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Electrostatic Control of the Rate-Determining Step of the Copper, Zinc Superoxide Dismutase Catalytic Reaction

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ABSTRACT: The dependence of the activity of bovine Cu,Zn superoxide dismutase on pH and ionic strength was extensively investigated in the ranges of pH 7.4–pH 12.3 and of ionic strength of 0.02–0.25 M. The results obtained indicate that two positively charged groups having pK values of approximately 10.1 and 10.8 are involved in the control of the activity. On the basis of previous work on the three-dimensional structure and on the chemically modified enzyme, these groups are likely to be lysine side chains, in particular Lys-120 and Lys-134. The oxidation state of the enzyme-bound copper ion at the steady state was found to be the same at either pH 7.4 or pH 11.5. The diffusion of superoxide ion into the active site, which is controlled by the positive charges around the active site itself, appears to be the rate-determining step of the dismutation reaction. NMR measurements of the relaxation rates of F^- showed that this control also applies to the access of F^- to the active site. Comparison of the nuclear relaxation rates of F^- with the enzyme activity indicates that F^- relaxation is controlled by the deprotonation of the group with p$K \sim 10.8$, which appears to be responsible for about 50% of the total activity measured at neutral pH.

The dismutation of the superoxide ion by Cu,Zn superoxide dismutase (SOD)[1] occurs according to (Fielden et al., 1974)

$$ECu^{2+} + O_2^- \xrightarrow{k_+} ECu^+ + O_2 \quad (1)$$

$$ECu^+ + O_2^- + 2H^+ \xrightarrow{k_-} ECu^{2+} + H_2O_2 \quad (2)$$

resulting in the overall process

$$2O_2^- + 2H^+ \xrightarrow[SOD]{k} H_2O_2 + O_2$$

The enzymatic reaction is very sensitive to ionic strength (Rigo et al., 1975; Cudd & Fridovich, 1982). This behavior, the decrease of the enzyme activity observed when positively charged lysines are modified to give neutral or negatively charged residues by carbamylation (Cocco et al., 1982) or succinylation (Marmocchi et al., 1982) and the lack or reversal of the ionic strength effect in enzyme samples with lysine charges neutralized or inverted, respectively (Cudd & Fridovich, 1982), indicate that the rate of the enzyme-catalyzed

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[1] Abbreviations: ECu^{2+}, oxidized form of the active site of Cu,Zn superoxide dismutase; ECu^+, reduced form of the active site of Cu,Zn superoxide dismutase; I, ionic strength; SOD, Cu,Zn superoxide dismutase.

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dismutation is, at least partially, dependent on the electrostatic interaction between O_2^- and some solvent-accessible lysines of the SOD molecules. An open question is if, or to what extent, the copper ion is involved in the rate-determining step. Two facts are to be considered in this regard: (i) the reversible decrease of the activity of the bovine enzyme in the range pH 9–12, where both the spectroscopic parameters of the enzyme-bound Cu^{2+} (Rotilio et al., 1971; Terenzi et al., 1974; Boden et al., 1979) and the lysine side chains of proteins are titrated; (ii) the equal, nearly diffusion-controlled, value of k_+ and k_-, at pH 8.7 (Fielden et al., 1974), which, on account of the different physicochemical characteristics of the Cu^{2+} and Cu^+ ions, suggests the entrance of the substrate into the active site channel as the rate-determining step of the enzyme-catalyzed dismutation.

In this work a new polarographic technique, which permits accurate measurements of SOD activity in the range pH 7–13 (Argese et al., 1984), has been used to study the dependence of the enzyme activity on pH and ionic strength. Similar studies have already been reported (Cudd & Fridovich, 1982) but never in such detail. Moreover, this work includes parallel investigations of the NMR relaxation processes, at the active site of SOD, of F^-, which can be considered as a substrate analogue (Rigo et al., 1979). The results obtained from both activity and nuclear magnetic relaxation measurements permit a more quantitative assessment of the fundamental role played by electrostatic charges in the control of the access of anions to the active site of Cu,Zn superoxide dismutase.

