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Role of the Dimeric Structure in Cu,Zn Superoxide Dismutase

pH-DEPENDENT, REVERSIBLE DENATURATION OF THE MONOMERIC ENZYME FROM *ESCHERICHIA COLI**

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To investigate the structural/functional role of the dimeric structure in Cu,Zn superoxide dismutases, we have studied the stability to a variety of agents of the *Escherichia coli* enzyme, the only monomeric variant of this class so far isolated. Differential scanning calorimetry of the native enzyme showed the presence of two well defined peaks identified as the metal free and holoprotein. Unlike dimeric Cu,Zn superoxide dismutases, the unfolding of the monomeric enzyme was found to be highly reversible, a behavior that may be explained by the absence of free cysteines and the highly polar nature of its molecular surface. The melting temperature of the *E. coli* enzyme was found to be pH-dependent with the holoenzyme transition centered at 66 °C at pH 7.8 and at 79.3 °C at pH 6.0. The active-site metals, which were easily displaced from the active site by EDTA, were found to enhance the thermal stability of the monomeric apoprotein but to a lower extent than in the dimeric enzymes from eukaryotic sources. Apo-superoxide dismutase from *E. coli* was shown to be nearly as stable as the bovine apoenzyme, whose holo form is much more stable and less sensitive to pH variations. The remarkable pH susceptibility of the *E. coli* enzyme structure was paralleled by the slow decrease in activity of the enzyme incubated at alkaline pH and by modification of the EPR spectrum at lower pH values than in the case of dimeric enzymes. Unlike eukaryotic Cu,Zn superoxide dismutases, the active-site structure of the *E. coli* enzyme was shown to be reversibly perturbed by urea. These observations suggest that the conformational stability of Cu,Zn superoxide dismutases is largely due to the intrinsic stability of the β-barrel fold rather than to the dimeric structure and that pH sensitivity and weak metal binding of the *E. coli* enzyme are due to higher flexibility and accessibility to the solvent of its active-site region.

Cu,Zn superoxide dismutases (Cu,Zn-SODs)[1] are metalloenzymes involved in the mechanisms of cellular defense against oxidative damage. They have been found in the cytoplasm of all the eukaryotic cells and in the periplasm of several bacterial species (1–2). Eukaryotic Cu,Zn-SODs are homodimers that contain one atom of zinc and one atom of copper per subunit and catalyze the dismutation of the superoxide anion at a diffusion-limited rate enhanced by electrostatic guidance of the substrate to the active site (3). Moreover, Cu,Zn-SODs possess a very compact structure that is highly resistant to denaturing agents such as urea and SDS and to attack by proteolytic enzymes. Several factors are thought to contribute to the enzyme stability, including the prosthetic metal ions (4), the intrasubunit disulfide bond (5), and the close packing of the hydrophobic interface between the subunits and the two halves of the β-barrel core (6). Structural and functional properties of bacterial Cu,Zn-SODs have not yet been studied in detail, but amino acid comparisons (7–9) and the analysis of the three-dimensional structure of the dimeric enzyme from *Photobacterium leiognathi* (10) have shown that prokaryotic and eukaryotic Cu,Zn-SODs share a conserved ligand stereochemistry and a very similar monomer fold, based on a flattened Greek-key eight-stranded β-barrel. However, despite these similarities, in *P.leiognathi* Cu,Zn-SOD the dimer interface is formed from β-strands that are different with respect to the eukaryotic enzymes (10). This finding suggests that, starting from a putative monomeric SOD precursor, prokaryotic and eukaryotic Cu,Zn-SODs have convergently evolved toward a dimeric structure, which may be important for the enzyme biological function.

Since the discovery of Cu,Zn-SOD, there has been interest in understanding if the dimeric structure contributes to the high catalytic efficiency and to the remarkable stability of this class of enzymes. All attempts to obtain monomeric Cu,Zn-SODs by treatments with detergents (11) or site-directed substitutions of hydrophobic residues at the dimer interface (12) have provided enzymes that display very low catalytic activity and gross alterations of the spectroscopic properties. These dramatic changes probably reflect changes in the tertiary structure consequent to rearrangements of the solvent-exposed hydrophobic dimer interface.

A valuable tool to investigate the role of the dimeric structure in Cu,Zn-SODs is represented by the recently discovered enzyme from *Escherichia coli* (13), which we have shown to be monomeric (9, 14, 15) and which possesses a catalytic activity very close to that of the human and bovine enzymes (9, 14, 16). This finding does not exclude the possibility of a more subtle regulation of activity in the dimeric enzymes but demonstrates that the dimeric structure is not necessary to ensure efficient catalytic activity. Moreover, the different organization of the dimer interface of *P. leiognathi* and eukaryotic Cu,Zn-SODs strongly argues against the existence of a common mechanism of communication between the subunits.

An alternative explanation that could account for a selective advantage of subunit association is that the dimeric structure

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[1] The abbreviations used are: Cu,Zn-SOD, Cu,Zn superoxide dismutase; DSC, differential scanning calorimetry; Cp, heat capacity.

