Adduct Formation between Sulfite and the Flavin of Phototrophic Bacterial Flavocytochromes *c*. Kinetics of Sequential Bleach, Recolor, and Rebleach of Flavin as a Function of pH†

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ABSTRACT: The kinetics of sulfite adduct formation with the bound flavin in flavocytochromes *c* from the purple phototrophic bacterium *Chromatium vinosum* and the green phototrophic bacterium *Chlorobium thiosulfatophilum* have been investigated as a function of pH. Both species of flavocytochrome *c* rapidly react with sulfite to form a flavin sulfite adduct ($k = 10^3$–10^5 M^{-1} s^{-1}) which is bleached at 450–475 nm and has associated charge-transfer absorbance at 660 nm. The rate constant for adduct formation in flavocytochrome *c* is 2–4 orders of magnitude faster than for model flavins of comparable redox potential and is likely to be due to a basic residue near the N-1 position of the flavin, which not only raises the redox potential but also stabilizes the negatively charged adduct. There is a p*K* for adduct formation at 6.5, which suggests that the order of magnitude larger rate constant at pH 5 as compared to pH 10 in flavocytochrome *c* is due the influence of another positive charge, possibly a protonated histidine residue. The adduct is indefinitely stable at pH 5 but decomposes (the flavin recolors) in a first-order process accelerating above pH 6 (at pH 10, $k = 0.1$ s^{-1}). The p*K* for recoloring is 8.5, which is suggestive of a cysteine sulfhydryl. On the basis of the observed p*K* and available chemical information, we believe that recoloring is due to a secondary effect of the reaction of sulfite with a protein cystine disulfide, which is adjacent to the flavin. At high pH and high sulfite concentration, the flavin bleaches a second time in a very slow reaction ($k = 10^{-3}$ s^{-1}), which suggests that the redox potential of the flavin, following reaction of sulfite with the presumed cystine, is much lower than that of native protein and probably close to that of free flavin.

Flavocytochromes *c* have been found in several species of phototrophic purple and green sulfur bacteria, which are able to utilize thiosulfate in addition to sulfide for growth (Meyer & Kamen, 1982; Cusanovich et al., 1991). These complex proteins have both flavin and heme-containing subunits. They appear to function as sulfide dehydrogenases and are inhibited by cyanide (Yamanaka & Kusai, 1976), which forms an adduct with the flavin, but not with the heme (Meyer & Bartsch, 1976). In addition, the flavocytochrome *c* flavin has been found to form stable adducts with sulfite, thiosulfate, and some mercaptans without reduction of either the flavin or the heme (Meyer & Bartsch, 1976). Not only is enzyme activity inhibited by ligands, which form adducts with the flavin, but also chemical reduction of the heme is inhibited. Heme reduction by exogenous reductants is mediated by the bound flavin, which is inhibited by formation of the sulfite adduct (Cusanovich et al., 1985).

It is known that free flavin and some flavoproteins form N-5 adducts with sulfite, and this is correlated with high flavin redox potential (Muller & Massey, 1969; Massey et al., 1969). We have found that the redox potentials of the flavocytochromes *c* are unusually high, which may explain their reactivity with sulfite (Meyer et al., 1991). By analogy, adduct formation with cyanide, thiosulfate, and mercaptans may also be facilitated by the high redox potential of the flavin (Meyer & Bartsch, 1976). However, there is a long-wavelength (650–690 nm) charge-transfer species associated with all four of the flavocytochrome *c* adducts, which has not been observed either with free flavin–sulfite adducts or with other flavoprotein sulfite adducts.

We previously examined the kinetics of adduct formation between sulfite and the bound flavin of *Chromatium vinosum* flavocytochrome *c* as a function of pH in phenomenological studies (Cusanovich & Meyer, 1982; Meyer et al., 1987). Sulfite appeared to be an order of magnitude more reactive with flavin at pH 5 than at pH 10, which is contrary to results obtained with free flavins which are more reactive at high pH (Muller & Massey, 1969). Another unique observation was that the sulfite adduct decomposed in a first-order reaction at high pH, resulting in recovery of flavin absorbance, although the adduct is indefinitely stable at pH 5. In order to understand adduct formation and subsequent reactions in more detail, we have reexamined and extended these observations with an additional species of flavocytochrome *c*, from *Chlorobium thiosulfatophilum*. Taken together, the results suggest a minimum three-step mechanism, involving adduct formation, decomposition leading to recovery of the flavin absorbance (recoloring), and subsequent loss of flavin absorbance (rebleach). On the basis of the kinetic scheme and the measured p*K* values for adduct formation and recoloring, the participation of a minimum for four amino acid side chains is suggested.

