A Histidine-rich Metal Binding Domain at the N Terminus of Cu,Zn-Superoxide Dismutases from Pathogenic Bacteria

A NOVEL STRATEGY FOR METAL CHAPERONING*

Andrea Battistoni‡§, Francesca Pacello‡, Anna Paola Mazzetti‡, Concetta Capo‡,
J. Simon Kroll¶, Paul R. Langford¶, Assunta Sansone¶, Giovanna Donnarumma∥,
Piera Valenti∥, and Giuseppe Rotilio‡

*From the ‡Dipartimento di Biologia, Università di Roma "Tor Vergata," Via della Ricerca Scientifica, 00133 Rome, Italy,
¶Molecular Infectious Diseases Group, Department of Paediatrics, Imperial College School of Medicine, St. Mary's
Hospital, Norfolk Place, London W2 1PG, United Kingdom, and ∥Istituto di Microbiologia-II, Università di Napoli,
Larghetto S. Aniello a Caponapoli, 2, 80138 Napoli, Italy*

A group of Cu,Zn-superoxide dismutases from pathogenic bacteria is characterized by histidine-rich N-terminal extensions that are in a highly exposed and mobile conformation. This feature allows these proteins to be readily purified in a single step by immobilized metal affinity chromatography. The Cu,Zn-superoxide dismutases from both *Haemophilus ducreyi* and *Haemophilus parainfluenzae* display anomalous absorption spectra in the visible region due to copper binding at the N-terminal region. Reconstitution experiments of copper-free enzymes demonstrate that, under conditions of limited copper availability, this metal ion is initially bound at the N-terminal region and subsequently transferred to an active site. Evidence is provided for intermolecular pathways of copper transfer from the N-terminal domain of an enzyme subunit to an active site located on a distinct dimeric molecule. Incubation with EDTA rapidly removes copper bound at the N terminus but is much less effective on the copper ion bound at the active site. This indicates that metal binding by the N-terminal histidines is kinetically favored, but the catalytic site binds copper with higher affinity. We suggest that the histidine-rich N-terminal region constitutes a metal binding domain involved in metal uptake under conditions of metal starvation *in vivo*. Particular biological importance for this domain is inferred by the observation that its presence enhances the protection offered by periplasmic Cu,Zn-superoxide dismutase toward phagocytic killing.

Cu,Zn-superoxide dismutases (Cu,Zn-SODs)[1] (encoded in bacteria by *sodC* gene(s)) are metalloenzymes that catalyze the dismutation of the superoxide anion into oxygen and hydrogen peroxide by the alternate reduction and oxidation of a copper ion that constitutes the catalytically active redox center (1). Although for a long time Cu,Zn-SODs have been considered almost exclusively eukaryotic cytosolic enzymes, more recent studies have established that this enzyme is present in a large number of bacterial species, where it is exported to extracytoplasmic compartments (2). Since the first step in the phagocytic oxidative burst is the single electron reduction of molecular oxygen to superoxide by a transmembrane NADPH-oxidase complex (3), and superoxide is unable to cross the cytoplasmic membrane (4), it has been proposed that Cu,Zn-SOD protects bacteria from oxygen free radicals generated by inflammatory cells and facilitates bacterial survival within the host. This hypothesis has gained support from different studies that have shown that *sodC* mutants of *Salmonella typhimurium* (5–8), *Neisseria meningitidis* (9), and *Haemophilus ducreyi* (10) are attenuated in animal models and by the demonstration that Cu,Zn-SOD protects bacteria from macrophage killing (6, 11) or extracellular superoxide generated *in vitro* (5, 6, 9, 12).

The discovery that, at least in some bacterial species, Cu,Zn-SOD is able to modulate virulence has suggested that this enzyme could represent a target for novel antimicrobial strategies and has stimulated studies on its structural and functional properties. In this context the x-ray structure of different bacterial Cu,Zn-SODs have been solved (13–17), and the spectroscopic and catalytic properties of some of these enzymes have been investigated (17–21). These studies have shown that prokaryotic and eukaryotic Cu,Zn-SODs derive from a common ancestor gene and share a similar three-dimensional fold, based on a flattened Greek-key eight-stranded β-barrel and a similar organization of the redox center. In the oxidized form the copper ion is coordinated by the nitrogen atoms of four histidine residues; the zinc ion is coordinated by three histidines and an aspartic acid residue, and the two metal ions are simultaneously coordinated by a single histidine residue (termed the "histidine bridge" or the "bridging imidazolate"), in a structural motif so far found only in Cu,Zn-SODs (22, 23). Relevant differences are, however, observed in the organization of the active site channel and in the way subunits are assembled. In particular, these studies have highlighted that bacterial Cu,Zn-SOD may be monomeric or dimeric (15) and that small differences at the dimer interface may finely modulate the enzyme activity and stability (17).

Interestingly, whereas all eukaryotic Cu,Zn-SODs conform to a single structural model that appears to have been strictly

[1] The abbreviations used are: Cu,Zn-SOD, Cu,Zn-superoxide dismutase; LB, Luria-Bertani broth; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; NTA, nitrilotriacetic acid; IDA, iminodiacetic acid.

preserved throughout evolution (24), analysis of amino acid sequences from Cu,Zn-SODs of different bacterial species suggests much greater variation, so individual enzyme variants may exhibit unique properties (15, 16). The most obvious differences between bacterial Cu,Zn-SODs include insertions and deletions in some of the major loops protruding from the β-barrel, which could plausibly result in differences in the active site channel architecture and subunit assembly, and the substitutions of some of the conserved metal ligands that are expected to affect significantly the enzyme activity. The functional implications of these variations are still to be explored, but it is likely that such differences may lead to modulation in the enzyme activity in different bacteria.

In general, the bacterial Cu,Zn-SODs are least alike at their N-terminal ends. In this study, we have investigated the metal binding ability of N-terminal extensions present in a subset of Cu,Zn-SODs from Gram-negative pathogenic bacteria. We have found that such extensions, which are in a highly mobile conformation and contain several histidine residues, bind divalent metal ions with high efficiency. We propose that they represent high affinity functional metal binding domains, perhaps involved in the uptake of the prosthetic metals of the enzyme in environments where their concentration is very low.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids—Plasmids pIT4, pJSK130, and pJSK40, bearing the *sodC* genes from *H. ducreyi*, *Haemophilus parainfluenzae*, and *Haemophilus influenzae*, respectively, have been described previously (25, 26). In order to ensure similar expression levels of the different enzyme variants, all sequences encoding mature Cu,Zn-SODs were cloned in plasmid pHEN-1 (27), under control of the *lacZ* promoter. Plasmid pPHduSOD1, expressing wild type *H. ducreyi* Cu,Zn-SOD, was obtained by PCR amplification with the oligonucleotides HduFor1 (5′-CTAAGCTTAAGGAGATAAAATGAAATTA) and HduRev (5′-CCTGAATTCTTATTTAATTACACCGCATGCC), using pIT4 as a DNA template and the high fidelity polymerase Expand™ (Roche Molecular Biochemicals). The amplified DNA was digested with *Hin*dIII and *Eco*RI and cloned in the corresponding sites of pHEN-1. Plasmid pJSK326, expressing a truncated form of *H. ducreyi* Cu,Zn-SOD (N-terminal deleted mutant) lacking the first 22 amino acids (HGDHMHNHDTKMDTMSKDMMSM), was obtained by a similar strategy, amplifying the coding sequence with primers HduFor2 (5′-TTCCATGGCCGAAAAAAATTGTAGTGCCT) and HduRev and subsequently cloning the amplified DNA in the *Nco*I and *Eco*RI sites of pHEN1. To construct plasmid pPHduSODFIRS, expressing a mutant *H. ducreyi* enzyme lacking residues 11–22, the sequence encoding residues 1–10 was amplified with primers HduFor1 and HduRevS (5′-GACCATGGTGTCATGATTATGCATA). The PCR product was restricted with *Hin*dIII and *Nco*I and inserted in the corresponding sites of plasmid pJSK326. Plasmids pPHpSOD and pPHiSOD expressing wild type Cu,Zn-SODs from *H. parainfluenzae* and *H. influenzae,* respectively, were constructed as follows. The sequences coding for the two mature enzymes were amplified with primers HiFor (5′-AACCATGGCCCATGACCATATGGCAAAAC) and HiRev (5′-CTGAATTCTTATTTAATCACGCCACATGC), using plasmids pJSK130 and pJSK40 as templates. The amplified DNA fragments were digested with *Nco*I and *Eco*RI. Since there is an internal *Nco*I site in these two *sodC* genes, the resulting *Nco*I-*Nco*I (279 base pairs) and *Nco*I-*Eco*RI (216 base pairs) DNA fragments were separated by agarose gel electrophoresis, and the *Nco*I-*Eco*RI fragment from *H. parainfluenzae* (identical to the corresponding DNA region of the *H. influenzae sodC* gene) was cloned in plasmid pHEN-1, thus obtaining plasmid pPHaeShort. Subsequently, the *Nco*I-*Nco*I fragments from the two distinct *sodC* genes were inserted in the *Nco*I site of pPHaeShort. To construct plasmids pPHpSOD-3 and pPHiSOD-3, expressing mutant proteins lacking the first three amino acid residues (HDH), DNA was amplified with primers hemeFor2 (5′-AACCATGGCTATGGCAAAACCAGCAGGT) and HiRev, using pPHpSOD and pPHiSOD as DNA templates, respectively. After digestion with *Nco*I, the 270-base pair *Nco*I-*Nco*I DNA fragments were purified and inserted in the *Nco*I site of plasmid pPHaeShort. Plasmids pPEcSODHis and pPXSODBHis, expressing *Escherichia coli* and *Xenopus laevis* Cu,Zn-SODs fused to the 22 N-terminal residues from *H. ducreyi* Cu,Zn-SODs, were obtained by inserting a DNA fragment obtained by amplification with HduFor1 and HduRevL into the *Hin*dIII

and *Nco*I sites of pPEcSOD81A (28) and pPXSODB (29). The complete nucleotide sequences of all the PCR amplified DNA fragments were checked by the dideoxy chain termination method.