provides a substantial contribution to the stability of the enzyme. The stability of the tertiary structure of the enzyme has been the focal point of several investigations. It was shown that eukaryotic Cu,Zn-SODs are characterized by a high conformational melting temperature and undergo irreversible denaturation at temperature values higher than 70 °C (17–21). To understand if the dimeric structure substantially contributes to the stability of Cu,Zn-SODs, we have undertaken an investigation of the thermal unfolding of the monomeric *E. coli* enzyme by differential scanning calorimetry (DSC). We have found that denaturation of this enzyme is reversible and that the temperature of unfolding is largely affected by small variations of pH. This pH-dependent thermal stability is consistent with a similar dependence of activity upon incubation of the enzyme at alkaline pH and by sensitivity of metal binding and EPR spectrum to pH variations. These findings, together with the observation that urea is able to induce modifications of the copper site, at variance with the insensitivity of the eukaryotic enzymes, suggest that the monomeric structure allows the solvent to alter the microenvironment of the active site more readily than in the dimeric enzymes.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification— E. coli Cu,Zn-SOD was purified from *E. coli* TOP10 cells harboring pPSEcSOD1 (9) as described previously, except that ion-exchange chromatographic steps were performed at pH 7.4 instead than 7.8. Purified Cu,Zn-SOD was dialyzed against 5 mM potassium phosphate, pH 7.0. Protein concentration was evaluated by the method of Lowry *et al.* (22) using bovine serum albumin as standard. Bovine Cu,Zn-SOD (obtained from Sigma) was dialyzed against 20 mM Tris-HCl, pH 7.0, and further purified by ion-exchange chromatography with a Mono-Q HR 5/5 fast protein liquid chromatography column (Pharmacia) equilibrated with the same buffer using a 0–0.1 M NaCl linear gradient. Bovine Cu,Zn-SOD concentration was determined spectrophotometrically using the extinction coefficient $\epsilon = 1.03 \times 10^4\ M^{-1}\ cm^{-1}$ (23). Copper content of protein samples was determined by double integration of the EPR spectra using a Cu^{2+}-EDTA solution as a standard (24).

Preparation of Apo-superoxide Dismutase—Apo *E. coli* Cu,Zn-SOD (devoid of both the copper and zinc ions) was prepared as follows using solutions prepared with Chelex 100 (Bio-Rad)-treated water and nitric acid-treated glassware. Samples containing 20 mg/ml recombinant Cu,Zn-SOD were initially dialyzed for 24 h at 4 °C 1:2,000 against 50 mM sodium acetate buffer, pH 3.8, 2 mM EDTA and then for 24 h against 50 mM sodium acetate, pH 3.8, 0.1 M NaCl to remove excess EDTA. Metal-devoid protein samples were subsequently dialyzed twice against 50 mM sodium acetate buffer, pH 5.0, and finally against 100 mM potassium phosphate buffer, pH 6.5 or 7.8. The complete removal of the zinc and copper ions was verified by metal content analysis of the apo-SOD samples, performed with a Perkin-Elmer 3030 atomic absorption spectrometer equipped with a graphite furnace. The zinc-containing derivatives were prepared by the addition of substoichiometric amounts of zinc to an apo-SOD dissolved in 100 mM potassium phosphate buffer, pH 6.5.

Differential Scanning Calorimetry—A MicroCal MC-2 ultrasensitive differential calorimeter (MicroCal Inc. Northampton, Ma) interfaced to a personal computer was used. Protein samples were dissolved at 1–3 mg/ml concentration, dialyzed against 0.1 M potassium phosphate buffer at the appropriate pH, and deaerated under mild vacuum for 10 min before loading in the sample cell. The reference cell was filled with deaerated dialysis buffer. A scan rate of 60 °C/h was used in all the experiments. At each pH, a buffer *versus* buffer base line run was first obtained and then subtracted from the sample curves. The reversibility of thermal transitions was checked by a second heating cycle of the same sample immediately after ending and cooling the previous scan. Data analysis was performed with the software package (Origin), also supplied by MicroCal, after subtracting a progress line or (in the case of the zinc-reconstituted proteins) a straight line connecting the initial and final temperatures of the overall transition. Thermodynamic data at all pHs were fitted assuming the calorimetric transition to be two-state. The validity of this assumption was confirmed by the good agreement between the experimental and the calculated curves for the native holoenzyme and for the apoenzyme at all pHs. Zinc-reconstituted enzymes were instead fitted assuming non-two-state thermal transitions. For each peak T_m (temperature of maximum heat capacity), ΔH_c (calorimetric enthalpy of denaturation), ΔH_{vH} (van't Hoff enthalpy of denaturation, equal to ΔH_c for a two-state transition), and ΔC_p (difference in heat capacity between the denatured and the native state) were obtained by deconvolution. Two different Origin software options of deconvolutions were used. Native Cu,Zn-SOD and apoprotein were deconvoluted according to a "two-state with ΔC_p" model, which fits the curve after creating an appropriate base line. Zinc-reconstituted proteins were deconvoluted according to a "non-two-state" model as described under "Results." Errors are estimated to be ±0.3 °C for T_m and ±10% for ΔH. The residual activity of scanned samples was measured at 30 °C using the pyrogallol method (25).

EPR Spectroscopy—EPR spectra were recorded at room temperature on a Bruker ESP 300 spectrometer operating at 9 GHz with 100-kHz field modulation. The pH titration was carried out adding small aliquots of diluted NaOH to a protein sample at 2 mM copper concentration. The pH of the sample solution was checked both after the addition of NaOH and at the end of the measurements. The pH of the alkaline-denatured protein was lowered by the addition of small amounts of diluted HCl.

Heat Stability of E. coli Cu,Zn-SOD Activity—Cu,Zn-SOD samples at a concentration of 0.04 mg/ml were incubated at 37 °C in 20 mM Tris-HCl buffers, pH 6.8, 8.0, and 8.8. Aliquots were withdrawn at different times and immediately assayed for residual activity by the pyrogallol method (25). The effect of metal chelators on the enzyme activity upon incubation at 37 °C was analyzed by incubating the enzyme in 20 mM Tris-HCl buffers containing 0.1 mM EDTA. The reversibility of pH-dependent loss of enzyme activity was checked by measuring the activity of the enzyme after the addition of small amounts of a diluted HCl solution.

Urea-induced Active-site Modifications—Experiments were carried out by diluting protein samples (at 0.3 mM copper concentration) in potassium phosphate buffer, pH 7.0, containing variable amounts of urea. Optical spectra of the *E. coli* enzyme were recorded 20 min after incubation at room temperature and checked after 2 h of incubation. The visible spectra did not appreciably change during this incubation period. UV spectra were checked at the end of the experiments. The optical and UV spectra of bovine Cu,Zn-SOD were found to be unaffected by incubation with 8 M urea. Spectrophotometric measurements were carried out with a λ2 Perkin-Elmer spectrophotometer.

