pH Dependence of the Reduction–Oxidation Reaction of Azurin with Cytochrome *c*-551: Role of Histidine-35 of Azurin in Electron Transfer†

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ABSTRACT: A fluorescence quenching experiment confirms that in the redox reaction between cytochrome *c*-551 and azurin, protein complexing is negligible. Azurin–pH indicator T-jump experiments show that *Pseudomonas aeruginosa* (*Ps.*) azurin exhibits a slow time constant, τ, in its return to pH equilibrium but *Alcaligenes faecalis* (*Alc.*) azurin does not. The decrease of $1/\tau$ with increasing pH shows that the rate-determining process is a slow transformation of the imidazolium form of histidine-35 from a conformation where it cannot ionize to one in which it can. The fast relaxation time constant of the redox reaction varies little with pH, but the slow time constant increased by a factor of ~2.5 with increasing pH between pH 5 and pH 8. The corresponding amplitudes, especially the slow one, vary with pH. On the basis of all the present evidence it is concluded that, while some differences of redox reactivity do occur on protonation, these differences are not major. In general, the two proteins cyt *c*-551 and azurin react with each other with rates only weakly dependent upon pH. A classical pH titration was carried out on the reduced and oxidized form of *Ps.* and *Alc.* azurin with the result that two protons were released between pH 6 and pH 8, in the former from His-35 and -83 and in the latter from His-83 and Ala-1.

Azurins are deep blue, small (14 000 daltons), bacterial copper proteins. By far the most studied is that from *Pseudomonas aeruginosa* (*Ps.*);[1] indeed the redox reaction between *Ps.* azurin and cytochrome *c*-551 from the same bacterium has been the subject of numerous experiments (Antonini et al., 1970; Pecht & Rosen, 1973; Brunori et al., 1974; Wilson et al., 1975; Rosen & Pecht, 1976; Wherland & Pecht, 1978; Silvestrini et al., 1981). What makes this system especially attractive is that both metalloproteins are small (cyt *c*-551 has M_r 9100), have about the same redox potential, and exhibit large spectral shifts with changes of oxidation state. The first remarkable aspect of the reaction

$$\mathrm{Az(II)} + \mathrm{Cyt(II)} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{Az(I)} + \mathrm{Cyt(III)} \qquad (1)$$

is its speed in both directions. For example, at 25 °C, k_1 is 6.1×10^6 M^{-1} s^{-1} and k_2 is 7.8×10^6 M^{-1} s^{-1}. The second remarkable aspect is that the swift reaction is coupled to a slow reaction. The nature of the latter is the main subject of this research.

The existence of a slow reaction is inferred from the fact that T-jump measurements on the reacting system exhibit both a fast relaxation and a slow relaxation. This slow process was first ascribed (Pecht & Rosen, 1973) to the slow reaction of a rapidly formed intermediate complex. However, more detailed studies (Rosen & Pecht, 1976) of the concentraction dependence of the relaxation times suggested instead that reduced azurin, Az(I), exists in two slowly interconverting forms, one reactive and the other nonreactive. NMR studies of *Ps.* azurin have shown that the protonated and deprotonated forms of the imidazole side chain of His-35 only slowly exchange with each other. Indeed the slow relaxation times (~100 ms) were comparable to the slow relaxation times of the redox reaction. The inference is that the "reactive" and "nonreactive" forms of *Ps.* Az(I) are the nonprotonated and protonated forms or perhaps the reverse. A study of the pH dependence of the redox reaction rates would test these tentative hypotheses.

Four different studies were carried out. The first was the determination by a fluorescence quenching experiment of an upper limit for the extent of complex formation between *Ps.* azurin and cyt *c*-551. The second study was a T-jump measurement of the rate of recovery of pH equilibrium of *Ps.* and *Alc.* azurin solutions in the presence of a relatively small amount of indicator. The third study was a set of pH titrations carried out on the oxidized and reduced forms of *Ps.* and *Alc.* azurins. Finally by the T-jump method the pH dependence was determined for the relaxation times of the redox reactions of these azurins with cyt *c*-551.

Experimental Procedures

Materials. A *Ps. aeruginosa* culture (ATCC 19429 or NCTC 6750) was obtained from the American Type Culture Collection. *Ps.* azurin, cyt *c*-551, and *Alc.* azurin were isolated at 4 °C according to procedures described by Mitra & Bersohn (1980, 1982). The last step in the isolation of all three proteins requires a CM-52 column preequilibrated with 0.05 M NH_4OAc buffer, pH 3.9. At this pH *Alc.* azurin is bound to this column and is eluted with pH 4.7 NH_4OAc. Azurin and cyt *c*-551 from *Pseudomonas* are simultaneously bound to the column. Cyt *c*-551 is eluted first at pH 4.45 and the azurin at pH 4.65. Only fractions with the following absorption rations were used: for reduced cyt *c*-551, A_{551}/A_{280} was between 1.10 and 1.17; for *Ps.* azurin, A_{625}/A_{280} was between 0.46 and 0.49. All proteins were dialyzed extensively against deionized distilled water. *Ps.* azurin and cyt *c*-551 were lyophilized and stored at −20 °C. *Alc.* azurin is more prone to denature on lyophilization. It was concentrated in a 50-mL Amicon concentration cell fitted with a Diablo ultrafiltration membrane with a M_r 10 000 cutoff. The protein was stored at −2 °C. Platinum black (Aldrich) was treated as described by Rosen & Pecht (1976). Hydrogen used for protein reduction was ultrahigh purity grade (Presto). N_2 and Ar were

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[1] Abbreviations: Az(I)/(II), reduced/oxidized forms of azurin; Cyt(II)/(III), reduced/oxidized forms of cyt *c*-551; cyt, cytochrome; *Ps.*, *Pseudomonas aeruginosa*; *Alc.*, *Alcaligenes faecalis*; CM, carboxymethyl.