Materials and Methods

All solutions were prepared from analytical-grade chemicals dissolved in doubly distilled water. SOD was isolated from bovine erythrocytes (McCord & Fridovich, 1969). Enzyme activity was measured in air-saturated solutions by a modified polarographic method of catalytic currents (Argese et al., 1984). $^{19}F^-$ NMR measurements were carried out as previously described (Viglino et al., 1979), in the presence of 0.15 M F^- to make negligible the competition of this ion with O_2^- for the active site copper ion (Rigo et al., 1977). Therefore, under these conditions, the effect of F^- on the enzyme activity can be almost completely ascribed to its effect on the ionic strength.

Tris(hydroxymethyl)aminomethane, borate, and *n*-propylamine buffers, I = 0.02 M, were used in the ranges pH 7.4–8.8, 8.8–10.5, and 10.5–12.3, respectively, to obtain a satisfactory buffer index. Activity and NMR relaxivity values were found to be independent of the type of buffer used. The ionic strength was increased starting from the initial value of 0.02 M by the addition of controlled amounts of $NaClO_4$. This salt was used since ClO_4^- does not bind directly to the enzyme-bound copper (Calabrese et al., 1983). All measurements were carried out at 22 °C.

Results

Activity and NMR Relaxation Measurements. The values of the kinetic rate constants, as calculated from activity measurements carried out in the ranges pH 7.4–12.3 and ionic strength of 0.02–0.25 M, are shown in Figure 1. No influence of I on the activity was observed at pH 12 while it was very large at neutral pH values. In particular, at pH 7.6 the increase of I from 0.02 to 0.25 M causes a 70% loss of activity, in line with the hypothesis that electrostatic interactions between O_2^- and the enzyme surface are involved in the rate-determining step of the SOD-catalyzed dismutation of superoxide. Therefore, the decrease of the kinetic rate constant, observed at higher pH values (Figure 1), is likely to be due

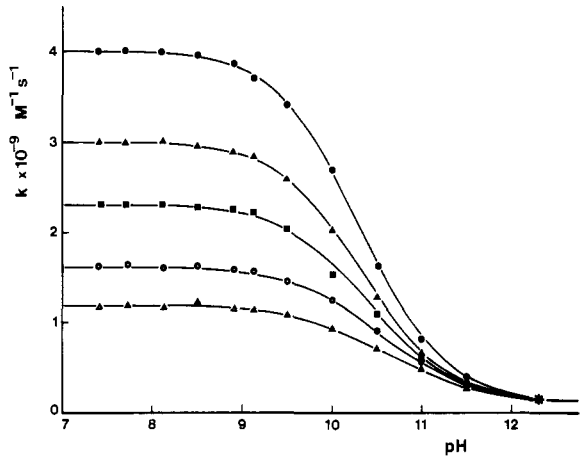

FIGURE 1: Variation of kinetic rate constant of O_2^- dismutation by SOD with ionic strength as a function of pH. The experiments were performed in solutions saturated with triphenylphosphine oxide (Argese et al., 1984) and equilibrated with air. Ionic strength was increased with $NaClO_4$. Full lines represent the best fit of eq 3 to the experimental activity data, see text. I = (●) 0.02, (▲) 0.05, (■) 0.09, (○) 0.16, and (△) 0.25 M.

to the vanishing of the electrostatic driving force as positively charged side chains around the active site are titrated. In fact, the plots of Figure 1 appear as the titration curves of positively charged amino acid groups interacting with O_2^-. The analysis of these plots was carried out on the following bases:

(a) n positively charged amino acid residues (RH^+) contribute to the electrostatic potential around the enzyme-bound copper ion. These residues are protonated at neutral pH.

(b) X_i is the contribution of the residue i to the fraction of the enzyme activity dependent on the electrostatic potential, being, at neutral pH

$$\sum_1^n X_i = 1$$

(c) At alkaline pH values the residues are progressively converted into the neutral form R and the contribution of the residue i becomes

$$X_i \frac{[RH^+]}{[R] + [RH^+]} = X_i \frac{1}{1 + K_i/[H^+]}$$

where K_i is the dissociation constant of the residue i. Therefore, the contribution C of the n charged groups to the enzyme activity is

$$C = \sum_1^n X_i \frac{1}{1 + K_i/[H^+]} \qquad (3)$$

where C ranges from 1, at neutral pH, to 0 at pH 12.4. It follows that the calculated k values of Figure 1 were obtained according to

$$k = (k_n - k_o)C + k_0 \qquad (4)$$

where k_n and k_0 are the experimental values of the kinetic rate constant, at pH 7.4 and pH 12.4, respectively, at a given ionic strength, see Figure 1. The final term of eq 4, k_0, represents the residual activity which is independent of the ionic strength.

