pH Dependence of Metal Ion Binding to the Native Zinc Site of Bovine Erythrocuprein (Superoxide Dismutase)

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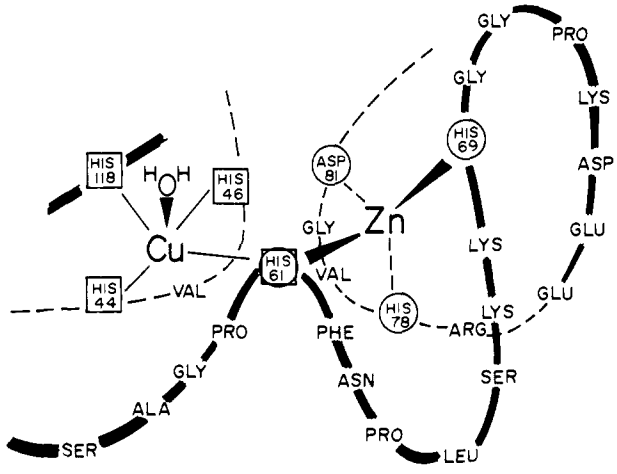
Abstract: A spectroscopic investigation of the behavior of bovine erythrocuprein and its four-copper-substituted derivative below pH 4.5 suggests strongly that the metal ion bound to the native zinc site is reversibly released in the pH range $3.0 \leq pH \leq 4.5$. Published spectra of the copper–cobalt derivative suggest that cobalt is similarly lost from that derivative in the same pH range. Dialysis of the native copper–zinc protein at pH 3.6 resulted in removal of 95% of the Zn but only 5% of the Cu, although the visible, UV, and ESR spectra were only slightly changed from the initial spectra at pH 3.6. The release of free cupric ion from the four-copper derivative at low pH was also determined potentiometrically with use of a copper-selective electrode. The pH dependence of the spectral changes observed for the copper–zinc, four-copper, and copper–cobalt derivatives was virtually identical. The zinc-free derivative of erythrocuprein was also observed to undergo small but distinct changes in its ESR and visible spectra when the pH was changed. These changes occurred with a midpoint pH of 5.0. Below pH 3, copper is also lost from the protein.

Bovine erythrocuprein is a relatively small (mol wt 31 200) globular protein consisting of two equivalent subunits, each of which binds one copper[2] and one zinc[3] in close proximity. X-ray crystallographic studies by the Richadsons[4-6] have shown that the major structural feature of each subunit is an eight-stranded barrel of β structure; two loops of irregular structure are also seen which are connected to the ends of the strands forming the barrel. The Cu^{II} and Zn^{II} ions in each subunit are separated by approximately 6 Å. The Cu^{II} is five coordinate, with the four histidyl imidazole rings that are bound to it (histidines 44, 46, 61, and 118) forming a distorted square plane, the fifth ligand being water. Three of these histidines (44, 46, and 118) have their α carbons in the β-barrel portion of the structure. The fourth, histidine 61, provides an imidazole ring that is deprotonated and bridges the Cu^{II} and Zn^{II} ions. That residue is found in the same single loop of irregular structure that contains those residues that provide the other ligands to Zn^{II}, i.e., histidines 69 and 78 and aspartate 81. The four ligands to Zn^{II} are arranged in a distorted tetrahedral arrangement.



The proximity of the Cu and Zn binding sites in Cu_2Zn_2BE[7]

and the imidazolate bridging ligand are structural features unique to this metalloprotein.[8,9] Whether or not this mode of metal binding is important for its physiological function has not been established. But, judging from the structural data alone, one would expect the chemical properties characteristic of one metal binding site to be influenced by the occupancy of the second site within a subunit. For example, the kinetics and thermodynamics of reactions occurring at the metal in the Cu site are anticipated to be dependent on the presence and nature of any metal ion bound at the adjacent Zn site. Moreover, the metal–protein equilibria of the respective metal binding sites and their pH dependence are also expected to be influenced by the occupancy of the second site with a subunit.

Recent work from our laboratory has shown that the metal ion affinities of the native Cu and Zn binding sites of Cu_2Zn_2BE are pH dependent and that metal ions will migrate from one site to another with considerable facility.[10,11] In this report we describe in detail the behavior of Cu_2Zn_2BE, Cu_2Cu_2BE, and Cu_2E_2BE at low pH and compare it with published reports of the low pH behavior of Cu_2Co_2BE,[12] E_2Co_2BE,[13] and apo BE.[14]

Experimental Section

Protein Preparations. Erythrocuprein was isolated from bovine erythrocytes and purified as described previously.[10] The apoprotein was prepared by dialysis of a solution of Cu_2Zn_2BE against several changes of a solution that was 50 mM in sodium acetate, pH 3.8, and 10 mM in the disodium salt of ethylenediaminetetraacetic acid (EDTA). This treatment was followed by exhaustive dialysis against 0.1 M NaCl in 50 mM sodium acetate (pH 3.8) to remove protein-bound EDTA[15,16] fol-

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(7) Abbreviations used in this paper are the following: Cu_2Zn_2BE designates bovine erythrocuprein as isolated from erythrocytes. In general, X_2Y_2BE designates derivatives of erythrocuprein in which the metal ions X and Y have been subsituted for Cu^{II} and Zn^{II}, respectively (X and Y may be the same). All metal ion oxidation states in these protein designations are 2+ unless denoted otherwise. Cu_2E_2BE designates the zinc-free derivative of bovine erythrocuprein in which the Zn site is vacant (E = empty).
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lowed by exhaustive dialysis against 10 mM sodium acetate buffered at pH 3.8 to remove salt. This treatment routinely yielded protein that contains 1–2% of the amount of Cu and Zn present in the native protein. The concentration of apoprotein was determined from the UV spectrum, using $\epsilon_{258} = 2920\ M^{-1}\ cm^{-1}$,[17] of solutions filtered through a 0.22 μm filter (Millipore). Apoprotein concentrations determined by using the protein assay method of Lowry et al.[18] agreed with those determined by using ϵ_{258}. The Zn-free derivative, Cu_2E_2BE, was prepared as described previously.[10] Unless otherwise mentioned, the derivative of erythrocuprein where Cu^{II} is bound at both the native copper and the zinc binding sites, Cu_2Cu_2BE, was prepared by titrating apoprotein in 0.25 M sodium acetate, pH 5.5, with 4 equiv of $CuSO_4$ as reported elsewhere.[10,19]

Copper and Zn content were determined by use of a Techtron AA4 atomic absorption (AA) photometer. Cu_2Zn_2BE was found to contain 0.85 ± 0.03 equiv of Cu and 0.83 ± 0.04 equiv of Zn per subunit. Metal analysis of the metal-hybrid derivatives of erythrocuprein showed that Cu_2E_2BE contained 0.95 equiv of Cu and 0.03 equiv of Zn per subunit. Similarly, Cu_2Cu_2BE was found to contain 1.8 equiv of Cu and 0.04 equiv of Zn per subunit.