Materials and Methods

Chlorobium thiosulfatophilum strain Tassajara flavocytochrome *c* was prepared as described by Meyer et al. (1968). The ratio of 280-nm to 410-nm absorbance was 0.94, and the ratio of 475-nm to 525-nm absorbance was 1.5. The first is indicative of pure protein, and the second ratio shows that the flavin is not complexed with any naturally occurring ligands. *Chromatium vinosum* strain D flavocytochrome *c* was prepared according to Bartsch (1971). The ratio of

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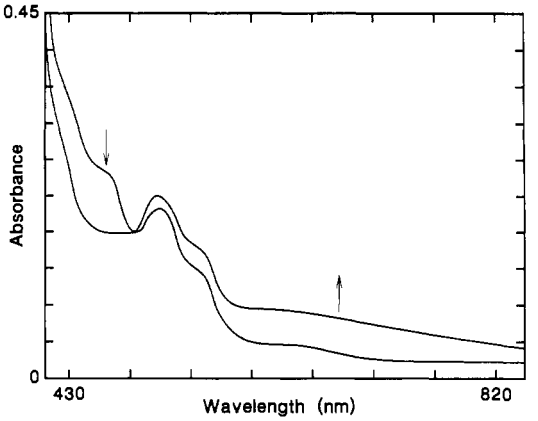

FIGURE 1: Absorption spectra of *Chromatium* flavocytochrome *c* and its sulfite adduct at pH 5. These spectra show the bleach at 475 nm and formation of charge-transfer absorbance at 660 nm. The 530-nm peak with a shoulder at ca. 550 nm plus the weak band at ca. 630 nm are due to the heme, which is unchanged in the presence of sulfite.

280-nm to 410-nm absorbance was 0.55, and the ratio of 475-nm to 525-nm absorbance was 1.3. Note that the ratios for *Chromatium* and *Chlorobium* are different because there are two hemes per flavin in *Chromatium* and only one in *Chlorobium*.

Kinetics were measured with a Durrum–Gibson stopped-flow spectrophotometer with a data acquisition system provided by On-Line Instrument Systems. All reactions were performed aerobically at 25 °C. The buffer contained 25 mM acetate, phosphate, Tris, and glycine adjusted to the appropriate pH. Sulfite solutions in buffer were prepared fresh daily. Unless otherwise noted, "sulfite" refers to the total of sulfite and bisulfite. No difference in the kinetics was observed in control experiments in the absence of oxygen. The protein concentration was about 6 μM before mixing.

Results and Discussion

At low pH, the reaction of sulfite with the flavocytochromes *c* results in a rapid and complete bleach of the flavin absorbance at 475 nm with formation of a charge-transfer band at 660 nm (Figures 1 and 2A). The loss of 475-nm absorbance (bleach) and the appearance of the charge-transfer band have identical kinetics. With the flavocytochrome *c* from *Chromatium*, the reaction is pseudo first order at all pH values and sulfite concentrations studied. Moreover, the observed rate constant (k_{obs}) varies with sulfite concentration in a second-order fashion. Measured second-order rate constants for initial reaction of sulfite with flavocytochrome *c* vary with pH as shown in Figure 3. However, it is important to note that at pH values above 7, the kinetics are difficult to analyze at low sulfite concentrations. This results because the flavin absorbance slowly returns (recolors) in a pH-dependent first-order reaction. At low sulfite concentrations, this recoloring reaction competes with the bleach reaction and results in a significant apparent increase in the pseudo-first-order rate constant. In turn, the second-order plots (k_{obs} vs sulfite concentration) appear nonlinear at high pH values. However, by careful choice of sweep times and by analyzing both bleaching and recoloring kinetics, the true pseudo-first-order rate constants were obtained.

In the case of *Chlorobium* flavocytochrome *c*, the bleach kinetics are somewhat more complicated at pH 6 and 7 with the kinetic data clearly biphasic at high sulfite concentrations. At both pH values, the second (slow) phase is second order; however, the magnitude of its contribution is concentration dependent, ranging from approximately 40% of the total ab-

