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THE POSITIVE CHARGE AT POSITION 189 IS ESSENTIAL FOR THE CATALYTIC ACTIVITY OF IRON- AND MANGANESE-CONTAINING SUPEROXIDE DISMUTASES

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We have previously shown (C.L. Borders, Jr. *et al.*, (1989) *Archives of Biochemistry and Biophysics*, **268**, 74–80) that the iron-containing (FeSOD) and manganese-containing (MnSOD) superoxide dismutases from *Escherichia coli* are extensively (> 98%) inactivated by treatment with phenylglyoxal, an arginine-specific reagent. Examination of the published primary sequences of these two enzymes shows that Arg-189 is the only conserved arginine. This arginine is also conserved in the three additional FeSODs and seven of the eight additional MnSODs sequenced to date, with the only exception being the MnSOD from *Saccharomyces cerevisiae*, in which it is conservatively replaced by lysine. Treatment of *S. cerevisiae* MnSOD with phenylglyoxal under the same conditions used for the *E. coli* enzymes gives very little inactivation. However, treatment with low levels of 2,4,6-trinitrobenzenesulfonate (TNBS) and acetic anhydride, two lysine-selective reagents that cause a maximum of 65–80% inactivation of the *E. coli* SODs, gives complete inactivation of the yeast enzyme. Total inactivation of yeast MnSOD with TNBS correlates with the modification of approximately 5 lysines per subunit, whereas 6–7 lysines per subunit are acylated with acetic anhydride on complete inactivation. It appears that the positive charge contributed by residue 189, lysine in yeast MnSOD and arginine in all other SODs, may be critical for the catalytic activity of MnSODs and FeSODs.

KEY WORDS: Superoxide dismutase, manganese, iron, arginine, lysine

INTRODUCTION

The manganese-containing (MnSOD) and iron-containing (FeSOD) superoxide dismutases are highly homologous to each other, but are unrelated to the more thoroughly characterized Cu,ZnSODs at all levels of structure.[1-4] Three-dimensional structures have been determined for the FeSODs from *Escherichia coli*[1,5] and *Pseudomonas ovalis*,[2,5] and the MnSODs from *Thermus thermophilus*[6] and *Bacillus stearothermophilus*.[7] The active site metal ligands have been identified as 3 histidines and 1 aspartate in all four structures, and these residues are conserved in the 13 members of this class sequenced to date (*vide infra*). A notable feature of MnSOD and FeSOD structures is a conserved apolar core of approximately 10 aromatic residues immediately surrounding the metal-ligand cluster.[6-8]

Chemical modification is an approach frequently used to identify amino acid residues that may be important for enzyme catalytic activity. Inactivation of *E. coli* FeSOD by H_2O_2 has used to confirm the importance of tryptophan for normal catalysis.[9] Recent work[10] has suggested that arginyl residues may be important for the catalytic activity of *E. coli* MnSOD and *E. coli* FeSOD. Examination of the aligned

amino acid sequences of these two proteins reveals that the only highly conserved arginyl residue is at position 189 (*vide infra*). This arginine is present in 12 of 13 sequences, with the only exception being *Saccharomyces cerevisiae* (yeast) MnSOD, where it is conservatively replaced by a lysine. We have studied the inactivation of yeast MnSOD, *E. coli* MnSOD, and *E. coli* FeSOD by phenylglyoxal, a reagent specific for the modification of arginyl residues in proteins, and TNBS and acetic anhydride, two lysine-selective reagents. The results strongly suggest that a positively charged amino acid side chain at position 189 in MnSODs and FeSODs is critical for catalysis.

EXPERIMENTAL

Materials

E. coli MnSOD and *E. coli* FeSOD were purchased from Sigma and were used without further purification, while *S. cerevisiae* MnSOD was purified and handled according to procedures described elsewhere.[11] Phenylglyoxal and TNBS were purchased from Aldrich and acetic anhydride was from J.T. Baker. [1-^{14}C]-Acetic anhydride (5.0 mCi/mmole) was a product of DuPont NEN.