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research grade. Bromothymol blue, indicator grade (Aldrich), was used as received.

Fluorescence Measurements. Stock solutions of the oxidized forms of both cyt and *Ps.* azurin were prepared by dissolving lyophilized protein into phosphate buffer (0.05 M, pH 6.8) with NaCl added to obtain a final ionic strength of 0.14. All fluorescence measurements were conducted in a Spex Fluorolog fluorometer used in the single photon counting mode. Samples were excited at 280 nm and the fluorescence monitored at 308 nm (the maximum of the azurin emission spectrum). The cytochrome–azurin mixtures used exhibited large absorption at both the excitation and emission wavelengths. This can give rise to large nonlinear effects (inner-filter effects), especially at the higher protein concentrations used (>150 μM azurin and >600 μM cyt). So that such effects could be minimized, a 0.1-mm path-length cell mounted at a 45° angle with respect to both the excitation beam and the detector was employed. In addition all fluorescence measurements were corrected for any remaining nonlinear effects caused by trivial absorption. A water-jacketed cell holder maintained the cell at 25 °C. So that good signal/noise ratios could be obtained, photon counts were integrated over a 5-s interval and electronically averaged.

Temperature-Jump Measurements. Ps. and *Alc.* azurin concentrations were determined by absorption at 625 nm where ϵ is 5700 (Rosen & Pecht, 1976) and 4000 M^{-1} cm^{-1} (Rosen et al., 1982), respectively. The cyt *c*-551 concentration was determined at 528 nm where ϵ = 11 700 M^{-1} cm^{-1} for both Cyt(II) and Cyt(III) [obtained assuming ϵ_{551} of Cyt(II) is 3.0 × 10^4 M^{-1} cm^{-1} (Dickerson & Timkovich, 1975)]. Stock phosphate buffer solutions were 0.2 M in NaH_2PO_4 and were adjusted in pH with solid NaOH. NaCl was added to each buffer to obtain the desired final ionic strength. It was more convenient to prepare reduced azurin than reduced cyt *c*-551 because the latter oxidizes much faster in air. Azurin was reduced by passing wet H_2 gas over the solution to which several milligrams of Pt black had been added. Details of the precautions taken to avoid atmospheric O_2 during the experiments with reduced azurin are given by Corin (1981).

Kinetic pH Titrations. Kinetic measurements were made on a double-beam temperature-jump spectrophotometer which is a modification of the instrument described by Crothers (1971). A Schoeffel Model GM 250 monochromator was used. The cell design has been given previously. As the cell and cell compartment of the instrument were not adapted for anaerobic work, oxygen-sensitive solutions were loaded into the cell inside a N_2-filled glove bag. The cell was then quickly transferred to the cell compartment which was continuously flushed with N_2. The instrument was calibrated by the method of Havsteen (1969). Temperature jumps from 25 to 28.4 °C were used throughout. Both references and main-beam photomultiplier outputs were delivered to a differential amplifier plug in of a Tektronix Model 7613 storage oscilloscope. Oscilloscope traces of kinetic decay curves were photographed, and kinetic data were extracted by plotting the traces of semilogarithmic paper and calculating lifetimes from the slopes. Amplitude data for a given sample are the average of two or more measurements.

pH Titrations. About 25 mg of freshly isolated azurin in 25 mL was dialyzed for 5 days against distilled deionized water with frequent changes. The protein was then concentrated in an Amicon concentration cell to 2 mL and brought to 5 mL with KCl solution so that the final KCl concentration was 0.15 M. Carbonate-free KOH in 0.15 M KCl was prepared (Kolthoff, 1923) and standardized against potassium acid phthalate. Standardization HCl was prepared with the KOH solution as a primary standard. All pH measurements were made at 25 °C, and when a reduced protein was titrated, water-saturated argon was passed over the solution.

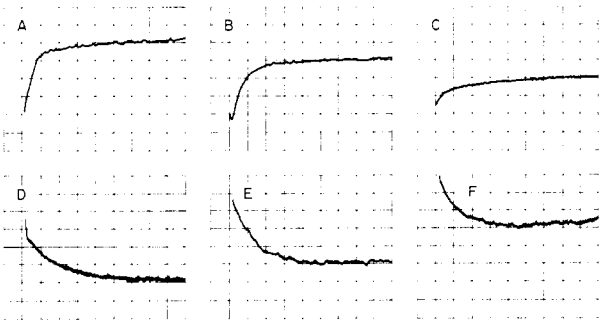

FIGURE 1: Oscilloscope traces of transient absorptions: (A–D) Reaction of cyt *c*-551 and *Ps.* azurin. (E, F) *Ps.* azurin and bromothymol blue. All vertical scales 10 mV/division except for (A) (5 mV/division). T_{final} = 28.4 °C. (A–D) 88 μM azurin, 45 μM cyt *c*-551, and 0.05 M phosphate buffer and NaCl added to obtain μ = 0.14 M. (E–F) 20 μM azurin, 100 μM bromothymol blue, and 0.14 M NaCl. (A) pH 5.50, horizontal 50 ms/division, 100 μs RC filter. (B) pH 7.00, horizontal 50 ms/division, 1 ms RC filter. (C) pH 8.35, horizontal 200 ms/division, 250 μs RC filter. (D) pH 7.00, horizontal 500 μs/division, 100 μs RC filter. (E) Oxidized azurin, horizontal 50 ms/division, 1 ms RC filter. (F) Reduced azurin, horizontal 50 ms/division, 1 ms RC filter.