Cu,Zn-SODs Expression and Purification—Overexpression of the different Cu,Zn-SODs was carried out in 71/18 *E. coli* cells (30) grown at 37 °C in Luria Bertani broth (LB) containing 100 μg/ml ampicillin. When cells reached an A_{600} of 0.5, cultures were supplemented with 0.2 mM isopropyl-β-D-thiogalactopyranoside, 0.25 mM CuSO$_4$, and 10 μM ZnSO$_4$. The periplasmic fraction was prepared as described previously (19). Wild type and mutant enzymes were purified by immobilized metal affinity chromatography on a nickel-nitrilotriacetic acid (NTA) resin (Qiagen). The column was equilibrated with a buffer containing 300 mM NaCl and 50 mM sodium phosphate, pH 7.8 (buffer A). Proteins bound to the resin were eluted by washing the column sequentially with 2–4 volumes of buffer A supplemented with 10, 20, 40, 80, and 250 mM imidazole. Fractions were analyzed by denaturing polyacrylamide gel electrophoresis (SDS-PAGE). The same procedure was used to test the affinity of Cu,Zn-SOD for different metal ions on an iminodiacetic acid (IDA) resin, charged with nickel, copper, zinc, cadmium, or cobalt according to the manufacturer's instructions (Amersham Pharmacia Biotech). Cu,Zn-SOD samples purified by immobilized metal affinity chromatography were analyzed for their metal content using the PerkinElmer Life Sciences spectrometer AAnalyst 300 equipped with the graphite furnace HGA-800. No significant nickel contamination was found in Cu,Zn-SODs purified from Ni-NTA, whereas the enzymes purified from IDA exhibited severe metal contamination.

After affinity purification on Ni-NTA columns, samples containing wild type Cu,Zn-SODs (usually >90% pure) were concentrated and subjected to gel filtration chromatography on a HiLoad™ 16/60 Superdex™ FPLC column (Amersham Pharmacia Biotech). Wild type *H. ducreyi* Cu,Zn-SOD purified from 71/18 was not suitable for a spectral analysis of copper binding (31). Therefore, for this purpose, the enzyme was expressed in H500 *E. coli* cells (32) and purified by the procedure described above.

To purify truncated proteins lacking N-terminal histidines (obtained from *E. coli* 71/18 cells harboring pJSK326 or pPHpSOD-3), periplasmic extracts were concentrated and dialyzed against 10 mM potassium phosphate, pH 7.0, and fractionated onto a Whatman DE52 column equilibrated with the same buffer. Fractions containing Cu,Zn-SOD were pooled, concentrated, and dialyzed against 20 mM Tris-HCl, pH 7.0, 0.15 M NaCl and injected onto a High Load 16/60 Superdex 75 gel filtration FPLC column and eluted with the same buffer. As a final step, the enzymes were further dialyzed against 20 mM potassium phosphate, pH 6.5, and subjected to ion exchange chromatography on a Mono-S HR 5/5 FPLC column (Amersham Pharmacia Biotech) equilibrated with the same buffer, using a 0–0.1 M NaCl gradient. Whereas the *H. parainfluenzae* mutant eluted in a single peak containing the pure enzyme, the *H. ducreyi* mutant eluted in two major peaks, one of which corresponded to the heme-containing enzyme (31). This Cu,Zn-SOD form was discarded, and the heme-lacking protein was subjected to a second round of ion exchange chromatography under identical conditions.

All purified proteins were concentrated and dialyzed against 10 mM potassium phosphate buffer, pH 7.0. Protein concentration was determined by the Lowry method (33). The zinc and copper content of purified Cu,Zn-SOD samples was verified by atomic absorption. The metal content of enzymes purified from metal enriched media was variable from one preparation to another. On average, the copper content of wild type *H. parainfluenzae* and *H. ducreyi* enzymes was between 1 and 2.5 copper ions/subunit, whereas zinc content was between 1.3 and 1.8 zinc ions/subunit. The metal content of these samples was diminished by treatment with EDTA (followed by dialysis), indicating that a significant fraction of these metal ions is loosely bound on the enzyme surface. Mutant enzymes contained lower amounts of both metals.

Limited Proteolysis and N-terminal Sequence Analysis—*H. ducreyi* Cu,Zn-SOD (0.25 mg/ml) was dissolved in 50 mM potassium phosphate buffer, pH 8.0, and incubated at 20 °C in the presence of increasing amounts of proteinase K (proteinase K/Cu,Zn-SOD ratios were between 0.01 and 1%). At different times aliquots were withdrawn, and the proteolytic digestion was stopped by boiling the sample in 2% SDS, 5% mercaptoethanol. A similar procedure was used for trypsin digestion, but in this case the reaction was carried out in ammonium bicarbonate buffer at 37 °C. Samples from limited proteolysis experiments were subjected to SDS-PAGE on 15% gels and electrotransferred onto polyvinylidene difluoride membranes (Immobilon P, Millipore). Protein bands were visualized by Coomassie Blue staining, excised from the membrane, and subjected to several cycles of automated Edman degradation performed with a model 473A pulsed liquid sequencer (Applied
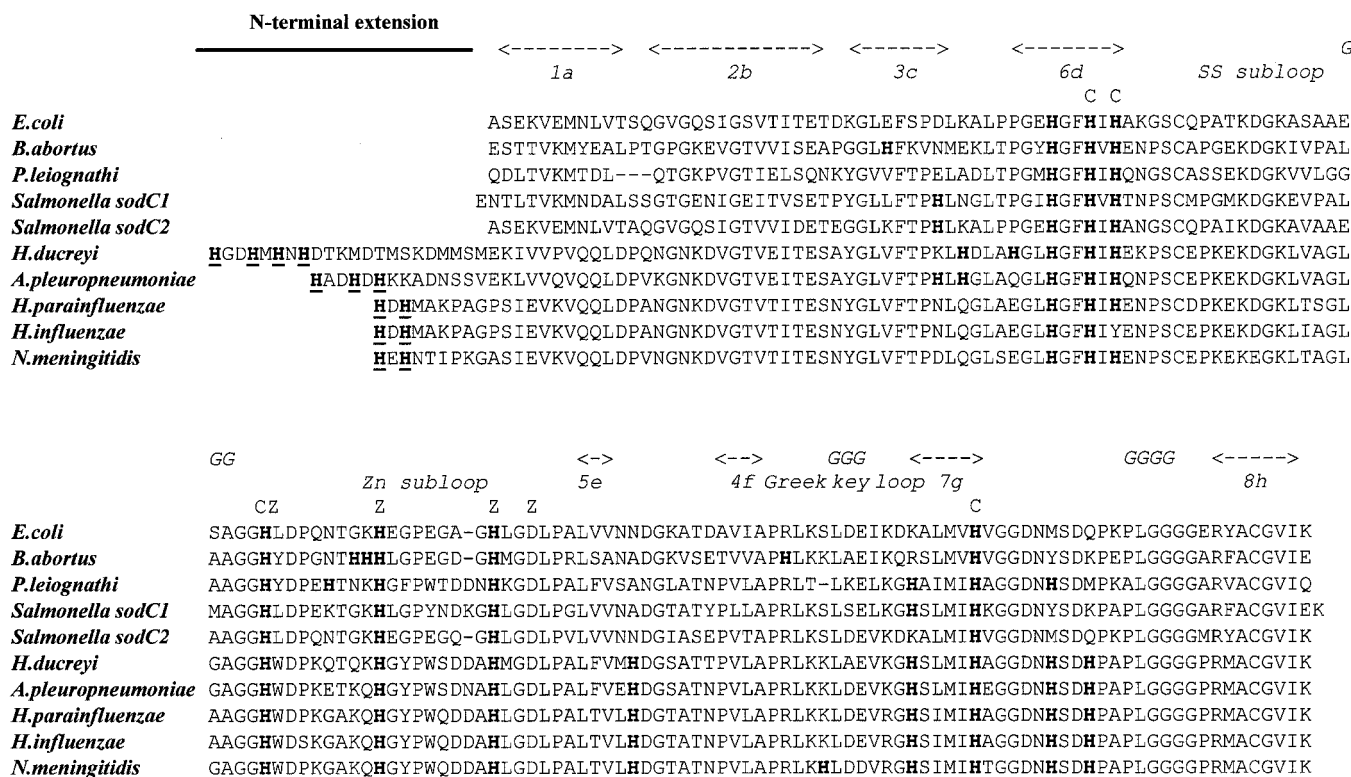
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                 N-terminal extension
                 _____
                                        <-------->  <------------>  <------>     <------->          G
                                           1a            2b          3c           6d      SS subloop
                                                                                  C C
E.coli                              ASEKVEMNLVTSQGVGQSIGSVTITETDKGLEFSPDLKALPPGEHGFHIHAKGSCQPATKDGKASAAE
B.abortus                           ESTTVKMYEALPTGPGKEVGTVVISEAPGGLHFKVNMEKLTPGYHGFHVHENPSCAPGEKDGKIVPAL
P.leiognathi                        QDLTVKMTDL---QTGKPVGTIELSQNKYGVVFTPELADLTPGMHGFHIHQNGSCASSEKDGKVVLGG
Salmonella sodC1                    ENTLTVKMNDALSSGTGENIGEITVSETPYGLLFTPHLNGLTPGIHGFHVHTNPSCMPGMKDGKEVPAL
Salmonella sodC2                    ASEKVEMNLVTAQGVGQSIGTVVIDETEGGLKFTPHLKALPPGEHGFHVHANGSCQPAIKDGKAVAAE
H.ducreyi                HGDHMHNHDTKMDTMSKDMMSMEKIVVPVQQLDPQNGNKDVGTVEITESAYGLVFTPKLHDLAHGLHGFHIHEKPSCEPKEKDGKLVAGL
A.pleuropneumoniae         HADHDHKKADNSSVEKLVVQVQQLDPVKGNKDVGTVEITESAYGLVFTPHLHGLAQGLHGFHIHQNPSCEPKEKDGKLVAGL
H.parainfluenzae             HDHMAKPAGPSIEVKVQQLDPANGNKDVGTVTITESNYGLVFTPNLQGLAEGLHGFHIHENPSCDPKEKDGKLTSGL
H.influenzae                 HDHMAKPAGPSIEVKVQQLDPANGNKDVGTVTITESNYGLVFTPNLQGLAEGLHGFHIYENPSCEPKEKDGKLIAGL
N.meningitidis               HEHNTIPKGASIEVKVQQLDPVNGNKDVGTVTITESNYGLVFTPDLQGLSEGLHGFHIHENPSCEPKEKEGKLTAGL
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                 GG                                  <->         <-->    GGG    <---->            GGGG    <----->
                       Zn subloop                    5e          4f Greek key loop 7g                      8h
                 CZ         Z      Z Z                                          C
E.coli           SAGGHLDPQNTGKHEGPEGA-GHLGDLPALVVNNDGKATDAVIAPRLKSLDEIKDKALMVHVGGDNMSDQPKPLGGGGERYACGVIK
B.abortus        AAGGHYDPGNTHHHLGPEGD-GHMGDLPRLSANADGKVSETVVAPHLKKLAEIKQRSLMVHVGGDNYSDKPEPLGGGGARFACGVIE
P.leiognathi     AAGGHYDPEHTNKHGFPWTDDNHKGDLPALFVSANGLATNPVLAPRLT-LKELKGHAIMIHAGGDNHSDMPKALGGGGARVACGVIQ
Salmonella sodC1 MAGGHLDPEKTGKHLGPYNDKGHLGDLPGLVVNADGTATYPLLAPRLKSLSELKGHSLMIHKGGDNYSDKPAPLGGGGARFACGVIEK
Salmonella sodC2 AAGGHLDPQNTGKHEGPEGQ-GHLGDLPVLVVNNDGIASEPVTAPRLKSLDEVKDKALMIHVGGDNMSDQPKPLGGGGMRYACGVIK
H.ducreyi        GAGGHWDPKQTQKHGYPWSDDAHMGDLPALFVMHDGSATTPVLAPRLKKLAEVKGHSLMIHAGGDNHSDHPAPLGGGGPRMACGVIK
A.pleuropneumoniae GAGGHWDPKETKQHGYPWSDNAHLGDLPALFVEHDGSATNPVLAPRLKKLDEVKGHSLMIHEGGDNHSDHPAPLGGGGPRMACGVIK
H.parainfluenzae AAGGHWDPKGAKQHGYPWQDDAHLGDLPALTVLHDGTATNPVLAPRLKKLDEVRGHSIMIHAGGDNHSDHPAPLGGGGPRMACGVIK
H.influenzae     AAGGHWDSKGAKQHGYPWQDDAHLGDLPALTVLHDGTATNPVLAPRLKKLDEVRGHSIMIHAGGDNHSDHPAPLGGGGPRMACGVIK
N.meningitidis   GAGGHWDPKGAKQHGYPWQDDAHLGDLPALTVLHDGTATNPVLAPRLKHLDDVRGHSIMIHTGGDNHSDHPAPLGGGGPRMACGVIK
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FIG. 1. **Multiple alignment of the amino acid sequences of 10 Cu,Zn-SODs from Gram-negative bacteria.** The global alignment has been deduced from optimal superimposition of available bacterial Cu,Zn-SOD structures, as described previously (15). The secondary structure elements are shown *above* the amino acid sequences, using the following symbols: β-strands, ↔ ; 3_{10} helix, G. The histidine residues present in these bacterial Cu,Zn-SODs are shown in *bold* and are *underlined*, and residues coordinating Cu^{2+} and Zn^{2+} are indicated by C and Z, respectively. Other known Cu,Zn-SOD sequences from Gram-negative bacteria (*Caulobacter crescentus*, *Legionella pneumophila*, and *Francisella tularensis*) have not been included in the alignment because they show significant variations in the primary structure which suggest a slightly modified enzyme architecture (15) and do not possess N-terminal extensions rich in histidine residues.

