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Single Mutation Induces a Metal-Dependent Subunit Association in Dimeric Cu,Zn Superoxide Dismutase

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Tryptophan 83, a residue strongly involved in the intersubunit interaction of the Cu,Zn superoxide dismutases from *Photobacterium leiognathi,* has been selectively mutated to phenylalanine or tyrosine. The recombinant mutant enzymes expressed in *Escherichia coli* were purified in two well distinct and stable forms, one dimeric and fully active and the other monomeric and devoid of metals. In agreement, *in vitro* experiments indicate that the removal and addition of zinc in the mutant enzymes induces monomerization and dimerization, respectively, while does not perturb the dimeric association of the native protein. This is the first unambiguous experimental proof of a direct communication between the intersubunit interface and the metal active site. © 2000 Academic Press

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Factors governing subunit–subunit interactions in multimeric proteins and the functional role of protein quaternary structure have been the object of intense studies over the past few years. An interesting class of homodimeric proteins is represented by Cu,Zn superoxide dismutases (Cu,ZnSODs), which are ubiquitous enzymes involved in the mechanisms of resistance against oxidative stress (1). All the eukaryotic Cu,ZnSODs so far characterized have a fully conserved dimeric structure. Bacterial Cu,ZnSODs have been recently characterized and it has been shown to have a distinct quaternary organization since in the dimeric enzyme from *Photobacterium leiognathi* the β-strands recruited to form the dimer interface are diametrically opposite to those used in the eukaryotic enzymes (2, 3). Moreover some bacterial Cu,ZnSODs have been shown to have a stable monomeric form (4–6). These findings suggest that, starting from a putative monomeric precursor, prokaryotic and eukaryotic Cu,ZnSODs have convergently evolved toward a dimeric structure which could be important for the enzyme biological function. The 3D structure of the Cu,ZnSOD from *P. leiognathi* has shown the presence of three clusters of residues which are important for the stability of the dimeric structure (3). Trp83 is central to the largest hydrophobic cluster located at the dimer interface of this enzyme and is conserved in several other bacterial Cu,ZnSODs known to be dimeric (3). In order to weaken the stability of subunit-subunit interaction in *P. leiognathi* Cu,ZnSOD and to investigate the monomer dimer equilibrium, we have constructed two site-specific mutants in which Trp83 has been selectively substituted with phenylalanine or tyrosine. We have found that the mutants do not display any monomer-dimer equilibrium, but that their quaternary structure is strongly metal-dependent. In detail, the metallated wild type and mutated proteins have a stable and active dimeric assembly, while metal depletion brings the mutant proteins in a stable and inactive monomeric state, at variance on what found in the wild type enzyme. This is the first unambiguous experimental proof of a direct communication between the intersubunit interface and the metal cluster constituting the active site, i.e., two regions which are more than 15 Å away.

MATERIALS AND METHODS

Site-directed mutagenesis has been carried out by a PCR based approach which has already been described (7), using the mutagenic primers Phe83 5′-ATCATCAGTAAATGGGAAACC and Tyr83 5′-ATCATCAGTATATGGGAAACC and the high fidelity polymerase Expand (Boehringer Mannheim). The nucleotide sequence of the mutated DNAs was verified by the dideoxy chain termination method. Overexpression of wild type and mutants Cu,ZnSODs was carried out in 71/18 *E. coli* cells grown in Luria Bertani broth containing 100 μg/ml ampicillin, 0.25 mM $CuSO_4$ and 50 μM $ZnSO_4$. Periplasmic extracts were prepared as previously described (8), concentrated by ultrafiltration and loaded onto a gel filtration FPLC column previously equilibrated with 20 mM Tris–HCl, pH 7.0, 0.15 M NaCl, in order to separate monomeric and dimeric forms of the enzyme. Further purification of the two enzyme forms has been

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carried by ion exchange chromatography, accordingly to previously described procedures (8, 9). The metal dependent subunit association of metal devoid and the zinc reconstituted proteins was studied by gel filtration chromatography under the above described conditions. Protein concentration was evaluated by the method of Lowry (10), using bovine serum albumin as standard. Cu,ZnSOD activity was measured using the pyrogallol method (11). Copper content was evaluated by double integration of the EPR spectra, using a Cu^{2+} EDTA solution as a standard (12).