Results

Fluorescence Quenching Titrations. To determine whether a complex is formed between *Ps.* azurin and cyt *c*-551 we can exploit the fact that upon absorption at 280 nm the former fluoresces but the latter does not. A complex of the two proteins would have a decreased fluorescence yield, the extent of decrease depending on the distance of the primary emitter Trp-48 from the energy-accepting heme. Accordingly a solution of azurin (75–155 μM) was titrated with cyt *c*-551 (44–690 μM), but within an experimental error of about 10% no fluorescence quencing was found. In this way a lower limit for the equilibrium constant for the dissociation of a complex is found to be 1 × 10^{-3}. The actual value might, of course, be much larger. This result argues against the need to incorporate a complexation step in the kinetic scheme; see Corin (1981) for a more detailed discussion.

Indicator–Azurin Kinetic Measurements. (a) Ps. Az(I) and Ps. Az(II) with Bromothymol Blue. Temperature-jump measurements were made on solutions consisting of azurin, with *Ps.* or *Alc.*, in 0.14 M NaCl with bromothymol blue (p*K* 7.1) as a pH indicator. Although no buffering agent was present aside from the protein, the pH of a sample measured before and after runs of typically six temperature jumps rarely varied by more than 0.3 pH unit.

Two well-resolved relaxation times were detected for *Ps.* Az(I) and *Ps.* Az(II). The slower mode observed for each protein is shown in Figure 1E,F. Both oxidation states at pH 7.0 show a fast relaxation time, τ_{fast}, of 50 μs and a slow relaxation time, τ_{slow}, of 65 ms. The indicator has two broad absorption maxima, one at 615 nm for the deprotonated anionic form and a second at 430 nm for the protonated neutral molecule. As these maxima are broad, absorption was monitored at 578 and 436 nm, wavelengths of intense mercury lines which provide better signal-to-noise ratios. For both the slow and the fast relaxations an increase in absorption is seen at 436 nm and a decrease at 578 nm. Both changes indicate a net release of protons to the solution as monitored by the indicator.

Both the fast and the slow relaxation times are dependent

Table I: pH Titration of *Pseudomonas* Azurin with Bromothymol Blue[a]

pH[b]	$1/\tau_{fast}$ (s^{-1}) $\times 10^{-3}$	amplitude[b] of τ_{fast} (OD) $\times 10^4$	$1/\tau_{slow}$ (s^{-1})	amplitude[b] of τ_{slow} (OD) $\times 10^4$
		Reduced		
5.00	N/A[c]	N/A	N/A	N/A
5.55	N/A	N/A	43	8
6.00	36	28	41	15
6.57	23	11	31	43
7.00	19	22	15	54
7.50	16	26	12	36
8.07	23	29	N/A	N/A
8.56	29	26	N/A	N/A
		Oxidized		
5.60	N/A	N/A	37	18
6.00	42	24	24	22
6.50	26	11	18	41
7.00	20	16	16	34
7.50	18	24	15	5
8.00	19	24	N/A	N/A

[a] Solution conditions: 20 μM azurin, 100 μM bromothymol blue, and 0.14 M NaCl. [b] Amplitudes are not corrected for the titration of the indicator. [c] N/A indicates that the signals were too small to extract data from.

on pH but not on dye concentration for measurements made over the range 10–100 μM in bromothymol blue at 20 μM protein (data not shown). The amplitudes corresponding to τ_{fast} are rather large. At pH 7.0 a 4% decrease in absorption was found. The fast decaying signal has, we believe, two causes. There are "fast-exchange" protons which titrate near neutral pH such as that of His-83 and/or the N-terminal amino group. In addition, a protein–dye complexation step may contribute, as a difference spectrum was found in reduced azurin–indicator mixtures vs. indicator and in oxidized azurin–indicator vs. indicator (Corin, 1981). Because the main focus of these indicator experiments is on the investigation of the "slowly exchanging" histidine in the millisecond time range, further studies of the faster microsecond process were not pursued.

The slower relaxation process exhibited approximately a 1% decrease in absorption at 578 nm of a 20 μM protein and 100 μM indicator, pH 7.0, solution. Under these conditions, the rate $1/\tau_{slow}$ is 15 s^{-1}. This rate is similar in magnitude to that of the slow process in the redox reaction of *Ps.* azurin with cyt *c*-551 where $1/\tau_{slow}$ is 28 s^{-1}. Both solutions were 0.14 M in ionic strength, but the salt in the former was only NaCl whereas the latter consisted of 0.05 M phosphate buffer and NaCl. However, when *Ps.* azurin–cyt *c*-551 reaction is studied in 5 mM phosphate, pH 7.0, μ = 0.14 M, the rate of the slower mode is 13.2 s^{-1}. This same buffer effect perhaps due to phosphate binding to the protein has been found by Rosen et al. (1981). NMR investigations estimate that the acid–base exchange rate for a slowly exchanging histidine of reduced azurin is between 1 and 35 s^{-1} when measured in 20 mM phosphate and 0.1 M NaCl, pH 6.0 at 36 °C (Hill & Smith, 1979). This value is clearly within the range observed for the slower mode in both the *Ps.* azurin–indicator and *Ps.* azurin–cyt *c*-551 temperature-jump experiments. The data for the amplitudes and relaxation rates vs. pH for both *Ps.* Az(I) and *Ps.* Az(II) with the indicator are given in Table I (additional material is available; see paragraph at end of paper regarding supplementary material). Figure 2 shows the pH dependence of the slow rate for the two oxidation states.