Biosystems) with an on-line analyzer of phenylthiohydantoin amino acids.

Preparation of the Copper-free Enzyme and Copper Reconstitution Experiments—Copper-free Cu,Zn-SODs were prepared following a procedure described previously (34). Briefly, the enzymes were treated with excess potassium ferrocyanide to reduce copper and then dialyzed for 12–24 h at 4 °C against 0.1 M potassium phosphate buffer, 50 μM KCN, pH 6.0. The samples were further dialyzed twice for 24 h at 4 °C against phosphate buffer to remove KCN. The metal content of copper-free proteins was evaluated by atomic absorption spectroscopy. The final copper content of samples used for reconstitution experiments contained between 0.3 and 0.6% of the initial value, whereas no significant loss of zinc was observed.

Reconstitution experiments were carried out by adding stoichiometric or sub-stoichiometric amounts of $CuCl_2$ to the copper-free SOD dissolved in water at 25 or 37 °C. The reconstitution process was monitored spectrophometrically by registering protein absorption in the visible region as a function of time with a PerkinElmer Life Sciences Lambda 2 spectrophotometer thermostated at 25 or 37 °C, using 1-cm path length cuvettes. In these experiments Cu,Zn-SODs were dissolved at a concentration of 2.5×10^{-4} M.

The rate of copper binding at the active site was also evaluated by measuring the increase in catalytic activity following the addition of copper to copper-free Cu,Zn-SODs dissolved in water. Reconstitution experiments at copper/subunit ratios below or equal to 1 were carried out by adding variable amounts of proteins to a 5.8×10^{-7} M copper solution. Reconstitution experiments at higher copper/subunit ratios were obtained by adding the proteins (at a final concentration of 2.9×10^{-7} M) to copper solutions of higher concentrations. The activity assays were carried out by the pyrogallol method (35). To rule out a possible contribution to enzyme activity of copper bound outside the active site, 0.1 mM EDTA was added to the assay buffer. The percent activity values were obtained by the ratio between the activity of reconstituted enzymes and that of an equal amount of fully reconstituted Cu,Zn-SODs. All the activity values obtained during reconstitution experiments were corrected by subtracting the contribution to activity of residual copper present in copper-free proteins. Fully metallated *H. parainfluenzae* and

mutant *H. parainfluenzae* and *H. ducreyi* enzymes showed nearly identical activity values. An approximate activity value for the fully reconstituted wild type *H. ducreyi* enzyme was obtained using as a standard the reconstituted N-terminal deleted mutant.

Macrophage Killing Experiments—The mouse macrophage-like cell line J774 and the human macrophage-like line THP-1 were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in an atmosphere of 5% CO_2. 3 h before the experiments the THP-1 cell suspension was supplemented with 1 μg/ml phorbol 12-myristate 13-acetate. Macrophage cell concentration was adjusted to 2×10^7 cells/ml for killing experiments.

E. coli GC4468 (36) expressing either wild type or mutant (devoid of N-terminal histidines) *H. parainfluenzae* and *H. ducreyi* Cu,Zn-SODs were grown overnight at 37 °C in LB medium or in LB supplemented with 0.25 mM $CuSO_4$ and 10 μM $ZnSO_4$. After growth cells were washed three times in phosphate-buffered solution without Ca^{2+} and Mg^{2+}. The bacterial cell concentration was then adjusted to 1×10^8 cells/ml in phosphate-buffered solution without Ca^{2+} and Mg^{2+}.

Before presentation to macrophages, microorganisms were pre-opsonized by exposing 2×10^7 bacteria (200 μl) to 50 μl of fresh pooled serum plus 750 μl of phosphate-buffered saline (pH 7.4), for 25 min at 37 °C with moderate agitation. 200 μl (~2×10^6) of opsonized bacteria were added to a mixture of 5% fresh serum (40 μl), phosphate-buffered solution (560 μl), and 200 μl of culture medium with or without macrophages (4×10^6). 100-μl samples were withdrawn at 0, 30, 60, and 90 min in order to determine bacterial survival in serum and in macrophages by counting colony-forming units in trypticase soy agar (Oxoid). The number of surviving bacteria was obtained from at least three independent experiments. In each experiment, the macrophage killing assay was done in triplicate.

RESULTS

Histidine-rich N-terminal Sequences in Bacterial Cu,Zn-SODs—Fig. 1 shows an alignment of the amino acid sequences of 10 Cu,Zn-SODs from Gram-negative bacteria, which includes the four enzymes whose three-dimensional structures

Natural His Tagging of Bacterial Cu,Zn-SODs

have been solved and a few other enzyme variants that are expected to possess a similar structure (15). Some of these enzymes, namely those from the mucosal colonists and pathogens *H. ducreyi*, *H. parainfluenzae*, *H. influenzae*, *N. meningitidis,* and *Actinobacillus pleuropneumoniae,* show two features that were ignored during previous analyses; they are characterized by an unusually large number of histidine residues (which in the case of the *H. ducreyi* and *A. pleuropneumoniae* enzymes approximates the 10% of total residues) and by amino acid extensions at their N-terminal sequence. These N-terminal extensions are of variable length and display low sequence identity, but all of them initiate with a cluster of histidines (2–4 residues) interspersed with negatively charged residues. Similar His-rich regions have been found in several other proteins able to bind nickel, zinc, or other transition metals. These proteins include UreE from *Klebsiella aerogenes* (37), HypB from *Rhizobium leguminosarum* (38), CooJ from *Rhodospirillum rubrum* (39), SlyD from *E. coli* (40), ZnuA from different bacteria (Ref. 41 and references therein), CzrB from *Staphylococcus aureus* (42), and Zrc1 from *Saccharomyces cerevisiae* (43). For some of these proteins, it has been shown that the His-rich region is directly involved in metal binding, suggesting that the N-terminal sequence found in some bacterial Cu,Zn-SOD may represent a protein domain able to bind divalent metals.