The solid lines of Figure 1 are the best fit to the experimental data with eq 4. These lines are the superimposition of the titration curves of two charged groups having $X_1 = X_2 = 0.5$ and $pK_1 = 10.1$ and pK_2 10.8, respectively. The pK_1 resulted independent of I, while pK_2 increased steadily from 10.5 to 11 with increasing ionic strength. The plot of pK_2 vs. $I^{1/2}$ was a straight line with a slope of 1.3, correlation coefficient = 0.99.

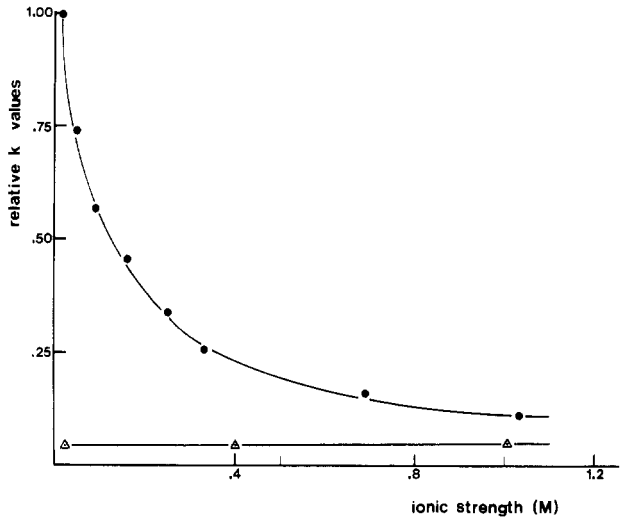

FIGURE 2: Dependence of SOD activity on ionic strength. The measurements were carried out in borate buffer, I = 0.02 M, at pH 9.8 (●) and pH 12.3 (△). Other conditions as in Figure 1.

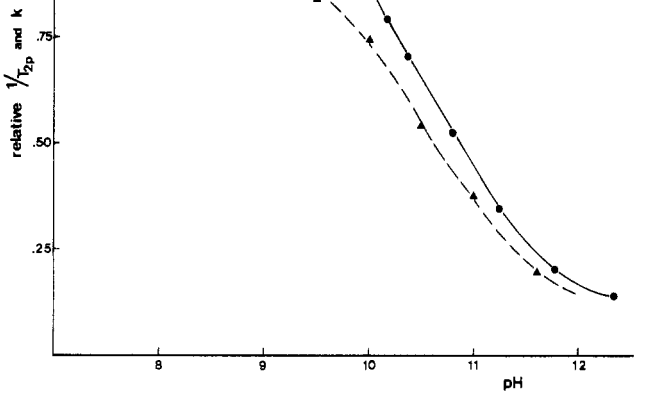

FIGURE 3: Activity and $^{19}F^-$ spin–spin relaxation rate dependence on pH. (●) Activity and (▲) $1/T_{2p}$ measurements were carried out at I = 0.25 M. The F^- concentration was 0.15 M.

Figure 2 shows the results obtained by measuring the SOD activity as a function of ionic strength at pH 9.8 and 12.3, respectively. The k values measured at pH 9.8 approached, by increasing I, the values obtained at pH 12.3, which, on the other hand, were found to be independent of the ionic strength up to values as high as 1 M. These data confirm that the activity is mostly controlled by electrostatic interactions and rule out any secondary effect of I on SOD activity, such as changes of protein conformation, under the experimental conditions used.

