Resolution of Two Distinct Electron Transfer Sites on Azurin†

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ABSTRACT: *Pseudomonas aeruginosa* azurin is stoichiometrically and specifically labeled upon reduction by $Cr(II)_{aq}$ ions, yielding a substitution-inert Cr(III) adduct on the protein surface. We investigated the effect of this chemical modification on the reactivity of azurin with two of its presumed partners in the redox system of the bacterium. The *Pseudomonas* cytochrome oxidase catalyzed oxidation of reduced native and Cr(III)-labeled azurin by O_2 was found to be unaffected by the modification. The kinetics of the electron exchange reaction between native or Cr(III)-labeled azurin and cytochrome c_{551} were studied by the temperature-jump method. Though similar chemical relaxation spectra were observed for native and modified systems, they differ quantitatively. Analysis of the concentration dependences of the relaxation times and amplitudes showed that both obey the same mechanism but that the specific reaction rates of the Cr(III)-modified protein are attenuated. This decreased reactivity of Cr(III)-labeled azurin toward one of its physiological partners suggests the involvement of the labeled region in the electron transfer reaction with cytochrome c_{551}. Furthermore, the presence of a second active site, involved in the reduction of cytochrome oxidase, is suggested by the results.

Azurins are single blue copper proteins found in different species of *Pseudomonas* and *Alcaligenes* where they serve as electron carriers in the energy conversion systems of these bacteria. Although the exact place of their action is not fully established, it is generally thought that they mediate electrons between cytochrome c_{551} and cytochrome oxidase. The electron transfer reaction between *Pseudomonas* azurin and cytochrome c_{551} has been extensively studied (Antonini et al., 1970; Pecht & Rosen, 1973; Wilson et al., 1975; Rosen & Pecht, 1976), and a detailed mechanistic scheme has been presented. However, these studies did not provide insight into the chemical nature of the electron transfer routes of azurin.

Recently the three-dimensional structure of *Pseudomonas aeruginosa* azurin has been determined and refined to 2.7 Å (Adman & Jensen, 1981). The identification of the coordination sphere of the copper ion and its location in a hydrophobic core in the protein, together with other structural features, provided the basis for examining its mechanism of electron transfer in that context. To probe for potential electron transfer loci on azurin, we have employed an affinity labeling procedure that allowed the identification of an electron transfer path leading from a specific site on the surface to the copper center (Farver & Pecht, 1981a).

The identification by a chemical procedure of such sites on the azurin and more recently also on plastocyanin (Farver & Pecht, 1981b) raises the question of their relevance to the electron transfer processes carried out by these proteins in vivo. In order to examine whether the reduction site labeled by Cr(III) in azurin is also involved in reactions with the proposed physiological partners, namely, cytochrome c_{551} and *Pseudomonas* cytochrome oxidase, we investigated the reactivities of these proteins with native and Cr(III)-labeled azurin. While no change could be resolved in the reactivity of Cr(III)-modified azurin with *Pseudomonas* cytochrome oxidase, an attenuation of the reactivity with cytochrome c_{551} has been observed. This effect of the Cr(III) label is interpreted as a result of modifying the region involved in the reaction with cytochrome c_{551}. That a different locus on the azurin is involved in the reaction with cytochrome oxidase is implied by the lack of any effect on the reactivity with the latter.

Experimental Procedures

Materials

Azurin was isolated from *P. aeruginosa* according to the procedure of Ambler & Brown (1967). The A_{625}/A_{280} ratio was always greater than 0.6. Concentrations of the oxidized protein were determined from the absorbance at 625 nm with $\epsilon = 5700\ M^{-1}\ cm^{-1}$ (Rosen & Pecht, 1976). Cr(III)-Labeled Az(I)[1] was produced by reduction of native Az(II) with Cr(II) under anaerobic conditions as described earlier (Farver & Pecht, 1981a). Cr(III)-Labeled azurin(II) was obtained by oxidation of Cr(III)-Az(I) with $IrCl_6^{2-}$ or with O_2 in the presence of a catalytic amount of *Pseudomonas* cytochrome oxidase. No damage to the protein is caused by $IrCl_6^{2-}$ as evidenced by the kinetic and spectroscopic properties of the product.

Cytochrome c_{551} was isolated from the same source as azurin and purified according to the procedure of Ambler (1963). Reduced azurin and cytochrome c_{551} were prepared by catalytic reduction with hydrogen in the presence of a small amount of Pt black (Rosen & Pecht, 1976). The extent of reduction was determined by measuring the absorbancies at 625 nm for the former and 520 and 551 nm for the latter protein by using published extinction coefficients (Rosen & Pecht, 1976). The final product always contained >99% of the reduced form. All solutions contained 10 μM EDTA.

