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Impaired copper binding by the H46R mutant of human Cu,Zn superoxide dismutase, involved in amyotrophic lateral sclerosis

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Abstract Several point mutations in the gene coding for human Cu,Zn superoxide dismutase have been reported as being responsible for familial amyotrophic lateral sclerosis (FALS). However, no direct demonstration has been provided for a correlation between total superoxide dismutase activity and severity of the FALS pathology. In order to get a better insight into the mechanism(s) underlying the FALS phenotype, we have investigated the activity and the copper binding properties of the single mutant H46R, which is associated with a Japanese form of FALS. We have shown that this mutant is structurally stable but lacks significant enzyme activity and has impaired capability of binding catalytic copper. The mutant protein can be fully reconsituted with copper in vitro but its ESR spectrum displays an axial shape quite different from that of the wild-type.

Key words: Amyotrophic lateral sclerosis; Cu,Zn superoxide dismutase; Oxidative damage

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder occurring both sporadically (SALS) and as a familial, age-dependent autosomal dominant disorder (FALS). Since histological features and clinical symptoms are indistinguishable between the two forms, studies of FALS at the molecular level could provide a better understanding of all ALS pathologies. It has recently been demonstrated that several (but not all) FALS families carry single mutations in *SOD1*, the gene coding for Cu,Zn superoxide dismutase (SOD) [1–4]. This enzyme is a 32 kDa homodimer, with one Cu and one Zn atom/monomer, which plays a major role in cell defense against toxic superoxide radical [5]. SOD is constitutively expressed in all tissues, although its level is quite variable in different cell types, and it is as yet unclear why motor neurones constitute the primary target of the *SOD1* genetic defect [1]. FALS patients show a wide range of age of onset of symptoms and variable survival after onset [3]. A clear correlation between severity of disease and degree of decrease in the cells' ability to scavenge O_2^-, however, has not been established [4]. To date, 16 different mutations in *SOD1* have been reported, all of them causing a reduction in SOD activity in blood of FALS patients [6]. This loss of activity is believed to be linked to loss of both specific activity and stability of mutant SOD [2,3,6]. Most of the mutations (15 out of 16) are localized in sites believed to be relevant either for correct protein folding or dimer contact [2], with the only exception being the mutation H46R which involves one of the residues binding catalytic copper at the active site. This mutation is typical of the 'mild' Japanese form of FALS [3], although one would expect that substitution of one copper ligand would severely affect the catalytic activity of Cu,ZnSOD.

In view of these considerations, in the present work we have measured the copper-binding properties and the enzyme activity of this mutant, as a first attempt in establishing the extent of the correlation between the molecular defects and the pathology of ALS.

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2. Materials and methods

2.1. Expression and purification of wild-type and mutated human Cu,ZnSOD

Plasmid pKH expressing wild-type human Cu,ZnSOD (hSODwt) was previously described [7]. Plasmid pEMBLHSOD, containing the 600 bp *PstI–PstI* DNA fragment from plasmid pS61–10 [8] and a *NcoI* restriction site at the 5′ end of the hSODwt coding region was used to generate single-stranded DNA to be used in oligonucleotide-directed mutagenesis experiments according to Kunkel et al. [9]. The *NcoI–PstI* DNA fragment from plasmid pEMBLHSod46R (containing the H46R46 mutation) was inserted into the corresponding sites of plasmid pKH, thus obtaining plasmid pKH46R.

Expression of wild-type and mutated human Cu,ZnSOD was performed in *E. coli* strain QC779 (*sodAsodB*; a kind gift of Dr. Danielle Touati) [11], which is defective in both bacterial SODs, grown in LB medium containing 10 μM $ZnSO_4$ and 1 mM $CuSO_4$ as previously described [10]. Purification was performed as reported in [10]. Both enzymes were >95% homogeneous, as judged by SDS-PAGE. The yield was comparable in both cases.

2.2. Complementation assay in SOD defective E. coli

E. coli QC779 cells were transformed by standard procedures either with control plasmid pKK233-2, or with plasmids pKH and pKH46R expressing hSODwt and H46R, respectively, and plated on standard LB agar containg 100 μg/ml ampicillin, 50 μg/ml chloramphenicol and 50 μg/ml kanamycin. Single colonies from each transformation were then grown overnight in LB medium containing 100 μM $CuSO_4$, and diluted to 10^3 cells/ml; 100 μl of each were plated either on selective LB agar or on M9 minimal medium (containing 0.4% glucose, 1 μg/ml thiamine, 50 μg/ml ampicillin, 25 μg/ml chloramphenicol, 25 μg/ml kanamycin, 0.2 mM IPTG, 10 μM $CuSO_4$ and 1 nM paraquat). Plates were incubated at 30°C up to several days. Every day the number of colonies on each plate was recorded.

2.3. Measurement of copper binding by ESR

ESR spectra were recorded at 100 K on a Bruker ESP300 spectrometer operating at X band with 100 kHz field modulation.