The nuclear magnetic spin–spin relaxation rate ($1/T_{2p}$) of $^{19}F^-$ in the presence of SOD was strongly increased by increasing the temperature at both pH 11.4, this work, and neutral pH (Viglino et al., 1979). This result indicates that the $1/T_{2p}$ values are controlled only by the chemical exchange of F^-, in the inner coordination sphere of the active site Cu^{2+}. Measurements of activity and F^- T_2 were carried out in parallel at different pH values, in order to evaluate the extent of overlap of their titration curves. The plots of normalized activity values and of $^{19}F^-$ relaxation rates vs. pH are reported in Figure 3. No overlap was observed in the range pH 8.5–10.5. A similar behavior was also observed for the T_{1p} of the water protons (Terenzi et al., 1974; Boden et al., 1979) and for the $1/T_{1p}$ values of $^{19}F^-$ (Viglino et al., 1979). At pH values higher than 10.5, the activity and $1/T_{2p}$ plots displayed a parallel trend

Table I: $1/T_{2p}$ Values of $^{19}F^-$ at Different pH Values in the Presence of Cu,Zn SOD[a]

ionic strength (M)	T_{2p}^{-1} (s^{-1}) pH 9.0	pH 11.4
0.169	231	13.4
0.361	96	13.3
0.553	43	13.6

[a] The measurements were carried out in the presence of 0.15 M NaF; the ionic strength was increased by the addition of $NaClO_4$. The concentration of SOD was 1.0×10^{-6} M.

Table II: Copper Oxidation State of Cu,Zn SOD under Turnover Conditions

O_2^- generating system	pH	ECu^{2+} (%)
xanthine–xanthine oxidase[a,d]	7.4	54
KO_2–NaOH[b,d]	9.2	52
KO_2–NaOH[c,d]	11.5	49

[a] Conditions: 10^{-3} M xanthine and 0.05 unit/mL xanthine oxidase in phosphate, 0.1 M. [b] Conditions: 0.1 mL of 0.1 M KO_2 in 10^{-2} M NaOH injected into 1 mL of 0.1 M borate. [c] Conditions: buffer was 0.1 M 2-hydroxypyridine; other conditions as in footnote *b*. [d] The reaction mixtures contained also 10^{-4} M EDTA, 2×10^{-6} M catalase, 0.15 M KF, and 2×10^{-6} M SOD. No significant change of the SOD activity was observed after the enzyme turnover.

(Figure 3). The dependence of F^- nuclear spin–spin relaxation rate on I was studied at pH 9.0 and 11.4. The results (Table I) showed that F^- behaved like superoxide, with respect to the enzyme, since increasing the ionic strength decreased the nuclear relaxation rate and the influence of I on the relaxation rate became negligible at pH 11.4.

Turnover Measurements. The fraction of the enzyme-bound copper, which is present at the steady state in the higher oxidation state (ECu^{2+}), was determined by a procedure utilizing $^{19}F^-$ as NMR probe (Viglino et al., 1981). Measurements were carried out at different pH values and in particular at pH 7.4 and 11.5, where, according to the results of Figure 1, electrostatic interactions between O_2^- and the enzyme appear to be predominant and negligible, respectively. Xanthine–xanthine oxidase and an alkaline solution of KO_2, slowly perfused in the reaction vessel (Marklund, 1985), were used to generate O_2^-. From Table II it appears that an equal content of oxidized and reduced copper ion at the enzyme active site is present during turnover, and therefore, $k_+ = k_-$ in the overall pH range explored, irrespective of the varied enzyme activity.

Discussion

The results obtained from turnover measurements (Table II) demonstrated that the constants k_+ and k_- of eq 1 and 2 are equal to each other even at pH values where the lysine-dependent electrostatic guidance of O_2^- into the active site must be scarcely effective. Since the rates of electron exchange between O_2^- and either ECu^{2+} or ECu^+ are very unlikely to be the same, in view of the different charge and ligand geometry of the metal ion in the two oxidation states, this experiment suggests that both the reduction and oxidation of the enzyme copper by O_2^- are controlled by the rate of access of the anion to the active site. The data of Figures 1 and 2 are detailed evidence for the electrostatic factor that dominates this interaction. In fact, they allow estimation of the average charge of the enzyme Z_e experienced by the superoxide molecule approaching the enzyme active site. Z_e values were calculated according to