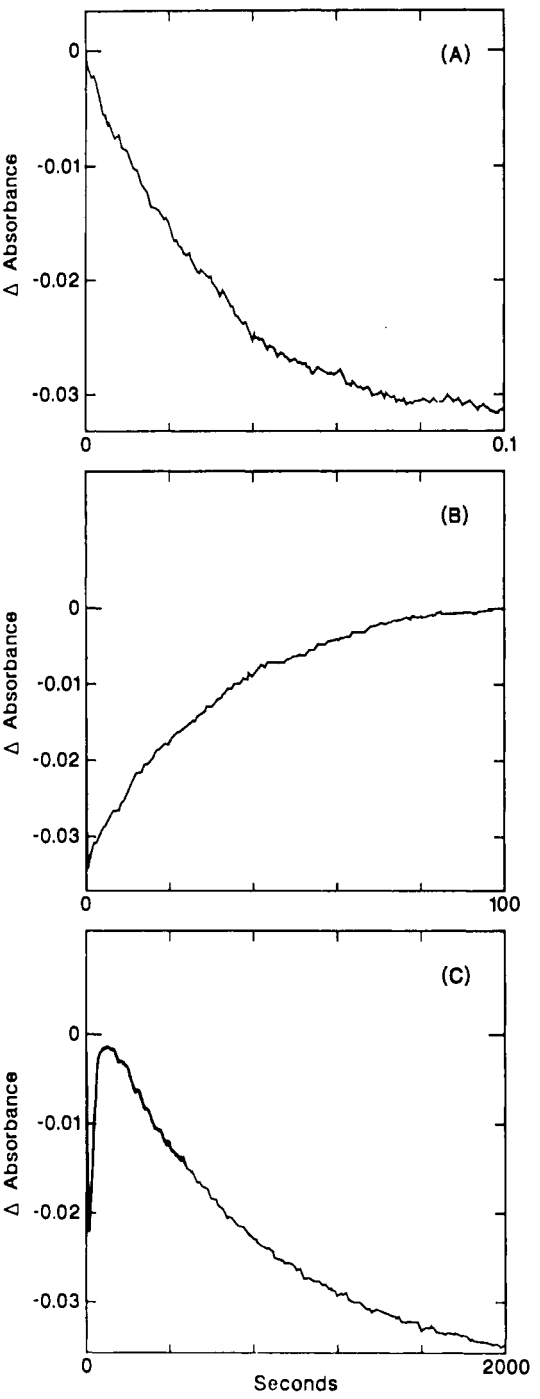

FIGURE 2: Reaction of sulfite with *Chlorobium* flavocytochrome *c* at pH 9. (A) Initial bleach of flavin absorbance at 475 nm (observed at all pH values). (b) Secondary recoloring of flavin at 475 nm (only observed at high pH). (C) Final bleach of flavin at 475 nm (seen only at high pH).

sorbance change at 5 mM sulfite to zero at 156 μM sulfite at pH 6. Similarly, at pH 7, the second phase is 20% of the total absorbance change at 5 mM sulfite and is no longer detectable at 1.25 mM sulfite. The apparent second-order rate constant for the fast phase is 10^5 M^{-1} s^{-1} at pH 6 and 3×10^4 M^{-1} s^{-1} at pH 7, whereas for the slow phase it is 12 M^{-1} s^{-1} at pH 6 and approximately 7 M^{-1} s^{-1} at pH 7. The absorbance changes at both pH 6 and pH 7 at low sulfite concentrations, where no slow phase is observed, are approximately equal to the sum of the slow and fast phases observed at higher sulfite concentrations. Because the slow phase is not observed above pH 7 and only present at the highest sulfite concentrations at pH 7, it was not studied in detail. The fast phase of *Chlo-*

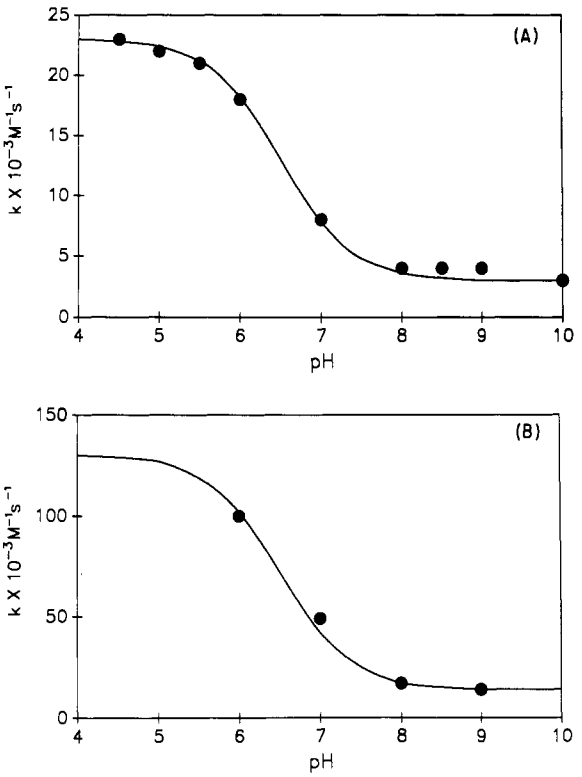

FIGURE 3: Effect of pH on the second-order rate constants for initial bleach of flavin absorbance at 475 nm in (A) *Chromatium* and (B) *Chlorobium*. The solid lines were calculated by using rate constants at the lower pH limit of 2.3×10^4 and 1.3×10^5 M^{-1} s^{-1} for (A) and (B) and those at an upper pH limit of 3×10^3 and 1.4×10^4 M^{-1} s^{-1} for (A) and (B), respectively, and assuming a pK of 6.5.

robium flavocytochrome *c* kinetics will be the focus of subsequent discussion.