RESULTS

Thermal Unfolding of E. coli Cu,Zn-SOD at pH 7.8—To compare the stability of the *E. coli* enzyme to that of dimeric Cu,Zn-SODs, we have initially performed DSC experiments under conditions (100 mM phosphate buffer, pH 7.8, scan rate = 60 °C/h) that have already been used to study the unfolding of several SODs (10, 19–21). The DSC profile of *E. coli* Cu,Zn-SOD under the above-mentioned conditions is shown in Fig. 1*A*. No aggregates were observed after the heating cycle, and a second scan of the same sample showed that, at variance with all the other Cu,Zn-SODs, the denaturation of the enzyme is highly reversible.

Deconvolution of the thermogram shows that it may be described by the sum of two independent two-state transitions (Table I). The first scan profile, its calculated progress base line, and the theoretical curve fitted with the data reported in Table I are shown in Fig. 1*B*, whereas the two deconvoluted transitions, characterized by melting temperature (T_m) values of 52.6 and 65.9 °C, respectively, are shown in Fig. 1*C*. The presence of two peaks in the differential scanning calorimetry profile could be indicative of a two-step denaturation process or of the presence of two species with different stability. Previous studies carried out in different experimental conditions have provided evidence that in the case of the bovine Cu,Zn-SOD, the thermal denaturation of the enzyme is characterized by two partially resolved transitions, representing the oxidized and reduced forms of the enzyme (18, 19). However, we have previously found that under the experimental conditions used in this work, the denaturation of the bovine enzyme as well as of the enzymes from sheep, shark, yeast, and of the two variants from *Xenopus laevis*, is characterized by a single endotherm (21). As the copper content of the protein samples used for DSC

studies was found to be about 0.72 mol of copper/mol of protein and this metal amount was consistent with the ratio of the ΔH_c of the second peak to the total ΔH_c, we analyzed the thermal stability of a protein sample completely devoid of both copper and zinc. Fig. 2*B* shows that the DSC profile of apo-SOD at pH 7.8 is characterized by a single transition with a T_m of 53.6 °C (Table I), very close to that of the less stable species observed in Fig. 1. This finding suggests that the Cu,Zn-SOD purified from *E. coli* consists of a mixture of a holo- (T_m 66 °C) and of an apoenzyme (T_m 53 °C).

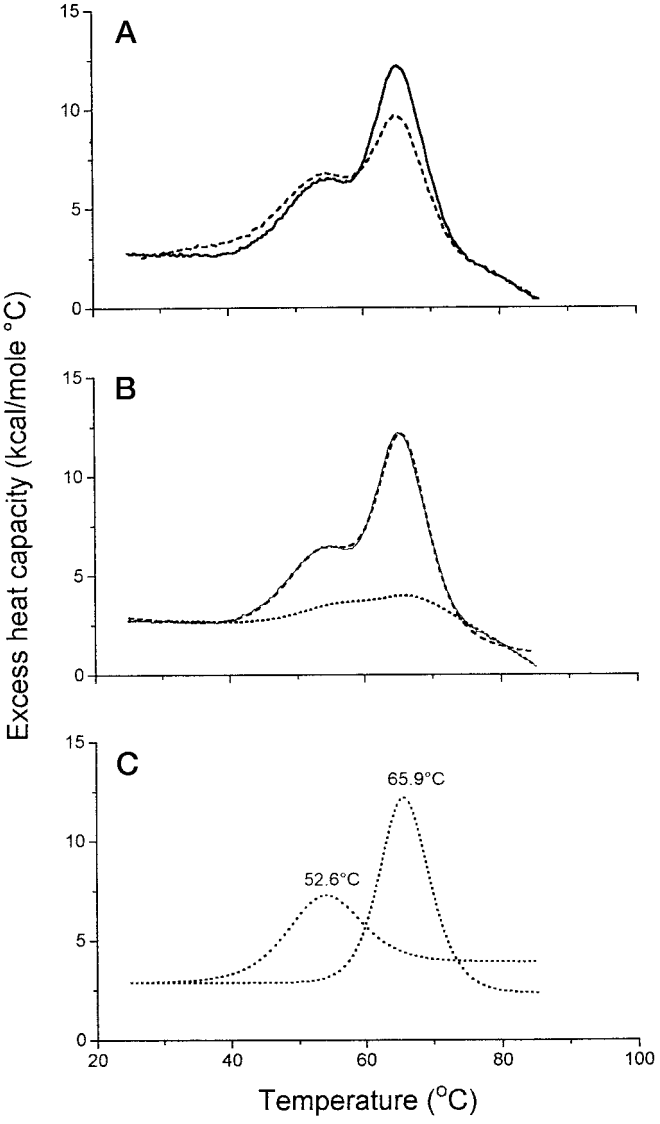

FIG. 1. **DSC scan of *E. coli* Cu,Zn-SOD of *E. coli* in 100 mM phosphate buffer, pH7.8.** All calorimetric traces were corrected by a buffer-buffer base line. *Panel A*, first heating scan (*solid line*); reheating (*dotted line*). *Panel B*, first scan (*solid line*); progress baseline *(dotted line*). *Dashed line*, theoretical curve fitted with the data in Table I. *Panel C*, fitted transition curves.

pH-dependence of E. coli Cu,Zn-SOD Denaturation—Previous preliminary studies on the *E. coli* Cu,Zn-SOD have suggested that the catalytic and structural properties of the enzyme may be negatively affected by alkaline pH. In fact, the enzyme is inactivated in native gels at pH 8.8 (13, 14), and gel filtration chromatography indicates an increase in the hydrodynamic volume with increasing pH values (14). Therefore, DSC scans were carried out in the physiologically relevant pH range of 6.0–7.8, and the results are shown in Fig. 2*A* and in Table I. At variance with the bovine enzyme whose T_m is stable in this pH interval (18), the stability of both peaks that characterize the thermal unfolding of the *E. coli* enzyme progressively increases at pH values closer to the pI (5.6), reaching at pH 6.0 the values of 58.8 and 79.3 °C, respectively. The reversibility of denaturation was also analyzed at pH 6.5, with results similar to those obtained at pH 7.8. The T_m of the apoenzyme at pH 6.5 (see Fig. 2*B* and Table I) is nearly superimposable to that of the first peak at this pH, further confirming the hypothesis that this is due to a molecular species with an incomplete metal content. Interestingly, the denaturation of apo-SOD at pH 6.5 is more than 90% reversible (Table I).

