Spectroelectrochemistry of blue copper proteins: pH and temperature dependences of the reduction potentials of five azurins

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Abstract

The pH and temperature dependences of the reduction potentials of *Alcaligenes denitrificans* (Ade), *Pseudomonas aeruginosa* (Pae), *Alcaligenes faecalis* (Afa), *Alcaligenes sp.* (Asp) and *Bordetella bronchiseptica* (Bbr) azurins have been determined by spectroelectrochemistry. Potentials at pH 8.0 (25 °C, μ 0.1 M, NaP_i) are: 267.0 (Ade); 291.9 (Pae); 251.5 (Afa); 278.1 (Asp); 250.3 (Bbr) mV versus NHE. The variations in the potentials with pH are interpreted in terms of interactions between the copper site and titrating histidine residues. Variations in the reduction potential of Bbr azurin with both pH and temperature indicate that the copper site is distorted significantly by a redox-linked conformational change in this protein.

Introduction

Azurins are bacterial blue copper proteins that possess relatively high reduction potentials (~300 mV versus NHE) [1, 2]. Crystal structure analyses have shown that the copper ligands are two histidine imidazoles and a cysteine thiolate; in addition, there are longer bonds to a methionine thioether and a main-chain carbonyl [3–7]. In a site that lies 6 Å below the surface of the protein, an extensive network of hydrogen bonds and other interactions with neighboring residues hold the copper ligands fixed [8, 9]. Preferential stabilization of Cu(I) at this rigid site is attributable to a weak ligand field in combination with Cu–S backbonding interactions [10, 11]. Work on azurin derivatives such as Met121→Met(Se) [12] and Met121→Leu [13] has shed additional light on the role of the coordination environment in tuning the copper reduction potential.

The reduction potentials of azurins must also be influenced by interactions of charged neighbors with the copper center. To examine this matter, we have employed spectroelectrochemistry to obtain the pH dependences of the reduction potentials of five azurins. In addition, the temperature dependences of the potentials at low and high pH have been determined.

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Experimental

Materials and instrumentation

The cultures of *Alcaligenes denitrificans* (NCTC 8582 or ATCC 15173), *Alcaligenes faecalis* (ATCC 8750) *Pseudomonas aeruginosa* (ATCC 10145) and *Bordetella bronchiseptica* (NCTC 8344 or ATCC 10145) were supplied by the American Type Culture Collection (ATCC). *Alcaligenes* sp. (NCIB 11015, subspecies *xylosoxidans*) was obtained from the National Collection of Industrial and Marine Bacteria (NCIMB), Torry Research Station, Scotland.

Hexaammineruthenium(III) chloride (Strem) was recrystallized from 1 M HCl. All other chemicals were reagent grade and were used without further purification. Water used in the preparation of all aqueous solutions was purified by passage through a Barnstead NANO pure system (model D2794). $[Ru(NH_3)_5(py)]Cl_3$ (py = pyridine) was prepared according to a standard method [14]. The crude material was purified by chromatography on SP Sephadex C-25, with elution by stepwise increases in [HCl]; the desired product elutes at 0.3 M HCl. $[Co(phen)_3]Cl_3$ (phen = 1,10-phenanthroline) was prepared by a modification of a previous method [15]. A solution of 1 equiv. $CoCl_2 \cdot 6H_2O$ and 3 equiv. phenanthroline monohydrate in water was refluxed for 90 min. The solution was then removed from heat, and chlorine gas was bubbled through the solution for 60 min. After rotary evaporation to dryness, the product was collected and recrystallized from

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MeOH/ether. Na[Co(edta)] was prepared according to Kirschner [16] and recrystallized from water.

Absorbance measurements were carried out using a Shimadzu UV-260 or a Varian Cary 219 spectrophotometer. The FPLC (fast protein liquid chromatography) system was supplied by Pharmacia. Azurin concentrations were determined by absorbance measurements at 625 nm ($\epsilon_{625}=5700\ M^{-1}\ cm^{-1}$) [17].