P. aeruginosa cytochrome oxidase was purified by using a combination of the methods of Gudat et al. (1973), Parr et al. (1976), and Kuronen & Ellfolk (1972) employing both CM-cellulose and DEAE-cellulose columns. The purified enzyme had, in the oxidized state, the following absorbancy ratios: $A_{411}/A_{280} = 1.15$ and $A_{640}/A_{520} = 1.1$.

Methods

Kinetic measurements were performed with a temperature-jump spectrophotometer (Rigler et al., 1974). A capacitor discharge of 20 kV raised the temperature of the solution from 20 °C by 5.2 ± 0.1 °C within <50 μs. Chemical relaxation measurements were all performed in 0.1 M Hepes–0.2 M KCl, pH 7.0, unless specifically stated otherwise. The tempera-

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[1] Abbreviations: Az(I)/(II), reduced or oxidized forms of azurin; Cr(III)-Az(I)/(II), the Cr(III)-labeled form of reduced or oxidized azurin; Cyt(II)/(III), reduced or oxidized forms of cytochrome c_{551}; EDTA, ethylenediaminetetraacetic acid; CM, carboxymethyl; DEAE, diethylaminoethyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EPR, electron paramagnetic resonance.

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ture-jump cell used was adapted for anaerobic work (I. Pecht, unpublished data), and a constant flow of deoxygenated argon, saturated with water, was maintained over the solution during all experiments. Also, solutions were made anaerobic prior to transfer into the cell. The change in transmittance followed at 551 nm by one photomultiplier was divided by the signal from a reference photomultiplier and fed into a Biomation 802 transient recorder operating on two time bases, each employing 500 channels. Time-base durations were chosen so as to obtain the maximal information from the signal. Two different rise-time filters were used, each less than 1% of its corresponding time base. Routinely, at each concentration of reactants, the sum of four to seven relaxation curves was recorded on a magnetic tape operated by a Hewlett-Packard 2100 minicomputer. The data collected from four to seven individual jumps were then transferred to an IBM 370/165 computer for detailed analysis similar to that described earlier (Zidovetzki et al., 1980). The concentration dependences of the relaxation times and amplitudes were analyzed with a simulation subroutine written by L. Avery (at the Max-Planck-Institut for Biophysical Chemistry, Göttingen, West Germany) and were fitted to the experimental data with a VA04A subroutine of the Harwell Fortran library (Powell, 1971). Both times and amplitudes were included as separate variables in every fit (Zidovetzki et al., 1980). The initial concentrations of Az(II) were gradually increased by more than 1 order of magnitude (from ~15 to 200 μM) beginning at about half of the initial concentration of Cyt(II) (15–35 μM).

The steady-state kinetic measurements of *Pseudomonas* cytochrome oxidase catalyzed oxidation of reduced native and Cr-labeled azurin were measured spectrophotometrically on a Cary 118C by monitoring the appearance of the 625-nm band of oxidation azurin. The reaction mixture was air-saturated 0.1 M Hepes buffer, pH 7.0. Oxidase concentration was 10 nM, and azurin concentration was varied from 1 to 50 μM.

Results

Static Experiments. The copper site in Cr(III)-labeled azurin undergoes the same redox changes as the native protein. This is evidenced by its characteristic EPR and absorption spectra. Thus, when azurin is fully reduced under anaerobic conditions by Cr(II) and converted into Cr(III)-Az(I), it can be fully reoxidized to the blue Cr(III)-Az(II) either with $IrCl_6^{2-}$ or enzymatically by *Pseudomonas* cytochrome oxidase under aerobic conditions (cf. Materials). In both cases, reoxidation caused no loss of the Cr(III) label, as monitored by measurements using ^{51}Cr as the tracer, even after dialysis of Cr(III)-Az(II) for several hours against the buffer (0.1 M Hepes–0.2 M KCl, pH 7.0). Further, upon reoxidation of the labeled protein, absorbance at 625 nm reached the same specific value as that of the native oxidized protein, i.e., ϵ_{625} = 5700 M^{-1} cm^{-1} (Rosen & Pecht, 1976).