On the basis of analogy with the work of Muller and Massey (1969), the reaction product of the second-order bleach is apparently a flavin N-5 sulfite adduct. However, Muller and Massey (1969) did not observe a charge-transfer band associated with free flavin sulfite adducts in the absence of an acceptor, nor was it observed with the protein-bound flavin sulfite adducts which were studied (Massey et al., 1969). Thus, the flavocytochromes *c* are unique in regard to the presence of a charge-transfer band associated with the sulfite adduct. Although the charge-transfer acceptor has not been identified, it could be a cystine disulfide, an aromatic amino acid side chain, or even the heme. Coenzyme A persulfide in acyl-CoA dehydrogenase (Williamson et al., 1982) and a cysteinyl anion in lipoamide dehydrogenase (Matthews & Williams, 1976) are charge-transfer *donors* to *oxidized* flavin. Thus, a cystine disulfide would appear to be a likely candidate for charge-transfer *acceptor* from the flavin sulfite adduct in flavocytochrome *c*, except for the reactivity of disulfides with sulfite as discussed below. In studies with D-amino acid oxidase (Massey & Ganther, 1965) and old yellow enzyme (Abramovitz & Massey, 1976), added aromatic molecules containing heteroatoms were better charge-transfer *donors* to *oxidized* flavin than was benzoate. Tryptophan was also found to be a good charge-transfer *donor* to *oxidized* flavin (Isenberg & Szent-Gyorgyi, 1958). Tryptophan forms strong complexes with oxidized, reduced, and semiquinone forms of lumiflavin, whereas cystine and tyrosine will only complex the semiquinone (Yeh & Ingraham, 1976). Phenylalanine and histidine do not form a complex with any of the three oxidation states of the flavin. Because the flavin sulfite adduct is equivalent to fully freduced flavin, it appears that tryptophan is the most likely charge-transfer *acceptor* from the flavocytochrome *c* sulfite adduct. We considered the heme as a possible charge-transfer acceptor, but the heme is on a separate subunit, which is structurally related to cytochrome *c* (Van Beeumen et al., 1991), and is probably too distant to fill that role.

Sulfite is more reactive with free flavins at high pH than at low pH, and there is a strong correlation between sulfite binding and the redox potential of the flavins (Muller & Massey, 1969). We recently showed that the flavocytochromes *c* have some of the highest flavin two-electron redox potentials of any known flavoprotein (−26 and +28 mV for *Chromatium* and *Chlorobium*, respectively; Meyer et al., 1991) which may partially explain their reactivity with sulfite. The high flavin redox potentials, the stabilization of the anionic semiquinone (Meyer et al., 1991), and the reactivity with sulfite may all result from the presence of a positively charged amino acid residue (Lys or Arg) adjacent to the N-1 position of the flavin (Meyer et al., 1991). For example, three-dimensional structures show that there is such an active-site lysine in glycolate oxidase (Lindqvist & Branden, 1989) and yeast lactate dehydrogenase (Xia & Mathews, 1990). There is also evidence for the presence of an arginine near the N-1 position of the flavin in D-amino acid oxidase (Fitzpatrick & Massey, 1983). All three of these flavoproteins have relatively high redox potentials and readily form sulfite adducts, although they apparently do not display charge-transfer absorbance nor are there potential charge-transfer acceptors near the flavin.