Analytical Procedures

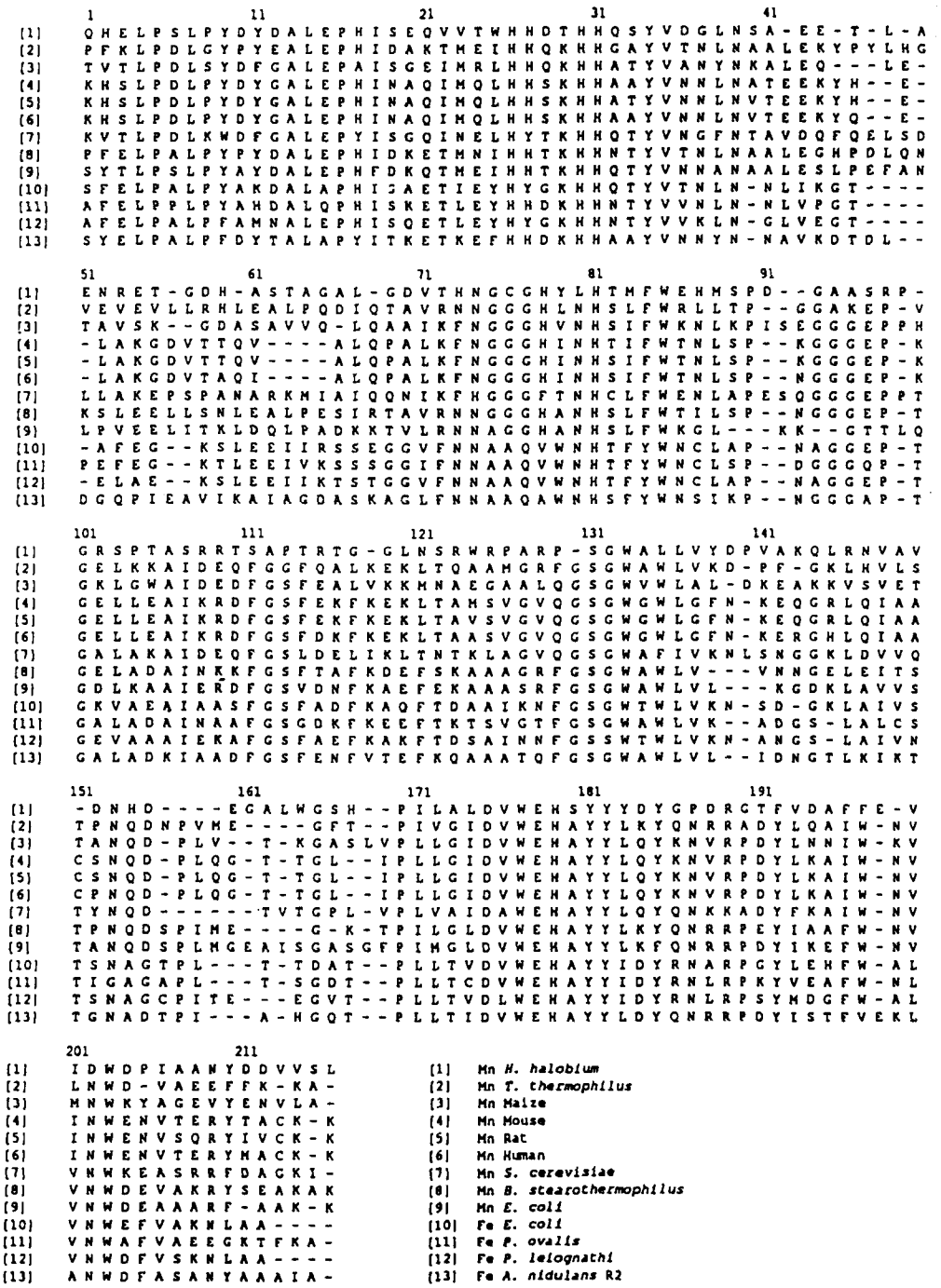
SOD activity was determined by the ability of the protein to inhibit pyrogallol autoxidation[12] as described elsewhere.[13,14] Appropriate corrections in the blanks were made for the small changes in the rate of autoxidation caused by phenylglyoxal, TNBS, and/or buffer salts. ^{14}C Incorporation was determined using Ecolume scintillation fluid and a Beckman LS-100C scintillation counter. For stoichiometry experiments, the concentration of *S. cerevisiae* MnSOD was determined from the absorbance at 280 nm ($\varepsilon = 1.92 \times 10^5\ M^{-1} \cdot cm^{-1}$)[15] and a molecular weight of 90,800 daltons for the tetrameric enzyme.[16] The number of lysine residues modified by TNBS was calculated from the increase in absorbance at 367 nm and a molar absorptivity at this wavelength[17] of $1.05 \times 10^4\ M^{-1}\ cm^{-1}$.