(*b*) *Alc. Az(II) and Bromothymol Blue.* No slow relaxation process was observed. However, as with *Ps.* azurin a fast process was observed with τ_{fast} = 50 μs.

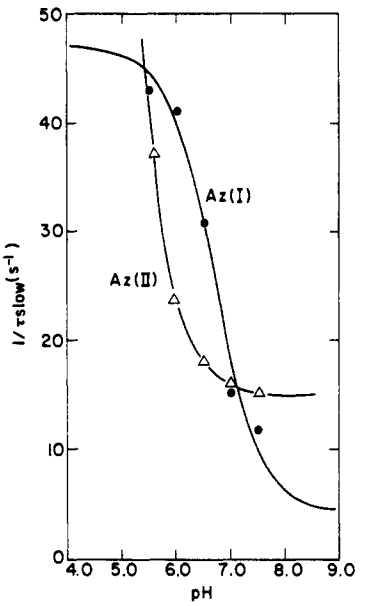

FIGURE 2: Variation with pH of the slow relaxation rate observed with 20 μM azurin, 100 μM bromothymol blue, and 0.14 M NaCl for the reduced and oxidized protein. The solid line is a theoretical curve.

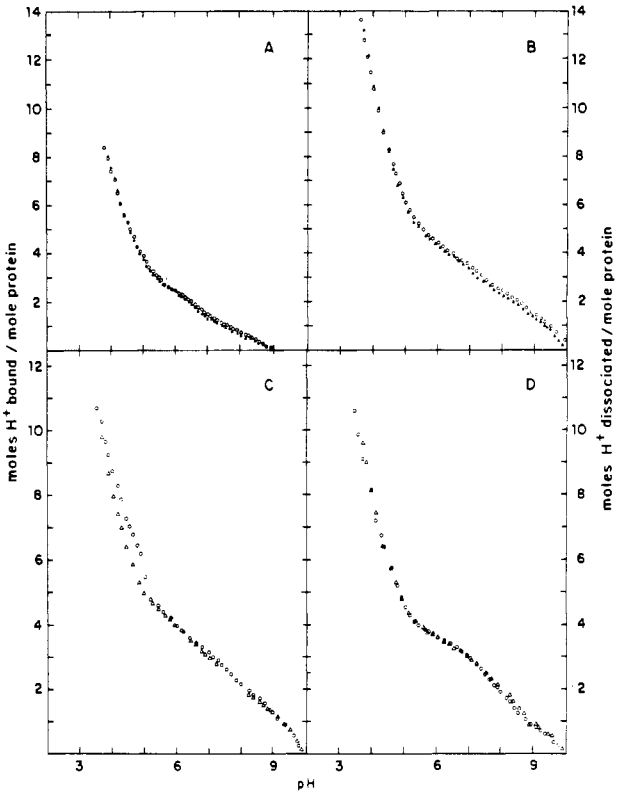

FIGURE 3: pH titration of azurins. (A) *Alc.* Az(II): 28 mg of protein/4.5 mL, 0.15 M KCl. (B) *Alc.* Az(I): 25 mg of protein/4.5 mL, 0.15 M KCl. (C) *Ps.* Az(II): 22.1 mg of protein/4.5 mL, 0.15 M KCl. (D) *Ps.* Az(I): 21.3 mg of protein/4.5 mL, 0.15 M KCl. (○) H^+ bound upon addition of HCl. (△) H^+ dissociated upon addition of KOH.

pH Titration of Alc. and Ps. Azurins. Classical acid–base titration curves of both azurins are shown in Figure 3. Between pH 6 and pH 8 approximately 2 protons are labile for *Alc.* Az(I), *Alc.* Az(II), and *Ps.* Az(II) and ~1.5 protons for reduced *Ps.* azurin.

Kinetic Measurements of the Redox Reaction of Azurin with Cyt c-551. Typical time-dependent absorptions at 551

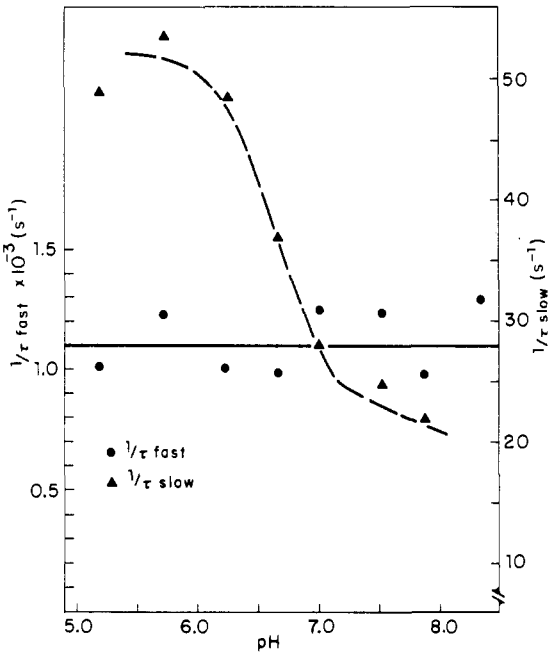

FIGURE 4: Variation with pH of the relaxation rates of cyt *c*-551 and *Ps.* azurin reaction. Conditions as in Figure 1. Solid and dashed lines passing through the data have no theoretical significance.