Analysis of the kinetic data for adduct formation in flavocytochrome *c* as a function of pH indicates that both flavocytochromes *c* can be fit with a single pK of 6.5 (Figure 3). The limiting second-order rate constants at high and low pH differ by nearly an order of magnitude. The pK at 6.5 is apparently only coincidentally similar to that for bisulfite ionization (pK = 6.7). This results from the fact that the apparent pK for the sulfite reaction is similar to that for the reaction between thiosulfate, which has no ionization in this pH region, and *Chromatium* flavocytochrome *c* (pK = 6.7) (Cusanovich & Meyer, 1982), supporting the conclusion that the pK at 6.5 is a result of a protein-linked ionization and not a ligand ionization. Moreover, we observe a *decrease* in the rate constant for adduct formation at high pH, a result which is the opposite of the situation with free flavin where sulfite is *more* reactive at high pH. We have recently shown by redox titrations that the flavin itself has no pK values (oxidized, reduced, or semiquinone) in the pH range 6–8 (Meyer et al., 1991). The flavin N-5 sulfonate should also be completely ionized within the pH 5–10 range of this study, and thus cannot be responsible for the pK at 6.5. It is interesting to note that our lowest rate constant obtained at pH 8 and above (3×10^3 M^{-1} s^{-1}) is more than 2 orders of magnitude faster than that found for the reaction of free flavins of comparable redox potential (7 M^{-1} s^{-1}; Muller & Massey, 1969) and demonstrates that flavocytochrome *c* is intrinsically more reactive with sulfite than are free flavins.

On the basis of the data presented here, we conclude that a positively charged ionizable side chain with a pK of approximately 6.5 is in the immediate vicinity of the flavin N-5 position which provides electrostatic attraction for the sulfite and additionally stabilizes the sulfite adduct. On the basis of this pK value, histidine is a likely choice for the ionizable amino acid side chain present. Glycolate oxidase and lactate dehydrogenase both have active-site histidines (Lindqvist & Branden, 1989; Xia & Mathews, 1990) and, as discussed above, form strong sulfite adducts. Both the histidine and an arginine, which are near the N-5 position, form salt bridges

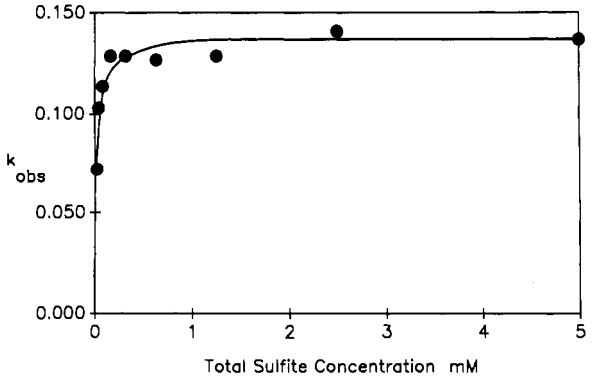

FIGURE 4: Effect of sulfite concentration on the pseudo-first-order rate constant for recovery of flavin absorbance in *Chromatium* flavocytochrome *c* at pH 9. The solid line was calculated by assuming a two-step mechanism with $k_{12} = 7 \times 10^3$ M^{-1} s^{-1}, $k_{21} = 0.1$ s^{-1}, and $k_{23} = 0.13$ s^{-1}.

or hydrogen bonds with the bound sulfite in lactate dehydrogenase (Tegoni & Mathews, 1988). We are not aware of any study of the effect of pH on formation of sulfite adducts with these enzymes, but predict that it will be similar to what we observe with flavocytochrome *c*.

There are other amino acid side chains which might ionize at an unusual pH in the appropriate environment, such as a buried glutamate or aspartate, but they would be destabilizing at high pH rather than stabilizing at low pH. An α helix could provide a positive charge in the vicinity of the flavin, if appropriately oriented, but an ionization of an amino acid in this helix would not change the helix dipole sufficiently to account for the order of magnitude change in kinetics. This is true of any amino acid ionization at a distance from the flavin and requires that the charge be nearby. The necessity to have a positive charge near the flavin with a pK of 6.5 indicates histidine as the most reasonable possibility.

At pH values of 6 and above, the kinetics of the reaction of sulfite with flavocytochrome *c* are more complicated than at pH 5 in that the adduct is unstable and decomposes in a concentration-independent fashion at high substrate concentration (see Figures 2B and 4). The reaction may be second order at low sulfite concentrations (150 μM) as shown in Figure 4. We performed only one experiment at sufficiently low sulfite concentrations where the data could be analyzed by a two-step mechanism shown in eq 1. For *Chromatium*

$$A + B \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} [AB] \xrightarrow{k_3} C \qquad (1)$$

flavocytochrome *c* at pH 9, $k_{12} = (7 \pm 4) \times 10^3$ M^{-1} s^{-1}, $k_{21} = 0.1$ s^{-1}, and $k_{23} = 0.13$ s^{-1}. All other experiments appeared to be first order over the concentration range studied, which indicates that the initial step (k_{12}) is no longer rate-limiting. The charge-transfer absorbance is lost with the same rate constant as for recovery of the flavin absorbance (recoloring). The decomposition of the adduct is pH dependent and accelerated at high pH (see Figure 5). The latter effect has not been observed with free flavins or with other flavoproteins, although not specifically addressed (Muller & Massey, 1969; Massey et al., 1969). At equilibrium, the D-amino acid oxidase sulfite adduct is slightly more stable at pH 7–8 than at higher and lower pH (Massey et al., 1969), and the glucose oxidase sulfite adduct is 3 orders of magnitude more stable at low pH than at high pH (Swoboda & Massey, 1966). We have not measured equilibria as a function of pH, but it appears that the flavocytochrome *c* sulfite adduct is least stable at pH 7 (see below).

