 (for flavodoxin semiquinone), and r_{12} = 3.5 Å. These are similar to those used previously (Tollin et al., 1984).

patch than are found for plastocyanin and azurin.

The k_∞ values for the FMN reactions shown in Table II are plotted in Figure 1. Again, plastocyanin, the two azurins, and nitrite reductase cluster together, with stellacyanin being significantly above and laccase being significantly below this grouping. For the former proteins, a theoretical curve has been drawn parallel to the curve for lumiflavin semiquinone reduction. Although the scatter about the curve is considerably greater for the FMN data, as we have consistently observed for other redox protein systems (Meyer et al., 1984, 1986; Przysiecki et al., 1985) and which we have ascribed to a greater degree of steric discrimination resulting from the bulkier ribityl phosphate side chain, the correlation is not unreasonable. Also as we have previously found, the FMN curve lies below the lumiflavin curve (i.e., the ν_{ET} value is smaller), again for steric reasons. The results obtained for stellacyanin and laccase are consistent with this.

Plots of the apparent second-order rate constants vs. the square root of the ionic strength for the reduction of four copper proteins by flavodoxin semiquinone are shown in Figure 4a. The solid lines again represent the theoretical fits, and again the agreement is good. The parameters used in the calculations, as well as the results, are given in Table III. The ρ value was the same as we have previously used for reactions between other redox proteins and flavodoxin semiquinone (Tollin et al., 1984; Meyer et al., 1986; Przysiecki et al., 1985), and we have used the same effective dielectric constant (D_e) as in our previous study of *c*-type cytochromes (Tollin et al., 1984). This latter value assumes a nonpolar interaction domain with water largely excluded, which is consistent with the hydrophobic patch about the copper site in the plastocyanin and azurin structures. As we have invariably observed in our earlier work, the signs of the electrostatic interactions are the same for both FMN and flavodoxin, whereas the magnitudes are different. Most frequently, the V_{ii} values have been larger for flavodoxin than for FMN (e.g., with the cytochromes *c* and *c'* and for most HiPIP's), although for some HiPIP's the V_{ii} values were the same for these two reductants. As can be seen from a comparison of Tables II and III, for plastocyanin, nitrite reductase, and stellacyanin, Z_1 is larger for flavodoxin than for FMN. With *Pseudomonas* azurin, however, this is reversed; i.e., the strength of the electrostatic interaction with flavodoxin is smaller than that with FMN. This unusual behavior for azurin can be rationalized as follows. FMN, being a smaller molecule, interacts with a more restricted region of the azurin surface than does flavodoxin (reflected in the smaller ρ value) and thus is likely to be influenced mainly by charged groups lying close to the electron-transfer site. Flavodoxin, on the other hand, sees a more global surface. As is shown by the distribution of charged side chains illustrated in Figure 3, azurin has a negatively charged group (Asp-11) located relatively close to the exposed histidine, but as one moves further away from this site the overall charge distribution becomes quite uniform. This would account very nicely