Protease Sensitivity of the N-terminal Extension of Bacterial Cu,Zn-SODs—Cu,Zn-SODs are in general very stable enzymes, highly resistant to protease digestion. However, we found that preparations of the *H. ducreyi* enzyme underwent rapid fragmentation, accumulating a major form with a molecular mass (~16 kDa) comparable to that of the enzyme produced by cells bearing pJSK326, which lacks the initial 22 amino acids. It has been found previously that, due to presumed proteolytic cleavage, the *A. pleuropneumoniae* Cu,Zn-SOD used in a recent crystallographic study lacked the seven initial amino acids and that the following residues were in a disordered conformation (16). Since proteolytic enzymes usually cleave the polypeptide chain within highly flexible regions and not in rigid structural elements (44), these observations suggest that the N-terminal extension of bacterial Cu,Zn-SODs could be in an exposed and mobile conformation. To probe the conformational state of its N-terminal region, the *H. ducreyi* enzyme was subjected to limited proteolysis experiments with proteinase K and trypsin. Fig. 2*A* shows the SDS-PAGE analysis of *H. ducreyi* Cu,Zn-SOD digested with proteinase K as a function of proteinase K/Cu,Zn-SOD ratio. The N-terminal sequence of each digestion product was determined in order to map the sites of higher accessibility. The N-terminal sequence of band a is Ser-Lys-Asp, indicating that initial cleavage occurs within the N-terminal domain and removes 15 amino acids from the enzyme. Band b, which appears after digestion with large amounts of proteinase K, is a mixture of three distinct protease digestion products whose amino acid sequences begin with Val-Gly-Thr, Glu-Ser-Ala, and Tyr-Gly-Leu, respectively, and derive from cleavages within the first β-strand elements of the enzyme. Incubation with trypsin (Fig. 2*B*) led to the appearance of two digestion products (bands c and d), which were both mapped within the N-terminal domain. The N-terminal sequence of band d is Asp-Met-Met. Such a sequence indicates that the proteolytic attack occurred at a position very close to the site initially recognized by proteinase K, leading to the removal of 17 residues. Band c sequence starts with Met-Asp-Thr and is due to a trypsin cleavage after the first lysine residue in the polypeptide.

It is noteworthy that the sites and rates of Cu,Zn-SOD cleavage by trypsin and proteinase K were neither affected by pre-
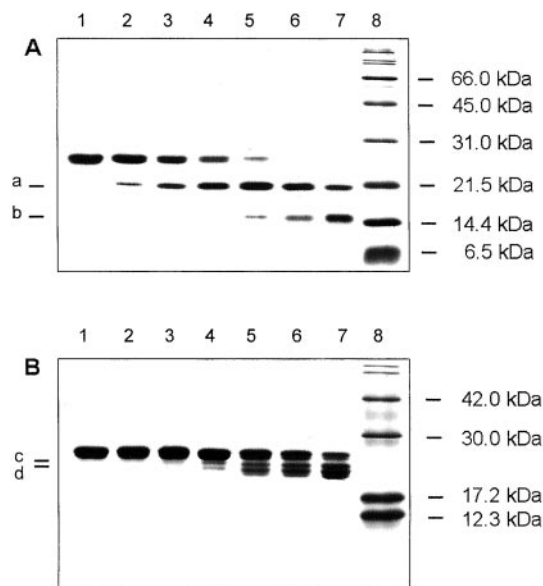

FIG. 2. **Limited proteolysis of *H. ducreyi* Cu,Zn-SOD.** *A,* SDS-PAGE analysis of proteolytic fragments obtained after digestion with proteinase K. Proteolysis was carried out for 10 min at the following proteinase K/Cu,Zn-SOD ratios (w/w): *lane 1,* 0; *lane 2,* 0.01; *lane 3,* 0.05; *lane 4,* 0.1; *lane 5,* 0.2; *lane 6,* 0.5; and *lane 7,* 1. *Lane 8,* molecular weight markers. *B,* SDS-PAGE analysis of proteolytic fragments obtained after digestion with trypsin. Proteolysis was carried out for 15 min at the following trypsin/Cu,Zn-SOD ratios (w/w): *lane 1,* 0; *lane 2,* 0.01; *lane 3,* 0.05; *lane 4,* 0.1; *lane 5,* 0.2; *lane 6,* 0.5; and *lane 7,* 1. *Lane 8,* molecular weight markers.

incubation of the enzyme with zinc nor by preincubation with chelating agents, suggesting that metal binding does not confer significant rigidity to the N-terminal domain.

Such experimental results are consistent with a theoretical analysis of *H. ducreyi* Cu,Zn-SOD stability carried out by the method described by Guruprasad and co-workers (45) which is based on the occurrence of specific dipeptides in stable and unstable proteins. According to this analysis, the enzyme was found to have a high instability index due to the presence in the N-terminal region of several dipeptides typical of unstable proteins (Met-His, Met-Ser, and Lys-Met).

The His-rich Region Confers High Affinity for Divalent Metal Ions—The metal binding ability of the His-rich regions of the Cu,Zn-SODs from *H. ducreyi*, *H. parainfluenzae,* and *H. influenzae* was tested by immobilized metal affinity chromatography. The three wild type enzymes (Fig. 3, *A, F,* and *G*) are all characterized by high affinity for Ni-NTA and may be purified nearly to homogeneity in a single chromatographic step, starting from periplasmic extracts. The removal of the whole N-terminal extension (22 amino acids) from the *H. ducreyi* enzyme drastically reduces its affinity for the resin (Fig. 3*B*). In contrast, a mutant enzyme that retains the first 10 residues (including the four N-terminal histidines), but lacks residues 11–22, possesses a metal affinity comparable to that of the wild type protein (Fig. 3*C*), thus indicating that residues 11–22 do not significantly contribute to the enzyme affinity for the metal chelate resin. Similarly, mutant Cu,Zn-SODs from *H. parainfluenzae* (Fig. 3*H*) and *H. influenzae* (not shown) lacking the first three residues (His-Asp-His) display low affinity for nickel. Fusion of the *H. ducreyi* N-terminal region to the Cu,Zn-SODs from *E. coli* (Fig. 3*E*) and *X. laevis* (not shown) confers high affinity for Ni-NTA also to these two enzymes. It should be noted, however, that the N-terminal deleted *H. ducreyi* Cu,Zn-SOD (Fig. 3*B*) shows a greater affinity for Ni-NTA than the *E. coli* (Fig. 3*D*) or mutant *H. influenzae* and *H. parainfluenzae* enzymes, indicating that other surface-exposed histidines also

FIG. 3. **Purification of Cu,Zn-SODs by immobilized metal affinity chromatography.** Periplasmic extracts (1 ml) from *E. coli* cells producing different Cu,Zn-SODs were loaded onto a Ni-NTA column (formed by 1 ml of Qiagen resin). Proteins were eluted by serial washing of the column with buffers (4 ml) containing increasing amounts of imidazole and analyzed by SDS-PAGE. The *eight panels* show purification of the following enzymes: *A, H. ducreyi* Cu,Zn-SOD; *B,* mutant *H. ducreyi* Cu,Zn-SOD lacking the initial 22 residues; *C,* mutant *H. ducreyi* Cu,Zn-SOD lacking residues 11–22; *D, E. coli* Cu,Zn-SOD; *E,* mutant *E. coli* Cu,Zn-SOD containing the *H. ducreyi* His-rich domain at the N terminus; *F, H. parainfluenzae* Cu,Zn-SOD; *G, H. influenzae* Cu,Zn-SOD; *H,* mutant *H. parainfluenzae* Cu,Zn-SOD lacking the His-rich region. *Lane 1,* molecular weight markers; *lane 2,* periplasmic extract; *lane 3,* flow-through; *lane 4,* 10 mM wash; *lane 5,* 20 mM wash; *lane 6,* 40 mM wash; *lane 7,* 80 mM wash; and *lane 8,* 250 mM wash.
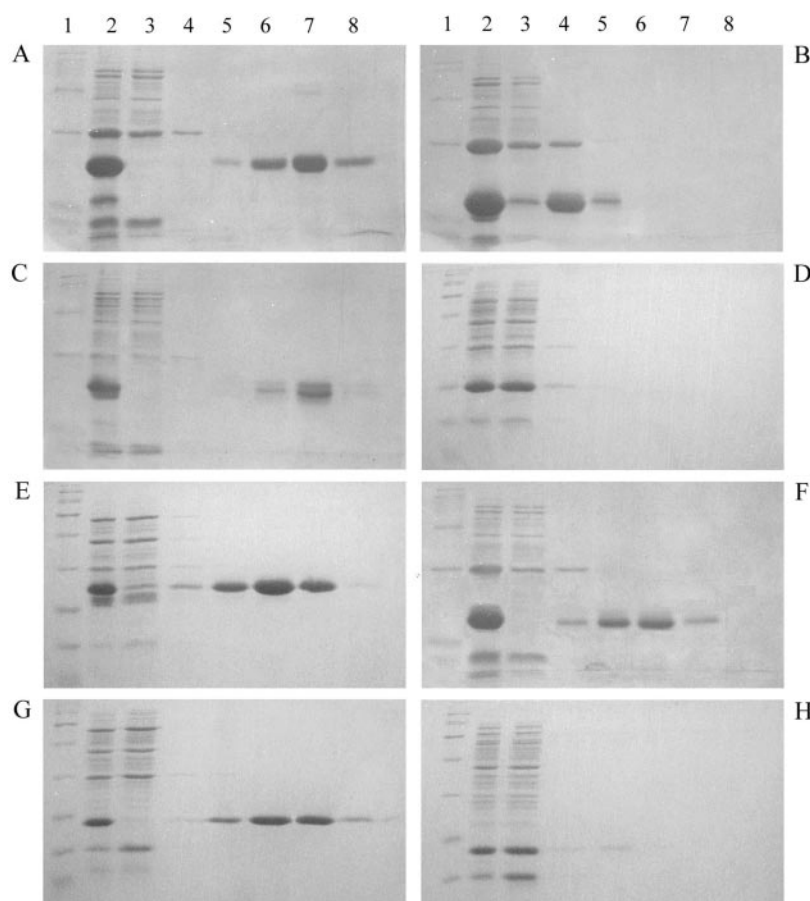
contribute to its affinity for metal chelate resins.

Similar experiments were also carried out with an IDA resin charged with different metal ions (nickel, zinc, copper, cadmium, and cobalt). We have found that the *H. ducreyi* enzyme binds to this resin with an affinity that is independent of the divalent metal ion used to charge the resin.