$$\log k = Z_e Z_O \sqrt{I} + \text{constant} \qquad (5)$$

where Z_O is the charge of O_2^- and k is the measured kinetic

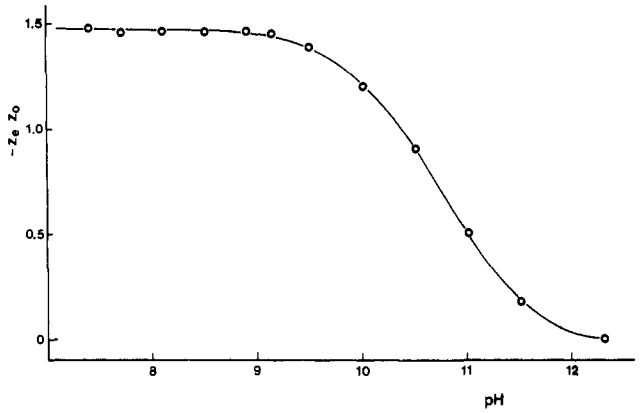

FIGURE 4: Plot of log k vs. Z_eZ_O at various pHs. Z_eZ_O values were calculated according to eq 4 from the experimental data of Figure 1.

rate constant of O_2^- dismutation by SOD. Alberty and Hammes (1958, 1959) derived eq 5 in the case of the interaction between a charged low molecular weight substrate and the active site of an enzyme, the reaction of which was diffusion-controlled. This equation is similar to the Bronsted–Bjerrum equation, which was obtained for small charged molecules in diluted solutions. Even though eq 5 cannot be considered theoretically valid at $I > 10^{-2}$ M, Alberty and Hammes (1958, 1959) found that the relationship between substrate binding rates and I was described as in eq 5 even at ionic strength values as high as 0.2 M. Also in our case the plots of log k vs. $\sqrt{I}$ resulted in straight lines at any pH value, with a correlation coefficient of 0.985.

The Z_e and Z_O values calculated from these straight lines should only be considered as empirical parameters since (i) the ionic strengths used in this work are outside the limits of validity of the Bronsted–Bjerrum theory and (ii) the change of the electrolyte concentration results in variation of the electrostatic interactions within the enzyme, which may alter the three-dimensional structure of the enzyme and the electrostatic potential around the active site. However, in spite of these limitations, the plot of the Z_eZ_O values vs. pH (Figure 4) was almost superimposable to that of the dependence of activity on pH. This is strong evidence for the fundamental role played by electrostatic charge in controlling the enzyme activity in the pH range explored and indicates Cu,Zn SOD as an interesting case of an enzyme, the catalytic action of which obeys just physical control.

The theoretical analysis of the plots of Figure 1 according to eq 4 suggests that two ionizations in the range pH 10–11 account for the full electrostatic control of the enzyme activity. On the basis of a refined crystallographic model of the enzyme (Getzoff et al., 1983), these residues are likely to be identified with Lys-120 and Lys-134. The independence of pK_1 on I and the increase of pK_2 with I suggest that the protonated form of the group having pK_2 in the range 10.5–11.0 interacts with a negatively charged amino acid residue, while the group with pK_1 = 10.1 does not. The computer graphical analysis (Getzoff et al., 1983) of the crystallographic data indicate that both, Lys-120 and Lys-134, are close to a carboxylate residue but only Lys-134 forms a salt link with Glu-131. This would suggest that pK_1 may reflect ionization of Lys-120 and pK_2 is more likely to belong to Lys-134. The relative importance of the two pK's in the control of the rate of access of anions to the active site can be further evaluated from the NMR relaxation measurements of F^-. The data of Figure 3 suggest that the group with pK = 10.8 controls also the rate of $^{19}F^-$ relaxation. The Z_eZ_F value, relative to the fluoride relaxation process, with Z_F being the charge of F^-, can be calculated according to eq 4, from the data of the Table I. The Z_eZ_F value was found to be −2.0 at pH 9.0 and to be zero at pH 11.4. The independence of I of the spin–spin relaxation rate at the higher pH value is interesting since the T_2 values appear to be controlled only by the exchange rate of F^- on the copper ion. The similar control of the access of anions such as O_2^- and F^- to the active site by positively charged amino acid residues explains why F^- was found to be a good probe of the enzyme activity (Rigo et al., 1979).