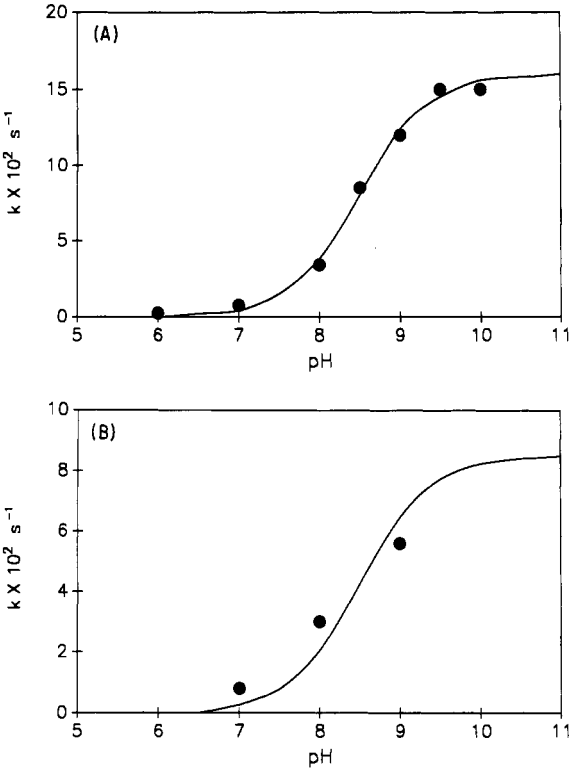

FIGURE 5: Effect of pH on the first-order rate constants for recovery of flavin absorbance at 475 nm following initial bleach of (A) *Chromatium* FC and (B) *Chlorobium* FC. The solid lines were calculated by using rate constants at the lower pH limit of 0 and those at the upper pH limit of 0.16 and 0.085 s^{-1} for (A) and (B), respectively, and assuming a pK of 8.5. It was also assumed that (A) and (B) are related.

Because the decomposition of the flavocytochrome *c* sulfite adduct is due to a first-order process (at least at high substrate concentrations), a structural or chemical change of the protein, which alters the flavin environment, is suggested. Fitting the pH dependence of the first-order rate constant yields an apparent pK of approximately 8.5 for both flavocytochromes *c*. The pK for the effect of pH on decomposition of the adduct is too high to be due to the ionization of bisulfite (6.7) but is within the range expected for a free sulfhydryl (8–9) (Houk et al., 1987). It is important to note that other flavin binding ligands, for example, cyanide or thiosulfate, form stable adducts at all pH values studied. Thus, the recoloring reaction is characteristic of sulfite. Moreover, there is no heme reduction in the presence of sulfite at high or low pH either in aerobic or in anaerobic solutions, excluding electron transfer to the heme as an explanation for recoloring. Because sulfite is known to be reactive with protein disulfides (McPhee, 1956), it is likely that the decomposition of the flavin sulfite adduct in flavocytochrome *c* is due to a secondary effect of the reaction of sulfite with a cystine disulfide near the flavin. To explain the observed kinetics, the first-order adduct decomposition reaction would have to be slower than the second-order reaction of sulfite with the disulfide at high concentrations and would thus be rate-limiting in the overall decomposition reaction. The second-order reaction observed at low sulfite concentrations (*Chromatium* flavocytochrome *c*, pH 9, see above) could be that for reaction of sulfite with the disulfide. This reaction would necessarily have to be slower than the initial reaction of sulfite with the flavin to form adduct (4×10^3 M^{-1} s^{-1}). Thus, the lower estimate for second-order recoloring ($k_{12} = 3 \times 10^3$ M^{-1} s^{-1}) is consistent with the reaction of sulfite with a disulfide followed by first-order decomposition. It is known that sulfite, but not bisulfite, is reactive with disulfides (k =

18 M^{-1} s^{-1} for neutral disulfides) (McPhee, 1956), which is consistent with our observations.

The postulated disulfide in flavocytochrome *c* is 2 orders of magnitude more reactive than is free cystine. Highly reactive cystine disulfides have been observed in several proteins, such as in thioredoxin, insulin, and α-lactalbumin (Holmgren, 1979a,b; Kuwajima et al., 1990), which can be as much as 4 orders of magnitude more reactive than model disulfides with mercaptans (k = 28 M^{-1} s^{-1}) (Creighton, 1975). The increased reactivity may be due to strain in the disulfide bond or to a positively charged environment. Both factors could be present in flavocytochrome *c*.