Copper Binding by the N-terminal Domain—The electronic spectra in the visible region of wild type Cu,Zn-SODs from *H. ducreyi* and *H. parainfluenzae* (Fig. 4) exhibited unusual absorption bands with respect to other bacterial or eukaryotic enzyme variants. The spectrum of *H. parainfluenzae* Cu,Zn-SOD (Fig. 4*A*) displays two maxima at 680 and 525 nm, whereas the *H. ducreyi* enzyme (Fig. 4*B*) is characterized by a broad absorption peak centered at 630–650 nm. The precise location of the peak varied from preparation to preparation, and the peak was blue-shifted in samples containing more copper. The addition of sub-stoichiometric amounts of copper to the *H. ducreyi* enzyme induced a strong increase in absorbance and a further blue shift of the absorption maximum up to 615 nm, whereas the addition of small amounts of copper to the Cu,Zn-SOD from *H. parainfluenzae* slightly increased the sample absorbance without affecting the maximum wavelengths (not shown). In contrast, the *H. ducreyi* and *H. parainfluenzae* mutants lacking the N-terminal histidines exhibited a copper absorption band centered at 680 nm, typical of Cu,Zn-SODs naturally lacking the N-terminal histidines. In line with these observations, which suggest that the anomalous electronic spectra of the two enzymes are due to copper bound at the N-terminal domain, we observed that, upon pretreatment with EDTA, both the enzymes exhibited a single absorption maximum close to 680 nm (Fig. 4, *A* and *B*).

To investigate the possible relationship between the His-rich metal binding domain and the active site of the enzyme, we prepared copper-free derivatives of the *H. parainfluenzae* and *H. ducreyi* Cu,Zn-SODs and monitored the process of copper binding *in vitro*. One eq of copper (*i.e.* two copper ions/enzyme dimer) was added to the *H. parainfluenzae* copper-free enzyme, and the electronic spectrum in the 450–800 nm region was followed over time (Fig. 4*C*). Immediately after the addition of copper, we observed the appearance of an absorption peak centered close to 525 nm with only a small shoulder at longer wavelengths. During incubation, a slow but progressive increase in absorbance of the copper band at 680 nm, indicative of copper binding in the active site, was observed, with the concomitant disappearance of the 525-nm peak. The rate of this process was influenced by temperature, being more than 5-fold faster at 37 than at 25 °C, suggesting that higher temperatures may increase motions of the N-terminal domain and favor metal translocation from the His-rich region to an active site. Immediately after the addition of a second equivalent of copper, the protein spectrum showed two well resolved absorption bands at 525 and 680 nm, similar to those found with the Cu,Zn-SOD purified from *E. coli* extracts (see Fig. 4*A*). The copper absorption peak at 525 nm disappeared following the addition of EDTA (Fig. 4*A*). In contrast, reconstitution of copper-free *H. parainfluenzae* mutant enzyme with an equivalent of copper led to the rapid development of the 680 nm peak and showed no evidence of additional copper-binding sites. Although the spectra reported in Fig. 4*C* do not identify a clean isosbestic point, they suggest that copper is rapidly bound at the N-terminal domain and then transferred to an active site. We suggest that the lack of an isosbestic point might be due to an increase in absorbance at low wavelengths following copper binding in an active site due to a ligand-to-metal charge transfer transition between the imidazole of His-61 (the bridging histidine) and copper (46). Alternative explanations may be
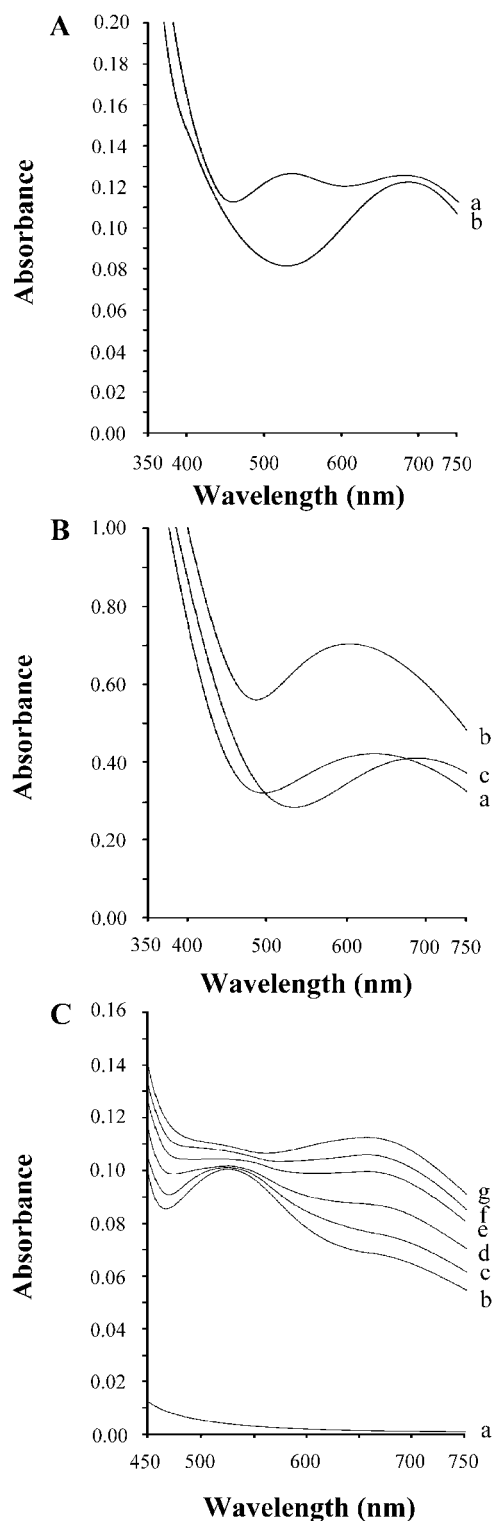
FIG. 4. **Electronic absorption spectra of *H. parainfluenzae* and *H. ducreyi* Cu,Zn-SODs.** *A, trace a, H. parainfluenzae* Cu,Zn-SOD as isolated (~12 mg/ml); *trace b, trace a* 1 min after EDTA addition. *B, trace a, H. ducreyi* Cu,Zn-SOD as isolated (~40 mg/ml); *trace b, trace a* after the addition of 0.5 mM CuCl$_2$; *trace c, trace b* 1 min after the addition of 1 mM EDTA. *C,* copper binding by copper-free *H. parainfluenzae* Cu,Zn-SOD. The process of copper binding by copper-free *H. parainfluenzae* Cu,Zn-SOD at 25 °C was monitored by electronic absorption spectroscopy. Copper reconstitution was started by the addition of 1 copper eq (5 × 10^{-4} M) to the enzyme dissolved in water at a concentration of 8.6 mg/ml. Cu,Zn-SOD absorption spectra in the 450–750 nm region were registered before the addition of copper (*trace a*) and at the following times after copper supplementation: *trace b,* 5 min; *trace c,* 15 min; *trace d,* 30 min; *trace e,* 60 min; *trace f,* 80 min; *trace g,* 120 min.
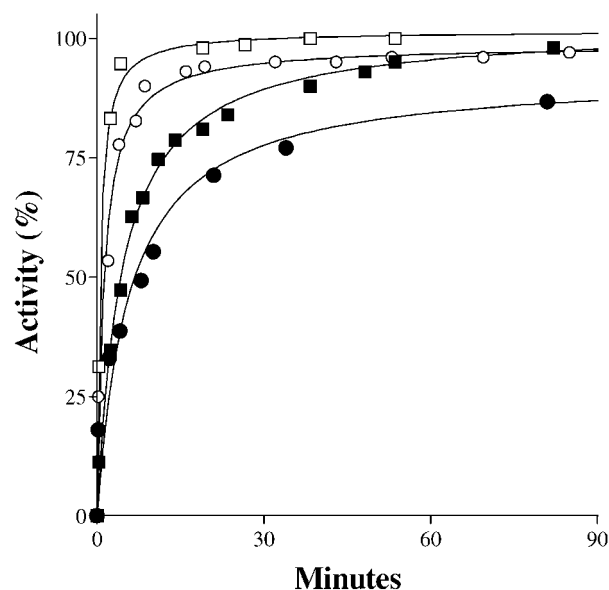
FIG. 5. **Kinetics of regaining activity by the copper-free enzymes.** Copper-free proteins at a concentration of 2.9 × 10^{-7} M were incubated with an equivalent of copper (5.8 × 10^{-7} M), and the increase in catalytic activity over time was measured by the pyrogallol method. ●, *H. ducreyi* Cu,Zn-SOD; ■, *H. parainfluenzae* Cu,Zn-SOD; ○, mutant *H. ducreyi* Cu,Zn-SOD; □, mutant *H. parainfluenzae* Cu,Zn-SOD.

that this is due to the presence of copper bound to additional sites on the enzyme surface or to the simultaneous presence of copper in both sites in a subset of molecules. Similar reconstitution experiments were performed with the copper-free *H. ducreyi* enzyme (data not shown). In this case, however, the large overlap between the absorption peaks due to copper binding at the N terminus and at the active site (and possibly at other sites on the enzyme surface) prevented a clear spectral demonstration of metal exchange between the two copper-binding sites. In fact, addition of 1 eq of copper to the wild type copper-free enzyme produced an increase in absorption over a wide region with no well defined peaks, whereas upon addition of a further equivalent a peak close to 615 nm appeared, similar to that observed in the enzyme as isolated after addition of copper (not shown). Also in this enzyme the removal of loosely bound copper by EDTA rapidly produced a modification of the spectrum, with appearance of the typical active site copper absorption band centered close to 680 nm. The same band was observed after reconstitution with 1 copper eq of the *H. ducreyi* mutant lacking the N-terminal domain.