At all pH values, a sharp drop in heat capacity (Cp) was observed after the high temperature transition (Fig. 2*A*). ΔCp values obtained by deconvolution (which are always affected by large errors, depending on the drawing of the progress base line) revealed that although the ΔCp is always positive for the apoprotein peak, it is always negative for the holoenzyme peak (Table I). It appears that an exotherm is superimposed on the unfolding endotherm, probably due to protonation of basic residues of the protein. This phenomenon prevents accurate calculations of the unfolding ΔCps and of other related thermodynamic data.

Zinc Contribution to Cu,Zn-SOD Thermal Stability—The above reported results show that metal ions contribute to the thermal stability of the monomeric Cu,Zn-SOD from *E. coli* to a much lower extent than in the eukaryotic enzymes. In fact, although metal presence increases the T_m of bovine Cu,Zn-SOD nearly 35–40 °C (17, 18, 21), the presence of the two metals increases the T_m of the *E. coli* enzyme only 12–20 degrees, depending on the pH value. The contribution of the zinc ion to the stability of the bacterial enzyme has been further studied by DSC scans on apoprotein solutions treated with variable amounts of zinc at pH 6.5. Upon substoichiometric addition of zinc (0.45 equivalents/mol), the thermal profile (Fig. 3) shows an endotherm centered approximately at 69 °C, which is not

TABLE I

Thermodynamic parameters for holo- and apo-Cu,ZnSOD from E. coli reversible unfolding

The data represent the best fit obtained after subtraction of a base line calculated from the progress of the reaction according to a two-state model ($\Delta H_c = \Delta H_{vH}$) with ΔCp. 1 and 2 refer to the low and high temperature transitions, respectively.

Enzyme	pH	T_m1	$\Delta H1$	ΔCp1	T_m2	$\Delta H2$	ΔCp2
		°C	*kcal/mol*	*kcal/mol °C*	*°C*	*kcal/mol*	*kcal/mol °C*
Holo I scan	7.8	52.6	56.2	1.0	65.9	93.4	−0.5
Holo rescan	7.8	52.9	54.7	0.2	66.6	78.0	−1.8
Holo	7.4	53.1	51.4	1.1	70.2	91.7	−2.9
Holo	7.0	54.4	50.3	1.5	72.6	91.2	−1.0
Holo I scan	6.5	58.5	53.8	0.3	76.0	89.5	−0.8
Holo rescan	6.5	62.8	46.7	−1.8	77.1	69.3	−2.3
Holo	6.0	58.8	45.5	1.0	79.3	85.5	−2.7
Apo	7.8	53.6	61.0	1.0			
Apo I scan	6.5	59.2	66.0	0.7			
Apo rescan	6.5	58.1	60.0	1.3			

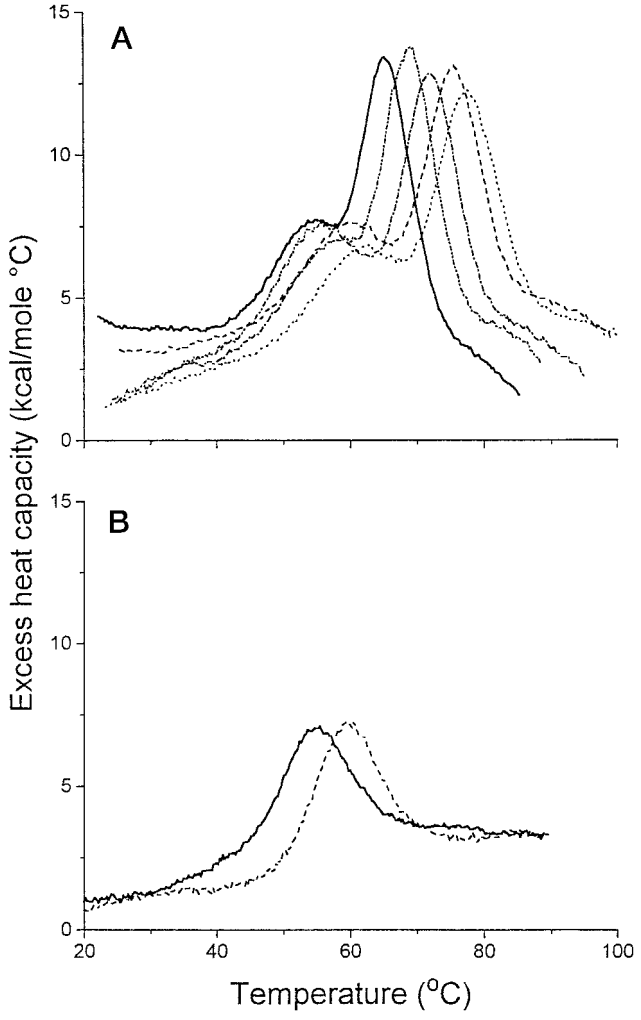

FIG. 2. **pH sensitivity of Cu,Zn-SOD unfolding.** All calorimetric traces were corrected by a buffer-buffer baseline. *Panel A*, native holoenzyme thermal profile in 100 mM phosphate buffer pH 7.8 (—), 7.4 (·· - ··), 7.0 (· - · - ·), 6.5 (- - -), 6.0 (····). *Panel B*, apoenzyme in 100 mM phosphate buffer, pH 7.8 (—), and 6.5 (- - -). Thermodynamic data obtained by deconvolution are reported in Table I.