Optically transparent thin-layer electrode cells were prepared by modifications of a design by Heineman *et al.* [18, 19]. A single minigrid (500 line/in gold electroformed mesh, Interconics, St. Paul, MN) was sandwiched between two spacers of 0.1 mm Teflon tape (Dilectrix Corporation, Farmingdale, NY) to give an optical pathlength of ~0.3 mm. A copper wire was connected to the gold minigrid to provide an external contact for the working electrode. Potentials were applied using a Princeton Applied Research polarographic analyzer (model 174A) and measured with a microvolt digital multimeter (Keithley 177). The reference electrode was a miniature saturated calomel electrode (Sargent-Welch), and the counter electrode was a platinum wire. Sample temperature was monitored during each spectroelectrochemical experiment by a Fluke digital thermometer (2175A) via a microthermocouple (Omega Engineering) placed inside the wall of the working electrode compartment. Sample conditions for spectroelectrochemical measurements were as follows: [azurin] = 0.5–1.5 mM; [mediator] = 3[azurin]. Mediators employed: $[Ru(NH_3)_5(py)]Cl_3$, <300; $[Co(phen)_3]Cl_3$, >300 mV versus NHE; in regions where both mediators were used, no mediator dependence was found. At least six points were used for each Nernst plot, in addition to the absorbance readings for the fully reduced and fully oxidized proteins.

Protein isolation and purification

Preparations of azurins from all five species of bacteria were based on modifications of previously described procedures [17, 20–22]. A general scheme is described below, followed by details specific to each species.

Culture media consisted of (per liter) 5.0 g sodium citrate (dihydrate), 5.0 g $NaNO_3$, 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 6.5 g Difco yeast extract, 10 mg $FeCl_3 \cdot 6H_2O$, 10 mg $CuSO_4 \cdot 5H_2O$ and 2 g Bacto Peptone. Bacteria were grown anaerobically at 35 °C in a 350 l fermenter and harvested at 5–10 °C at a flow rate of 60–90 l/h. Cell paste was used immediately or frozen at −50°C. The cell paste was converted to acetone powder and then extracted with buffer according to literature procedures [20, 22]. All steps in the isolation and purification after this point were carried out at 5 °C, except for purification by FPLC. Solids were removed by centrifugation for 90 min at 18 000 x *g*. The supernatant was dialyzed against three changes of a ten fold volume excess of 0.05 M ammonium acetate buffer, pH 4.0 (loading buffer). The pH of the dialysate was adjusted to 4.0 if necessary, and the precipitate which forms at low pH was removed by centrifugation as above. Na[Co(edta)] (~1 mg/g acetone powder) was added to the supernatant, followed by cold distilled water to lower the conductivity of the solution to that of the loading buffer. The resulting brown solution was loaded onto a column of CM52 cation exchange resin (Whatman) equilibrated with loading buffer; the column was approximately 4.0 cm diameter × 20 cm for a 200 g batch of acetone powder. The column was washed with loading buffer until the eluent was colorless. Periodically during elution, 20–30 mg portions of Na[Co(edta)] were dissolved in buffer and passed down the column, in order to prevent autoreduction of the proteins. Azurin and other proteins were eluted by stepwise increases in the pH of the 0.05 M ammonium acetate buffer. At this stage, the azurin preparation contained considerable amounts of cytochromes and other proteins and nucleic acids. Gel filtration chromatography (standard techniques or FPLC) was used as an intermediate step in purification. After gel filtration chromatography, a combination of anion and/or cation exchange chromatography steps was used to purify each azurin to the proper purity ratio ($A_{\sim 620}/A_{280}$).

Gel filtration chromatography (standard techniques)

The protein solution was concentrated to a small volume, and ~2 ml aliquots were loaded onto a 2.5 cm diameter × 90 cm column of Sephadex G-75-150 (Sigma) on 0.1 M sodium phosphate buffer, pH 7.0. A flow rate of 7.7 ml/h gave baseline separation of an orange band from the azurin band; azurin eluted at ~180 ml.

Gel filtration chromatography (FPLC)

The solution of azurin was concentrated and loaded in ≦0.1 ml aliquots on a 25 ml column of Superose 12. Using $\mu = 0.1$ M sodium phosphate, pH 7.0 with a flow rate of 0.5 ml/min, near baseline separation of a cytochrome band from azurin could be achieved; azurin eluted at ~17 ml.