Spectral Studies. Absorption spectra were recorded with a Cary 17D spectrophotometer at ambient temperature. Protein solutions were initially filtered through a 0.22 μm filter (MF-Millipore) and centrifuged after pH adjustments to reduce the amount of light scattering due to small amounts of undissolved protein. All spectra were obtained at least 1 h after pH adjustments were made. Extinction coefficients were calculated on the basis of the concentrations of Cu found by AA analysis, unless mentioned otherwise. Liquid-nitrogen and room-temperature ESR spectra were obtained with a Varian E-12 spectrometer. The microwave frequency was measured with a Hewlett Packard Model 5255A frequency meter, and the magnetic field was calibrated with the signal of Mn^{II} naturally present as an impurity in strontium oxide.[20] The ambient temperature (30 ± 2 °C) ESR spectra were obtained with use of an aqueous ESR cell (Wilmad, WS-812). All ESR parameters are defined according to Malmström and Vänngård.[21] The amount of ESR detectable Cu^{II} in the 30 °C spectra was estimated by comparison of the doubly integrated spectra with those of 1:10 $CuSO_4$:EDTA solutions with Cu^{II} concentrations very close to those of the samples.

Potentiometric Studies. Cu_2Cu_2BE was prepared by titration of apoprotein (buffered at pH 5.5 in 0.1 M sodium acetate) with 4 equiv of $Cu(NO_3)_2$. The resulting protein was dialyzed against 0.1 M $NaNO_3$ for several hours with no change in dialyzate. AA analysis indicated that approximately 4 equiv of copper were present per mol of protein. In two separate experiments, the pH of 3 mL of this solution (0.8 mM in subunits) was lowered by adding 0.01-mL increments of 0.1 M HNO_3. The concentration of free cupric ion was determined using a solid-state copper ion-selective electrode (Orion 94-29) and digital millivolt meter (Orion 901 microprocessor ionalyser). A manual electrode switch (Orion 605) was used to monitor both the copper and pH electrode simultaneously. The pH electrode was calibrated with "certified buffer solutions" (Fisher Scientific) at pH 4.00 and 7.00. The copper electrode was calibrated with solutions prepared by the stepwise dilution of a 0.1 M $Cu(NO_3)_2$ stock solution (Orion) from 10^{-3} to 10^{-8}M. Calibrations were performed immediately before and after the experiment. The copper electrode calibrations and the experiment were carried out under a nitrogen atmosphere. Data were recorded only after the pH and $mVCu^{2+}$ readings had stabilized. All solutions were thermostrated at 25.0 °C.

General Procedures. Doubly distilled deionized water was produced by passing oridinary distilled water through a multibed, high-capacity, demineralization cartridge (Barnstead), followed by a second distillation using a Kontes WS-2 all glass still. All aqueous solutions were prepared with this water. Glassware (cuvettes, pipets, flasks, etc.) was rendered metal free by soaking for at least 2 h in a concentrated H_2SO_4–No Chromix (Ace) solution followed by extensive rinsing with doubly distilled deionized water. Electronic grade (low metal content) acetone (Fisher) was used to dry cuvettes. Dialysis tubing (Spectra/Por 2) was prepared by the method of McPhie[22] to remove metal ions and sulfurous compounds. In general, precautions, as outlined by Thiers,[23] were taken throughout to avoid contamination by adventitious metals. Reagent grade chemicals were used throughout without further purification except where specified. A Corning Model 12 pH meter with a thin (6 mm) Sensorex combination glass electrode was employed for pH measurements. Adjustments of pH were made by adding no more that 5 μL of 1.0 N H_3PO_4 or 1.0 M NaOH at a time to protein solutions. Larger additions were found to produce excessive amounts of denatured protein.

Results

Properties of Cu_2Zn_2BE at Low pH. Lowering the pH of solutions of Cu_2Zn_2BE below pH 4.5 causes the visible, UV, and ESR spectral changes shown in Figure 1. The optical spectral changes include loss of the 420-nm shoulder, a small (~15%) diminution of the ligand field maximum at 680 nm, and a loss of a shoulder at ~290 nm. The ESR spectral changes that occur with decreasing pH include an increase in the hyperfine coupling constant, $\mathbf{A}_{\parallel}$, from ~129 G to ~150 G at pH 3.1, and the coalescing of the $\mathbf{g}_x$ and $\mathbf{g}_y$ values to give a more nearly axial Cu^{II} ESR signal. In each case, the spectra at low pH are very similar to those of Cu_2E_2BE in the same pH region (see below). The ESR and visible spectral changes induced by conditions of low pH for Cu_2Zn_2BE can be reversed by raising the pH from 3.1 back up to 5.6 (see Figure 1C).

Ambient temperature (~30 °C) rather than frozen solution ESR spectra were employed in order to avoid possible temperature-dependent pH fluctuations.[24,25] In addition, in our hands, the X-band ESR spectra at 30 °C of Cu_2Zn_2BE at pH >4.5 gave spectra that were better resolved in the region of $\mathbf{g}_x$ and $\mathbf{g}_y$ than those we obtained at liquid nitrogen temperature. We also made use of the 30 °C ESR spectra to distinguish between protein-bound and free aqueous Cu^{2+}. Copper(II) bound specifically to protein gives an anisotropic ESR signal at room temperature as in frozen solution, since it has a rotational correlation time, τ_R, that is equal to that of the slowly tumbling protein and therefore too long to cause any significant averaging of $\mathbf{g}$ and $\mathbf{A}$ tensors.[26] By contrast, free aqueous Cu^{2+}, or Cu^{II} bound adventitiously to a part of the protein characterized by rapid segmental movement, have τ_R small enough to average $\mathbf{g}$ and $\mathbf{A}$ tensors almost completely and therefore give isotropic ESR signals at room temperature that are not observed in frozen solution. The significance of this will become apparent in the discussion of experiments below.