In conclusion, the data and the analysis reported here give a first quantitative description of the general role of the surface positive charges in determining the rate of access of anions to the active site of Cu,Zn SOD. The pK's of activity-linked groups, most likely lysines-134 and -120, have been calculated to be approximately 10.8 and 10.1, respectively. The rate of the two half-reactions of the catalytic process have been established to be approximately equal between neutral pH and pH 12. Therefore, the reorganization of the first coordination shell of the copper ion occurring in the range pH 10–12 (Rotilio et al., 1978) has no apparent role under the conditions used in this work. At the upper limit of this pH range, other factors such as the charge on Arg-141 (Malinowski & Fridovich, 1979; Cudd & Fridovich, 1982) may come into play. At the lower limit (pH 5–5.5), irreversible alteration of the copper coordination sphere (Fee & DiCorleto, 1973; Rigo et al., 1978) may affect the activity. Therefore, the large majority of studies on Cu,Zn SOD enzyme activity in the range pH 5–12, including anion inhibition and formation of enzyme–substrate complex, should be revisited in light of a mechanism controlled by a merely physical interaction of charges.

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Isolation and Characterization of a New Zinc-Binding Protein from Albacore Tuna Plasma†

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ABSTRACT: The protein responsible for sequestering high levels of zinc in the plasma of the albacore tuna (*Thunnus alalunga*) has been isolated by sequential chromatography. The glycoprotein has a molecular weight of 66 000. Approximately 8.2% of its amino acid residues are histidines. Equilibrium dialysis experiments show it to bind 3 mol of zinc/mol of protein. The stoichiometric constant for the association of zinc with a binding site containing three histidines was determined to be $10^{9.4}$. This protein is different from albumin and represents a previously uncharacterized zinc transport protein.

High levels of zinc, relative to other trace elements, in the tissue of fish have been recognized for a long time (Vinogradov, 1953). Plasma of the albacore tuna (*Thunnus alalunga*), for example, has a zinc level more than 12 times higher than human serum. It is clear from the work of many investigators [see Vallee (1959)] that in biological systems zinc must exist as complexes with organic ligands rather than as free ions in solution. This paper presents our isolation and investigation of the plasma protein in albacore which is responsible for complexing the large amount of endogenous zinc. Fletcher and Fletcher (1978) described a similar Zn-binding protein(s) in winter flounder responsible for binding almost all plasma zinc.

Since zinc in plasma is chelated to endogenous ligands, only high-affinity sites on a plasma-binding protein are likely to be occupied under biological conditions. We have therefore designed experiments to characterize such sites by chelating the zinc with a well-characterized ligand in order to prevent hydrolysis and to form a mathematical basis with which to quantitate the competition for protein uptake of this metal. Experiments using competitive chelation to modulate zinc binding to proteins have been carried out with some enzymes (Cohen & Wilson, 1966; Billo et al., 1978). We have generalized this approach to multicomponent zinc-binding systems and have adapted the traditional Scatchard analysis of equilibrium binding to investigate the high-affinity Zn-binding sites on the tuna protein.

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Materials and Methods

Albacore Plasma. Albacore were captured with hook-and-line in the Pacific Ocean within 200 miles of San Diego during the months of July and August of 1979-1983 aboard the N.O.A.A. research vessel *David Starr Jordan.* Blood was collected immediately in syringes by cardiac puncture. Because blood clotting and clot dissolution occur almost simultaneously in this fish, true "serum" was not obtained; rather, plasma was collected within 30 min of bleeding after centrifugation of red cells at approximately 2000*g* for 15 min at room temperature. Plasma was stored frozen at −18 °C.

Radiozinc Solutions. $^{65}ZnCl_2$ ("carrier-free"; New England Nuclear Corp., Boston, MA; specific activity 6-7 mCi/mg) was diluted into 0.1 N HCl prior to use. Radioactivity measurements were made in a Nuclear-Chicago Model 1085 γ well counter with the spectrometer calibrated to capture the 1.11-MeV emission (±10%) of ^{65}Zn.

Protein Purification. (A) Rationale for Purification. At the beginning of our studies, we were able only to distinguish the tuna plasma Zn-binding protein as one of the two most anodal "albumin-like" bands in sodium dodecyl sulfate (SDS) gel electrophoresis (data not shown). For purification, we therefore explored chromatographic systems that would fractionate these two bands, which seemed similar in size and electrophoretic mobility, on the basis of properties other than charge alone. Metal-chelating resins with iminodiacetate functionalities have been used successfully for affinity chromatography of other metal-binding proteins (Muszynska et al., 1986). Lectin affinity chromatography has been of value in the separation of various non-glycoprotein plasma albumins

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