Anion exchange chromatography (standard techniques)

The azurin solution was washed into 0.01 M Tris–HCl, pH 8.7 (loading buffer), and concentrated to ~3 ml. The solution was loaded onto a 1.5–2.5 diameter × 20 cm column of DE52 (Whatman) in loading buffer. Protein was eluted with 0.01–0.05 M Tris HCl, pH 8.7.

Cation exchange chromatography (FPLC)

Samples were loaded on a Mono S 10/10 column in 0.05 M ammonium acetate buffer, with elution by increasing NaCl concentration. Specific conditions are given below for each bacterial species.

Alcaligenes denitrificans. Several changes were made to the general culture medium described above (per liter): Bacto Peptone was increased to 10 g, sodium citrate was replaced by 10 g sodium glutamate, $FeCl_3$ was replaced by 2 mg $FeSO_4$, $MgSO_4$ was replaced by 8 mg $MgCl_2$ and the $NaNO_3$ was deleted. Bacteria were grown with aeration at 34 °C for 30 h.

A tendency toward autoreduction at low pH, especially during temperature changes, was noted for Ade azurin. Thus, pH 4.5 (rather than 4.0) ammonium acetate was used as the dialysis buffer. The loaded column was washed with pH 4.5 buffer, followed by pH 4.8 and 5.1. pH 5.5 buffer was used to elute the azurin. If large amounts of cytochromes co-eluted with azurin, the majority of impurities were removed by concentrating the protein to a small volume and passing it down a CM52 column in pH 5.65 ammonium acetate buffer, using isocratic elution conditions. The protein was further purified by gel filtration chromatography (standard techniques or FPLC) as described above. Final purification was by FPLC cation exchange, using pH 4.5 buffer. Gradients were 0 to 0.18 M NaCl in 180 ml. In some cases, an additional chromatographic step was performed to purify the azurin, using FPLC cation exchange with pH 5.2 buffers; the gradient was 0 to 25 mM NaCl in 78 ml. Yields for Ade were far higher than those obtained with the other four species of bacteria, and were typically 2.5 kg cell paste/350 l fermenter run, 800 g acetone powder/2.5 kg cell paste, and 2.4 g azurin/800 g acetone powder. The purity ratio (A_{621}/A_{280}) was $\geqq 0.28$.

Pseudomonas aeruginosa. Cultures were grown at 37 °C for 24–48 h, without aeration or agitation. After loading the crude protein solution on the initial CM 52 column at pH 4.0, the pH of the eluting buffer was raised stepwise to pH 4.9. The azurin was purified by gel filtration chromatography as described above, and further purified either by anion exchange followed by cation exchange chromatography, or by two cation exchange steps. FPLC cation exchange was performed using pH 4.3 buffer, with a gradient to 0.10 M NaCl in 114 ml. Pae azurin could be further purified by crystallization from 0.10 M sodium acetate buffer, pH 5.5 by bringing a very concentrated solution of azurin to 65–70% $(NH_4)_2SO_4$ saturation at room temperature over a few hours. Yields were typically 2 kg cell paste/350 l fermenter, 500 g acetone powder/2 kg cell paste, and 600 mg azurin/500 g acetone powder; the purity ratio (A_{621}/A_{280}) was $\geqq 0.58$.

Bordetella bronchiseptica. Cells were grown as described for Ade. Bbr azurin showed a tendency toward autoreduction on the CM52 column at pH $\leqq 5.0$; thus, Na[Co(edta)] was added to the elution buffer to maintain oxidizing conditions. A small cytochrome band was eluted at pH 4.5, followed by washing at pH 4.8 and elution of azurin (with a second small cytochrome band) at pH 5.0. A minor blue or green band remained bound to the top of the column at pH 5.0, and was not characterized. After gel filtration chromatography as described above, final purification was performed by FPLC cation exchange, using pH 4.4 buffers and [NaCl] increasing from 0 to 0.13 M in 130 ml; azurin elutes at 0.12 M NaCl. The final azurin solution had a purity ratio of $\geqq 0.55$.