Static redox titrations were performed by adding aliquots of a concentrated solution of Cr(III)-Az(II) to a solution of reduced cytochrome c_{551} in 0.1 M Hepes–0.2 M KCl, pH 7.0 at 25 °C. The redox reaction was monitored at 520, 551, and 625 nm on a Zeiss PMQ2 spectrophotometer. An equilibrium constant

$$K_{tot} = [\text{Cyt(III)}][\text{Cr(III)-Az(I)}]/([\text{Cyt(II)}] \times [\text{Cr(III)-Az(II)}]) = 2.5 \quad (1)$$

was determined. This result is in good agreement with the values previously obtained for native azurin at pH 7.0 in 0.05 M phosphate [K_{tot} = 2.9 (Rosen & Pecht, 1976)] and in 0.1 M phosphate [K_{tot} = 3.7 (Wilson et al., 1975)] buffers. The redox potential of Cr(III)-labeled azurin is thus very close to that of the native protein. The similarity of redox potentials and extinction coefficients of both native and Cr-labeled azurins is not surprising, considering that the chromic ion is bound ≥10 Å away from the copper center (Farver & Pecht, 1981a).

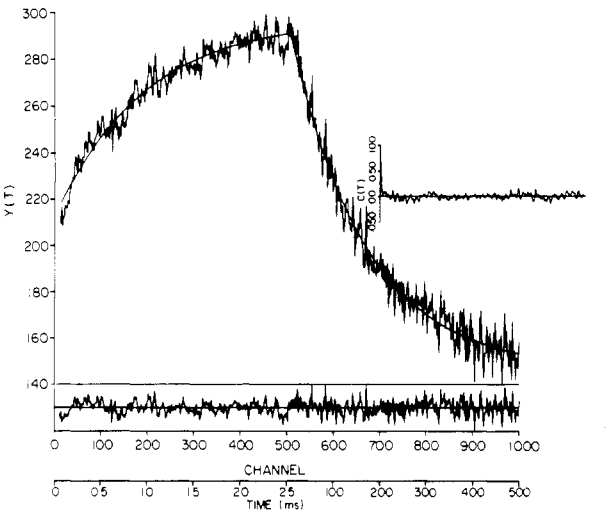

FIGURE 1: Example of analysis and fitting of temperature-jump relaxation curves monitored via changes in transmittance at 551 nm. Concentration of Cr(III)-Az(II) was 180 μM, and that of Cyt(II) was 26 μM. The medium was 0.1 M Hepes–0.2 M KCl, pH 7.0. The data presented are the sum of five individual temperature jumps. The ordinate shows the Biomation units and the abscissa the channel numbers of the Biomation memory together with the corresponding time in milliseconds. In the first 2.5 ms after the temperature jump (20 → 25 °C) the time base was 5 ms/1000 channels (TB1), and after 500 channels were recorded, the time base was switched to 1000 ms/1000 channels (TB2). At the same time, the rise-time filter was changed from 50 μs to 10 ms. The data were fitted to two exponents, and the resulting relaxation times are 0.94 and 200 ms and amplitudes are −5.97 and 10.35 mV, respectively. The solid line is drawn by using those parameters. The total signal after the jump was 5500 mV. The inset, upper right, is the autocorrelation analysis of the fit. The lower part shows the deviation between experimental and fitted curves.

Kinetic Measurements. Temperature-jump chemical relaxation measurements on equilibrium mixtures of native azurin and cytochrome c_{551} were performed in 0.05 and 0.1 M phosphate buffers, pH 7.0, over a wide range of azurin concentrations. The results (not shown) were in complete agreement with those reported earlier (Pecht & Rosen, 1973; Wilson et al., 1975; Rosen & Pecht, 1976). However, since a previous study had shown that the Cr(III) label becomes labile in the presence of high phosphate concentration (Farver & Pecht, 1981a), comparative experiments with Cr(III)-Az could not be made in this medium. Instead, 0.1 M Hepes–0.2 M KCl at pH 7.0 was used, the KCl added to achieve the conductance required for a fast heating of the system.

Cr(III)-Labeled Azurin plus Cytochrome c_{551}. Temperature-jump measurements of the reaction between the above two proteins (in 0.1 M Hepes–0.2 M KCl, pH 7.0) followed at 551 nm exhibited two chemical relaxations. A typical chemical relaxation spectrum for this system is shown in Figure 1. It is seen that the absorbance changes during the two processes are of opposite sign and each corresponds to a single exponent. The reciprocal of the faster relaxation time showed a linear dependence on azurin concentration with no tendency to level off at the higher range, while the slower relaxation time was essentially independent of the azurin concentration. This is demonstrated in Figure 2A,C, where the solid points represent results of experiments carried out with Cr(III)-Az.