Chemical modification

Modifications were carried out under conditions described in the table legend. Reactions were initiated by the addition of a stock solution of the enzyme to an aqueous solution of the reagent, or by the addition of a stock solution of the reagent in acetonitrile to an aqueous solution of the enzyme. At appropriate times after mixing the enzyme and modifying reagent, aliquots were diluted and assayed for SOD activity. The controls, including those with 10% (v:v) acetonitrile, were stable throughout all experiments.

RESULTS AND DISCUSSION

Figure 1 shows the aligned amino acid sequences of nine MnSODs and four FeSODs reported to date. We have previously shown[10] that *E. coli* MnSOD and *E. coli* FeSOD are extensively inactivated by phenylglyoxal, a reagent that is highly selective for the



[1]	Mn	*H. halobium*
[2]	Mn	*T. thermophilus*
[3]	Mn	Maize
[4]	Mn	Mouse
[5]	Mn	Rat
[6]	Mn	Human
[7]	Mn	*S. cerevisiae*
[8]	Mn	*B. stearothermophilus*
[9]	Mn	*E. coli*
[10]	Fe	*E. coli*
[11]	Fe	*P. ovalis*
[12]	Fe	*P. leiognathi*
[13]	Fe	*A. nidulans* R2

FIGURE 1 Aligned amino acid sequences of the nine MnSODs and four FeSODs sequenced to date. The references (source and metal ion utilized) are: *Halobacterium halobium* Mn;[18] *Thermus thermophilus* Mn;[19] maize Mn;[20] mouse Mn;[21] rat Mn;[22] human Mn;[23–25] *Saccharomyces cerevisiae* Mn;[16,26] *Bacillus stearothermophilus* Mn;[27] *Escherichia coli* Mn;[28,29] *Escherichia coli* Fe;[8,30] *Pseudomonas ovalis* Fe;[31] *Photobacterium leiognathi* Fe;[4] *Anacystis nidulans* Fe.[32]

TABLE I
Inactivation of *E. coli* FeSOD, *E. coli* MnSOD and *S. cerevisiae* MnSOD by arginine-selective and lysine-selective reagents.[a]

SOD	Reagent	conditions	% Activity
E. coli FeSOD	Phenylglyoxal	1.5 hr	31
		3.0 hr	7
	TNBS	1.0 hr	36
		4.0 hr	29
	Acetic Anhydride	0.6 mM	51
		7.0 mM	27
E. coli MnSOD	Phenylglyoxal	1.5 hr	24
		3.0 hr	12
	TNBS	1.0 hr	53
		4.0 hr	35
	Acetic anhydride	0.6 mM	45
		7.0 mM	18
S. cerevisiae MnSOD	Phenylglyoxal	1.5 hr	80
		3.0 hr	70
	TNBS	0.3 hr	8
		1.0 hr	3
	Acetic anhydride	0.6 mM	2
		3.5 mM	0

[a]Modifications were carried out at 25°C in 45–80 mM sodium pyrophosphate, 45–80 mM $NaHCO_3$, 0.1 mM EDTA, pH 9.0. When acetic anhydride was used, 10% (v:v) acetonitrile was also present. Inactivation by phenylglyoxal (20 mM) and TNBS (0.5 mM) was time dependent, and the values given are residual activities after the indicated time of reaction. Acetic anhydride hydrolyzes very rapidly under the conditions of the experiment, with an estimated half-time of < 2 min. For modifications with acetic anhydride, reactions were carried out with the indicated initial concentration of reagent and the activity was determined after one hour.

modification of arginyl residues in proteins.[33] A comparison of the two *E. coli* enzymes shows that Arg-189 is the only conserved arginine and is the prime candidate for the critical residue. This arginine is also conserved in ten of the additional eleven SODs shown in Table I, with the single exception being *S. cerevisiae* MnSOD, where it is conservatively replaced by a lysine. If the positively charged residue at position 189 plays an important role in the catalytic function of MnSODs, its covalent modification should cause extensive loss of activity. More importantly, the pattern of sensitivity of yeast MnSOD to arginine-selective and lysine-selective reagents should be dramatically different than those of the *E. coli* SODs.