Alcaligenes faecalis. Cell cultures were allowed to grow for 48 h. The crude protein solution was loaded on the CM52 column at pH 4.0; the column was washed at pH 4.5, then pH 4.7; azurin was eluted at pH 5.0. The protein was further purified by FPLC using pH 4.3 buffers, with a gradient to 0.14 M NaCl in 140 ml. Typical yields were 850 g cell paste/350 l fermenter; 250 g powder/850 g paste; 300 mg azurin/250 g powder. The purity ratio of the final protein solution was 0.56.

Alcaligenes sp. The culture medium was as described initially, with the addition of (per liter) 8 g Bacto Peptone, 2.5 g NaCl, 3.6 g Na_2HPO_4, 6.4 g K_2HPO_4, 15 g KNO_3. Approximately 4.5 ml per liter of 1 N NaOH was added to the broth to bring the pH to 7. The initial cation exchange column was loaded at pH 4.0, then washed extensively at pH 4.4 and 4.7; azurin eluted at pH 5.0. A faint blue band eluted before azurin; this material was not characterized. Final purification was by FPLC cation exchange at pH 4.35; [NaCl] was increased from 0 to 0.135 M in 180 ml. Typical yields were 680 g cell paste/200 l fermenter, 170 g powder/680 g cell paste, and 60 mg azurin/170 g acetone powder; the purity ratio was $\geqq 0.58$.

Results and discussion

Reduction potentials

The results of a typical thin-layer spectroelectrochemical experiment with Afa azurin at pH 7.0 and 25 °C are shown in Fig. 1. The uppermost trace ($E_{app} = 225$ mV versus SCE) is the visible spectrum of fully oxidized azurin, and the bottom trace ($E_{app} = -175$ mV) is that of the fully reduced protein. At each applied potential, the approach to equilibrium between the oxidized and reduced forms of the protein was observed by monitoring the absorbance at 625 nm. Data points were collected in both the reducing and oxidizing directions, and the absorbance of the fully oxidized protein was rechecked at the end of the experiment in order to detect possible decomposition of the protein. The mediators $[Ru(NH_3)_5(py)]Cl_3$ and $[Co(phen)_3]Cl_3$ are

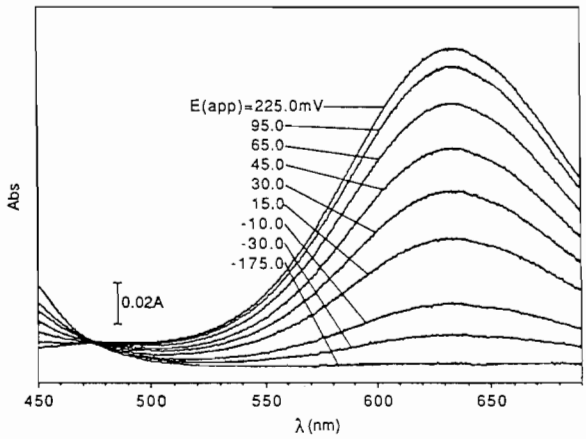

Fig. 1. Overlay spectra showing the reduction of Afa azurin (pH 7.0, 25 °C, μ 0.1 M, NaP_i). Applied potentials are referenced to SCE.

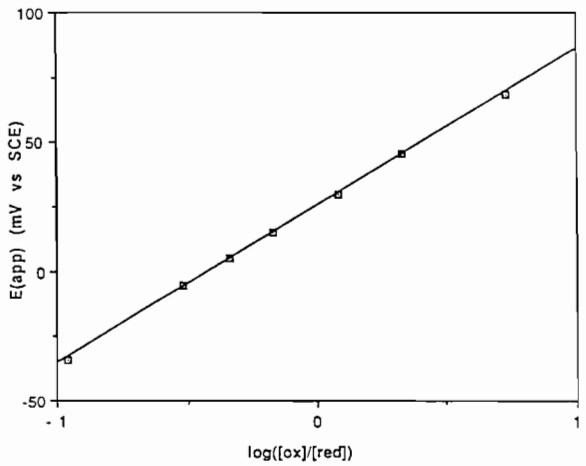

Fig. 2. Nernst plot of the Fig. 1 data (least-squares fit yields $E^{\circ\prime} = 25.4$ mV vs. SCE and $n = 1.03$).

relatively transparent in both redox states at 625 nm; thus, the recorded A_{625} values could be analyzed directly to determine the amounts of oxidized and reduced azurin present at each potential. The Nernst plot obtained from these data is shown in Fig. 2.