Metal-free Cu,ZnSODs (devoid of both the copper and zinc ions) were prepared by extensive dialysis against 50 mM sodium acetate buffer, pH 3.8, 2 mM EDTA followed by a further dialysis against 50 mM sodium acetate, pH 3.8, 0.1 M NaCl to remove excess EDTA, as previously described (8). Before reconstitution with zinc metal devoid proteins were dialyzed twice against 50 mM Na acetate buffer, pH 5.5. Zinc reconstituted samples were dialyzed against 0.15 M NaCl, 20 mM Tris–HCl, pH 7.0 and loaded on a gel filtration column equilibrated with the same buffer plus 50 μM EDTA.

RESULTS AND DISCUSSION

Purification by gel filtration chromatography of wild type *P. leiognathi* Cu,ZnSOD and of the two mutant enzymes Trp83Phe and Trp83Tyr from periplasmic extracts of *E. coli* cells, gives rise to different elution patterns. Wild type Cu,ZnSOD elutes in a single peak with an apparent molecular weight of about 32 kDa (9, 13). On the contrary, the elution profiles of the two mutants are characterized by two clearly resolved peaks at 32 and 17 kDa, corresponding to the dimeric and monomeric forms of the enzymes, respectively. The dimeric form of both the mutants is fully active, while the monomeric forms lack Cu,ZnSOD activity. The EPR spectra of the dimeric Trp83Phe and Trp83Tyr mutants are indistinguishable to that of the wild type enzyme, while the monomeric forms are totally devoid of copper, thus explaining their lack of activity, and contain only a very low amount of zinc. According to this observation the mutant enzymes purified from bacteria grown in LB containing 1 mM EDTA, display only the monomeric form which is completely devoid of metals.

The influence of the metal site occupancy on the dimeric interaction in *P. leiognathi* Cu,ZnSOD was further studied by gel filtration chromatography using metal-depleted derivatives of the native and mutated proteins. Wild type Cu,ZnSOD always elutes as a dimeric enzyme, irrespectively of the presence of copper and zinc in the active site (Fig. 1A). On the contrary the mutants devoid of both copper and zinc elute mainly as monomers (Fig. 1B). The addition of a stoichiometric amount of zinc allows dimerization of the mutants (Fig. 1C) while the subsequent addition of copper does not influence their quaternary structure, but it confers full catalytic activity.

This is the first clear proof of a direct communication between the dimer interface and the metal site in Cu,ZnSODs. Some evidences in this sense came from reconstitution experiments on the eukaryotic enzymes which indicated that the binding of copper to apo-SOD