Cleavage of a disulfide bond alone should not have such a large effect on the stability of the flavin sulfite complex, especially at a distance, unless the redox potential of the flavin was dramatically lowered, either through a conformational change, which would result in a large increase in the exposure of the flavin to solvent or which would neutralize the stabilizing positively charged environment of the flavin. One such conformational change might be dissociation of heme and flavin subunits in the presence of sulfite, although we failed to detect dissociation by gel filtration. The flavin sulfite adduct in flavocytochrome *c* could be destabilized by the resultant nearby negative charge on the cysteinyl anion or that on the cysteinyl-*S*-sulfonate resulting from reaction of a proximal disulfide with sulfite. The recoloring reaction is only significant at high pH, with a pK of 8.5, suggesting that the cysteinyl anion is much more destabilizing than the cysteinyl-*S*-sulfonate, which should have a much lower pK. The pK of a cysteinyl residue near the flavin in flavocytochrome *c* may be lower than that of free cysteine because of the positively charged environment, but the negative charge on the nearby cysteinyl-*S*-sulfonate resulting from reaction with sulfite would cancel this effect, giving a more or less normal pK for the cysteinyl residue. It is possible that the basic residue, which we have suggested stabilizes the adduct by forming a salt bridge with the N-1 position of the flavin, forms a new salt bridge with the cysteinyl anion. In this model, once the basic residue has moved away from the flavin N-1 position and has been neutralized, the flavin would be in a much lower redox potential environment and would behave more like free flavin, resulting in decomposition of the adduct (see below for further discussion). Alternatively, the rearrangement involving the basic residue could be the rate-limiting step in adduct decomposition. In either case, the neutralization or movement of the base would trigger adduct decomposition.

The reaction of sulfite with the flavin in flavocytochrome *c* is most complicated at pH 8–10. In this pH range, the flavin is slowly bleached a second time following initial bleaching and recoloring as shown in Figure 2C. The rebleach rate constant is 10^{-3} s^{-1} at pH 9 for *Chromatium* and 1.5 × 10^{-3} s^{-1} at pH 8 and 2.7 × 10^{-3} s^{-1} at pH 9 for *Chlorobium*. In general, the rate constants for rebleach appear to be first order at high sulfite concentrations (0.6–5 mM), although we have fewer data than for initial adduct formation and decomposition. The rebleach kinetics (k = 10^{-3} s^{-1}) are comparable in magnitude to those for free FAD at 5 mM and higher sulfite concentrations (10^{-4} s^{-1}; Muller & Massey, 1969), which suggests that the redox potential of the flavin in the recolored flavocytochrome *c* is similar to that of free FAD. However, the flavocytochrome *c* rebleach appears to be first order, and that with free flavins is second order. Thus, a rate-limiting first-order process is required with flavocytochrome *c* prior to formation of the second flavin sulfite adduct. The second flavin sulfite adduct formed at high pH also has the charge-transfer absorbance, which is characteristic of the first adduct. The cystine which we postulate to be near the flavin thus cannot be the charge-transfer acceptor if it has reacted with sulfite prior to the second bleach and no longer able to fill this role. This is evidence in favor of some other protein side chain being the charge-transfer acceptor, such as an aromatic residue as discussed above.