The time course of the pH-dependent spectral changes was briefly investigated. It was found that equilibrium was reached slowly ($t_{1/2} \simeq 27$ min, 20 °C) when the pH of a 1.0 mM (subunits) solution of Cu_2Zn_2BE in 0.1 M potassium phosphate was rapidly dropped from 4.5 to 3.8. The sluggish rate of this reaction dictated that pH and spectral measurements be made at least 1 h after adjusting the pH with acid in order to ensure that equilibrium had been reached. Raising the pH back to 4.5, however, resulted in a rapid ($t_{1/2} \simeq 15$ s, 20 °C) reversion to the original optical spectrum. Similar results for Cu_2Zn_2BE have been described by Fee and Phillips.[14]

The percent relative change in the optical and ESR spectral parameters for Cu_2Zn_2BE are plotted as a function of pH in Figure 2A. It is clear from this figure that the pH-dependent changes in the optical spectrum occurred concomitantly with those for the ESR spectrum. The midpoint for this transition is difficult to assess accurately since the plot indicates that the maximum change occurred below pH 3.0 where spectral measurements were complicated by the apparent dissociation of Cu^{2+} from its native binding site (see below). The absence of data below pH 3 unfortunately makes it impossible to find a unique fit for a theoretical titration curve. By assuming that the spectral parameters obtained at pH 3 are the maximum relative change possible, we can calculate that the midpoint is pH 3.8, but this can only be taken as an approximation. It should be noted that this pH-dependent transition is clearly too steep to fit a theoretical acid–base titration curve for a single ionizing group, suggesting that the origin of this

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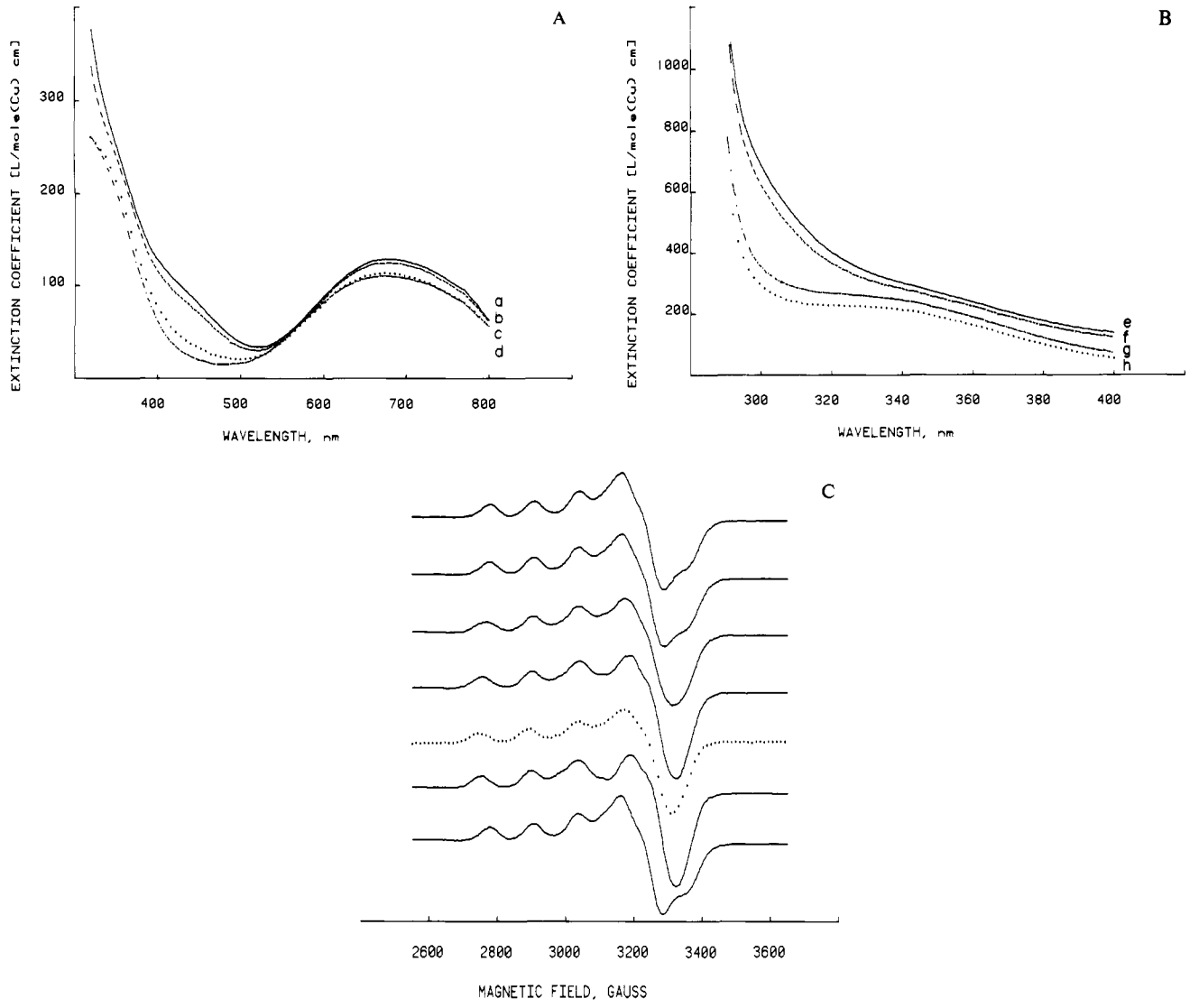

Figure 1. (A) pH dependence of the visible spectrum of Cu_2Zn_2BE. Approximately 0.7 mL of a 2.06 mM (subunits) solution of Cu_2Zn_2BE in 0.1 M potassium phosphate, initially buffered at pH 6.1, was titrated with 1.0 N H_3PO_4. The resulting spectra are of (a) Cu_2Zn_2BE at pH 6.1, (b) Cu_2Zn_2BE at pH 3.9, and (c) Cu_2Zn_2BE at pH 3.6. The bottom spectrum, (d), is of a 2.02 mM (subunits) solution of Cu_2E_2BE in 0.1 M potassium phosphate at pH 3.8. All spectral and pH measurements were made at least 1 h after adjusting the pH. (B) pH dependence of the near-ultraviolet spectrum of Cu_2Zn_2BE. These spectra are for the same Cu_2Zn_2BE and Cu_2E_2BE solutions as in A. In this case, the protein spectra are of (e) Cu_2Zn_2BE at pH 5.8, (f) Cu_2Zn_2BE at pH 3.9, (g) Cu_2Zn_2BE at pH 3.6, and (h) Cu_2E_2BE at pH 3.8. These spectra and those in A above were obtained with microcuvettes so that a small (15%) error exists for the calculated extinction coefficient at each wavelength. (C) pH dependence of the ESR spectrum of Cu_2Zn_2BE at 30 ± 2 °C. These spectra were obtained as part of the same experiment described for A and B. The spectra drawn with a solid line (—) are for Cu_2Zn_2BE and were obtained at the following pH values: from top to bottom pH 6.1, 4.2, 3.9, 3.6, 3.0, and 5.6 (readjusted up from pH 3.0 with 16 μL of 1.0 N NaOH). An ESR spectrum of a 0.85 mM (subunits) Cu_2E_2BE solution at pH 3.6 (···) is also shown for comparison. The instrument settings for the Cu_2Zn_2BE spectra were as follows: microwave power = 30 mW; microwave frequency = 9.41 GHz; modulation amplitude = 8.0 G; receiver gain = 2500; field modulation = 100 kHz; time constant = 0.1 s; and sweep rate = 180 G/min. The Cu_2E_2BE spectrum was similarly obtained except that the microwave power, modulation amplitude, receiver gain, and time constant were 20 mW, 10.0 G, 6300, and 0.3 s, respectively.