Representative reduction potentials in the pH range 4.0–8.5 and values of other thermodynamic parameters for the five azurins are set out in Table 1. The enthalpies of reduction (approximately −13 to −16 kcal/mol) are comparable to previously reported ΔH° values for blue copper proteins [19, 23]. With the exception of Bbr azurin at pH 8, the ΔS° values are all between −23 and −29 e.u.

The pH dependences of the reduction potentials of the five azurins are shown in Figs. 3–7 (25 °C; μ 0.1 M NaOAc for pH 4 to 5.5; μ 0.1 M NaP_i for pH 6 to 8.5; μ 0.1 M Na glycinate for pH 9). In each case, data were collected over as wide a pH range as possible. At pH values above or below those for which data are given, an accurate potential could not be determined, either because the absorbance of the protein decayed at constant applied potential, or because the Nernst plots had low correlation coefficients or slopes outside the range of ±5%.

Analysis of pH dependences

The reduction potential of a redox-active protein is affected by the charges on residues near the active site [24]. Owing to the influence of neighboring protonated residues, the reduction potential of the copper center in a blue copper protein will increase as the pH is lowered. It follows that the pK_a at which any given residue titrates will depend on the oxidation state of the metal ion, with $pK_a^{red} > pK_a^{ox}$. These effects are incorporated in eqn. (1) [25]:

$$E_m(\text{pH}) = E_m(\text{low pH}) + \frac{RT}{nF} \ln \frac{K_a^{red} + [H^+]}{K_a^{ox} + [H^+]} \quad (1)$$

We attribute the general increase in E_m with decreasing pH observed for all five azurins to protonations of the non-ligated histidines [26–31] (Fig. 8). Histidines that are coordinated to the copper (His 46 and His 117) should not contribute to the pH dependence, as their pK_a values are $\leqslant 4$ [26].

Both non-ligated histidines in Pae azurin have been shown by ^1H NMR spectroscopy to titrate in the pH range under consideration (Table 2). In addition, each histidine pK_a is known to be affected by the oxidation state of the copper. A fit of our data to eqn. (1) gives $pK_a^{red} = 7.31$ and $pK_a^{ox} = 6.26$ (Fig. 3); these values fall between the pK_a values of His 35 and His 83 determined by NMR (Table 2), and they are not very different from those extracted from measurements of redox potentials in hexacyanoferrate solutions of the protein [32].

At pH values where both histidines are in the deprotonated state, $[H^+]$ will be negligible relative to K_a^{red} and K_a^{ox} (Eqn. (2)):

$$E_m(\text{low pH}) = E_m(\text{high pH}) + \frac{RT}{nF} \ln \frac{K_a^{red}}{K_a^{ox}} \quad (2)$$

Based on the NMR-derived pK_a values (Table 2), the deprotonations of His 83 and His 35 in Pae azurin should produce shifts in the potential of 13 and 70 mV, respectively. The observed 60 mV change in potential (Fig. 3) is in reasonable agreement with the 83 mV prediction, particularly in light of the large uncertainty (±0.4) in the pK_a^{ox} value of 5.9 for His 35 [27].

It is likely that variations in the Pae protein structure contribute significantly to the relatively large pH dependence of the potential. In reduced Pae azurin, ^1H NMR line-broadening studies indicate that a shift in