substantially changed in the presence of 0.9 equivalents of zinc. Deconvolution revealed the presence of two transitions under the peak (Table II), probably corresponding to the apo and the zinc-containing enzyme. The finding that the zinc-containing protein is more stable than the apoenzyme confirms the hypothesis that the recombinant enzyme purified from the periplasm of *E. coli* cells is a mixture of holo- and apo- (devoid of both metals) enzyme. Unlike the bovine enzyme, the addition of 1.35 mol of zinc/mol did not further increase the stability of the enzyme but rather caused the appearance of a new species showing an irreversible melting transition at low temperature (43.7 °C). Such a novel peak could be due to the binding of zinc to a spurious site or to a zinc-induced distortion of the copper binding site. Analysis of the thermal profiles obtained from the zinc-reconstituted protein was complicated by the difficulty of drawing appropriate base lines. Indicative data, shown in Table II, were obtained by subtracting from the thermograms a linear base line connecting the initial and final temperatures of the whole transition. Deconvolution data show that ΔH_c and ΔH_{vH} reach equal values (a necessary condition for a two-state transition) only when more than 1 zinc atom is bound/mol of protein.

Recovery of SOD Activity after DSC Scans and Effect of Incubation at Different pH on the Enzyme Activity—At the end of DSC scans at pH 6.5 and 7.8, the enzyme was diluted to 40 μg/ml with the same buffers and assayed for residual activity. The enzyme scanned at pH 6.5 regained approximately 85% activity, whereas the sample scanned at pH 7.8 recovered only 24% of its initial activity. Analysis of the scan profiles of the two samples indicated that the lower recovery of activity at pH 7.8 was not explained by a lower reversibility of unfolding at this pH (Table I) but suggested that at alkaline pH the enzyme refolded to a less active conformation. This was confirmed by the observation that the activity of the sample scanned at pH 7.8 increased more than twice upon lowering the pH to 6.5 by the addition of a few drops of a diluted HCl solution. This result prompted us to further investigate the effect of pH on the enzyme activity. Samples of the enzyme were incubated at 37 °C up to 3 h in different buffer conditions, and the activity of the enzyme at different times of incubation was assayed by the pyrogallol method. As shown in Fig. 4*A*, the enzyme underwent

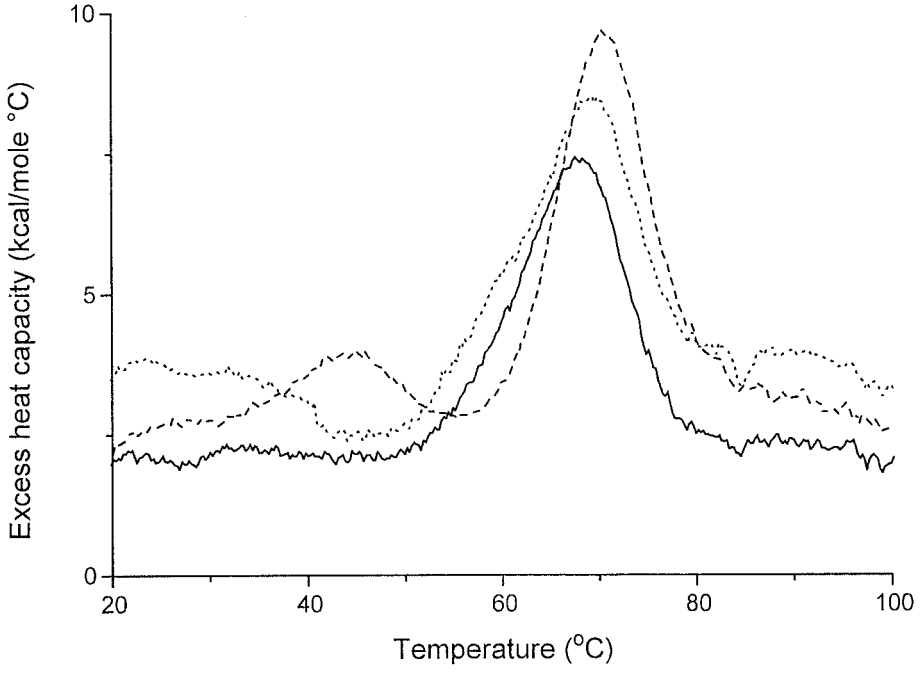

FIG. 3. **Zinc-induced stabilization of *E. coli* apo-Cu,Zn-SOD in 100 mM buffer pH 6.5.** All calorimetric traces were corrected by a buffer-buffer base line. Zinc ions/mol of protein: 0.45 (*dotted line*); 0.9 (*solid line*); 1.35, (*dashed line*). Thermodynamic data obtained by deconvolution are reported in Table II.

TABLE II
Thermodynamic parameters for the zinc-reconstituted protein unfolding

The data represent the best fit obtained by deconvolution according to a non-two-state model ($\Delta H_c \neq \Delta H_{vH}$). 1 and 2 refer to the low and high temperature transitions, respectively.