transition is not a simple titration of one amino acid residue of the protein. It is not possible to distinguish between reactions involving two or more protonations on the basis of our data, however.

Dialysis of Cu_2Zn_2BE. Dialysis of 2 mL of a 1.0 mM (subunits) solution of Cu_2Zn_2BE (adjusted to pH 3.6) against three 500-mL changes of 0.1 M potassium phosphate at pH 3.6 (adjusted with H_3PO_4) resulted in the removal of 95% of the Zn and only 5% of the Cu (as determined by atomic absorption photometry), although the visible, UV, and ESR spectra were only slightly changed from the initial spectra at pH 3.6. By contrast, dialysis of Cu_2Zn_2BE under the same conditions but at pH 5.8 resulted in a negligible loss of metals. Dialysis was also conducted at pH 3.2, but it was found that besides the loss of 95% of the Zn, the protein also lost 30% of the Cu.

Properties of Cu_2Cu_2BE at Low pH. Lowering the pH of solutions of Cu_2Cu_2BE below pH 4.5 causes the visible, UV, and ESR spectral changes shown in Figure 3. The optical spectral changes in this case are more dramatic than in the case of Cu_2Zn_2BE. They include an ~50% decrease in absorbance at ~800 nm and loss of the broad and moderately intense (ϵ = 2350 M^{-1} cm^{-1}) shoulder at ~300 nm. In addition, the 420-nm shoulder was lost, as in the case of Cu_2Zn_2BE. The visible and UV absorption spectra that resulted after lowering the pH to 3.1 also closely resemble those for Cu_2E_2BE at low pH (see below). In the ESR spectrum, lowering the pH of solutions of Cu_2Cu_2BE below 4.5 can be seen to uncouple the Cu^{II} ions and to produce an ESR spectrum that appears to be a superposition of the signal due to Cu_2E_2BE at low pH ($A_{\parallel}$ = 150 G, Figure 4) and the isotropic signal of free aqueous Cu^{2+}. The changes in the optical and ESR spectrum are shown to be almost completely reversed when the pH is adjusted back up to pH 5.4.

The appearance of free Cu^{2+} upon lowering the pH was also detected potentiometrically with use of a copper–selective electrode.