As shown in Table I, *E. coli* FeSOD and *E. coli* MnSOD are extensively inactivated by phenylglyoxal in a time-dependent manner, and > 98% inactivation is achieved if the reaction is allowed to proceed for a sufficient length of time (data not shown). Yeast MnSOD is very slowly inactivated by phenylglyoxal under identical conditions, but is rapidly and almost completely inactivated by treatment with acetic anhydride and TNBS. These two reagents show a high degree of selectivity for the modification of lysine in proteins, but can also modify other nucleophilic residues such as cysteine, tyrosine, and histidine. However, inactivation of yeast MnSOD by acetic anhydride is not reversed on treatment with 0.1 M NH_2OH, pH 9.0, for 2 hr (data not shown), strongly suggesting that inactivation is due to the modification of lysine.

Treatment of the *E. coli* enzymes with acetic anhydride and TNBS also reduces their catalytic efficiencies, but only to a residual activity of 20–45% that of the control, in line with a previous report on thse enzymes.[10] It has been proposed[34] that lysines

play a role in the electrostatic facilitation of catalysis in MnSODs and FeSODs. The reduced, but still significant, residual activity after modification with lysine-selective reagents suggests that this role may be important, but not a critical one, for the *E. coli* enzymes, In contrast, yeast MnSOD may have a lysine residue that is essential for normal catalysis.

We determined the stoichiometry of inactivation of yeast MnSOD with [^{14}C]-acetic anhydride and with TNBS, but in neither instance was a high degree of selectivity obtained (data not shown). With the former, incorporation of 6 acetyl groups per subunit reduced the activity to 10% that of the control, while with the latter complete inactivation was obtained on formation of approximately five 2,4,6-trinitrophenyllysines per subunit. This lack of selectivity, along with the inability to protect the critical lysine(s) with substrate or a competitive inhibitor, makes it very difficult to identify the critical lysine unequivocally by conventional protein chemistry. However, the different behaviors of yeast MnSOD, on one hand, and *E. coli* FeSOD and *E. coli* MnSOD, on the other, towards arginine and lysine reagents strongly suggests that modification of residue 189 may cause the complete loss of activity.

An examination of the recent high resolution (1.8 Å) structural data on *T. thermophilus* MnSOD suggests that Arg-189 is uniquely situated to play an important role in catalysis. This enzyme is a dimer of dimers, and the major dimer interface is nearly identical to that of the three dimeric SODs that have had their crystal structures determined to date.[1,2,7] Arg-189 in one subunit of *T. thermophilus* MnSOD is approximately equidistant from the two metals in the major dimer, 11.4 Å from the Mn in the same polypeptide (subunit A) and 12.1 Å from the Mn in the other subunit (subunit B). While the positively charged side chains of the other five arginines in the *T. thermophilus* enzyme fit into the almost universal "surface-exposed" mode that is found in most other proteins, Arg-189 forms five strong hydrogen bonds and appears to play a more critical structural role. Four of the hydrogen bonds, ranging from 2.6 to 3.4 Å, are to the backbone carbonyl oxygens of residues 125, 128, 130, and 176, while the fifth (3.09 Å) is to the OH of the fully conserved Tyr-181.

The highly ordered Arg-189 lies near the major dimer interface. It has been proposed[6] that superoxide anion may approach the Mn ion through a channel formed by the two dimer subunits from a direction that is trans to the Asp-175 ligand. One wall of this channel to the metal ion in subunit A is built around the side chain of Arg-189 from subunit B. If, as seems likely, this highly hydrogen-bonded arginine is positively charged, it would provide a diffuse electrostatic attractive force to guide superoxide to the catalytic metal center. It has been noted[10] that the rates of inactivation of *E. coli* FeSOD and *E. coli* MnSOD by phenylglyoxal are slow compared to the rates of inactivation of essential arginines in most proteins, and the relative inaccessibility of Arg-189 is consistent with this finding. Moreover, it is likely that covalent modification of Arg-189 would block the channel and/or dramatically alter the major dimer interface.