Zinc/protein	T_m1	ΔH_c1	$\Delta H_{vH}1$	T_m2	ΔH_c2	$\Delta H_{vH}2$
	°C	kcal/mol	kcal/mol	°C	kcal/mol	kcal/mol
0.45	61.2	24.7	72.8	69.7	48.6	89.9
0.90	62.9	30.6	67.8	69.1	40.4	92.8
1.35	43.7	15.3	65.3	70.7	80.5	80.5

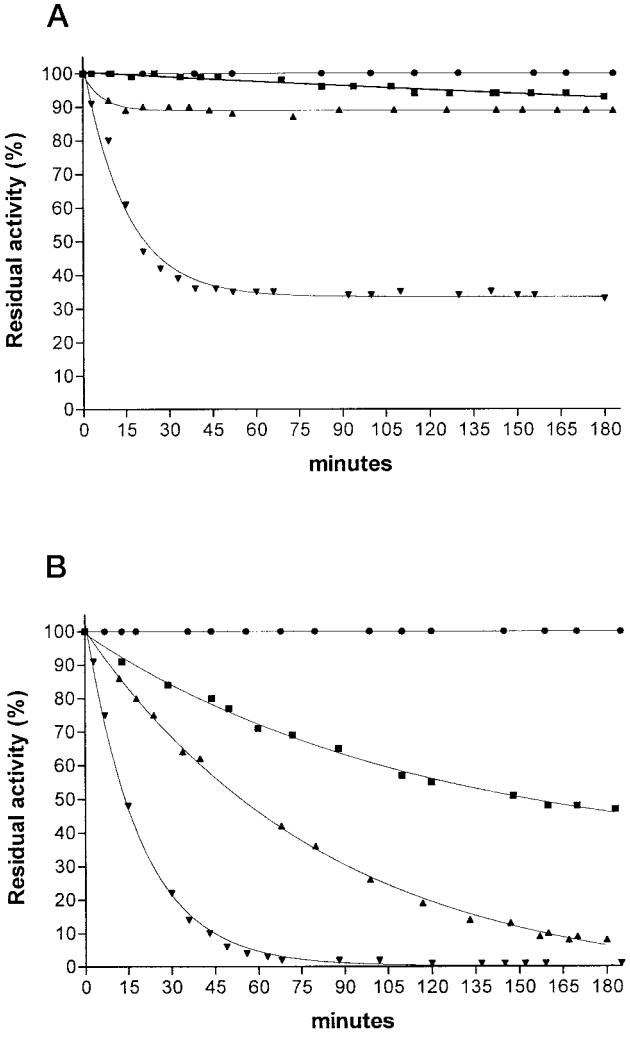

FIG. 4. **Decrease of *E. coli* Cu,Zn-SOD activity as a function of pH.** The enzyme was incubated at 37 °C in 20 mM Tris-HCl buffer (*A*) or in 20 mM Tris-HCl, 0.1 mM EDTA buffer (*B*). Aliquots were withdrawn at the indicated times and immediately assayed by the pirogallol method to measure residual activity. ■, pH 6.8; ▲, pH 8.0, ▼, pH 8.8, ●, bovine enzyme in the 6.8–8.8 pH range.

a progressive, pH-dependent loss of activity, leveling out at 38% of the starting activity value in Tris-HCl, pH 8.8. The pH-dependent loss of activity of the *E. coli* enzyme incubated at alkaline pH was more than 90% reversible. The bovine enzyme did not display any loss of activity in all conditions tested. When incubation of the enzyme was carried out in the presence of EDTA, the decrease of activity was much faster and completely irreversible (Fig. 4*B*).

Solvent-induced Perturbations of the Active-site Region—The reversibility of the pH-dependent denaturation of the enzyme was also studied at room temperature by EPR spectroscopy

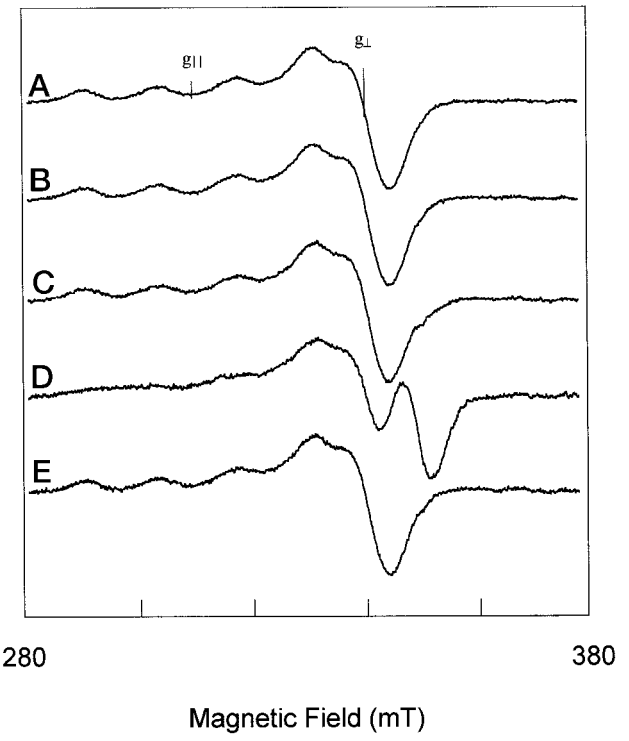

FIG. 5. **EPR spectra at room temperature of 2 mM *E. coli* Cu,Zn-SOD as a function of pH.** *A*, pH 7.0; *B*, pH 8.5; *C*, pH 9.5; *D*, pH 10.5; *E*, sample *D* lowered to pH 7.0. Setting conditions: 20 milliwatt microwave power, 9.83 GHz microwave power; 1.0 millitesla, modulation amplitude.

(Fig. 5). The EPR spectrum of the *E. coli* Cu,Zn-SOD is slightly more axial ($g_{\|}$ = 2.260, $g_{\perp}$ =2.064) than the corresponding spectrum of the eukaryotic enzymes (26). It did not change in the 5.5–10 pH range, but at pH 10.5 a copper-biuret-type EPR spectrum appeared, suggesting a regular square-planar coordination of the copper ion, as it is bound by four peptide nitrogens in a denatured protein (26). However, this pH-induced modification of the EPR spectrum, which occurs at much lower pH values with respect to other Cu,Zn-SODs (26, 27), is completely reversible by lowering the pH to neutrality, at variance with the cases of alkaline denaturation of other copper proteins. Such a change of the EPR spectrum was paralleled by alteration of the enzyme activity, which at pH 10.5 was below the detection limit of the assay method used and was fully recovered by adjusting the solution pH to neutral values.