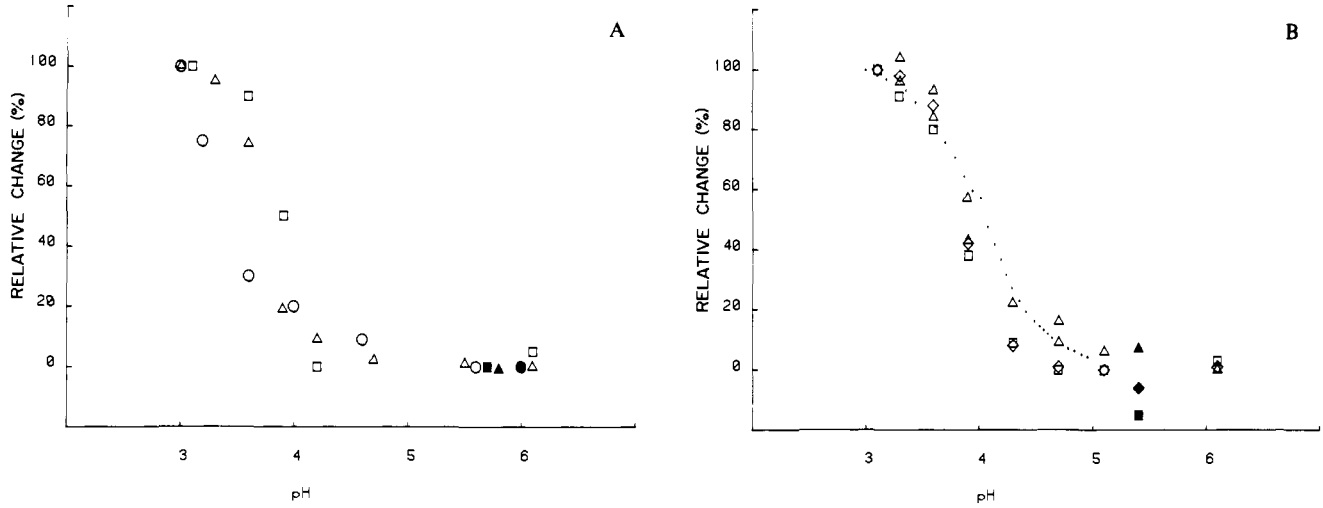

Figure 2. (A) Optical and ESR spectral parameters for Cu_2Zn_2BE and Cu_2Co_2BE as a function of pH: (□) percent relative increase in the hyperfine coupling constant, $A_{\parallel}$, with decreasing pH for Cu_2Zn_2BE, where 0% = 129 G and 100% = 149 G; (△) percent relative decrease in the extinction coefficient at 420 nm, ϵ_{420}, with decreasing pH for Cu_2Zn_2BE, where 0% = 106 M^{-1} cm^{-1} and 100% = 32 M^{-1} cm^{-1}; (○) percent relative increase in ESR detectable Cu with decreasing pH for Cu_2Co_2BE as found by Calabrese et al.[12] In each case the largest observed change was at pH 3.0 so that all measurements are quoted relative to the value at this pH. Data obtained below pH 3.0 are not included for reasons described in the text. The solid data points are for spectral parameters obtained after readjusting the pH up from 3.0 with 1.0 N NaOH. The largest measured range for $A_{\parallel}$ is ±5%. (B) Optical, ESR, and potentiometric parameters for Cu_2Cu_2BE as a function of pH: (△) percent relative increase in the fraction of ESR detectable Cu (at 30 °C) with decreasing pH, where 0% = 0.15 and 100% = 1.0 (all values are measured relative to Cu_2Cu_2BE at pH 3.3; (◇) percent relative decrease in the extinction coefficient at 300 nm, ϵ_{300}, with decreasing pH, where 0% = 2350 M^{-1} cm^{-1} and 100% = 469 M^{-1} cm^{-1}; (□) percent relative decrease in the extinction coefficient at 800 nm, ϵ_{800}, with decreasing pH, where 0% = 170 M^{-1} cm^{-1} and 100% = 105 M^{-1} cm^{-1}. The filled in data points are for the spectral parameters obtained after readjusting the pH up from 3.1 with 1.0 N NaOH. The dotted line represents actual data points for $[Cu^{2+}]$ as determined using a copper-selective electrode, where 100% represents release of 0.84 Cu per subunit (see text).

Lowering the pH of an unbuffered 0.75 mM (subunits) solution of Cu_2Cu_2BE from pH 5.0 to 3.0 caused the concentration of free Cu^{2+} to increase from 0.06 to 0.63 mM (corrected for dilution). This amount of copper corresponds to 84% of that predicted for loss of one Cu per subunit.

The time course for the pH-dependent spectral changes for Cu_2Cu_2BE was also investigated. Rapid decrease of the pH of an unbuffered 0.42 mM (subunits) solution of Cu_2Cu_2BE from 5.6 to 3.6 caused the broad and intense shoulder at ~300 nm to be bleached with $t_{1/2}$ = 20 s at 20 °C. Rapidly increasing the pH to 5.4 restored the original optical spectrum with $t_{1/2} < 1$ s. These rates for Cu_2Cu_2BE are considerably faster than those observed for Cu_2Zn_2BE; a meaningful comparison of these rates is not possible at this time, however, because of the different conditions (buffered vs. unbuffered) for the reactions. It is interesting to note that both derivatives show a much slower change in spectral properties when the pH is decreased than when it is increased.

The optical spectral parameters, amount of ESR detectable Cu^{II}, and amount of free Cu^{2+} detected potentiometrically for Cu_2Cu_2BE are plotted as a function of pH in Figure 2B and are shown to change concomitantly. They also show a pH dependence that is remarkably similar to that for the spectral parameters of Cu_2Zn_2BE (see Figure 2A). The midpoint for the low pH transition for Cu_2Cu_2BE is also estimated to be 3.8. In this case also, the data in Figure 2B clearly do not fit a theoretical curve for a single protonation. It is not possible to distinguish between reactions involving two or more net protonations using all of the data. The potentiometric data taken alone, however, do fit quite well to a theoretical titration curve for a highly cooperative reaction involving a net two-proton change, in agreement with the results of Hirose et al.[27]

Properties of Cu_2E_2BE at Low pH. The spectral properties of Cu_2E_2BE were also modified at low pH. The ESR spectral changes that occur upon decreasing the pH below 6.0 include the increase in the hyperfine coupling constant, $A_{\parallel}$, from ~140 G to ~150 G at pH 3.6, and the coalescing of the g_x and g_y parameters to give a more axial line shape (see Figure 4). In addition, the d–d absorption maximum shifted from ~700 to ~680 nm as the pH was decreased with isosbestic points appearing at ~490 and ~720 nm. The apparent midpoint of the transition was pH ~5. These changes were found to be reversible and independent of whether Cu_2E_2BE was prepared by adding Cu^{II} to apoprotein or by dialyzing Cu_2Zn_2BE at pH 3.6.

Cu_2Zn_2BE at pH below 3.0. When the pH of solutions of Cu_2Zn_2BE was lowered below pH 3.0, we observed optical and ESR spectral changes that were distinct from those that occurred in the range $3.0 \leq pH \leq 4.5$. Lowering the pH below 3.0 caused a bleaching of the ligand field maximum at 680 nm with the solution becoming colorless at pH 2.6. These spectral changes were accompanied by an increase in the isotropic ESR signal characteristic of free aqueous Cu^{2+}, and an attendant decrease in the anisotropic signal of Cu^{II} characteristic of Cu_2E_2BE at pH 3.6. Returning the pH to 4.0 was found to reverse most of these changes, but the isotropic ESR signal that remained indicated that approximately one-third of the Cu^{II} failed to bind to the protein, even after storage at 4 °C for 2 months.

Discussion

We have observed that the visible, ultraviolet, and ESR spectra of Cu_2Zn_2BE and Cu_2Cu_2BE undergo changes when the pH is lowered to pH ~3.0 that can be fully reversed by raising the pH above 4.5. The visible and UV spectra of Cu_2Zn_2BE and Cu_2Cu_2BE at low pH are virtually identical with those for Cu_2E_2BE in this same pH region. A similar observation was made for the ambient temperature (~30 °C) ESR spectra of Cu_2Zn_2BE and Cu_2Cu_2BE. These results suggest that the Zn^{2+} and Cu^{2+} ions at the zinc binding site of Cu_2Zn_2BE and Cu_2Cu_2BE respectively are no longer bound at low pH. The ambient temperature ESR spectral changes and the potentiometric detection of free Cu^{2+} that occurred when the pH of Cu_2Cu_2BE solutions was lowered provide the most dramatic evidence supporting this conclusion. Cu_2Cu_2BE was shown earlier[10] to be ESR silent at room temperature, as is expected for a magnetic triplet.[28] We observed, however, that lowering the pH below 4.5 resulted in a magnetic uncoupling of the Cu^{II} ions and generated an ESR signal