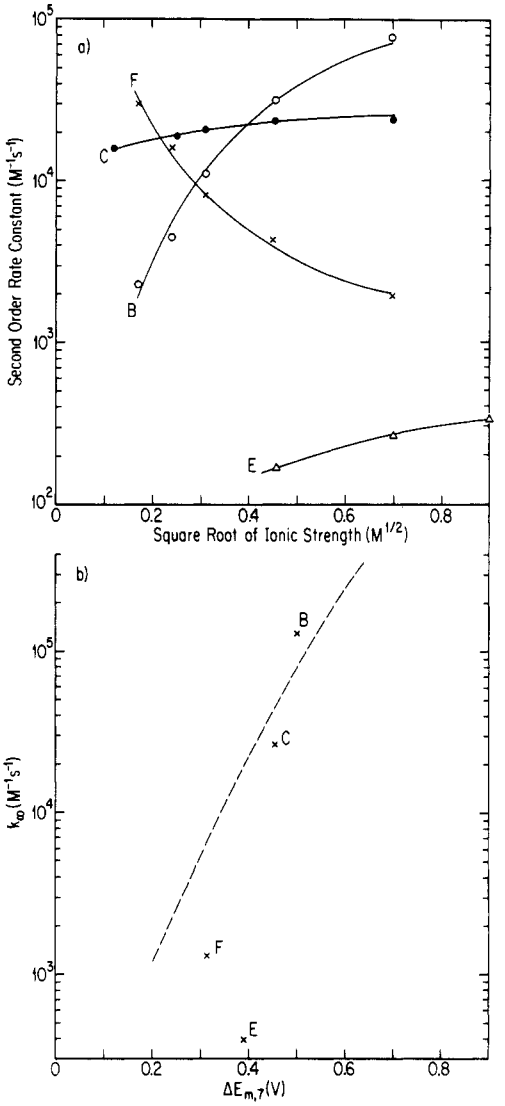

FIGURE 4: (a) Semilog plots of ionic strength dependence of second-order rate constants for reduction of various copper proteins by flavodoxin semiquinone (from *Clostridium pasteurianum*). Letter designation of proteins is as given in Table I. (b) Semilog plots of k_∞ values obtained from theoretical analysis of data in panel a vs. the difference in redox potential between reactants ($\Delta E_{m,7}$). The dashed line corresponds to a plot of the Marcus exponential equation using ν_{ET} = 2 × 10^9 M^{-1} S^{-1} and $\Delta G^*(0)$ = 10.7 kcal/mol. These parameter values were chosen to obtain a curve parallel to that found with *c*-type cytochromes (Tollin et al., 1984) and HiPIP's (Przysiecki et al., 1985), assuming that the k_∞ values for plastocyanin and azurin were on the same curve.

for the experimental results. The plastocyanin results can also be accounted for by the charged side chain distribution shown in Figure 3; i.e., with a larger interaction domain Glu-9 and Glu-68 would likely become involved, and the negative charge cluster of residues 42–45 might also contribute. On the basis of the results obtained with nitrite reductase and stellacyanin, we would predict nonuniform charge distributions for these proteins as well.

In Figure 4b we have plotted the k_∞ values obtained from the theoretical analyses (cf. Table III) vs. the difference in redox potentials between the reactants. Again, as we have observed in our previous work, the dependence of k_∞ upon redox potential is much larger for flavodoxin than for FMN (i.e., the slope of a Marcus curve that correlates the data is much greater, as reflected in the $\Delta G^*(0)$ and ν_{ET} values). A quantitative measure of this can be obtained from the following: upon going from FMN to flavodoxin, the ratio of rate

constants for reduction of plastocyanin and nitrite reductase changes from 2.7 to 325. Note also that stellacyanin, which was intrinsically more reactive toward LFH· and FMNH· than plastocyanin and azurin, is considerably less reactive toward flavodoxin semiquinone than these other two copper proteins. This is similar to what we have observed previously for cytochromes *c'* relative to cytochromes *c* (Meyer et al., 1986), which we have interpreted as resulting from steric factors; i.e., although the heme is more exposed at the protein surface in the cytochromes *c'*, it lies at the bottom of a deep cleft that restricts access to large molecules such as flavodoxin but not to small molecules such as the free flavins. We can therefore suggest that a similar situation exists for stellacyanin, with the steric hindrance to flavodoxin arising from the protein surface topography around the electron-transfer site. We can also predict, on the basis of the fact that nitrite reductase has a much lower intrinsic reactivity toward flavodoxin than expected relative to plastocyanin and azurin, that it too will have a copper site that is sterically less accessible to large reactants. This is in contrast to the surfaces of plastocyanin and azurin near the electron-transfer site, which are rather smooth and featureless (cf. Figure 3) and thus should not present any appreciable steric barriers to reactants.