The lower rhombicity of copper geometry of the *E. coli* enzyme is reflected by the blue shift of the peak of the copper absorption band to 663 nm (Fig. 6, spectrum *a*), instead of 680 nm, typical of all the eukaryotic Cu,Zn-SODs. At variance with the well established stability of eukaryotic Cu,Zn-SODs in the presence of high concentrations of urea (28), the copper absorption band of the *E. coli* enzyme was further blue-shifted as a function of the urea concentration, reaching 630 nm in 6 M urea (Fig. 6). The UV spectrum of the enzyme was not substantially affected by urea and displayed minor modifications only in 8 M urea (not shown). All the modifications were fully reversible, as the original spectrum of the *E. coli* enzyme was completely restored by dilution of the samples to urea concentrations lower than 4 M (not shown).

DISCUSSION

The DSC thermograms of the *E. coli* Cu,Zn-SOD purified from cells overexpressing the enzyme consist of two well resolved components whose transition temperatures are both highly influenced by pH. According to the denaturation profiles

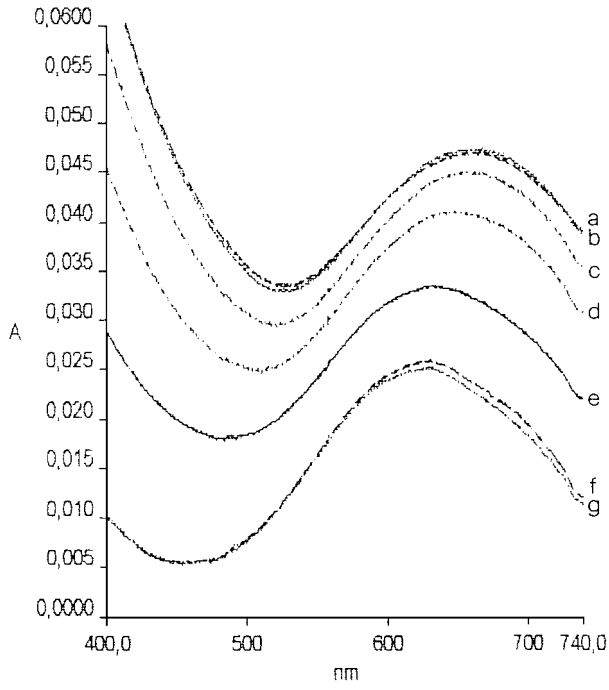

FIG. 6. **Urea-induced perturbation of the *E. coli* Cu,Zn-SOD active site.** The enzyme (0.3 mM copper concentration) was incubated at the following urea molar concentrations: *a*, 0; *b*, 2; *c*, 4; *d*, 5; *e*, 5.5; *f*, 6; *g*, 8. *A*, absorbance.

of the apo and of the copper-free zinc-containing derivatives and to the different ΔCp values of the two forms (negative for the first component and positive for the second one), we assign the first peak to the presence of a metal-free form and the second one to holo-Cu,Zn-SOD. The presence of a consistent amount of apoprotein is not usual in Cu,Zn-SOD preparations as the eukaryotic enzymes are generally purified with a nearly complete zinc and copper complement comprised of between 1.5 and 2.0 atoms of metal/protein dimer. However, the results reported in Fig. 4 indicate that, at variance with eukaryotic Cu,Zn-SODs, the *E. coli* enzyme has a lower affinity for the active-site metals, both of which can be removed by EDTA at pH > 6 (Fig. 4*B*). This finding is in agreement with a recent report showing that the metals are easily lost by the enzyme also at room temperature and that the addition of zinc or copper to the enzyme solution increases the recovery of activity of the heat-inactivated enzyme (29).

Several DSC studies have been focused on the thermal stability of different Cu,Zn-SODs (10, 17–21), but the results obtained by different authors are not always directly comparable to each other, as the unfolding of the enzyme is significantly influenced by solvent composition and scan rate (18). Therefore, to investigate the contribution of the quaternary structure to the stability of the enzyme, we have performed DSC experiments under a condition that has been used previously to study the thermal stability of a number of Cu,Zn-SODs. In Table III, the T_m values of some Cu,Zn-SODs variants are reported, showing that the *E. coli* enzyme is significantly less stable than all the other variants so far studied. The difference is particularly evident with respect to the bovine, ovine, and human enzymes, which possess a T_m about 20 °C higher, whereas the T_m of the monomeric enzyme is less than 8 degrees lower than that found for the Cu,Zn-SOD from yeast, *X. laevis*, and the prokaryote *P. leiognathi*. This finding suggests that subunit interactions may provide a contribution to the thermostability of this class of enzymes, but also that the compactness of the β-barrel fold is sufficient to confer a remarkable conformational stability to the monomeric Cu,Zn-SOD. This statement is reinforced by DSC experiments carried out at lower pH, which showed that the stability of the Cu,Zn-SOD from *E. coli* is heavily influenced by pH in the relatively narrow interval roughly corresponding to the physiological pH range of an enteric bacterium. In fact, we have found that the T_m of the holoprotein at pH 6.0 is 79.3 °C, about 14 °C higher than the T_m observed at pH 7.8, whereas the difference for the apoenzyme is 6 °C between this two pH values. Such a pH-dependent stability is a peculiar feature of the *E. coli* enzyme, as the T_m of the bovine enzyme is stable in the 6.0–8.0 pH range (18), and it is well established that the spectroscopic and catalytic properties of all the eukaryotic Cu,Zn-SODs are stable in this pH range (26, 30, 31). It is worth noting that the T_m at pH 7.8 of the apoenzyme from *E. coli* is very similar to that of the bovine enzyme, and that metals increase the T_m of the protein only 13 °C compared with the 35 °C increase reported for the bovine enzyme under the same conditions (21). Moreover, it was previously shown that metal-induced reorganization of the active site is critical for the pH stability of eukaryotic Cu,Zn-SODs, as the stability of the bovine apoenzyme is pH-dependent, reaching its maximum T_m value near pH 6.0 (18). These observations suggest that differences in the active-site structure of eukaryotic and prokaryotic Cu,Zn-SODs are major determinants of their different thermal stability.