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References

1. W.C. Stallings, T.B. Powers, K.A. Pattridge, J.A. Fee and M.L. Ludwig (1983) Iron superoxide dismutase from *Escherichia coli* at 3.1 Å resolution: A structure unlike that of copper/zinc protein at both monomer and dimer levels. *Proceedings of the National Academy of Sciences U.S.A.*, **80**, 3884–3888.
2. D. Ringe, G.A. Petsko, F. Yamakura, K. Suzuki and D. Ohmori (1983) Structure of iron superoxide dismutase from *Pseudomonas ovalis* at 2.9 Å resolution. *Proceedings of the National Academy of Sciences U.S.A.*, **80**, 3879–3883.
3. W. Stallings, C. Bull, K.A. Pattridge, T.B. Powers, J.A. Fee, M.L. Ludwig, D. Ringe and G.A. Petsko (1984) The three-dimensional structure of iron superoxide dismutase: Kinetic and structural comparisons with Cu/Zn and Mn dismutases. In *Oxygen Radicals in Chemistry and Biology* (eds. W. Bors, M. Saran and D. Tate), de Gruyter, Berlin, pp. 779–792.
4. D. Barra, M.E. Schininà, W.H. Bannister, J.V. Bannister and F. Bossa (1987) The primary structure of iron-superoxide dismutase from *Photobacterium leiognathi*. *Journal of Biological Chemistry*, **260**, 1001–1009.
5. D.R. Ponzi, G.A. Petsko, F. Yamakura, D. Ohmori, K. Suzuki, W.C. Stallings, K.A. Pattridge, T.B. Powers, J.A. Fee and M.L. Ludwig (1983) Three-dimensional structure of iron superoxide dismutase. In *Oxy Radicals and Their Scavenger Systems* (eds. G. Cohen and R.A. Greenwald), Vol. I: Molecular aspects, Elsevier, New York, pp. 340–343.
6. W.C. Stallings, K.A. Pattridge, R.K. Strong and M.L. Ludwig (1985) The structure of manganese superoxide dismutase from *Thermus thermophilus* HB8 at 2.4 Å resolution. *Journal of Biological Chemistry*, **260**, 16424–16432.
7. M.W. Parker and C.C.F. Blake (1988) Crystal structure of manganese superoxide dismutase from *Bacillus stearothermophilus* at 2.4 Å resolution. *Journal of Molecular Biology*, **199**, 649–661.
8. A. Carlioz, M.L. Ludwig, W.C. Stallings, J.A. Fee, H.M. Steinman and D. Touati (1988) Iron superoxide dismutase: Nucleotide sequence of the gene from *Escherichia coli* K12 and correlations with crystal structures. *Journal of Biological Chemistry*, **263**, 1555–1562.
9. W.F. Beyer, Jr and I. Fridovich (1987) Effect of hydrogen peroxide on the iron-containing superoxide dismutase of *Escherichia coli*. *Biochemistry*, **26**, 1251–1257.
10. C.L. Borders, Jr., P.J. Horton and W.F. Beyer, Jr. (1989) Chemical modification of iron-containing and manganese-containing superoxide dismutases from *Escherichia coli*. *Archives of Biochemistry and Biophysics*, **268**, 74–80.
11. M.J. Bjerrum (1987) Structural and spectroscopic comparison of manganese-containing superoxide dismutases. *Biochimica et Biophysica Acta*, **915**, 225–237.
12. S. Marklund and G. Marklund (1974) Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *European Journal of Biochemistry*, **47**, 469–474.
13. C.L. Borders, Jr. and J.T. Johansen (1980) Essential arginyl residues in Cu,Zn superoxide dismutase from *Saccharomyces cerevisiae*. *Carlsberg Research Communications*, **45**, 185–194.
14. D.M. Blech and C.L. Borders, Jr, (1983) Hydroperoxide anion, HO_2^-, is an affinity reagent for the inactivation of yeast Cu,Zn superoxide dismutase. Modification of one histidine per subunit. *Archives of Biochemistry and biophysics*, **224**, 579–586.
15. C. Ditlo and J.T. Johansen (1982) Isolation of manganese-superoxide dismutase from *Saccharomyces cerevisiae*. *Carlsberg Research Communications*, **47**, 71–79.
16. C. Ditlow, J.T. Johansen, B.M. Martin and I. Svendsen (1982) The complete amino acid sequence of manganese-superoxide dismutase from *Saccharomyces cerevisiae*. *Carlsberg Reearch Communications*, **47**, 81—91.
17. A.R. Goldbarb (1966) A kinetic study of the reactions of amino acids and peptides with trinitrobenzenesulfonic acid. *Biochemistry*, **5**, 2570–2574.
18. M.L. Salin, M.V. Duke, D. Oesterhelt and D.-P. Ma (1988) Cloning and determination of the nucleotide sequence of the Mn-containing superoxide dismutase gene from *Halobacterium halobium*. *Gene*, **70**, 153–159.
19. S. Sato, Y. Nakada and K. Nakazawa-Tomizawa (1987) Amino-acid sequence of a tetrameric, manganese superoxide dismutase from *Thermus thermophilus* HB8. *Biochimica et Biophysica Acta*, **912**, 178–184.
20. J.A. White and J.G. Scandalios (1988) Isolation and characterization of a cDNA for mitochondrial manganese superoxide dismutase (SDD-3) of maize and its relation to other manganese superoxide dismutases. *Biochimica et Biophysica Acta*, **951**, 61–70.