Conclusions

The results of the present investigation have shown that the commonly studied blue copper electron-transfer proteins appear to fall into three separate intrinsic reactivity groupings (stellacyanin > plastocyanin, azurin, nitrite reductase > laccase) with respect to free flavins and that surface topography is an important determinant of reactivity for nitrite reductase and stellacyanin when flavodoxin semiquinone is used as a reductant. Electrostatic interactions during electron transfer are generally dominated by charged groups lying approximately 9–17 Å from the predominantly nonpolar active site. The results are quite consistent with our earlier studies of other classes of electron-transfer proteins (cytochrome *c*, cytochromes *c'*, HiPIP's), thus demonstrating that a common set of structural parameters are involved in controlling reaction rate constants for a wide range of prosthetic group types. This permits a high level of predictability with regard to structure/function relationships.

Acknowledgments

Drs. Harry Gray and Walter Ellis generously supplied the *Rhus* acetone powder for preparation of laccase and stellacyanin.

Registry No. LFH·, 34533-61-4; FMNH·, 34469-63-1; LFH, 1088-56-8; FMN, 5666-16-0; riboflavin semiquinone, 35919-91-6; laccase, 80498-15-3; nitrite reductase, 9080-03-9.

References

Adman, E. T. (1985) in *Metalloproteins, Part 1: Metal Proteins with Redox Roles* (Harrison, P., Ed.) pp 1–42, Verlag Chemie, Weinheim, FRG.

Adman, R. T., Stenkamp, R. E., Sieker, L. C., & Jensen, L. H. (1978) *J. Mol. Biol. 123*, 35–47.

Ahmad, I., Cusanovich, M. A., & Tollin, G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6724–6728.

Ambler, R. P. (1963) *Biochem. J. 89*, 341–348.

Augustin, M. A., Chapman, S. K., Davies, D. M., Watson, A. D, & Sykes, A. G. (1984) *J. Inorg. Biochem. 20*, 281–289.

Beoku-Betts, D., Chapman, S. K., Knox, C. V., & Sykes, A. G. (1985) *Inorg. Chem. 24*, 1677–1681.

Bergman, C., Gaudvik, E., Nyman, R. O., & Strid, L. (1977) *Biochem. Biophys. Res. Commun. 77*, 1052–1059.

Bernstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, E. F., Brice, M. D., Rogers, J. R., Kennard, O., Shimanouchi, T., & Tasumi, M. (1977) *J. Mol. Biol. 112*, 535–542.

Boulter, D., Haslett, B. G., Peacock, D., Ramshaw, J. A. M., & Scawen, M. D. (1977) *Int. Rev. Biochem. II 13*, 1–40.

Cho, K. C., Blair, D. F., Banerjee, U., Hopfield, J. J., Gray, H. B., Pecht, I., & Chan, S. I. (1984) *Biochemistry 23*, 1858–1862.

Colman, P. M., Freeman, H. C., Guss, J. M., Murata, M., Norris, V. A., Ramshaw, J. A. M., & Venkatappa, M. P. (1978) *Nature (London) 272*, 319–324.

Connolly, M. L. (1983) *Science (Washington, D.C.) 221*, 709–713.

Connolly, M. L., & Olson, A. J. (1985) *Comput. Chem. 9*, 1–6.

Cummins, D., & Gray, H. B. (1977) *J. Am. Chem. Soc. 99*, 5158–5167.

Ellefson, W. L., Ulrich, R. A., & Krogmann, D. W. (1980) *Methods Enzymol. 69*, 223–228.

Farver, O., Blatt, Y., & Pecht, I. (1982) *Biochemistry 21*, 3556–3561.

Getzoff, E. D., Tainer, J. A., & Olson, A. J. (1986) *Biophys. J. 49*, 191–204.

Goldberg, M., & Pecht, I. (1976) *Biochemistry 15*, 4197–4208.

Guss, J. M., & Freeman, H. C. (1983) *J. Mol. Biol. 169*, 521–563.

Holwerda, R. A., Wherland, S., & Gray, H. B. (1976) *Annu. Rev. Biophys. Bioeng. 5*, 363–396.

Masuko, M., Iwasaki, H., Sakurai, T., Suzuki, S., & Nakahara, A. (1984) *J. Biochem. (Tokyo) 96*, 447–454.