TABLE 1. Thermodynamic parameters for azurin reductions

Azurin	pH	E_m (mV vs. NHE)[a]	$\Delta S°$ (e.u.)	$\Delta H°$ (kcal/mol)	$\Delta G°$ (kcal/mol)[b]
Ade	5.4	283.7	−24 (±2)	−13.8 (±0.7)	−6.5
	8.0	267.0	−24 (±2)	−13.2 (±0.7)	−6.2
Pae	5.0	348.6	−27 (±1)	−16.1 (±0.4)	−8.0
	8.0	291.9	−29 (±2)	−15.4 (±0.6)	−6.7
Afa	5.0	299.4	−27 (±1)	−15.1 (±0.4)	−6.9
	8.5	250.6	−23 (±3)	−12.6 (±0.9)	−5.7
Asp	4.0	358.7	[c]	[c]	[c]
	8.0	278.1	[c]	[c]	[c]
Bbr	4.0	343.7	−24 (±2)	−15.2 (±0.6)	−7.9
	8.0	250.3	−36 (±4)	−16.4 (±1.4)	−5.8

[a] ±2.0 mV; 25 °C; μ 0.1 M. [b] ±0.1 kcal/mol. [c] Not determined.

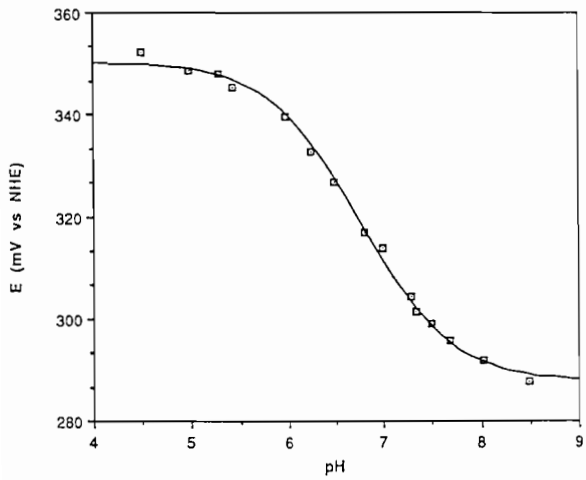

Fig. 3. pH dependence of the reduction potential of Pae azurin (the curve is a fit to eqn. (1)): $pK_a^{ox} = 6.26$; $pK_a^{red} = 7.31$.

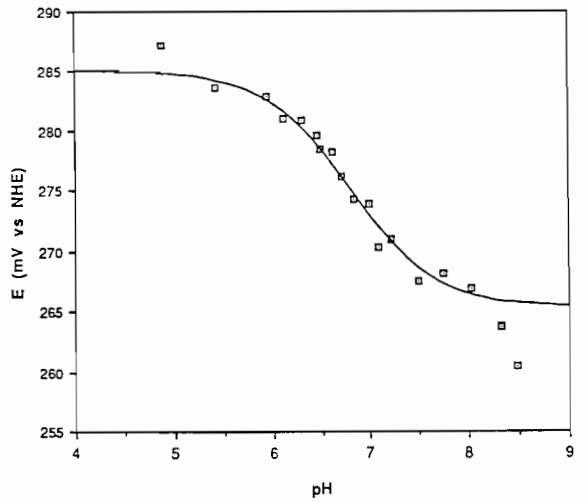

Fig. 4. pH dependence of the reduction potential of Ade azurin (points at pH 4.8, 8.3, and 8.5 were excluded from the fit): $pK_a^{ox} = 6.63$; $pK_a^{red} = 6.97$.

pH from 4.6 to 8.2 induces a change in the copper–His 35 distance of about 1 Å [33, 34]; in addition, the position of the C4 proton of His 46 relative to the His 35 ring changes by 0.5–0.9 Å [35]. These changes occur with a pK_a of 7.3, approximately the same as the pK_a of His 35 in Cu(I) azurin. The crystal structure analysis of Pae azurin indicates that the imidazole ring of His 46 lies parallel to and adjacent to the ring of His 35; thus, His 46 may relay the effects of the movement of His 35 to the copper site (the relative positions of His 46 and His 35 in the Ade protein are shown in Fig. 8). ^{1}H NMR spectra of the reduced protein also indicate pH-dependent behavior for the ligand methionine (Met 121) methyl resonance, which is reduced in intensity and shifts slightly between pH 4.6 and 8.2 [33, 35, 36]. This shift has been interpreted as a movement of ≥ 0.15 Å in the position of the methyl group with respect to the aromatic ring of a nearby phenylalanine (Phe 15) and/or His 46. In addition, ^{13}C NMR results [26] show that the Gly 45 carbonyl ligand resonance titrates with pH, suggesting either that the carbonyl moves slightly, and thus the copper–oxygen bond length probably changes, or that a nearby residue (perhaps His 46) moves with respect to the carbonyl. These changes also occur at the same pK_a as the titration of His 35, implying that the motion of this residue is coupled to the copper site.