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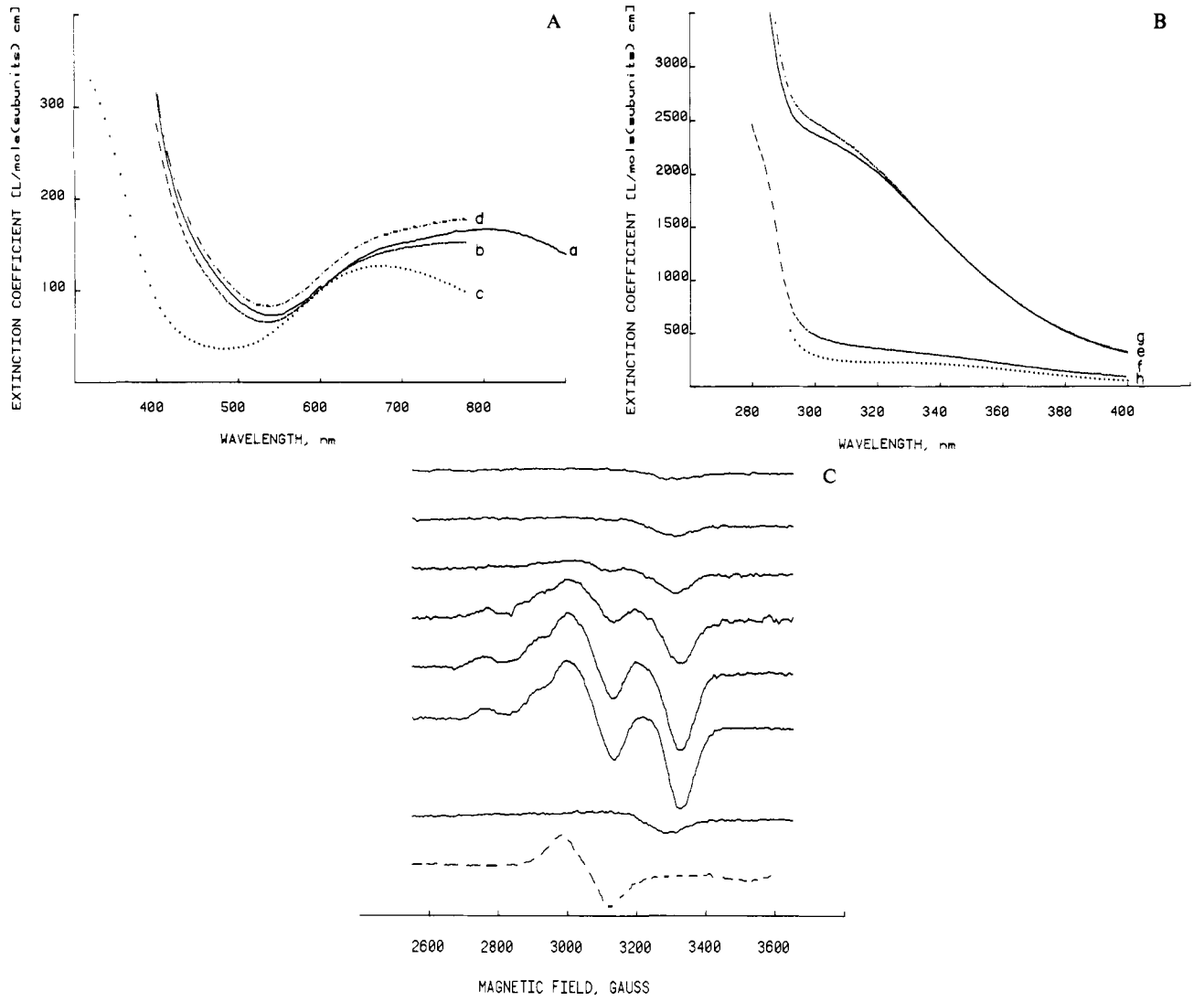

Figure 3. (A) pH dependence of the visible spectrum of Cu_2Cu_2BE. A 3-mL solution of 0.42 mM (subunits) Cu_2Cu_2BE in unbuffered water was titrated with a total of 15 μL of 1.0 N H_3PO_4. The resulting spectra at intermediate pH values are of (a) pH 6.1, (b) pH 4.3, (c) pH 3.1, and (d) pH 5.4 (readjusted up from pH 3.1 with 7.5 μL of 1.0 N NaOH). A matched set of 3-mL quartz cuvettes were used to obtain these spectra. Otherwise all other conditions are the same as described for Figure 1A. (B) pH dependence of the near-ultraviolet spectrum of Cu_2Cu_2BE. The spectra shown here were obtained as part of the same experiment described in A. The spectra at various pH values are as follows: (e) pH 5.1; (f) pH 3.1; and (g) pH 5.4 (readjusted up from pH 3.1). Also, a spectrum of Cu_2E_2BE at pH 3.6 (h) is shown for comparison. (C) pH dependence of the ESR spectrum of Cu_2Cu_2BE at 30 ± 2 °C. The spectra shown here are part of the same experiment described in A and B. The pH values for the spectra drawn with a solid line (—) are from top to bottom: 6.1, 4.7, 4.3, 3.9, 3.6, 3.3, and 5.4 (readjusted up from pH 3.1). The dashed line spectrum is that for a 0.23 mM aqueous $CuSO_4$ solution (---). The ESR instrument settings are as described in Figure 1C for Cu_2E_2BE except that the microwave power was 30 mW.

that appeared to be a superposition of the signal characteristic of Cu_2E_2BE at low pH and the isotropic signal of free aqueous Cu^{2+}. This transformation was almost totally reversed when the pH was raised. Our conclusion that Cu^{2+} was released from Cu_2Cu_2BE at low pH has also been confirmed qualitatively by Hirose et al.[27] by means of equilibrium dialysis. Their results differ from ours in that their experiments were carried out in 0.2 M acetate, a chelator of Cu^{2+}, while our experiments were carried out in unbuffered solutions. The effect of 0.2 M acetate is apparently to shift the onset of copper release to higher pH, i.e., pH 5.3 as opposed to pH 4.5.

In light of the experiments described above for Cu_2Cu_2BE, the low pH transition for Cu_2Zn_2BE was examined further. It was discovered that dialysis at pH 3.6 resulted in the loss of 95% of the zinc and only 5% of the Cu originally present, although the visible, UV, and ESR spectra were only slightly changed from the initial spectra at pH 3.6. Dialysis of Cu_2Zn_2BE under the same conditions but at pH 5.8, however, produced negligible loss of metal ions. Moreover, addition of 1 equiv of zinc acetate per subunit to solutions of Cu_2E_2BE at pH 3.4 caused only small changes in the vis–UV spectra of this sample, but returning the pH to 6.0 gave spectra identical with those for Cu_2Zn_2BE at this pH. We conclude from these experiments that either Zn^{2+} is not bound to the protein at low pH or it is bound in such a way that its presence does not affect the spectral properties of the Cu^{II} chromophore and it is readily removed by dialysis. The dissociation of Zn^{II} from the bridging imidazolate at low pH has also been inferred from electron spin echo and X-ray absorption experiments.[29]