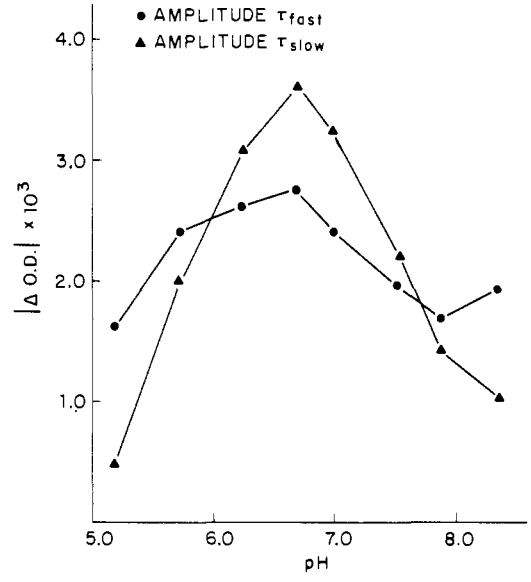

FIGURE 5: Variation with pH of the amplitudes of the slow and fast relaxation mode for the cyt *c*-551 and *Ps.* azurin reaction. Conditions as in Figure 1. Curves have no theoretical significance.

nm, a wavelength at which Cyt(II) has an absorption maximum, are shown in Figure 1. Two well-resolved relaxation times were observed over the accessible range of 20 μs to 1 s. Plots of $1/\tau$ vs. pH (Figure 4) show that the fast rate, $1/\tau_{fast}$, is essentially independent of pH, while the slow rate, $1/\tau_{slow}$, decreases by a factor of 2.5 with increasing pH between 5 and 8. The sigmoidal decrease of $1/\tau_{slow}$ with pH resembles a titration curve with an inflection at pH 6.8. However, the curve is much broader than the theoretical prediction for the pH titration of a single weak acid. Amplitudes corresponding to both relaxation modes are plotted against pH in Figure 5. The pH profile of the amplitude of the fast relaxation is very broad; absorbance changes vary less than a factor of 2 between pH 5 and pH 8 with a slight maximum at pH 6.6. The pH profile of the amplitude of the slow relaxation is also broad and displays a maximum at pH 6.7. Silvestrini et al. (1981)

Table II: Deuterium Isotope Effect on the Relaxation Modes for the Reaction between Cytochrome *c*-551 and Azurin from *Pseudomonas aeruginosa*[a]

pH	$1/\tau_{fast}$ (s^{-1}) $\times 10^{-1}$	amplitude of τ_{fast} (OD) $\times 10^4$	$1/\tau_{slow}$ (s^{-1})	amplitude of τ_{slow} (OD) $\times 10^4$
6.98	110	26	37	36
7.09[b] (D_2O)	88	27	17	34

[a] Solution conditions: 0.05 M phosphate buffer, 35 μM azurin, 45 μM cyt *c*-551. [b] Meter reading uncorrected for the deuterium isotope effect.

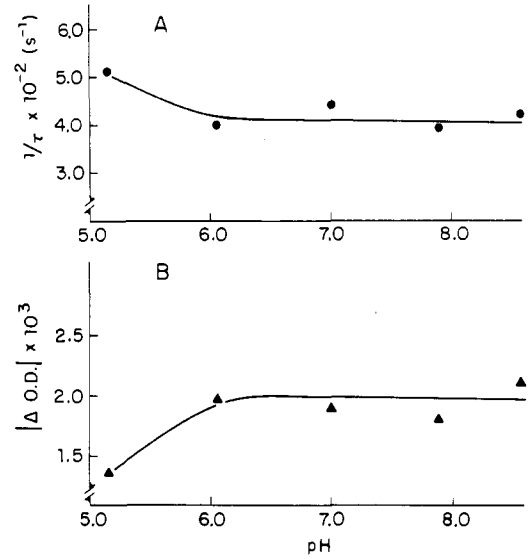

FIGURE 6: Variation with pH of the relaxation rate (A) and amplitude (B) of the transient absorption in the reaction of cyt *c*-551 and *Al.* azurin; 70 μM azurin, 86 μM cyt *c*-551, and 0.05 M phosphate and NaCl added to obtain a final ionic strength of 0.15. Curves have no theoretical significance.

reported similar result with a maximum at pH 7.0. The measurements were confined to the pH range 5–8 because outside of this range the amplitude of the slow relaxation was too small to be determined reliably.

In D_2O at pH* (uncorrected for the isotope effect) 7.09 (Table II), $1/\tau_{fast}$ decreased by 20% whereas $1/\tau_{slow}$ decreased by a factor of 2. The decrease in $1/\tau_{fast}$ is only a little larger than experimental error, but the decrease in $1/\tau_{slow}$ is far outside the expected experimental error. The pH* can be corrected for the deuterium isotope effect to obtain pD 7.49. It might, therefore, be more appropriate to compare $1/\tau_{slow}$ for D_2O with pH 7.5 for H_2O. As shown in Figure 5, a difference of about 30% in $1/\tau_{slow}$ is expected between pH 7.0 and pH 7.5, not a factor of 2. It thus appears that the isotope effect is real. Amplitudes for both relaxation modes do not show any significant change between D_2O and H_2O.

As also reported by Rosen et al. (1981) only a fast relaxation time is found for the cyt *c*-551–*Alc.* azurin reaction. Both the rate of relaxation (Figure 6A) and the corresponding amplitudes (Figure 6B) are essentially independent of pH in the range 5–9. There is, however, 25% increase in the rate and 30% decrease in the amplitude as the pH is reduced from 6 to 5.