TABLE III
T_m of different Cu,Zn-SODs

With the exception of the T_m value of the Cu,Zn-SOD from *E. coli*, all the other T_m values reported here are taken from literature data (10, 20, 21) and refer to Cu,Zn-SODs scanned under identical experimental conditions (100 mM phosphate buffer, pH 7.8; scan rate = 60 °C/h).

SOD	T_m
	°C
Ox	88.0
Sheep	87.1
Human	83.6
Shark	84.1
Yeast	73.1
X. laevis A	71.1
X. laevis B	76.8
P. leiognathi	71.0
E. coli	65.9

The high pH sensitivity of the monomeric enzyme is confirmed by a titration of the EPR spectrum as a function of pH, showing that the protein-bound copper assumes a biuret type of conformation at much lower pH values (10.5 *versus* 12.5) than the Cu,Zn-SODs from ox (26) and *P. leiognathi* (27). These findings, together with the previously reported inactivation in native polyacrylamide gel electrophoresis at pH 8.8 (13, 14), clearly demonstrate that the enzyme from *E. coli* is more sensitive to alkaline denaturation than the highly homologous dimeric protein from the bacterium *P. leiognathi*. Moreover, we have also observed that the enzyme loses its catalytic activity upon incubation at 37 °C, and that the extent of this inactivation is pH- and buffer-dependent. The enzyme activity is stable at pH 6.0, but at higher pH values, the activity significantly drops to reach 38% of its initial value in Tris-HCl buffer, pH 8.8. Also the eukaryotic Cu,Zn-SODs are inhibited at alkaline pH, but this inhibition, due to protonic equilibria of basic residues near the active site of the enzyme (31), occurs at higher pH values. Therefore, the time-dependent loss of activity of *E. coli* Cu,Zn-SOD at much lower pH values is rather indicative of a conformational change involving the active-site structure. Inspection of *E. coli* Cu,Zn-SOD amino acid sequence (8, 9) and of the recently solved three-dimensional structure of the dimeric enzyme from *P. leiognathi* (10) may provide some clues in this regard. Bacterial enzymes share with the monomer of

eukaryotic Cu,Zn-SODs a conserved β-barrel topology (7–10) but display differences in the organization of the major loops. In particular, the architecture of the active site is largely modified by a 4-amino acid deletion in loop 7,8 and a 7-amino acid insertion in the "S-S" subloop that creates a very long and solvent-exposed loop, containing a cluster of charged residues that are strictly conserved in all the bacterial Cu,Zn-SODs. These charged residues have been proposed to be involved in substrate steering to the active site (10) but could also play an important structural role. The lack of dimeric structure in the *E. coli* enzyme, which is characterized by a highly polar molecular surface (9), could increase flexibility of this loop and explain its unusual active-site accessibility. Alterations in the active-site structure of the monomeric enzyme may be inferred by the EPR and optical spectra that are clearly different with respect to that of the eukaryotic and *P. leiognathi* enzymes (26, 27). All these observations suggest that the copper environment of *E. coli* Cu,Zn-SOD may be more susceptible to solvent modifications than that of the dimeric enzymes. This is confirmed by the urea-induced alteration of the copper site of the *E. coli* enzyme, as deduced by the changes of its optical spectrum as a function of urea concentration. It is interesting to note that the monomeric derivatives of human and wheat germ Cu,Zn-SODs display a more axial coordination of the copper chromophore (11, 12) and an increased distance of the water-coordinated molecule (12), thus suggesting that the dimeric structure may be important to reduce flexibility of the active site also in Cu,Zn-SODs lacking the 7-residue insertion found in the bacterial enzymes.

One of the most intriguing features of the *E. coli* enzyme evidenced by DSC experiments, pH titration of the EPR spectrum, and recovery of activity of the enzyme incubated at alkaline pH is its efficient reversibility of unfolding. All dimeric Cu,Zn-SODs have been shown to undergo irreversible aggregation after exposure to temperatures higher than T_m, with the only exception being the yeast enzyme, which partially refolds to a new conformation of lower stability than the native one (10, 17–21). At least two factors are known to be involved in this phenomenon: metal ions and free cysteines. It is well established that incorrect disulfide bond formation, concomitant with cysteine oxidation probably enhanced by metals, is a cause of aggregation of heat-denatured proteins (32). In the case of Cu,Zn-SODs it has been observed that the denaturation of the bovine apoenzyme is partially reversible (18) and that the substitution by site-directed mutagenesis of Cys residues not involved in disulfide bonds, although producing moderate effects on conformational stability (19, 20, 33), greatly increases the reversibility of thermal denaturation of human and bovine Cu,Zn-SODs thermal denaturation. Moreover, the only dimeric Cu,Zn-SOD, which shows a partial reversibility after a DSC scan is the yeast enzyme, which does not contain free cysteines (17). The Cu,Zn-SOD from *E. coli* does not contains cysteines that could cause formation of incorrect disulfide bonds, and this undoubtedly contributes to its efficient refolding. However, the dimeric enzyme from *P. leiognathi* also denatures irreversibly (10) despite lack of free cysteines. Probably, solvent-induced distortion of the hydrophobic subunit interface plays a role in the aggregation of heat-denatured dimeric Cu,Zn-SODs, whereas the polar nature of the *E. coli* Cu,Zn-SOD surface favors its efficient refolding to the native structure.

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Role of the Dimeric Structure in Cu,Zn Superoxide Dismutase: pH-DEPENDENT, REVERSIBLE DENATURATION OF THE MONOMERIC ENZYME FROM ESCHERICHIA COLI

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