Reexamination of the published[12] vis–UV and ESR spectra of solutions of Cu_2Co_2BE at low pH reveals that they also strongly resemble those of Cu_2E_2BE and free aqueous Co^{2+}. The disappearance of the visible spectral features due to Co^{II} bound at an approximately tetrahedral site suggests that Co^{II} has moved to an octahedral site, such as in $Co(H_2O)_6^{2+}$, where the extinction coefficients of the visible absorption bands are expected to be much lower.[30] Furthermore, the increase in the amount of ESR detectable Cu^{II} in Cu_2Co_2BE as the pH is decreased below pH 4.5 is similar to the situation found for Cu_2Cu_2BE and is consistent

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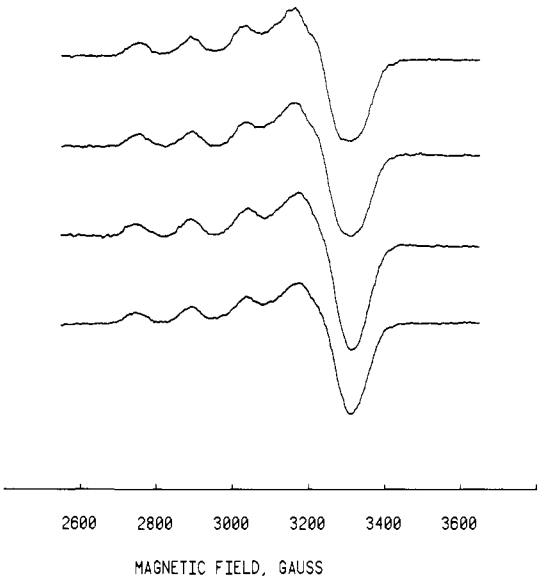

Figure 4. The pH dependence of the ESR spectrum of Cu_2E_2BE at 30 ± 2 °C. The pH values are, from top to bottom: 6.4, 5.6, 4.0, and 3.6. These spectra were obtained for a 0.85 mM (subunits) solution of Cu_2E_2BE in 50 mM potassium phosphate. The ESR instrument settings are as described for Cu_2E_2BE in Figure 1C.

with an uncoupling of the magnetic interaction between the two vicinal paramagnetic ions, Cu^{II} and Co^{II}. The percent relative increase in the amount of ESR detectable Cu^{II} in Cu_2Co_2BE is replotted from the data of Calabrese et al.[12] in Figure 2A. Comparison with the data for Cu_2Zn_2BE (also Figure 2A) and Cu_2Cu_2BE (Figure 2B) reveals that the reversible low pH transition for Cu_2Co_2BE occurs in approximately the same pH region. The apparently slightly lower midpoint (pH 3.3) for the Cu_2Co_2BE transition may be deceptive since by analogy with Cu_2Zn_2BE the rate of spectral changes may be slow and the system may not have reached equilibrium before the spectral determinations were made.

All of the observations described above strongly suggest that the metal ion bound at the native zinc binding site in Cu_2Zn_2BE, Cu_2Cu_2BE, or Cu_2Co_2BE is released in the range $4.5 \geq pH \geq 3.0$ and that the metal ion is rapidly rebound when the pH is raised. Furthermore, the fact that the low pH transition for these three protein derivatives occurs at nearly coincidental pH values regardless of whether Zn^{II}, Cu^{II} or Co^{II} is bound at the zinc binding site suggests the possibility that the reaction may not be a simple competition between metal ions and protons for the donor atoms of the protein ligand. A simple competition reaction is expected to display a different dependence on pH for the three metal ions since consideration of the Irving–Williams order predicts that individual stability constants for binding of Zn^{2+}, Cu^{2+}, and Co^{2+} should be significantly different.[31] Stability constants for binding of metal ions to apo carboxypeptidase and carbonic anhydrases suggest that a modified form of the Irving–Williams order applies even in the case of the relatively rigid, low symmetry metal binding sites of zinc metalloproteins.[32] Further work is required to test the possibility that a pH-dependent conformational change is involved here.

The changes that occur in the absorption, CD,[14] and ESR spectra in the range $3.0 < pH < 4.5$ for Cu_2Zn_2BE certainly reflect a change in the symmetry and/or the ligand field for the Cu^{II} chromophore. Whether this change is due to a change in the protein conformation or is simply the result of substitution of a proton for Zn^{II} at the bridging imidazolate anion is difficult to assess. The 420-nm shoulder in the absorption and CD spectra has been assigned to an imidazolate to Cu^{II} ligand-to-metal charge transfer transition,[9] and its disappearance in the spectra at low pH is consistent simply with protonation of the bridging histidine 61 which is expected to occur when Zn^{2+} is lost. On the other hand, proton NMR spectra of Cu_2Zn_2BE have been found to undergo minor changes in both the aliphatic and aromatic regions as the pH is varied from 6.9 to 3.4.[14] These changes must reflect overall alterations in protein structure because the resonances due to protons near the metal binding region are not visible due to broadening by paramagnetic Cu^{II}.

It is interesting in this regard to compare the pH-dependent behavior of these derivatives of bovine erythrocuprein with derivatives of human carbonic anhydrase.[33] This metalloprotein was found to undergo a sharp pH-dependent transition in its metal ion affinity when the pH is lowered below 4.0, with most of the change occurring between 4.0 and 3.0. This transition is identical for both $^{65}Zn^{II}$ and $^{60}Co^{II}$, in spite of the fact that the stability constants for the binding of these two metals to the apoprotein differ by approximately three orders of magnitude at pH 5.5.[34] In these respects, the pH-dependent release of metal ions from carbonic anhydrase bears a striking resemblance to that observed for binuclear derivatives of erythrocuprein. In the case of carbonic anhydrase, the loss of metal ions is accompanied by major changes in secondary and tertiary structure in both the Zn and Co enzymes, as judged by optical rotatory dispersion (ORD) measurements in this pH range.[33] (But unlike Cu_2Zn_2BE, the transition for carbonic anhydrase is irreversible.[33])

The Cu_2E_2BE derivative was also found to undergo a reversible change in its optical and ESR spectral parameters upon lowering the pH. The midpoint for this sharp transition occurs at pH 5 which is 1.2 pH units more basic than the transition observed for Cu_2Zn_2BE, Cu_2Cu_2BE, and Cu_2Co_2BE. Although it is possible that Cu_2E_2BE may adopt a conformation at pH 6.0 that is significantly different from that of the other derivatives and that the transition that is observed may be unique to this derivative, it is tempting to speculate that these pH-dependent changes in the spectra of Cu_2E_2BE are related in some fashion to the pH-dependent changes observed for Cu_2Zn_2BE, Cu_2Cu_2BE, and Cu_2Co_2BE.