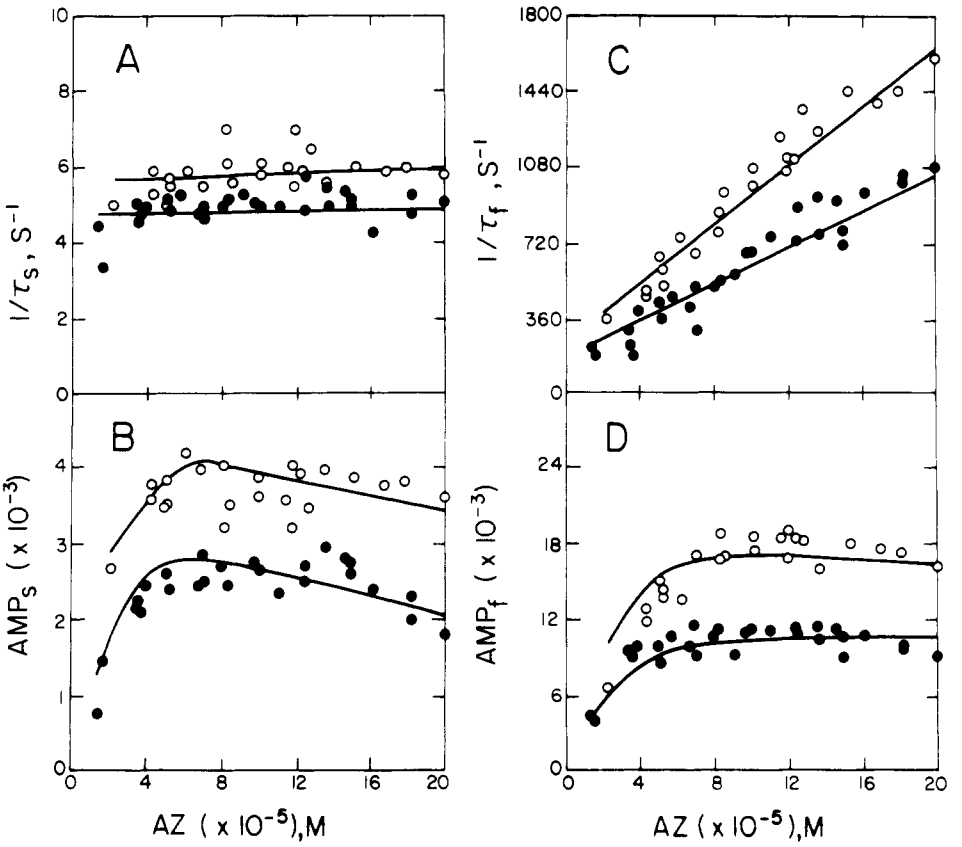

FIGURE 2: Dependence of slow (A and B) and fast (C and D) reciprocal relaxation times and amplitudes on total azurin and Cr(III)-Az concentrations, respectively: (○) experiments with native azurin; (●) experiments with Cr(III)-labeled azurin. The scatter of the points is not only due to experimental errors but also reflects the different cytochrome c_{551} concentrations. Solid lines are drawn by using the best-fit parameters from Table I, with an average cytochrome c_{551} concentration. However, the fitting was carried out with the actual cytochrome c_{551} concentration employed at each point.

The amplitudes are functions of equilibrium reactant concentrations, as well as of the enthalpy and extinction changes of the individual steps. Hence, the amplitude data have to be consistent with any suggested fraction scheme (Eigen & De Maeyer, 1974). The dependence of the amplitudes on Cr(III)-Az concentrations for the fast and slow relaxations is shown in Figure 2B,D. A detailed numerical analysis based on our mechanistic proposal has been performed and is presented below.

Native Azurin plus Cytochrome c_{551}. Temperature-jump measurements of the reaction between native azurin and cytochrome c_{551} in the same medium (0.1 M Hepes–0.2 M KCl) have been performed for comparison. Throughout the whole concentration range examined, this system also showed two chemical relaxations and with the same features as described above (Figure 2, open circles). Furthermore, this behavior is fully compatible with the earlier reports (Pecht & Rosen, 1973; Wilson et al., 1975; Rosen & Pecht, 1976), with the exception that the rate of the slow relaxation is markedly decreased in the Hepes medium. Thus, we find a reciprocal relaxation time $\tau^{-1} \sim 6\ s^{-1}$ compared to 29 s^{-1} in 0.05 M phosphate (Rosen & Pecht, 1976) and 80 s^{-1} in 0.1 M phosphate (Wilson et al., 1976) buffers (all at pH 7.0 and 25 °C). This finding demonstrates a specific effect that phosphate ions have on the slow monomolecular step, which will be discussed further below. To check whether the Cr(III) binding causes any irreversible change in azurin, we carried out the following experiments: Azurin, which had first been reduced and labeled by Cr(III), was reoxidized with $IrCl_6^{2-}$ followed by extensive dialysis against 0.1 M phosphate. This procedure effectively removed the Cr(III) label, and the azurin produced was found to behave like native azurin in its reaction with cytochrome c_{551}, thus excluding the above problem.