Meyer, T. E., Przysiecki, C. T., Watkins, J. A., Bhattacharyya, A., Simondsen, R. P., Cusanovich, M. A., & Tollin, G. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6740–6744.

Meyer, T. E., Watkins, J. A., Przysiecki, C. T., Tollin, G., & Cusanovich, M. A. (1984) *Biochemistry 23*, 4761–4767.

Meyer, T. E., Cheddar, G., Bartsch, R. G., Getzoff, E. D., Cusanovich, M. A., & Tollin, G. (1986) *Biochemistry 25*, 1383–1390.

Nagy, J., Knoll, J., Kenney, W. C., & Singer, T. P. (1982) in *Flavins and Flavoproteins* (Massey, V., & Williams, C. H., Jr., Eds.) pp 573–578, Elsevier/North-Holland, New York.

Norris, G. E., Anderson, B. F., & Baker, E. N. (1983) *J. Mol. Biol. 165*, 501–521.

O'Donnell, T. J., & Olson, A. J. (1981) *Comput. Graphics 15*, 133.

Przysiecki, C. T., Cheddar, G., Meyer, T. E., Tollin, G., & Cusanovich, M. A. (1985) *Biochemistry 24*, 5647–5652.

Reinhammer, B. (1970) *Biochim. Biophys. Acta 205*, 35–47.

Rosenberg, R. C., Wherland, S., Holwerda, R. A., & Gray, H. B. (1976) *J. Am. Chem. Soc. 98*, 6364–6368.

Sailasuta, N., Anson, F. C., & Gray, H. B. (1979) *J. Am. Chem. Soc. 101*, 455–458.

Sano, M., & Matsubara, T. (1984) *Chem. Lett.*, 2121–2124.

Simondsen, R. P., & Tollin, G. (1983) *Biochemistry 22*, 3008–3016.

Sisley, M. J., Segal, M. G., Stanley, C. S., Adzamli, I. K., & Sykes, A. G. (1983) *J. Am. Chem. Soc. 105*, 225–228.

Solomon, E. I., Penfield, K. W., & Wilcox, D. E. (1983) *Struct. Bonding (Berlin) 53*, 2–57.

Suzuki, H., & Iwasaki, H. (1962) *J. Biochem. (Tokyo) 52*, 193–199.
Takabe, T., Ishikawa, H., Niwa, S., & Tanaka, Y. (1984) *J. Biochem. (Tokyo) 96*, 385–393.
Tollin, G., Cheddar, G., Watkins, J. A., Meyer, T. E., & Cusanovich, M. A. (1984) *Biochemistry 23*, 6345–6349.
Ugurbil, K., & Mitra, S. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2039–2043.

Mechanism of Action of Aromatic Amines That Short-Circuit the Visual Cycle†

Paul S. Bernstein, Brian S. Fulton, and Robert R. Rando*
Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115
Received December 19, 1985; Revised Manuscript Received January 28, 1986

ABSTRACT: DAPP [1,5-bis(*p*-aminophenoxy)pentane] is an antischistosomal drug that can inhibit dark adaptation in vertebrates by impairing formation of 11-*cis*-retinoids in the eye and by depleting preformed stores of them [Bernstein, P. S., & Rando, R. R. (1985) *Vis. Res. 25*, 741–748]. It has recently been shown that *p*-phenetidine and other monofunctional analogues of DAPP (a symmetric bifunctional molecule) can duplicate DAPP's effects, and it was proposed that these retinotoxic compounds exert their effects in vivo by "short-circuiting" the visual cycle, catalyzing the thermodynamically downhill isomerization of 11-*cis*-retinal to *all-trans*-retinal [Bernstein, P. S., Lichtman, J. R., & Rando, R. R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1632–1635]. In this paper, the "short-circuit" hypothesis is investigated more fully. Numerous phenetidine-like molecules are assayed for their ability to inhibit rhodopsin formation and 11-*cis*-retinyl palmitate formation in the living frog eye. It is found that virtually any aromatic amine with a moderately hydrophobic alkyl chain "tail" is an active inhibitor in vivo. The tail can be in either the para or the meta position and can be attached to the aromatic ring either by direct linkage or by an ether linkage. Compounds that can be metabolized in vivo to such active compounds are also inhibitory. Amino group modification studies demonstrate an absolute requirement for structures that can form a Schiff base with retinal. Further support for the short-circuit hypothesis is evidenced by the detection of Schiff bases of aromatic amines with retinal in the eyes of frogs injected with the amines; these are the catalytic intermediates postulated in the short-circuit hypothesis. Additionally, it is demonstrated that Schiff base formation in vitro in phosphatidylcholine-based liposomes enhances the catalysis of retinal isomerization by aromatic amines by a factor of 10^3 over the rate in *n*-heptane. This work introduces a novel, mechanistically defined class of inhibitors of dark adaptation that can be used to probe the biochemistry of the visual cycle. It also provides a model that predicts possible ocular toxicity of drugs in advance of clinical trials and animal studies.