Discussion

Fluorescence Quenching Titration. The determination that the equilibrium constant for dissociation of a *Ps.* azurin–cyt *c*-551 complex must be $<10^{-3}$ means that association is negligible. This does not mean that the two molecules do not have

to make contact in order to react or that they do not spend a little time while in contact finding a desirable mutual orientation. It simply means that the equilibrium concentration of complex is exceedingly small and therefore the slow process does not involve complex formation.

Indicator–Azurin Kinetic Measurements. The central result of our T-jump studies on azurins in the presence of an indicator is that *Ps.* azurin exhibits a slow step in its return to pH equilibrium but *Alc.* azurin does not. NMR measurements show that in *Ps.* azurin His-35 slowly exchanges between its protonated and nonprotonated forms (Hill & Smith, 1979; Ugurbil & Bersohn, 1977; Ugurbil et al., 1977) but in *Alc.* azurin His-35 remains in its neutral form in the pH range 4–10 (Mitra & Bersohn, 1982). In the redox reaction of *Ps.* azurin with cyt *c*-551 there is a slow process, but in the redox reaction of *Alc.* azurin with cyt *c*-551 there is no such slow step (Rosen et al., 1981).

In both azurins the two forms of His-83 are in fast exchange. The X-ray structure of *Ps.* Az(II) crystals (Adman et al., 1978; Adman, 1981) grown at pH 5.5–5.8 shows clearly that the imidazole group of His-83 juts out into the solution whereas that of His-35 lies in the crevice near the surface. The structural and kinetic data collectively suggest that the fast reaction of imidazole protonation is coupled to a slower protein conformation change involving His-35.

There are two particularly simple mechanisms which could be used to interpret the kinetic data. In one model, A, His-35 imidazolium ion is present in two different configurations, only one of which can quickly equilibrate with hydrogen ion and neutral imidazole. In the other model, B, the imidazole is present in two different configurations, only one of which can quickly equilibrate with hydrogen ion and imidazolium ion. These two models can be represented by the equations:

$$\mathrm{HAz(I)^+} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{HAz(II)^+} \overset{K_A}{\rightleftharpoons} \mathrm{H^+ + Az(II)} \qquad (2a)$$

$$\mathrm{Az(I)} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{Az(II) + H^+} \overset{K_A}{\rightleftharpoons} \mathrm{HAz(II)^+} \qquad (2b)$$

k_1 and k_2 are the slow rate constants, and K_A is the ionization constant of the rapidly equilibrating form $HAz(II)^+$. A short calculation using the method of French & Hammes (1965) leads to the following expressions for the relaxation time constant:

$$1/\tau = k_1 + k_2[1 + K_A/(H^+)]^{-1} \qquad (3a)$$

$$1/\tau = k_1 + k_2[1 + (H^+)/K_A]^{-1} \qquad (3b)$$

Equation 3a predicts that with increasing pH the relaxation rate, $1/\tau$, should diminish; eq 3b predicts the opposite. As shown by Figure 2, of the two models only model A is consistent with the observations.

Equation 3a has the form of a mass action expression, and it would seem straightforward to extract the various parameters k_1, k_2, and K_A by fitting an experimental curve. Unfortunately, in the T-jump experiment the amplitude of the slow relaxation goes to zero at the asymptotic pH values of the titration. Thus the parameters can only be rather coarsely determined. The constant which we are most interested in obtaining is

$$K_{A0} = \frac{(H^+)(Az(II))}{(HAz(I)^+) + (HAz(II))} = K_A k_1 (k_1 + k_2)^{-1}$$

which is the overall equilibrium constant for ionization of His-35. Table III lists the best values of the parameters. These parameters were obtained by a least-squares analysis fit of the data to eq 3b. Best values of k_1 and k_2 correspond to those values of pK_A which minimize the root mean square error of fit. A plot of this fit criterion vs. pH (not shown) shows that the fitting is a sensitive function of the pK values used in the neutral pH range of the kinetic and NMR results. It is difficult to assess the probable error in the pK_{A0} values. However, for reduced azurin, the pK of His-35 was found to be 7.1 ± 0.3 at 31 °C. From ^{13}C NMR measurements (Ugurbil et al., 1977), Hill & Smith (1979) estimated that at 25 °C the pK was between 6.9 and 7.3. In view of the difficulty referred to, the pK of 7.7 extracted from the T-jump indicator experiment is not in bad disagreement with the value obtained from the NMR experiments. The latter suffers from the opposite difficulty in that the intensity of the imidazole peaks are well defined at the extremes of pH but not near pH ≃ pK where the lines are extremely broad.

Table III: Best Values of the Theoretical Rate Constant for the *Pseudomonas* Azurin Conformational Exchange[a]

oxidation state	k_1 (s^{-1})	k_2 (s^{-1})	pK_A	pK_{A0}
oxidized	15	130	4.9	5.9
reduced	4.4	43	6.7	7.7

[a] Solutions were 20 μM azurin, 100 μM bromothymol blue, and 0.14 M NaCl.