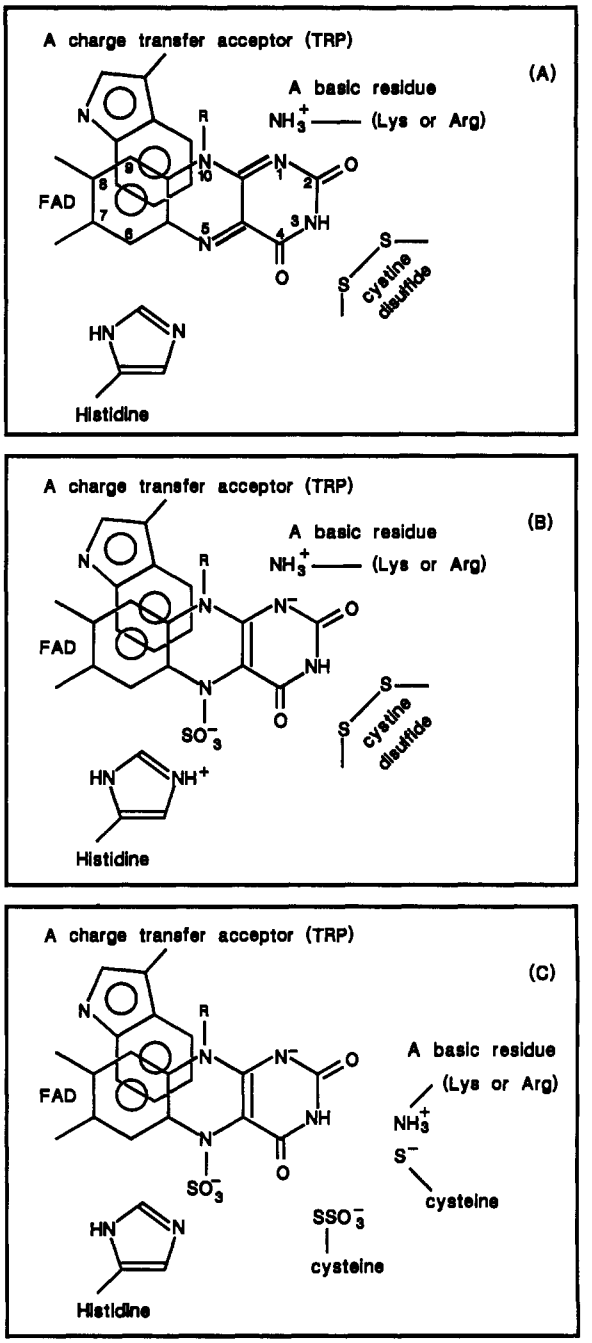

FIGURE 6: Model for adduct formation and amino acid side chains thought to be near the flavin in flavocytochromes *c*. (A) Native enzyme at pH 7; (B) flavin sulfite adduct at pH 5; (C) secondary flavin sulfite adduct at pH 10 after reaction of two molecules of sulfite.

The dissociation constant (K_d) suggested by the kinetics for the initial reaction of sulfite with the two flavocytochromes *c* studied is less than 10 μM at all pH values studied. Although not directly measured, we found little or no change in the magnitude of the absorbance change, determined in the stopped flow, associated with the initial bleach as the sulfite concentration was decreased. The lowest concentrations of sulfite used were 20 μM, suggesting that the dissociation

constant must be smaller than 10 μM. An equilibrium titration at pH 5.5 for *Chromatium* flavocytochrome *c* indicated a dissociation constant of 4 μM, although there was a small amount of recoloring (ca. 10%), which suggests that this number may be an upper limit. For comparison, the dissociation constant for the flavocytochrome b_2 sulfite adduct is 1.5 μM, and the inhibitor constant for sulfite from enzyme assays is 1.4 μM (Lederer, 1978).

A model of the flavin environment in flavocytochrome *c* and its reactions with sulfite are summarized in Figure 6. We postulate the presence of four active-site amino acid side chains near the flavin to explain our kinetic results. First, by analogy to other systems, there is apparently a basic residue (shown as lysine) near the flavin N-1 position, which raises the redox potential of the flavin, stabilizes the anionic form of the semiquinone, and increases reactivity with sulfite (Meyer et al., 1991). The three-dimensional structures of enzymes which share these properties such as glycolate oxidase (Lindqvist & Branden, 1989) and yeast lactate dehydrogenase (Xia & Mathews, 1990) establish the presence of such a basic residue, predicted by Massey and Hemmerich (1980). Second, there must be a basic residue near the flavin which has a pK of approximately 6.5 and which additionally stabilizes the sulfite adduct at low pH, which we believe is a histidine. Third, there has to be another site near the flavin, which is reactive with sulfite. Either the product of this reaction or a consequent rearrangement due to this secondary reaction causes the decomposition of the flavin sulfite adduct by lowering the redox potential of the flavin. We believe that a cystine disulfide is the site of secondary reaction with sulfite. It is only when the cysteinyl thiol, which results from reaction of sulfite with the disulfide, ionizes with a pK of 8.5 that the anion forms a salt bridge with the active-site basic residue and thereby destabilizes the flavin sulfite adduct, and causes it to dissociate in a presumably rate-limiting step. The flavin then resides in a neutral or negatively charged low redox potential environment and is only able to react slowly with sulfite to form a secondary adduct of relatively low stability. Fourth, the charge-transfer absorbance associated with both adducts is present at high and low pH. An aromatic side chain such as tryptophan could fill that role. There are of course other explanations for our results, and Figure 6 should be viewed as a preliminary model requiring verification by three-dimensional structural determination as well as by other experiments.

Registry No. His, 71-00-1; cystine, 56-89-3; flavocytochrome *c*, 61419-36-1.

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