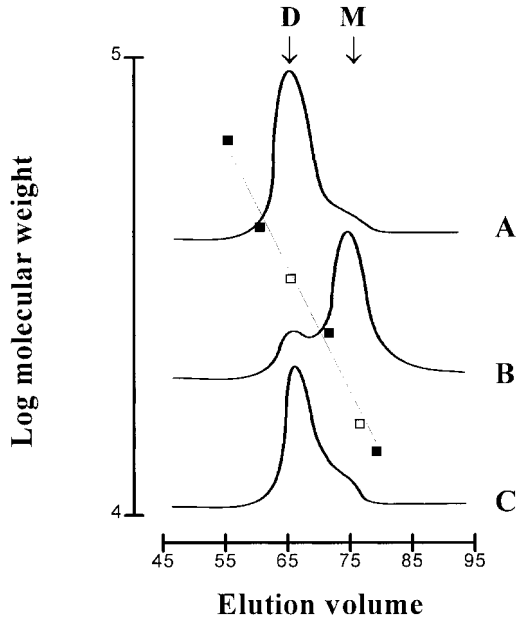

FIG. 1. Elution profile of metal-free and zinc-reconstituted wild-type and Trp 83Phe *P. leiognathi* CuZnSOD injected onto an HiLoad 16/60 Superdex 75 gel filtration FPLC column. (A) Metal-free wild-type *P. leiognathi* Cu,ZnSOD; (B) metal-free Trp83Phe mutant Cu,ZnSOD; (C) sample B reconstituted with zinc. In the case of wild-type Cu,ZnSOD the elution profile is identical to that shown in A, independently of the enzyme metal content. The column was calibrated with the following molecular weight markers (indicated by black squares): bovine serum albumin (67,000 Da), ovalbumin (43,000 Da), chymotrypsinogen A (25,000 Da), and ribonuclease A (13,700 Da). The two major peaks characterizing the elution profiles of wild-type and mutant *P. leiognathi* Cu,ZnSOD (indicated by open squares on the calibration line) were of approximately 33,000 and 17,000 Da, respectively, corresponding to the dimeric (D) and monomeric (M) forms of the enzyme. The elution profile of the Trp83Tyr mutant was essentially identical to that of the Trp83Phe mutant here shown.

is affected by the occupancy of the copper site in the other subunit (14). More recently, molecular dynamics simulations have indicated the occurrence of a dynamical correlation between the intersubunit interface and the active site, since significant movements of the loops surrounding the active site of one monomer were found to be correlated to motions in the other subunit, near to the dimer interface (15, 16). The results here reported confirm the MD suggestion and demonstrate the occurrence of a direct communication between intersubunit surface and metal active site. In fact, the Trp83Phe and Trp83Tyr mutants reach the native like dimeric state only in presence of the zinc atom indicating that this atom has an important role, not only in providing the correct structure to the metal cluster (17), but also in conferring the right conformation for the dimerization process. This results acquires a particular interest since the loss of zinc from either wild type or mutant Cu,ZnSODs associated to amyotrophic lateral sclerosis

has been shown to induce apoptosis in cultured motor neurons (18).

Our findings also imply that mutations at the subunit interface can modulate the shape of the active site and then the catalytic activity of the enzyme. In agreement, it has been shown that the monomeric mutants of the human enzyme obtained by site directed mutagenesis at the dimeric interface display an activity much lower than that of the native enzyme (19). Moreover it has been shown that even different protein contact in crystals can tune the coordination geometry of the active site copper ion with modulation effects on its redox properties (20, 21). We suggest that mutations of the residues involved in subunit association should not necessarily reduce catalytic activity, but could even positively perturb the active site shaping the surrounding loops in a favorable position to better attract the incoming superoxide anion.