Kinetics of Activity Recovery by Reconstituted Copper-free Enzymes—The process of metal transfer from the N-terminal domain to the active site was also monitored by following the kinetics of activity recovery by the copper-free enzyme. The buffer used to measure SOD activity by the pyrogallol method contains chelating agents that allow discrimination between activity due to copper bound at the active site and that due to spurious or loosely bound metals. In fact, as chelating agents rapidly remove the copper bound to His-rich domains, the catalytic activity observed during the reconstitution process must be exclusively attributable to copper bound in the active sites. Reconstitution experiments in which a stoichiometric amount of copper (1 copper eq/SOD subunit) was added to wild type and mutant copper-free enzymes from *H. parainfluenzae* and *H. ducreyi* were initially performed. The kinetics of activity recovery of the four proteins are shown in Fig. 5. Mutant *H. parainfluenzae* enzyme regained more than 90% activity in less than 5 min, indicating that this protein is able to bind copper very rapidly in the catalytic site. In contrast, presumably due to copper trapping by the His-rich domain, the increase of
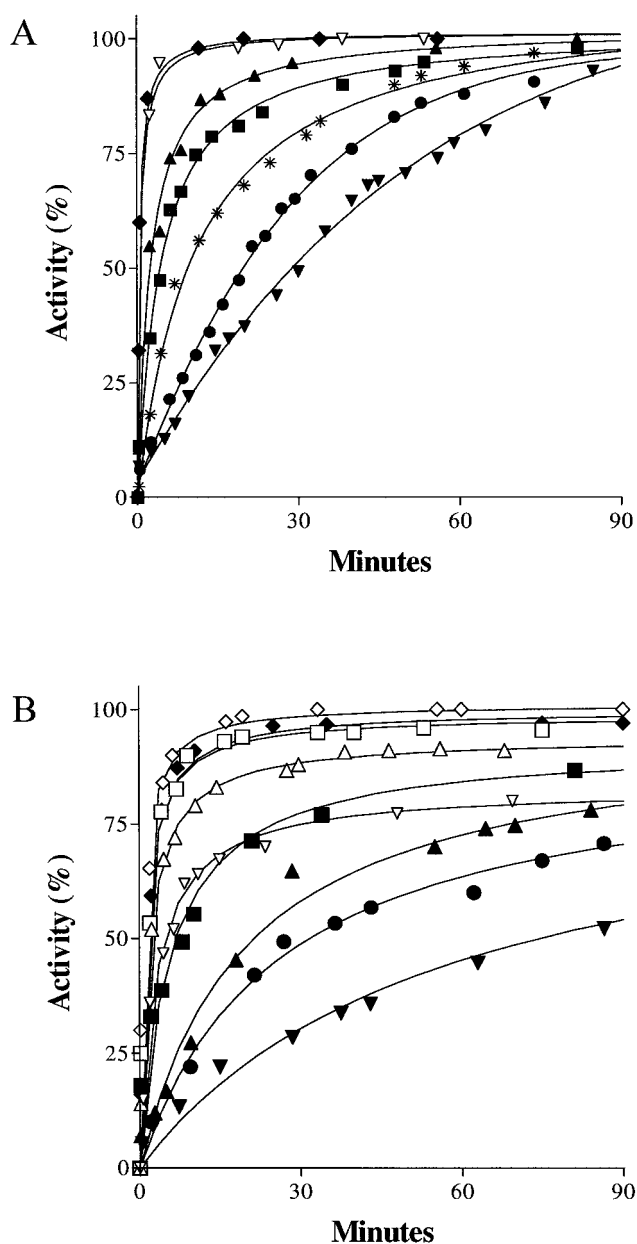
FIG. 6. **Kinetics of regaining activity by the copper-free enzymes at variable copper/subunit molar ratios.** *A,* reconstitution of copper-free wild type *H. parainfluenzae* SOD carried out at the following copper/SOD subunit molar ratios: ◆, 2:1; ▲, 1.4:1; ■, 1:1; *, 1:5; ●, 1:20; ▼, 1:40. Reconstitution of mutant *H. parainfluenzae* enzyme at the same copper/protein molar ratios was also performed obtaining in all cases a nearly identical curve (▽). *B,* reconstitution of copper-free wild type (*filled symbols*) and N-terminal deleted mutant (*open symbols*) *H. ducreyi* enzyme, carried out at the following copper/SOD subunit molar ratios: ◆, 4:1; ◇, 2:1; ■, □, 1:1; ▲, 1:5; △, 1:10; ●, 1:20; ▼, ▽, 1:40.

catalytic activity of wild type enzyme was much slower (it reached 90% activity only after more than 30 min of incubation). Under the same experimental conditions, the N-terminal deleted *H. ducreyi* enzyme regained activity more slowly than mutant *H. parainfluenzae* enzyme, but differences between the two enzymes were abolished when the reconstitution experiments were carried out using 2 copper eq/SOD subunit (see also Fig. 6). This finding suggests, in line with affinity chromatography experiments (see Fig. 3), that other site(s) able to compete with the active sites for copper binding are present on the surface of mutant *H. ducreyi* enzyme devoid of the His-rich domain. Compared with the other enzymes, wild type *H. du-*

creyi Cu,Zn-SOD showed much slower kinetics of activity recovery.

The process of copper incorporation into the active site was further investigated by measuring the kinetics of recovery of activity as a function of the copper/subunit ratio (Fig. 6). These experiments confirmed that the recovery of activity that follows copper addition to mutant *H. parainfluenzae* copper-free protein is extremely fast, as 50% of the enzyme maximal activity was reached in less than 30 s under all conditions tested (the rate of reactivation of this enzyme is too fast to show differences related to the copper/protein ratio by the method we have used). Nearly identical kinetics of activity recovery were observed for the wild type *H. parainfluenzae* enzyme when reconstitution was carried out with two or more copper ions/SOD subunit. At lower copper/subunit molar ratios, the rates of wild type *H. parainfluenzae* enzyme reactivation were slower (Fig. 6A); the process of copper incorporation in an active site was negatively affected by decreasing the copper/subunit ratio. The most likely explanation for such results is that copper-deficient N-terminal sites compete with each other and with the active sites for the copper ion bound at the N-terminal domain of another Cu,Zn-SOD molecule. Also, it is possible that more stable copper complexes involving histidine ligands from different molecules may form when protein is in excess with respect to copper. In any case, despite the long time required to activate the enzyme (at a copper/subunit molar ratio equal to 1:40 about 28 min were necessary to reach 50% maximal activity), we have observed that wild type *H. parainfluenzae* Cu,Zn-SOD is eventually able to reach an activity value close to 100% of its maximal activity even at low copper/protein ratios (see Fig. 6A and data not shown).

Reactivation kinetics of the *H. ducreyi* enzyme are much slower than those of *H. parainfluenzae* Cu,Zn-SOD (Fig. 6B). The N-terminal deleted enzyme rapidly reached 50% of its maximal activity when it was reconstituted with two copper ions/SOD subunit, but at lower copper/subunit molar ratios, the times required to reach 50% maximal activity slightly increased, and the enzyme failed to reach full activation. This result is consistent with the hypothesis that, unlike the case of the mutant *H. parainfluenzae* enzyme, there must be another site with significant copper affinity (or more sites with low affinity) on the enzyme surface that compete(s) with the active sites for copper binding. It is possible that this additional site might involve ligands from different SOD dimers, as already observed in the crystal of *A. pleuropneumoniae* Cu,Zn-SOD (16) or the two facing histidine residues (His-64) located at the dimer interface (31).

As in the case of the Cu,Zn-SOD from *H. parainfluenzae*, the wild type *H. ducreyi* copper-free enzyme recovered activity very slowly, and a strong decrease in the rate of catalytic reactivation was obtained by lowering the copper/protein ratio in the reconstitution reaction. For example, at a copper/subunit ratio equal to 1:40 about 75 min are necessary to achieve 50% maximal activity (compared with 28 min for the *H. parainfluenzae* enzyme and 5 min for the N-terminal deleted *H. ducreyi* mutant). On the whole, the kinetics of *H. ducreyi* Cu,Zn-SOD reconstitution suggest that the metal-binding sites present on the surface of this enzyme compete with very high efficiency with the active sites for copper binding. Moreover, although the fully reconstituted N-terminal deleted *H. ducreyi* protein (obtained at a Cu/subunit ratio of 2) showed an activity identical to that of the reconstituted mutant and wild type *H. parainfluenzae* enzymes, we never succeeded in obtaining the same activity value for reconstituted *H. ducreyi* Cu,Zn-SOD. When reconstitution was carried out at a copper/subunit ratio equal to 4, the *H. ducreyi* enzyme reached 97% activity of the copper-
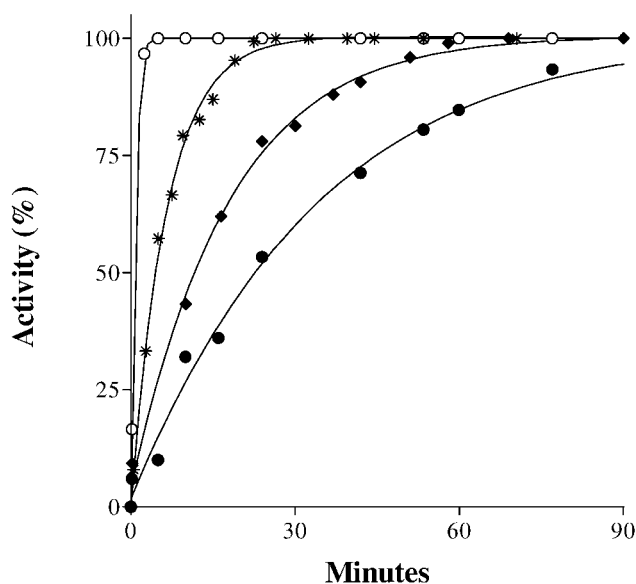
FIG. 7. **Intermolecular copper transfer.** Reconstitution was started by the addition of copper (5.8×10^{-7} M) to copper-free wild type *H. parainfluenzae* enzyme (2.9×10^{-6} M), thus obtaining a mixture with a copper/subunit ratio of 1:10. After 2.5 min 2.9×10^{-6} M mutant *H. parainfluenzae* enzyme lacking the N-terminal histidines was added to the copper-enzyme complex (where copper is still largely bound at the N terminus of the protein), thus obtaining a sample with a copper/subunit ratio of 1:20. The recovery of activity of this sample (*) was monitored in parallel with that of the native enzyme reconstituted at 1:10 (♦) and 1:20 (●) copper/subunit ratios and of the mutant enzyme reconstituted at the copper/subunit ratio of 1:20 (○).
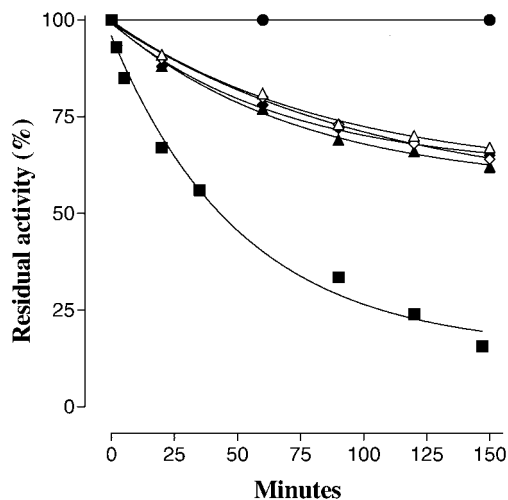
FIG. 8. **Copper loss by bacterial Cu,Zn-SODs.** Enzymes were incubated in 100 mM phosphate buffer, 0.1 mM EDTA, pH 7.0, at 37 °C. Aliquots were withdrawn at the indicated times and immediately assayed by the pyrogallol method to measure residual activity. Each data point is the mean of three independent measurements. ■, *E. coli* Cu,Zn-SOD; ▲, *H. ducreyi* Cu,Zn-SOD; ♦, *H. parainfluenzae* Cu,Zn-SOD; △, mutant *H. ducreyi* Cu,Zn-SOD; ◇, mutant *H. parainfluenzae* Cu,Zn-SOD; ●, bovine Cu,Zn-SOD.

saturated N-terminal deleted mutant. Lower copper/subunit ratios resulted in lower levels of activity recovery, whereas higher amounts of copper induced significant protein aggregation. This result suggests that each *H. ducreyi* N-terminal domain might be involved in the binding of two or more copper ions.