More dramatic changes occur at the copper binding site upon lowering the pH below 3.0. The ESR and visible spectral data for Cu_2Zn_2BE clearly illustrate the dissociation of Cu^{2+} from its native binding site to give free aqueous Cu^{2+} and apoprotein at pH 2.6. This reaction apparently occurs over an unusually narrow pH range since most of the observed changes occur between 3.0 and 2.6. The half-dissociation point for this transition can be approximated at pH ~2.7. Although the midpoints of the pH-dependent metal–protein equilibria associated with the Zn and Cu binding sites are separated by only ~1 pH unit, the sharpness of their pH profiles, especially that for Cu, causes them to be more differentiated than would otherwise be expected. In fact, it is only near the low end of the pH transition associated with the metal ion dissociation from the Zn site that one observes any indication of dissociation from the Cu site such as the small isotropic ESR signal at pH 3.1 for Cu_2Zn_2BE (Figure 1C) and loss of 30% of Cu when dialyzed at pH 3.2.

The loss of Cu from Cu_2E_2BE at pH <3.0 is presumably accompanied by large changes in secondary, tertiary, and quarternary structure of the protein since there is considerable evidence that the subunits of the apoprotein dissociate into two randomly coiled peptide chains at this pH.[14] Thus even the partial reversibility of such a multistep event involving refolding of the polypeptide chain and reassociation of protein subunits, in addition to binding of metal ions, to give native-like spectroscopic properties is remarkable. Other metalloproteins, such as carbonic anhydrase and liver alcohol dehydrogenase, that undergo major changes in secondary and tertiary structure in the pH range where the metal ion dissociates have been found to do so irreversibly.[33,35]

The pH-dependent nature of the metal ion affinities of the two metal ion binding sites of erythrocuprein explains some of the inconsistencies in the early work on metal ion reconstitution of this protein,[14,36,37] and the preparation of metal-substituted de-

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rivatives.[38,39] It also explains the success of the reconstitution of Beem et al.[5,37] in which either Cu^{2+} or Ag^{+} is added to apoprotein at pH 3.8 where it binds predominantly at the copper binding site. Our results indicate that the copper binding site is the only strong binding site available at the pH so that competition with a second binding site in the subunit is thereby avoided.

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Registry No. Superoxide dismutase, 9054-89-1; Zn, 7440-66-6; Cu, 7440-50-8.

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Rates of Cytidine Amino Mercuration by Methylmercury(II) Determined by ^1H NMR Saturation Recovery

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Abstract: Saturation recovery of the amino ^1H resonance is a useful and necessary alternative to line-shape analysis for the determination of rates of cytidine amino mercuration by methylmercury. Reciprocal lifetimes of the free ligand could be obtained by correction of recovery data for dipolar contributions, which were determined from the temperature dependence of recovery in D_2O-H_2O mixtures. Reciprocal lifetimes fall in the range of 2-11 s^{-1} and are influenced by two separate rate processes: At high levels of total CH_3HgOH they are a measure of the forward rate constant of amino mercuration, $k_1 = 1.2 \pm 0.1 \times 10^3$ s^{-1} M^{-1} (free CH_3HgOH), and conform to an activation energy of 17 ± 2 kcal mol^{-1}; at low total [CH_3HgOH], they reflect an additional amino-to-water proton exchange mechanism under conditions associated with significant concentrations of N_3-protonated Cyd (free amino ligand). This latter route is proportional to total cytidine concentration.

Introduction

The interaction of methylmercury with DNA is of interest with respect to both its biological effects and its great value as a tool to study DNA in vitro. Chronic intake of methylmercury at subtoxic levels can produce chromosome damage in humans,[1] presumably through its direct interaction with DNA.[2] In vitro experiments show that methylmercury in millimolar concentrations will melt DNA and at lower concentrations will trap preformed melted DNA segments that are too small to be observed spectrophotometrically.[3,4] The aqueous chemistry of the methylmercury cation, CH_3Hg^+, is very similar to that of the proton, which assumes the crucial role in DNA base recognition through hydrogen bonding.[5,6,20]

In anticipation of detailed kinetic studies at the polymer level, recent studies have been undertaken in this laboratory to provide a *kinetic* comparison of methylmercury and the proton in their interactions with mononucleotides. Mixtures of methylmercuric hydroxide and adenosine, adenylic acid, and cytosine provide ^1H NMR spectra characteristic of low rates of mercuration at the amino site.[7,8] ^1H NMR line-shape analysis of separate resonances of the free amino and mercurated amino exchange pair provided mercuration rates in the range of 2-100 s^{-1}. Resonances of the amino protons involved in the mercuration could be studied directly in the case of adenine but not in the cytidine system, owing to the marked frequency dependence of the amino ^1H NMR line shape in Cyd-CH_3HgOH mixtures, which originate from C_4-NH_2 bond rotation.[8]

The present study was undertaken to explore the method of saturation recovery as a direct means to utilize the amino resonances of cytosine for the estimate of mercuration rates. Because of the large (~1 ppm) frequency separation of the cytidine amino proton resonances of the free and bound (-NH_2) forms,[8] saturation recovery by selective presaturation of either resonance should be sensitive to exchange coupling through mercuration and would be initiated by proton transfer from magnetically unsaturated water as the third site. This should provide estimates of mercuration rates independent of the rotamer equilibria that are characteristic of cytosine. In this report it is shown that dipolar contributions determined from the temperature dependence of recovery in D_2O and H_2O are used as corrections to obtain reciprocal lifetimes in agreement with those of the previous study.[8] These reciprocal lifetimes are a measure of amino mercuration and conform to an activation energy of 17 ± 2 kcal mol^{-1}. At low concentrations of methylmercuric hydroxide, recovery is dominated by an additional amino-to-water exchange mechanism proportional to the C(N_3)-protonated form of cytidine.

Experimental Section

Cytidine (Sigma Chemical Co.) was used as supplied. Methylmercuric hydroxide samples were diluted from a 16.8% stock solution kindly provided as a gift from the Agricultural Division of Morton Chemical Co. Methylmercury concentration was determined by the procedure of Libich and Rabenstein[9] with the Varian HA-100 NMR spectrometer of the Department of Chemistry, University of Hawaii. All pH adjustments were made with perchloric acid with the use of Ingold and Beckman pH electrodes as discussed previously.[7] Nominal pH values of 3, 4, and 5 are all within 0.1 pH unit.

All NMR experiments were performed on the Bruker HXS 360 NMR spectrometer at the Stanford Magnetic Resonance Laboratory, Stanford

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