Enzymatic Oxidation of Azurin by Cytochrome Oxidase. *Pseudomonas* cytochrome oxidase catalyzed oxidation by O_2 of both reduced native and Cr-labeled azurin has been compared under experimental conditions where the Az(I) oxidation was the rate-determining step. This has been done by measuring the steady-state kinetics of the process as described under Methods. No difference was resolved between the native and Cr-labeled azurins as substrates, both yielding identical values of $K_m = 4.2 \times 10^{-5}$ M and $V_{max} = 1.6$ mol of oxidized azurin (mol of enzyme)$^{-1}$ s^{-1} (T = 25 °C, 0.1 M Hepes, pH 7.0). These values are similar to those observed in earlier studies of native azurin as the substrate, though in a different medium (Yamanaka & Okunuki, 1963; Gudat et al., 1973; Barber et al., 1976).

In summary, several noteworthy features emerge from the comparison between the parameters obtained for electron exchange reactions of cytochrome c_{551}, with native and Cr(III)-labeled azurin. First, the slope of the linear plot of the reciprocal relaxation time vs. total azurin concentration is significantly steeper for native azurin than for the Cr(III)-labeled protein (Figure 2A). Second, the relaxation amplitudes for both the fast and slow chemical relaxations are almost twice as large for the native protein (Figure 2B,D). The extinction coefficient of azurin is not influenced by the Cr(III) label. Further, the reaction is followed via the cytochrome c_{551} absorption at 551 nm, where the azurin contribution is relatively small. These results therefore indicate that the equilibria and/or enthalpy changes differ significantly between reactions of native and Cr-labeled azurin. Finally, it should be noted

that in contrast to the relaxation amplitudes of both steps, the relaxation times of the slow monomolecular step are only slightly affected by the presence of Cr(III) on azurin.

Discussion

Examination of the concentration dependence of the relaxation times and amplitudes presented in Figure 2 suggest that the same mechanism is operative in the electron exchange reactions of native and Cr(III)-labeled azurin with cytochrome c_{551}. Since only the absolute magnitudes of the reaction parameters are different, both systems will be analyzed and discussed together.

The linear dependence of $1/\tau_{fast}$ on total azurin concentration (Figure 2A), without a leveling off at high protein concentration, is assigned, in agreement with the earlier studies (Pecht & Rosen, 1973; Wilson et al., 1975; Rosen & Pecht, 1976), to a bimolecular electron transfer step of the type

$$\mathrm{Az(II)} + \mathrm{Cyt(II)} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{Az(I)} + \mathrm{Cyt(III)} \qquad (2)$$

This assignment implies that any complex formation between the reactants prior to the electron transfer is kinetically insignificant. The slow, concentration-independent relaxation is again assigned to a conformational transition between a reactive conformer and a nonreactive conformer of reduced azurin that is coupled to the electron transfer reaction

$$\mathrm{Az(I)} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{Az^*(I)} \qquad (3)$$

where the asterisk (*) denotes the less-reactive form of azurin. This is also in agreement with the earlier reports (Wilson et al., 1975; Rosen & Pecht, 1976).

We now have two reactions and two sets of relaxation data. However, a numerical analysis of the experimental data based on this two-step mechanism did not yield any reasonable fit. In addition, we found it impossible to analytically reproduce the experimentally observed amplitude pattern with this reaction scheme by computer simulation. It was therefore necessary to include in the scheme an additional step, which we assume to be undetected since only two relaxations were observed. One simple and obvious hypothesis would be proposing an analogous isomerization step for oxidized azurin with the same time constants as those for reduced azurin. However, including this additional step in the reaction mechanism did not improve the fit significantly. Therefore, we had to assume the existence of a conformational equilibrium of oxidized cytochrome c_{551}. This could be uncoupled from the electron exchange, i.e., with a relaxation time smaller than or of the same order of magnitude as the heating time of ~ 15 μs. This yields a reaction scheme (eq 4) that is identical with the one proposed by Rosen & Pecht (1976):

$$\begin{array}{ccc} \mathrm{Az(II)} + \mathrm{Cyt(II)} & \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} & \mathrm{Az(I)} + \mathrm{Cyt(III)} \\ \Big\| k_{-3} \quad k_3 \Big\downarrow & & \Big\| k_{-1} \quad k_1 \Big\downarrow \\ \mathrm{Az^*(I)} & & \mathrm{Cyt^*(III)} \end{array} \qquad (4)$$