Spectroscopic analyses and reconstitution experiments indicate that the copper ions bound by the N-terminal domains are subsequently slowly transferred to an active site. Different mechanisms, including intramolecular and intermolecular copper transfer from the N-terminal domain to an active site, could be invoked to explain these results. To investigate further the mechanism of copper translocation from the N-terminal domain to the active site, a different kind of reconstitution experiment was carried out (Fig. 7). Copper was added to wild type *H. parainfluenzae* enzyme at a 1:10 copper/subunit ratio, and after 2.5 min the still largely inactive copper-enzyme complex (possessing about 6% maximal activity) was added to the mutant protein devoid of the N-terminal histidines at a final copper/subunit ratio equal to 1:20 (50% subunits of wild type and 50% subunits of mutant enzyme). The recovery of activity was monitored in parallel with that of the mutant (copper/subunit ratio of 1:20) and wild type (copper/subunit ratios of 1:10 and 1:20) enzymes. A net increase in the rate of enzyme reactivation with respect to that of the wild type enzyme was observed. This result strongly suggests that intermolecular copper transfer may occur from the N-terminal domain of an SOD molecule to an active site of an enzyme subunit located in a distinct Cu,Zn-SOD dimer.

Copper Release from the Active Site of Bacterial Cu,Zn-SODs—After extensive dialysis, the EDTA-treated *H. ducreyi* Cu,Zn-SOD (either the as-isolated enzyme or the copper-free reconstituted form) showed an absorption maximum at 660 nm, whereas the dialyzed EDTA-treated *H. parainfluenzae* enzyme showed a shoulder close to 525 nm. Moreover, a consistent decrease in copper content after dialysis was observed

in all Cu,Zn-SOD samples (wild type and mutants lacking N-terminal His-rich domains). These observations suggest that the active site copper can be lost by these bacterial Cu,Zn-SODs, as described previously for the *E. coli* enzyme (19, 47). To test this hypothesis, Cu,Zn-SODs from *H. parainfluenzae*, *H. ducreyi,* and *E. coli* were incubated at neutral pH in the presence of 0.1 mM EDTA at 37 °C, and the enzyme activity was assayed by the pyrogallol method at a series of time points. As shown in Fig. 8, all Cu,Zn-SODs underwent a progressive decrease in activity, indicative of copper loss from the active site. Under the same conditions bovine Cu,Zn-SOD activity was totally unaffected by incubation in the presence of EDTA. The rate of loss of enzyme activity was similar in wild type and mutant *Haemophilus* enzymes, indicating that the N-terminal domain is not directly involved in the process of metal loss from the active site. It should be noted that the rate of inactivation of the *E. coli* enzyme was much faster than that of the *Haemophilus* enzymes, possibly due to higher mobility of loops forming the active site channel in the monomeric enzyme (19).

Macrophage Killing Assays—We have tested the ability of wild type and mutant Cu,Zn-SODs from *H. parainfluenzae* and *H. ducreyi* to protect *E. coli* K12 cells from phagocytic killing. The assays were carried out using murine or human macrophages. Control experiments were performed to determine the serum resistance of each bacterial strain in the presence of 5% fresh pooled serum but without macrophages.

Bacteria used in phagocytic killing experiments were obtained by culturing cells until they reached the stationary phase either in standard LB medium or in LB supplemented with copper and zinc. In fact, as observed previously for other Cu,Zn-SODs (28), the enzymes from *H. ducreyi* and *H. parainfluenzae* produced in *E. coli* cells grown in LB medium display a modest catalytic activity, whereas both wild type and mutant enzymes show maximal activation (about a 20-fold higher activity) when produced in media supplemented with 200 μM CuSO$_4$ and 10 μM ZnSO$_4$. Under these conditions the Cu,Zn-SOD activity expressed by bacteria producing wild type or mutant Cu,Zn-SOD was comparable (data not shown). Fig. 9 shows that the survival of recombinant *E. coli* cells expressing wild type or mutant *H. parainfluenzae* Cu,Zn-SOD within the mouse macrophage-like line J774 was similar to that of control
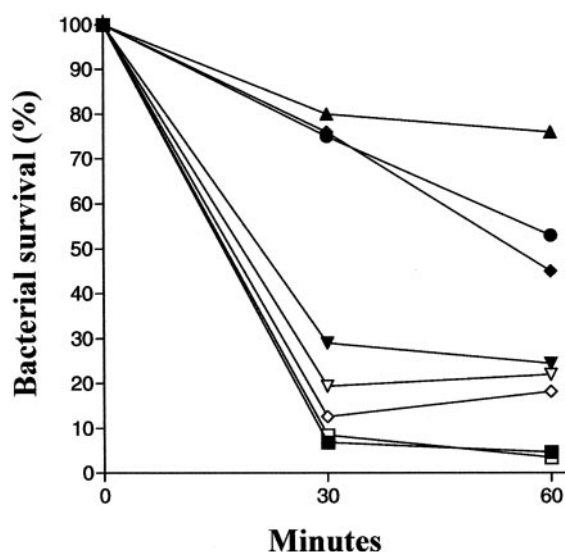
FIG. 9. **The His-rich domain influences *E. coli* cells resistance to phagocytic killing.** Macrophage killing of *E. coli* GC4468 cells expressing wild type and mutant *Haemophilus* Cu,Zn-SODs. Bacteria were grown in standard LB (*open symbols*) or in LB supplemented with copper and zinc (*filled symbols*). The phagocytosis experiment was carried out as described under "Experimental Procedures" with J774 murine macrophage-like cells. ▲, wild type *H. ducreyi* Cu,Zn-SOD; ●, mutant *H. ducreyi* Cu,Zn-SOD; ◆, ◇, *H. parainfluenzae* Cu,Zn-SOD; ▽, ▼, mutant *H. parainfluenzae* Cu,Zn-SOD ■, □; control cells bearing plasmid pEMBL18.

cells when bacteria were pre-cultured in standard LB medium. In contrast, wild type *H. parainfluenzae* Cu,Zn-SOD was able to confer significant protection from phagocytic killing to *E. coli* cells grown in a medium supplemented with metals. Interestingly, the mutant enzyme lacking the N-terminal histidines provided a significantly lower protection. Experiments carried out with the human macrophage-like line THP-1 gave essentially identical results.

Similar experiments were carried out with bacteria expressing the *H. ducreyi* Cu,Zn-SOD. Overexpression of this Cu,Zn-SOD conferred very efficient protection toward macrophage killing on *E. coli* grown in copper- and zinc-supplemented media. In this case, however, the difference in survival between *E. coli* cells expressing the wild type enzyme and the mutant protein devoid of the N-terminal histidines was less pronounced (Fig. 9).

DISCUSSION

This investigation was prompted by inspection of the amino acid sequences of a subgroup of Cu,Zn-SODs, all expected to have a nearly identical monomer fold (15). The sequences of some of these enzymes (from *A. pleuropneumoniae*, *H. ducreyi*, *H. parainfluenzae*, *H. influenzae,* and *N. meningitidis*) are characterized by the following two features that attracted our attention for their potential ability to modulate metal binding and/or uptake: the unusually large number of histidines and the presence of His-rich N-terminal extensions. The presence of an unusually flexible N-terminal domain could be predicted from the recently solved x-ray structure of *A. pleuropneumoniae* Cu,Zn-SOD (16). This enzyme was found to contain two copper ions/subunit, one of which, as expected, was bound in the active site, whereas the other was ligated in a tetrahedral geometry on the protein surface by two neighboring histidine residues from each of two adjacent SOD molecules. Such an x-ray analysis did not provide information on the metal binding ability of the N-terminal extension because the crystallized enzyme was devoid of the N-terminal histidines, due to protease digestion during protein purification. The aim of this work,

therefore, was to evaluate whether such N-terminal extensions effectively represent metal binding domains and to assess their ability to modulate metal uptake in the active site.

Immobilized metal affinity chromatography experiments clearly demonstrated that the N-terminal regions of the Cu,Zn-SODs from *H. ducreyi*, *H. influenzae,* and *H. parainfluenzae* are characterized by high affinity for divalent metal ions and that such an affinity is largely mediated by the His-rich region. The Cu,Zn-SOD from *H. ducreyi* showed some affinity for Ni-NTA also upon removal of the N-terminal extension, indicating that other residues (probably some other histidines located on the enzyme surface) are able to interact with divalent metal ions, as in the case of the *A. pleuropneumoniae* enzyme. The affinity for divalent ions provided by Cu,Zn-SODs N-terminal regions was comparable to that reported for several proteins engineered by the introduction of a 6-histidine tag (48) and was sufficient to allow purification of the enzyme in a single chromatographic step. Such a property can be transferred to other proteins that have no affinity for metal chelate resins.