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REFERENCES

1. Bannister, J. V., Bannister, W. H., and Rotilio, G. (1987) Aspects of the structure, function and application of superoxide dismutase. *Crit. Rev. Biochem.* **22,** 111–180.
2. Bourne, Y., Redford, S. M., Steinman, H. M., Lepock, J. R., Tainer, J. A., and Getzoff, E. D. (1996) Novel dimeric interface and electrostatic recognition in bacterial Cu,Zn superoxide dismutase. *Proc. Natl. Acad. Sci. USA* **93,** 12774–12779.
3. Bordo, D., Matak, D., Djinovic-Carugo, K., Rosano, C., Pesce, A., Bolognesi, M., Stroppolo, M. E., Falconi, M., Battistoni, A., and Desideri, A. (1999) Evolutionary constraints for dimer formation in prokaryotic Cu,Zn superoxide dismutase. *J. Mol. Biol.* **285,** 283–296, doi:10.1006/jmbi.1998.2267.
4. Battistoni, A., and Rotilio, G. (1995) Isolation of an active and heat-stable monomeric form of Cu,Zn superoxide dismutase from the periplasmic space of *Escherichia coli. FEBS Lett.* **374,** 199–202
5. Pesce, A., Capasso, C., Battistoni, A., Folcarelli, S., Rotilio, G., Desideri, A., and Bolognesi, M. (1997) Unique structural features of the monomeric Cu,Zn superoxide dismutase from *Escherichia coli. J. Mol. Biol.* **274,** 408–420, doi:10.1006/jmbi.1997.1400.
6. Chen, Y. L., Park, S., Thornburg, R. W, Tabatabai, L. B., and Kintnar, A. (1995) Structural characterization of the active site of *Brucella abortus* Cu-Zn superoxide dismutase: a ^{15}N and ^{1}H NMR investigation. *Biochemistry* **34,** 12265–12275.
7. Folcarelli, S., Battistoni, A., Falconi, M., O'Neill, P., Rotilio, G., and Desideri, A. (1998) Conserved enzyme-substrate electrostatic attraction in prokaryotic Cu,Zn superoxide dismutase. *Biochem. Biophys. Res. Commun.* **244,** 908–911, doi:10.1006/bbrc.1998.8364.
8. Battistoni, A., Folcarelli, S., Cervone, L., Polizio, F., Desideri, A., Giartosio, A., and Rotilio, G. (1998) Role of the dimeric structure in Cu,Zn superoxide dismutase. pH-dependent, reversible denaturation of the monomeric enzyme from *Escherichia coli. J. Biol. Chem.* **273,** 5655–5661.
9. Foti, D., Lo Curto, B., Cuzzocrea, G., Stroppolo, M. E., Polizio, F, Venanzi, M., and Desideri, A. (1997) Spectroscopic characterization of recombinant Cu,Zn superoxide dismutase from *Photobacterium leiognathi* expressed in *Escherichia coli:* Evidence for a novel catalytic copper binding site. *Biochemistry* **36,** 7109–7113.
10. Lowry, H. O., Rosebrough, N. J., Farr, L. A., and Randall, R. J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.
11. Marklund, S., and Marklund, G. (1974) Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur. J. Biochem.* **47,** 469–474.
12. Polticelli, F., Bottaro, G., Battistoni, A., Carrì, M. T., Djinovic-Carugo, K., Bolognesi, M., O'Neill, P., Rotilio, G., and Desideri, A. (1995) Modulation of the catalytic rate of Cu,Zn superoxide dismutase in single and double mutants of conserved positively and negatively charged residues. *Biochemistry* **34,** 6043–6049.
13. Stroppolo, M. E., Sette, M., O'Neill, P., Polizio, F., Cambria, M. T., and Desideri, A. (1998) Cu,Zn superoxide dismutase from *Photobacterium leiognathi* is an hyperefficient enzyme. *Biochemistry* **37,** 12287–12292.
14. Rigo, A., Terenzi, M., Viglino, P., Cocco, D., Calabrese, L., and Rotilio, G. (1978) The binding of copper ions to copper-free bovine superoxide dismutase. Kinetic aspects. *Biochem. J.* **169,** 277–280.
15. Chillemi, G., Falconi, M., Amadei, A., Zimatore, G., Desideri, A., and Di Nola, A. (1997) The essential dynamics of Cu, Zn superoxide dismutase: Suggestion of intersubunit communication. *Biophys. J.* **73,**1007–1018.
16. Falconi, M., Venerini, F., and Desideri, A.(1998) A spectroscopic and molecular dynamics study of native and of a mutant of *Xenopus laevis* Cu,Zn superoxide dismutase: Mechanistic consequences of replacing four charged amino acids on the 'electrostatic' loop. *Biophys. Chem.* **75,** 235–48.
17. Valentine, J. S., and Pantoliano, M. W. (1981) Protein–metal ion interactions in cuprozinc protein (superoxide dismutase). *In* Copper Proteins (Spiro, T. G., Ed.), pp. 291–358, Wiley-Interscience, New York.
18. Estevez, A. G., Crow, J. P., Sampson, J. B., Reiter, C., Zhuang, Y., Richardson, G. J., Tarpey, M. M., Barbeito, L., and Beckman, J. S. (1999) Induction of nitric oxide-dependent apoptosis in motor neurons by zinc-deficient superoxide dismutase. *Science* **286,** 2498–2500.
19. Bertini, I., Piccioli, M., Viezzoli, M. S., Chiu, C. Y., and Mullenbach, G. T. (1994) A spectroscopic characterization of a monomeric analog of copper, zinc superoxide dismutase. *Eur. Biophys. J.* **23,** 167–176.
20. Hart, P. J., Balbirnie, M. M., Ogihara, N. L., Nersissian, A. M., Weiss, M. S., Valentine, J. S., and Eisenberg, D. (1999). A structure-based mechanism for copper–zinc superoxide dismutase. *Biochemistry* **38,** 2167–2178.
21. Hough, M. A., and Hasnain, S. S. (1999) Crystallographic structures of bovine copper–zinc superoxide dismutase reveal asymmetry in two subunits: Functionally important three and five coordinate copper sites captured in the same crystal. *J. Mol. Biol.* **287,** 579–592, doi:10.1006/jmbi.1999.2610.