where the asterick denotes the less reactive forms of the proteins. With $K_1 = k_1/k_{-1}$, $K_2 = k_2/k_{-2}$, and $K_3 = k_3/k_{-3}$, the overall equilibrium constant becomes

$$K_{tot} = (1 + K_1)(1 + K_3)K_2 = ([\mathrm{Cyt(III)}] + [\mathrm{Cyt^*(III)}]) \times ([\mathrm{Az(I)}] + [\mathrm{Az^*(I)}])/([\mathrm{Az(II)}][\mathrm{Cyt(II)}])$$

and the corresponding overall enthalpy change is

$$\Delta H_{tot} = \Delta H_2 + \frac{K_1}{1 + K_1}\Delta H_1 + \frac{K_3}{1 + K_3}\Delta H_3$$

Table I: Kinetic and Thermodynamic Data for Azurin–Cytochrome c_{551} Reaction[a]

	native Az + cyt c_{551}	Cr(III)-Az + cyt c_{551}
K_1	0.59	0.62
k_2 (M^{-1} s^{-1})	6.88×10^6	4.68×10^6
k_{-2} (M^{-1} s^{-1})	1.47×10^7	6.96×10^6
K_2	0.47	0.67
k_3 (s^{-1})	1.67	0.58
k_{-3} (s^{-1})	4.52	4.40
K_3	0.37	0.13
ΔH_1 (kJ/mol)	44.0	38.5
ΔH_2 (kJ/mol)	−14.6	−14.0
ΔH_3 (kJ/mol)	−23.6	−31.8
K_{tot}	1.0	1.2
ΔH_{tot} (kJ/mol)	−4.7	−2.9

[a] Results are from the numerical analysis of the experimental data shown in Figure 2, using the proposed mechanism presented in eq 4, which also defines the symbols and subscripts. Measurements were done at 25 °C, pH 7.0, in 0.1 M Hepes–0.2 M KCl buffer. All other experimental conditions are given in the legend to Figure 1. The error range in the calculated parameters is estimated to be ±20%.

Applying this mechanism, we were able to perform a satisfactory numerical analysis that conformed with all the observed parameters. The resulting best fit is shown as the solid lines in Figure 2, and the numerical values are presented in Table I. The agreement between the K_{tot} calculated from the kinetic data for Cr(III)-azurin/cytochrome c_{551} (1.2) and the one obtained by static titrations (2.5) is not quite satisfactory. We assign the discrepancy to the fact that the calculated value is a product of three independent parameters and could therefore accumulate a relatively large error. A more complex scheme involving further steps that are undetected optically is also possible. However, with the present experimental information we feel that such an analysis is not justified. The other parameters obtained by the fit are in good agreement with the results reported earlier (Wilson et al., 1975; Rosen & Pecht, 1976), especially considering the different media (phosphate vs. Hepes buffers). It was reassuring that the numerical analysis, performed with no constraints on the parameters, yielded values of K_1 and ΔH_1 that are identical within experimental error for both native and Cr(III)-labeled azurin systems. An effect of Cr binding to azurin on the fast isomerization of Cyt(III) would have been difficult to rationalize.

The three-dimensional structure of *Pseudomonas* azurin (Adman et al., 1978; Adman & Jensen, 1981) along with the assigned position of Cr(III) on the azurin surface (Farver & Pecht, 1981a) allows for a detailed discussion of the present kinetic data in terms of the sites that are involved in the electron transfer reaction. In our earlier study of Cr(III)-labeled azurin, an electron transfer pathway from Cu(II) to the protein surface was identified (Farver & Pecht, 1981a). It was proposed that the electron is transferred from Cr(II) to Cu(II) via a water molecule hydrogen bonded to N_ϵ of a nonligating His-35. The imidazole ring of this histidine is oriented parallelly and in van der Waals contact with the imidazole ring of His-46, which is one of the Cu ligands.

In support of this proposal, a nonligating histidine, which has a pK of ~ 7, was found to be involved in an unusually slow proton exchange (Ugurbil & Bersohn, 1977), and an estimate of the proton exchange rate between 1 and 35 s^{-1} has been given (Hill & Smith, 1979).

The slow isomerization step of Az(I) observed in its electron exchange reaction with cytochrome c_{551} is therefore interpreted as a conformational transition that occurs in the region of

His-35. This transition causes a change in the solvent accessibility of the imidazole side chain, which is reflected in the coupling between the protonation equilibrium of His-35 and the conformational transition. The labeling of azurin by Cr(II) thus leads to a modification in the area proximal to His-35. The difference in the reaction parameters between native azurin and Cr(III) azurin with cytochrome c_{551} (Table I) is a result of this modification and constitutes an independent corroboration of the involvement of that region of azurin in the electron transfer (Farver & Pecht, 1981a) and of the molecular interpretation of the observed slow relaxation.