The value of pK = 5.9 for the His-35 of the oxidized azurin cannot be compared directly with a pK determined from NMR because the resonance of the C-2 H of His-35 is unobservable in the oxidized protein. (This photon is paramagnetically broadened by the Cu^{2+} whose average distance is 6.7 Å.) However, Cu^{2+} can be removed and replaced by Co^{2+} (McMillin et al., 1974), and there is considerable evidence that the overall structure of the protein is unaffected by the replacement. Hill et al. (1976) discovered that this cobalt derivative has a slowly exchanging imidazole (presumably that of His-35) whose pK is 6. If we assume that the difference in pK of the slowly exchanging histidine between the reduced and oxidized forms is due to the increased repulsion of the proton by the increased charge on the metal ion, His-35 of oxidized azurin and cobalt azurin should exhibit the same pK, which seems to be true. In any case it is clear from the data plotted in Figure 2 that the pK of His-35 is oxidized azurin is at least one pK unit less than that of His-35 in reduced azurin. We adopt the final value of pK = 5.9 ± 0.4 where 0.4 is a conservative estimate of the probable error.

pH Titration of Alc. and Ps. Azurins. In the physiologically interesting pH range 6–8 the only groups expected to release protons are the imidazole side chains of His-35 and -83 and the amino-terminal group of Ala-1. (The two other histidines, ligands of the Cu atom, do not titrate.) A priori one would expect these protons to be released in this range, but for each azurin only about two are released. One of these protons is surely due to His-83 which by NMR has been shown to titrate normally with a pK of 7.57 in *Ps.* Az(I), 7.35 in *Ps.* Az(II) at 24 °C, and 6.8 in *Alc.* Az(I and II) at 35 °C (Mitra & Bersohn, 1982). As previously discussed His-35 does not titrate in *Alc.* azurin but does in *Ps.* azurin albeit with a 0.1-s time delay. The fact that *Alc.* azurin loses two protons in the pH range 6–8 shows that both His-83 and Ala-1 ionize. However, in *Ps.* azurin again only two protons are released in this range. Because both its His-35 and His-83 have been shown to titrate here, the pK of the Ala-1 NH_3^+ group must be shifted to a higher value.

Fortunately, the sequences (Ambler, 1968, 1971) provide a clue. The azurins from both species have a disulfide bridge

connecting Cys-3 and -26. In *Ps.* azurin the first two amino acids are Ala-1 and Glu-2 which constitute a flexible side chain whose coordinates are not well determined by X-ray crystallography. The carboxylate group of Glu-2 may hydrogen bond to the NH_3^+ group of Ala-1, thus raising its pK. In *Alc.* azurin, Glu-2 has simply been deleted from the sequence, leaving an N-terminal amino group with a normal pK.

Kinetic Measurements on the Redox Reaction of Azurin with Cyt c-551. Consistent with their kinetic measurements on the *Ps.* azurin–cyt *c*-551 reaction at pH 7.0, Rosen and Pecht proposed the following mechanism:

$$\begin{array}{ccc} & \mathrm{Cyt(III)}^* \quad \mathrm{Az(I)}^* & \\ & \Big\downarrow \text{very fast} \quad \Big\downarrow \text{slow} & \\ \mathrm{Az(II)} + \mathrm{Cyt(II)} & \overset{\text{fast}}{\rightleftharpoons} \mathrm{Cyt(III)} + \mathrm{Az(I)} & \end{array} \qquad (4)$$

The asterisk denotes a nonreactive form of the protein. As we now know that the slow step corresponds to a protonation, it follows from eq 4 that the amplitude of the slow step should be maximum at a pH = pK of the ionizing group and should go to zero at higher and lower pH values. Such a bell-shaped curved is, in fact, obtained with a maxium at pH 6.7. Another consequence of eq 4 and the assumption that the unreactive form has one proton more or less than the reactive form is that the fast rate should decrease at either a low or a high pH when the reactive species has virtually disappeared. In fact (Figure 4) between pH 5.2 and pH 8.4 there is no appreciable change in the fast time constant. Silvestrini et al. (1981) showed that there was very little change in the equilibrium constant for reaction 1 in the pH range 4.8–9.1.

Silvestrini et al. explained some of the above observations by introducing an additional protonation equilibrium involving reduced cyt *c*-551.

$$\begin{array}{ccc} \mathrm{HCyt(II)} + \mathrm{HAz(II)} & \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} & \mathrm{HCyt(III)} + \mathrm{HAz(I)} \\ \Big\downarrow & & \Big\uparrow \\ \mathrm{Cyt(II)} + \mathrm{H}^+ & & \mathrm{H}^+ + \mathrm{Az(I)} \end{array} \qquad (5)$$

Their assumption was that the reactive forms of the two proteins are the protonated forms. Support for this mechanism comes from the observation by both optical absorbance and NMR experiments (Moore et al., 1980) (1) that a group on Cyt(II), probably a hemeproprionic acid, has a pK of 6.8 and (2) that the overall equilibrium constant is almost independent of pH. In contrast to the Az(I) $\rightleftharpoons$ HAz(I) interchange, the HCyt(II) $\rightleftharpoons$ Cyt(II) interchange is very fast.

Rosen & Pecht (1976) introduced a rapidly attained equilibrium between two forms of Cyt(III) to account for the following paradox: k_{21} increases with increasing temperature faster than does k_{12}, suggesting that the product of Cyt(III) is exothermic but the direction of the fast relaxation is toward producing more oxidized cyt rather than less. Thus an additional fast endothermic reaction was proposed between two forms of Cyt(II). Thus the model of Silvestrini et al. (1981) is not essentially different from that of Rosen and Pecht except for the fact that it explicitly identifies the reactive form of Az(I) and Cyt(II) as the protonated form and the nonreactive species as the unprotonated form.