The ability of the N-terminal region to bind transition metals with high affinity was confirmed by the electronic spectra of the wild type enzyme of *H. ducreyi* and *H. parainfluenzae* compared with mutant enzymes lacking their N-terminal histidines. The two mutant enzymes showed a copper absorption band centered at 680 nm, typical of all Cu,Zn-SODs. In contrast, the wild type enzymes from both organisms exhibited unusual spectra due to the overlapping of the copper absorption band centered at 680 nm with another due to copper bound to the N-terminal domain. The enzyme from *H. parainfluenzae* displayed two clearly distinct absorption maxima (680 and 525 nm), whereas the *H. ducreyi* enzyme showed a broad absorption band with a maximum affected by copper content of the sample. The difference between the two enzymes probably reflects a difference in the number or the nature of residues coordinating the metal ion as well as the possible presence of additional metal-binding site(s) on the surface of the *H. ducreyi* enzyme.

We have also investigated the possibility that metals initially bound by the N-terminal domains could be delivered to an active site. This hypothesis has been tested spectroscopically, monitoring the changes in the electronic spectra following copper addition, and by catalytic assays, measuring the regain of activity of copper-free proteins. Reconstitution experiments with copper-deficient *H. parainfluenzae* enzyme demonstrated that *in vitro* copper is rapidly bound at the N terminus of the enzyme and then is slowly transferred to an active site. The ability of the N-terminal domain to compete efficiently with the active sites for copper binding demonstrates that this protein region may act as a high affinity binding domain able to capture efficiently metals from the environment. The observation of a unidirectional transfer of copper from the N-terminal histidines to an active site suggests that copper binding by the N-terminal domain is kinetically favored, possibly due to the high mobility of the N-terminal extension but that the active site binds copper more tightly than the external domain. Similar reconstitution experiments were carried out with the *H. ducreyi* enzyme, although in this case the overlap between the absorption bands due to copper bound at the active site and at the N-terminal domain (and possibly at other site(s)) prevented a spectral demonstration of copper transfer from the N-terminal region to an active site.

The kinetics of activity recovery reported in Figs. 5 and 6 confirmed that the N-terminal domain rapidly traps the available copper ions as the two wild type enzymes showed a much slower recovery of activity than the mutant enzymes lacking N-terminal histidines. These experiments also suggested that

the N-terminal domains from different Cu,Zn-SOD molecules compete for copper binding. As a consequence, when protein reconstitution is carried out with a large molar excess of protein with respect to copper, the copper ion is transferred very slowly to an active site.

Either an intra- or an intermolecular mechanism of copper transfer from the N-terminal domain to an active site could be envisioned to explain the reconstitution data obtained with the two wild type Cu,Zn-SODs. However, the observation that the rate of copper transfer to the active site is enhanced by the addition of an equimolar amount of mutant protein to wild type *H. parainfluenzae* enzyme (with copper already bound at its N-terminal domain (Fig. 7)) demonstrates that copper transfer does not necessarily proceed via an intramolecular pathway. If copper bound by the N-terminal histidines were necessarily transferred intramolecularly, the rate of copper transfer should be insensitive to the addition of molecules of mutant enzyme. Although such a result does not totally exclude the possibility that intramolecular transfer might occur in a subset of molecules, it is more simply explained by an intermolecular mechanism of copper transfer. This interpretation is consistent with the observation that the N-terminal extension of the *H. parainfluenzae* enzyme is rather short (9 amino acids) so that, also assuming the total absence of constraints in its possibility to move, it is difficult to imagine, based on the available structures of bacterial Cu,Zn-SODs, that it could reach the active site cavity of the same subunit and mediate the direct transfer of the copper ion. Similar considerations may apply to the enzymes from *H. influenzae*, *N. meningitidis*, and *A. pleuropneumoniae*, in all of which the N-terminal extensions are probably too short to allow direct intramolecular transfer of the metal ion. Further work is required to understand whether the N-terminal histidines exert their action by mediating direct insertion of the copper ion into an active site of a neighboring SOD dimer or by increasing the local concentration of the metal ions around the catalytic site. Also, it is worth noting that the *H. ducreyi* enzyme may form multimeric complexes (31) and that production of this enzyme in media not supplemented with metal ions strongly induces formation of the oligomeric forms of the enzyme (not shown). The formation of these high molecular weight Cu,Zn-SOD complexes *in vivo* might favor the process of intermolecular metal transfer.

Little is known about metal trafficking in the periplasmic space and the mechanisms of metal uptake by periplasmic Cu,Zn-SOD, but the discovery of a high affinity metal-binding site located on a very mobile protein region in bacterial Cu,Zn-SOD suggests that this protein domain could play some role in the process of copper and zinc binding *in vivo*. In fact, recent work has established that several metalloproteins, although easily able to acquire their metal cofactor *in vitro*, require auxiliary factors to obtain the metal *in vivo* (49–51). In the cytoplasm of eukaryotic and prokaryotic cells, the delivery of copper to specific proteins is mediated by a family of copper-transporting proteins, collectively known as copper chaperones. Specific interactions between a chaperone and its target protein allows metal exchange between the two proteins, protecting the cell from the reactivity of free metal ions. This is the case, for example, of eukaryotic Cu,Zn-SOD that, while possessing an extraordinary high affinity for copper *in vitro*, is unable to obtain its metal cofactor *in vivo* under physiologically low metal conditions in the absence of a specific copper transporter (49, 52). Although the mechanism of copper exchange between Cu,Zn-SOD and its copper chaperone is still under investigation, it has been established that the chaperone for Cu,Zn-SOD is characterized by three distinct protein domains (53, 54). The central domain has a SOD-like structure that

physically interacts with Cu,Zn-SOD. The N- and C-terminal domains are both involved in metal binding, with the C-terminal domain, which is disordered in crystal structures and highly mobile in solution, playing a crucial role for Cu,Zn-SOD activation *in vivo*.

Bacteria do not possess genes encoding proteins homologous to the eukaryotic copper chaperone for Cu,Zn-SOD (55), and copper uptake by prokaryotic Cu,Zn-SODs is probably largely dependent on metal availability in the periplasmic space. This hypothesis is consistent with the observations that the activity of periplasmic Cu,Zn-SOD overexpressed in bacteria is roughly proportional to the amount of copper with which the medium is supplemented (28 and this work) and that the *E. coli* s*odC* promoter is down-regulated by extracellular copper chelators (55). Whereas the outer bacterial membrane is highly permeable to small molecules (including metal ions) present in the environment (56), copper availability in the periplasmic compartment is regulated by pumps that mediate copper transport into cells when its intracellular concentration is limiting or its excretion when it is in excess (51, 57). In the present study, we have investigated the metal-binding properties of the His-rich domains, following their ability to bind copper and mediate its transfer to an active site. This choice was prompted by the assumption that copper availability is limited in cells and by our ability to monitor copper binding either spectroscopically or by its effect on the enzyme activity. In contrast, zinc, which is usually considered an abundant element, is spectroscopically silent, and its contribution to activity cannot be detected by indirect solution assays. However, the possibility that the N-terminal domain could play a role also in the binding of zinc should not be discarded. In fact, complex mechanisms also regulate zinc concentration in the bacterial cell (58) and could potentially limit zinc availability in the periplasm. As a matter of fact, screening *Vibrio cholerae* and *Pseudomonas aeruginosa* for genes induced during infection have identified zinc-responsive regulators as putative virulence factors (59, 60).

The problem of obtaining adequate amounts of these trace metals must be particularly acute for those pathogens that colonize host compartments where free metal ions (defined as metal ions not sequestered by other metal-binding proteins) are virtually or entirely absent. We suggest that a likely function for the His-rich domain present in some Cu,Zn-SODs could be to facilitate metal uptake under conditions of metal starvation. By virtue of their high affinity for divalent ions, such N-terminal domains could efficiently trap the few available metal ions and subsequently deliver them to an active site. Through development of such a metal recruiting function, Cu,Zn-SODs may be enabled to acquire these crucial cations in the presence of other competing proteins in the crowded periplasmic environment. Although no data are available concerning free copper concentration in mammalian tissues, it has recently been shown that only a few mammalian cell types are able to support a low level of copper incorporation into cytosolic Cu,Zn-SOD in the absence of its copper chaperone (61), implying that in most tissues free copper availability is low. Interestingly, so far we have identified the His-rich metal binding domain exclusively in Cu,Zn-SODs from mucosal colonists and pathogens. These organisms inhabit an environment where free metal ions might be particularly scarce, in contrast to the situation in which enteric or free living bacteria (whose Cu,Zn-SODs lack this domain) are to be found.

The slow kinetics of copper transfer from the N-terminal domain to an active site *in vitro*, with a consequent retarding effect on the rate of catalytic activation of the protein compared with mutant Cu,Zn-SODs devoid of N-terminal histidines, suggests that such protein domains may provide a contingency

rather than an obligatory function, being needed perhaps only under conditions of severe metal deprivation.

Our results (Fig. 9) demonstrate that the ability of overexpressed Cu,Zn-SODs to protect *E. coli* from phagocytic killing requires pre-charging of the enzyme with catalytic copper before bacteria enter the phagocyte. In this context, overexpression of Cu,Zn-SODs containing the additional N-terminal domain protect *E. coli* more efficiently than overexpression of mutant enzymes devoid of this high affinity metal-binding site. Such enhanced protection might suggest a higher enzymatic activity of the wild type protein in the course of microbial infection. However, the metal binding function of the N-terminal domain may be invoked as a plausible alternative explanation. As reported previously for the *E. coli* enzyme (19), Cu,Zn-SODs from *Haemophilus* species readily lose copper from their active site. Reduction of divalent copper at the active site, which follows reaction with hydrogen peroxide generated at high concentrations during the oxidative burst, may further decrease copper affinity for the active site and favor its release. If such copper loss occurs in the course of infection, we speculate that, where present, the N-terminal domain could confer an advantage, either by favoring reacquisition of copper by the Cu,Zn-SOD active site or by shielding bacterial cells from toxic free radicals generated by the reaction of free copper ions with hydrogen peroxide.

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A Histidine-rich Metal Binding Domain at the N Terminus of Cu,Zn-Superoxide Dismutases from Pathogenic Bacteria: A NOVEL STRATEGY FOR METAL CHAPERONING

Andrea Battistoni, Francesca Pacello, Anna Paola Mazzetti, Concetta Capo, J. Simon Kroll, Paul R. Langford, Assunta Sansone, Giovanna Donnarumma, Piera Valenti and Giuseppe Rotilio

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