The protonation step can be monitored directly by pH indicators upon subjecting reduced azurin to a temperature-jump perturbation. The chemical relaxation due to that reaction has been resolved, and protons are found to be released upon temperature increase (S. Wherland and I. Pecht, unpublished data; Silvestrini et al., 1981; A. F. Corin and R. Bersohn, personal communication). From the present report and from that of Rosen & Pecht (1976), we know that the enthalpy change due to the slow isomerization reaction (eq 3) is negative. This means that the active conformer of azurin(I) is favored at higher temperatures. Hence, we conclude that formation of the active conformer is coupled to a deprotonation, i.e., that the inactive conformer is the protonated species. As also proposed by Corin (1981), one can assume a process in which a fast proton exchange of His-35 in the active conformer of Az(I) is coupled to a slow relaxation of the equilibrium between the two isomers:

$$\mathrm{Az^*(I)\cdot H} \underset{k_3'}{\overset{k_{-3}'}{\rightleftharpoons}} \mathrm{Az(I)\cdot H} \overset{K_a}{\underset{\text{fast}}{\rightleftharpoons}} \mathrm{Az(I)} + \mathrm{H^+} \qquad (5)$$

where k_3' and k_{-3}' are the intrinsic isomerization rate constants independent of pH and K_a is the acid dissociation constant. One can show that the measured K_3 is related to the intrinsic K_3' by the expression

$$K_3' = K_3(1 + K_a/[\mathrm{H^+}]) \qquad (6)$$

The pK_a for native azurin was measured to be 6.7 (Corin, 1981). Using our K_3 (Table I), we can calculate the intrinsic isomerization constant to be $K_3' = 1.11$. One should note that k_{-3} (Table I) hardly changes with the labeling of azurin while there is a change in k_3 as might be rationalized by the expression for the isomerization (French & Hammes, 1965):

$$1/\tau_{slow} = k_3'/(1 + K_a/[\mathrm{H^+}]) + k_{-3}' = k_3 + k_{-3} \qquad (7)$$

If the intrinsic isomerization constants are not affected by the Cr labeling [$K_3' = 1.11$, K_3(Cr-Az) = 0.13], we calculate a pK_a of 6.12 for the Cr-azurin. The pK of His-35 would therefore be lowered by about half a pH unit, a reasonable change considering the proposed proximity of the positively charged Cr(III) label.

The different relaxation times found for the slow isomerization of native Az(I) at the various phosphate concentrations are interesting and possibly of physiological relevance. Thus, in the present study we find a $\tau_{slow} \sim 160$ ms [$=(k_3 + k_{-3})^{-1}$] (0.1 M Hepes, pH 7.0) at 25 °C. In earlier studies a τ_{slow} of ~ 12 ms at 0.1 M phosphate (Wilson et al., 1975) has been reported, while a τ_{slow} of ~ 35 ms was found at 0.05 M phosphate (Rosen & Pecht, 1976), all at 25 °C and pH 7.0. Since we were able to reproduce the latter findings, the different values are not due to inhomogeneity of the different azurin preparations but rather reflect a pronounced influence of phosphate ions.

It is worth noting that the slow isomerization was not observed in the *Alcaligenes faecalis* azurin–*Pseudomonas* cytochrome c_{551} electron exchange reaction (Rosen, 1977; Wherland & Pecht, 1978; Rosen et al., 1981). It has also been shown that the homologous histidine residue (His-35; Ambler, 1971) is not involved in any acid–base equilibrium at neutral pH, most probably because the imidazole is even less accessible to the solvent in *Alcaligenes* as compared to *Pseudomonas* azurin (Mitra & Bersohn, 1982).

The difference seen in the fast relaxation time of the reaction between *Pseudomonas* azurin and cytochrome c_{551} (Figure 2C,D and Table I) clearly implies that the presence of Cr(III) on azurin is attenuating the electron exchange between the proteins. The exposed heme edge in cytochrome c_{551} is surrounded by lysyl side chains (Almassy & Dickerson, 1978), which are protonated at pH 7.0. The presence of the positively charged Cr(III) ion near His-35 could perturb the recognition site on azurin for cytochrome c_{551}, by either an electrostatic or a steric effect, which would limit the close approach necessary for an optimal interprotein electron transfer.