21. R.A. Hallewell, G.T. Mullenbach, M.M. Stempien and G.I. Bell (1986) Sequence of a cDNA coding for mouse manganese superoxide dismutase. *Nucleic Acids Research*, **14**, 9539.
22. Y.-S. Ho and J.D. Crapo (1987) Nucleotide sequences of cDNA coding for the rat Manganese-containing superoxide dismutase. *Nucleic Acids Research*, **15**, 10070.
23. D. Barra, M.E. Schininà, M. Simmaco, J.V. Bannister, W.H. Bannister, G. Rotilio and F. Bossa (1984) The primary structure of human liver manganese superoxide dismutase. *Journal of Biological Chemistry*, **259**, 12595–12601.
24. Y. Beck, R. Oren, B. Amit, A. Levanon, M. Gorecki and J.R. Hartman (1987) Human Mn superoxide dismutase cDNA sequence. *Nucleic Acids Research*, **15**, 9076.
25. Y.-S. Ho and J.D. Crapo (1988) Isolation and characterization of complimentary DNAs encoding human Manganese-containing superoxide dismutase. *FEBS Letters*, **229**, 256–260.
26. C.A.M. Marres, A.P.G.M. Van Loon, P. Oudshoorn, H. Van Steeg, L.A. Grivell and E.C. Slater (1985) Nucleotide sequence analysis of the nuclear gene coding for manganese superoxide dismutase of yeast mitochondria, a gene previously assumed to code for the Rieske iron-sulphur protein. *European Journal of Biochemistry*, **147**, 153–161.
27. C.J. Brock and J.E. Walker (1980) Superoxide dismutase from *Bacillus stearothermophilus*. Complete amino acid sequence of a manganese enzyme. *Biochemistry*, **19**, 2873–2882.
28. H.M. Steinman (1978) The amino acid sequence of mangano superoxide dismutase from *Escherichia coli* B. *Journal of Biological Chemistry*, **253**, 8708–8720.
29. Y. Takeda and H. Avila (1986) Structure and gene expression of the *E. coli* Mn-superoxide dismutase gene. *Nucleic Acids Research*, **14**, 4577–4589.
30. M.E. Schininà, L. Maffey, D. Barra, F. Bossa, K. Puget, and A.M. Michelson (1987) The primary structure of iron superoxide dismutase from *Escherichia coli*. *FEBS Letters*, **221**, 87–90.
31. T. Isobe, Y.-I. Fang, D. Muno, T. Okuyama, D. Ohmori and F. Yamakura (1987) Amino acid sequence of iron-superoxide dismutase from *Pseudomonas ovalis*. *FEBS Letters*, **223**, 92–96.
32. D.E. Laudenbach, C.G. Trick and N.A. Straus (1989) Cloning and characterization of an *Anacystis nidulans* R2 superoxide dismutase gene. *Molecular and General Genetics*, **216**, 455–461.
33. J.F. Riordan (1979) Arginyl residues and anion binding sites in proteins. *Molecular and Cellular Biochemistry*, **26**, 71–92.
34. J. Benovic, T. Tillman, A. Cudd and I. Fridovich (1983) Electrostatic facilitation of the reaction catalyzed by the manganese-containing and the iron-containing superoxide dismutases. *Archives of Biochemistry and Biophysics*, **221**, 329–332.

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