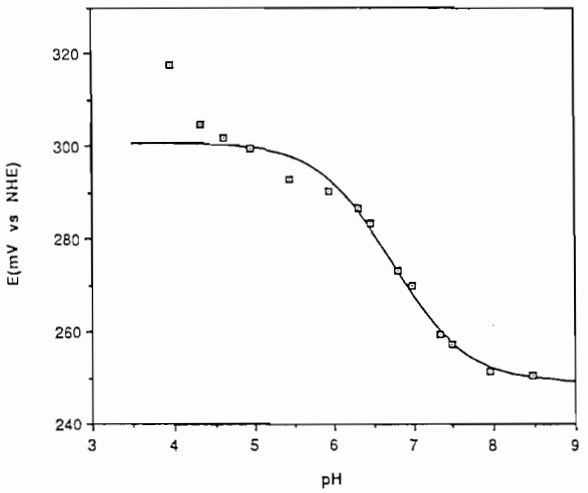

Fig. 5. pH dependence of the reduction potential of Afa azurin (the point at pH 4.0 was excluded from the fit): $pK_a^{ox} = 6.31$; $pK_a^{red} = 7.17$.

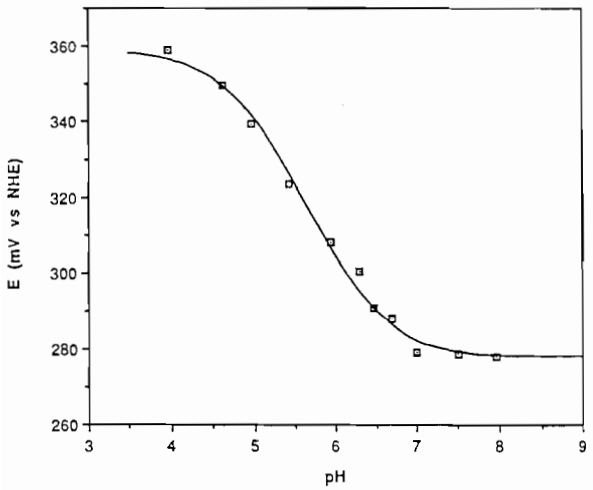

Fig. 6. pH dependence of the reduction potential of Asp azurin: $pK_a^{ox} = 4.95$; $pK_a^{red} = 6.32$.

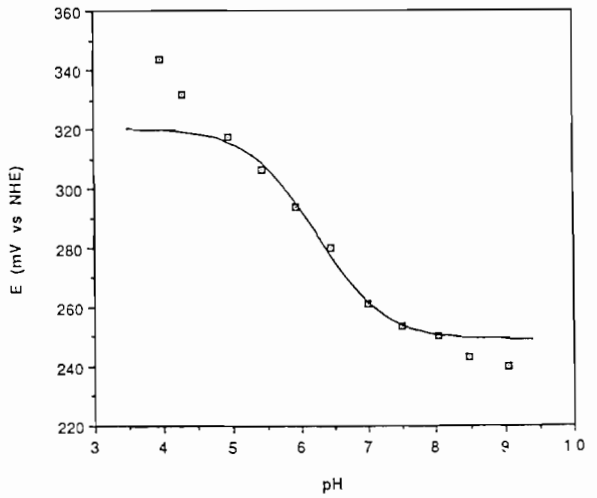

Fig. 7. pH dependence of the reduction potential of Bbr azurin (the two lowest and highest pH points were excluded from the fit): $pK_a^{ox} = 5.65$; $pK_a^{red} = 6.85$.