Significantly, the *Pseudomonas* cytochrome oxidase catalyzed oxidation of Cr(III)-Az(I) by O_2 is unaffected by the presence of Cr(III). This brings us to the conclusion that there are at least two physiologically operative reaction sites for electron transfer on azurin, one for cytochrome c_{551} and another for *Pseudomonas* cytochrome oxidase. From the three-dimensional structure it is known that the edge of the imidazole ring of one of the Cu-ligating histidines (His-117) is slightly exposed (Adman et al., 1978). The area around this imidazole is lined with hydrophobic residues (Adman, 1979), which confer on this site quite a different character from the one proposed for the cytochrome c_{551} interaction. Thus, the region proximal to His-117 could very well act as the reaction site with cytochrome oxidase, wherein the imidazole of this histidine being part of the copper coordination sphere allows for an electron transfer to cytochrome oxidase.

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Properties of the Prosthetic Groups of Rabbit Liver Aldehyde Oxidase: A Comparison of Molybdenum Hydroxylase Enzymes†

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ABSTRACT: Rabbit liver aldehyde oxidase (AO), like milk xanthine oxidase (XO) and chicken liver xanthine dehydrogenase (XDH), possesses the following prosthetic groups: FAD, a functional Mo center, and two spectroscopically distinct iron–sulfur centers, one with $g_{av} < 2.0$ (termed Fe/S I) and the other with $g_{av} > 2.0$ (termed Fe/S II) in the reduced enzyme. EPR spectra for the Mo^V species were found to be nearly identical in AO and XO for a number of enzyme complexes, and the midpoint reduction potentials for functional Mo^{VI}/Mo^V (−359 mV) and Mo^V/Mo^{IV} (−351 mV) were nearly the same in all three enzymes (50 mM phosphate, pH 7.8). A strong magnetic interaction between Mo^V and reduced Fe/S I, previously detected in XO and XDH, was also found in AO. No Mo^V–Fe/S II interaction could be detected in AO (nor in XO). In contrast, the order of reduction of Fe/S I and Fe/S II, as measured from their midpoint potentials, is reversed in AO (E_m = −207 and −310 mV, respectively) as compared to XO (E_m = −280 and −245 mV, respectively) in phosphate buffer at pH 7.8. The oxidized–reduced extinction coefficients at 450 and 550 nm for the two centers are also apparently reversed in AO and XO. Although magnetic interaction between FAD and one or both reduced Fe/S centers has been detected in both AO and XO, no magnetic interaction between the two reduced Fe/S centers themselves was found in AO (although such interaction has been seen in XO). The average FAD reduction potential is substantially more positive in AO (E_m for FAD/FADH·, −258 mV; FADH·/$FADH_2$, −212 mV at pH 7.8) than in XO or XDH. It can be concluded that although the properties and immediate environment of the functional Mo center are conserved in the three Mo hydroxylase enzymes, and all three enzymes possess the same set of prosthetic groups, the properties of the groups which transfer electrons from the Mo to the ultimate electron acceptor can vary substantially in AO, XO, and XDH.

Milk xanthine oxidase (XO)[1] and avian liver xanthine dehydrogenase (XDH) each contain two subunits (M_r 140000) (Coughlan, 1980). Each subunit contains one FAD, one Mo center with a novel pterin cofactor (Johnson et al., 1980), and two spectroscopically distinct Fe_2S_2 centers, termed Fe/S I and Fe/S II, which differ in their EPR properties (Bray, 1975), reduction potentials (Cammack et al., 1976; Barber et al., 1980), and visible absorption spectra (Olson et al., 1974b).

Rabbit liver aldehyde oxidase, which differs markedly in its substrate specificity from XO and XDH (Feldsted et al., 1973; Coughlan, 1980), apparently contains a similar molecular structure [M_r 280000; two FAD, two Mo, and eight non-heme Fe (Rajagopalan et al., 1962)]. The Mo center of AO contains a pterin cofactor like that found in XO/XDH (Johnson, 1980). In its functional form, the AO Mo center contains a cyanolyzable sulfur atom (Branzoli & Massey, 1974a) which EXAFS (Bordas et al., 1980; Cramer et al., 1981) and EPR studies (Malthouse & Bray, 1980) have shown to be due to

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[1] Abbreviations: AO, rabbit liver aldehyde oxidase; Fe/S I, iron–sulfur center with $g_{av} < 2.0$ in the reduced enzyme; Fe/S II, iron–sulfur center with $g_{av} > 2.0$ in the reduced enzyme; XO, milk xanthine oxidase; XDH, avian liver xanthine dehydrogenase; EXAFS, X-ray absorption fine structure, extended; EDTA, ethylenediaminetetraacetic acid.

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