The biochemical basis of the vertebrate visual cycle is an area of very active scientific research, and the initial events triggered by the absorption of a photon have been extensively characterized. Light induces the photochemical isomerization of the 11-*cis*-retinal Schiff base chromophore of rhodopsin to the *all-trans*-retinal form (Hubbard & Wald, 1952; Bownds, 1967). The activated rhodopsin can then catalyze the exchange of GTP for GDP in a G protein (Fung & Stryer, 1980), which in turn can activate a cGMP-specific phosphodiesterase (Wheeler & Bitensky, 1977), eventually leading to hyperpolarization of the rod cell. The isomerized retinal chromophore of rhodopsin is released, and it is reduced to *all-trans*-retinol. Under bright light conditions the released retinoid is esterified to long-chain fatty acids such as palmitic acid and stored in the pigmented epithelium (Bridges, 1976). As part of the process of dark adaptation, the *all-trans*-retinyl esters in the pigmented epithelium must be mobilized and converted to 11-*cis*-retinal, which can then combine with the apoprotein opsin to form rhodopsin once again. In contrast to the enormous amount known about the initial events of vision, very little is understood about the biochemistry of the conversion of *all-trans*-retinoids to 11-*cis*-retinoids in the eye. 11-*cis*-Retinoids are compounds whose free energies are approximately 4 kcal/mol higher than those of their *all-trans*-retinoid counterparts in both nonpolar and polar solvents (Rando & Chang, 1983; Futterman & Futterman, 1974), due primarily to steric crowding of the methyl group at C-13 with the hydrogen at C-10. The form of retinoid isomerized, whether alcohol, aldehyde, or ester, as well as the anatomical site of isomerization in the eye, is unknown, nor is it understood where the energy comes from to drive the all-trans to 11-cis conversion. Recent work has shown that the actual isomerization event may not even be enzyme-mediated (Bernstein et al., 1985).

One way of approaching the problem of how 11-*cis*-retinoids are formed in the eye is to study the biochemistry of pharmacological agents that inhibit dark adaptation in vivo. 1,5-Bis(*p*-aminophenoxy)pentane (DAPP,[1] **1**), an antischistosomal drug, is one of the most powerful and selective inhibitors of dark adaptation available (Goodwin et al., 1957). In addition to decreasing the rate of dark adaptation in frogs approximately 50-fold, this compound will also inhibit formation of

† This work was supported by U.S. Public Health Service Research Grant EY 04096 from the National Institutes of Health. P.S.B. was supported by U.S. Public Health Service Training Grant GM 07306 from the National Institutes of Health and by the Albert J. Ryan Foundation.
* Author to whom correspondence should be addressed.

[1] Abbreviations: DAPP, 1,5-bis(*p*-aminophenoxy)pentane; PE, pigmented epithelium; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HPLC, high-performance liquid chromatography; RSB, retinal Schiff base; ADH, alcohol dehydrogenase; PC-MLV, phosphatidylcholine multilamellar vesicle.

0006-2960/86/0425-3370$01.50/0