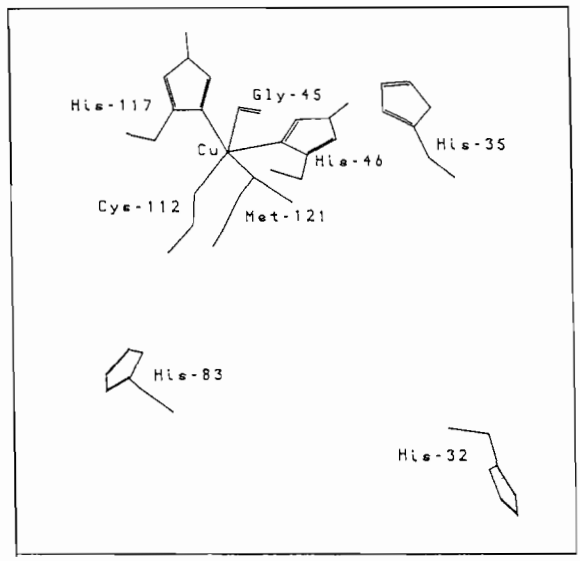

Fig. 8. The copper center and the non-ligated histidines in Ade azurin: Cu–H^+ (His) distances are 7–8 (His 35); 12–15 (His 83); 18–19 Å (His 32) (refs. 5 and 6).

The pH dependence of the Ade azurin reduction potential (Fig. 4) provides a sharp contrast with the Pae protein data. Over the pH range of 5 to 8, Ade azurin exhibits only an 18 mV change in potential, compared to 60 mV for the Pae protein. Although it contains a histidine (His 32) that is not present in Pae azurin (Fig. 8), the more important difference in the Ade protein is the finding from ^{1}H NMR [28] that its His 35 titrates with a pK_a of about 4.8. Because it is near the copper, His 35 is expected to make a much greater contribution to the pH dependence of the potential than either His 83 or His 32. Over the pH range 5 to 8, then, the pH dependence of the reduction potential should be much smaller in Ade than in Pae azurin.

The pK_a values obtained from NMR measurements (Table 2) predict that the protonations of His 32 and His 83 in Ade azurin will raise the potential by 4 and 15 mV, respectively, for a total of 19 mV (eqn. (2)). This calculated ΔE_m is in excellent agreement with the observed change of 18 mV, which means that the pH dependence of the potential can be explained entirely by the interactions of these two histidines with the copper site.

The reduction potential of Afa azurin shows a strong pH dependence (~50 mV change) in the range in which a histidine would be expected to titrate, and there also is a sharp increase below pH 4.5 that could be due to the protonation of carboxylate groups ($pK_a = 3.9$–4.3) (Fig. 5). The relatively large change in the reduction potential with pH appears to be at odds

TABLE 2. pK_a values for histidines of Ade, Pae and Afa azurins

Azurin	His 32		His 35		His 83	
	pK^{red}	pK^{ox}	pK^{red}	pK^{ox}	pK^{red}	pK^{ox}
Ade[a]	6.24	6.17	(4.8)[b]		7.05	6.80
Pae			6.9–7.3[c]		7.57[c]	7.35[c]
			6.9–7.3[d]		7.6[d]	
			6.8–7.8[e]		7.5[e]	
			~7.7[f]	5.5–6.3[f]		
Afa[g]			<4	<4	6.8	6.8

[a]Ref. 28; 36 °C. [b]Estimated value. [c]Ref. 29; 24 °C. [d]Ref. 30; 37 °C. [e]Ref. 26; 24 °C. [f]Ref. 27; 25 °C. [g]Ref. 31; 35 °C.

with NMR work [31] (Table 2). A possible explanation is that there is a pH-dependent structural change that is not linked to the protonation of either His 35 or His 83.

The pH dependence of the reduction potential of Asp azurin gives $pK_a^{red} = 6.32$ and $pK_a^{ox} = 4.95$. The relatively large change in potential (80 mV) indicates that His 35 titrates in this protein and the pK_a values suggest that His 35 is shielded from the solvent as in Ade azurin.

Bbr azurin exhibits a dramatic change (104 mV) in reduction potential over the pH range 4 to 9. Our finding that the potentials do not level off at low pH may mean that there is overlap between the pK_a values of carboxylic acid and histidine side chains. Both the large pH dependence of the potential and the strikingly negative entropy of reduction at pH 8.0 indicate that a redox-linked conformational change induces a particularly severe distortion in the copper center in this protein.

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