Unfortunately, there are several lines of evidence which suggest that the model of eq 5 is oversimplified. First of all at 25 °C the values of k_{12} and k_{21} are 6.1×10^6 and 7.8×10^6 M^{-1} s^{-1}, respectively (Rosen & Pecht, 1976) whereas for the redox reaction of *Alc.* azurin with cyt *c*-551 k_{12} and k_{21}

Scheme I

$$\begin{array}{lll} \mathrm{HAz(II)} + \mathrm{HCyt(II)} \underset{k_{b1}}{\overset{k_{f1}}{\rightleftharpoons}} \mathrm{HAz(I)} + \mathrm{HCyt(III)} & & \mathrm{pH} < 6 \\ \Big\downarrow\Big\uparrow & & \\ \mathrm{HAz(II)}^* & & \\ \Big\downarrow\Big\uparrow \mathrm{H}^+ & & \\ \mathrm{Az(II)} + \mathrm{HCyt(II)} \underset{k_{b2}}{\overset{k_{f2}'}{\rightleftharpoons}} \mathrm{Az(I)} + \mathrm{HCyt(III)} & & \\ \Big\uparrow\Big\downarrow \mathrm{H}^+ & & \\ \mathrm{HAz(I)}^* + \mathrm{HCyt(III)} & & \mathrm{pH}\ 6\text{-}8 \\ \Big\downarrow\Big\uparrow & & \\ \mathrm{HAz(II)} + \mathrm{Cyt(II)} \underset{k_{b3}}{\overset{k_{f3}}{\rightleftharpoons}} \mathrm{HAz(I)} + \mathrm{Cyt(II)} & & \\ \mathrm{Az(II)} + \mathrm{Cyt(III)} \underset{k_{b4}}{\overset{k_{f4}}{\rightleftharpoons}} \mathrm{Az(I)} + \mathrm{Cyt(III)} & & \mathrm{pH} > 8 \end{array}$$

at 25 °C are 1.8×10^6 and 3.0×10^6 M^{-1} s^{-1}, respectively (Rosen et al., 1981). The redox reactions of the two azurins, 65% of whose amino acids are identical, are presumed to be similar. Differences in the charge distribution on the surface of the two proteins, general steric effects, and the fact that His-35 is not protonated in *Alc.* azurin may be responsible for the lower redox rates (by a factor of 3). This factor is not large enough to justify the claim that the nonprotonated form is totally unreactive. Computer simulations of eq 5 using the method of Avery (1982) for the *Ps.* azurin–cyt *c*-551 reaction do not predict the bell-shaped curve observed for the amplitude of the slow relaxation time. Similar computer simulations of eq 5 for the reaction between *Alc.* azurin and cyt *c*-551 predict results which are contrary to the experimental data measured. Modeling predicts that the amplitude of this reaction should decrease with pH at pH higher than 7.0 (the amplitude would be expected to decrease by a factor of ~2.5 between pH 7.0 and pH 8.5) and remain constant below pH 5.0, but the data (Figure 6B) are essentially independent of pH between 6.0 and 8.5, and there appears to be an increase in the amplitude below pH 6.0.

The azurin–indicator T-jump experiments cause us to postulate that the protonated species of His-35 exists in two forms, only one of which exchanges protons rapidly with water. The strong deuterium isotope effect on the slow relaxation rate suggests the formation of a particularly strong hydrogen bond between the imidazolium ion and another group on the protein. In any case, according to the kinetic model about 90% of the protonated species exists in one form so we neglect the other. Thus in all we consider four forms of azurin in solution, Im and HIm^+, reduced and oxidized. (There are, of course, other forms depending, for example, on the state of protonation of groups such as His-83, but all these are assumed to have the same redox kinetics.) Thus we have four possible redox equilibria and accompanying proton and conformational equilibria of azurin (see Scheme I).

The slow interconversion between protonated and deprotonated forms of *Ps.* azurin manifests itself in a second slow time constant for the redox reaction. This slow interconversion, coupled to the redox reaction and represented by the equilibria written along the vertical direction, is shown for reduced azurin in the neutral pH range and for oxidized azurin at lower pH as these two azurin species exhibit pK_As of 5 and 7, respectively (this work). It may be that either not all the forward rate constants k_{fi} are equal, not all the backward rate constants k_{bi} are equal, or both. It does not follow as previous workers have assumed, for the sake of simplicity, that one of these

reactions is dominant and the others have negligible rate constants.

Farver et al. (1982) have recently shown that there are probably specific paths for electron transfer through azurin. The key finding was that when Cr^{2+} reduces Az(II), the Cr^{3+} attaches itself to a unique site not far from His-35. Moreover, once the Cr^{3+} is attached, the rate constants for the redox reaction with cyt *c*-551 are somewhat reduced. The present work shows that whether or not there is a specific pathway for electron transport to and from the copper ion, the redox rate does not depend strongly on the state of protonation of His-35.

In conclusion, our major findings are that (1) the p*K* of His-35 in Az(II) is substantially less than that of Az(I), (2) the slow protonation equilibrium of His-35 arises from the fact that most of the imidazolium ion exists in an unreactive form (inaccessible to fast proton exchange with the solvent), and (3) the variation of redox kinetics of azurin and cyt *c*-551 with pH does not suggest any strong dependence of reactivity on the state of protonation of the protein.

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Supplementary Material Available

pH titration data (table) of the relaxation spectrum for the reaction between cytochrome *c*-551 and azurin from *Pseudomonas aeruginosa* (1 page). Ordering information is given on any current masthead page.

Registry No. Cytochrome *c*-551, 9048-77-5.

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