Titration with copper of purified hSODwt and H46R was carried out by increasing additions of $CuCl_2$ to reach a 2-fold excess of available copper sites. Copper uptake by both proteins was estimated by double integration of the ESR signal. The resulting numerical value was compared to the one obtained from a Cu-EDTA standard solution.

2.4. Determination of SOD activity

Activity assays were performed by staining of native discontinuous PAGE [12] according to Beauchamp and Fridovich [13] or by the

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pyrogallol method at pH 8.2 [14]. Protein was determined according to Lowry et al. [15].

3. Results

Expression of hSODwt enables *E. coli* QC779 (*sodAsodB*) cells to grow in a selective medium ([16] and Table 1), conferring to this defective strain the ability to survive paraquat-induced oxidative stress. When mutant H46R was expressed in the same strain, however, the ability to overcome the SOD genetic defect was retained only to a slight extent. Table 1 shows that after 3 days of plating on selective medium a few cells were able to grow when pKH46R was expressed, while expression of pKH allowed 100% of colonies to appear after the same time of incubation.

It is worth noting that cells transformed with pKH46R reached 100% plating efficiency under these conditions only after 5 days of incubation, suggesting that a substantially lower SOD activity was retained by the mutant protein in vivo. Indeed, this was also demonstrated by PAGE analysis of crude extracts from *E. coli* QC779 cells transformed either with the wild-type or the mutant enzyme (Fig. 1), where the same amount of SOD was expressed, as judged by Coomassie staining (not shown). A band corresponding to hSODwt was easilly detectable in extracts from pKH-transformed cells in activity stained gels, while no activity band was observed in pKH46R cells (Fig. 1). It should be noted that a SOD activity band amounting to 10% of that from the wild-type would be clearly evident by such a technique, suggesting that residual activity of the mutant is less than a few percent of the wild-type. This was confirmed by direct measurement of SOD activity in the extracts by the pyrogallol method, where extracts containing H46R possessed undetectable enzyme activity.

In order to investigate the copper binding properties of H46R, we have also attempted to reconstitute the native Cu-bound form of the protein from the Cu-free form of the enzyme by copper addition either during cell growth or in cell extracts. As shown in Fig. 1 (lanes 2–4), while both treatments increase hSODwt activity, no significant gain in H46R activity was observed (lanes 6–8). These data were confirmed by direct SOD activity assay on copper-supplemented extracts, where activity

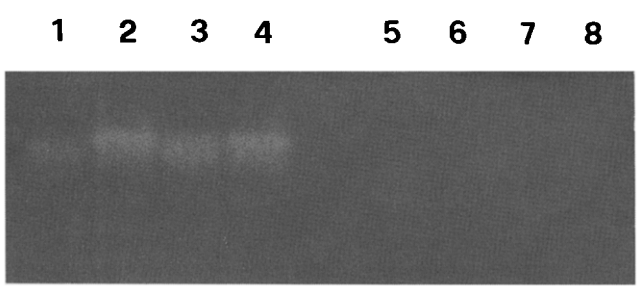

Fig. 1. Staining for SOD activity of native PAGE of extracts from *E. coli* QC779 (*sodAsodB*) cells expressing hSODwt (lanes 1–4) or cells expressing mutant H46R (lanes 5–8). (Lanes 1, 3, 5, 7) No copper supplementation in the medium during cell growth. (Lanes 2, 4, 6, 8) 1 mM copper sulphate was added to the medium during cell growth. (Lanes 3, 4, 7, 8) cell extracts were supplemented with 3 μM $CuCl_2$ and incubated on ice for 2 h prior to PAGE analysis. Zinc was supplemented as 10 μM $ZnSO_4$ during cell growth. Further addition of zinc in the extracts had no effect on SOD activity of all samples (not shown). 50 μg of total protein were loaded on each lane.

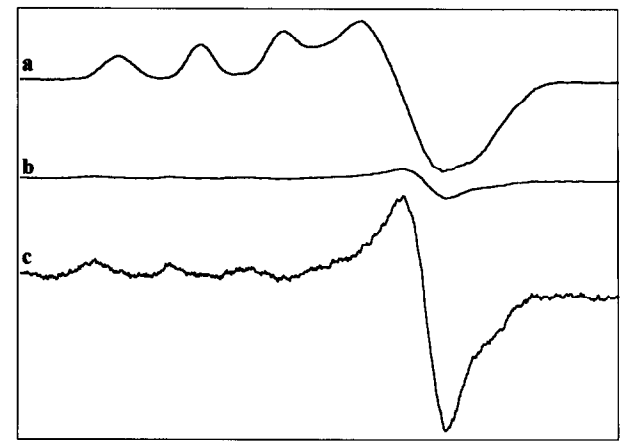

Fig. 2. ESR spectra of purified recombinant hSODwt (a) and mutant H46R (b and c), where the signal in b was magnified 8 times. All samples were in 10 mM K-phosphate buffer, pH 7.4. Protein concentration was kept at 2.1×10^{-4} M (hSODwt) or 2.2×10^{-4} M (H46R). Microwave power, 20 mW; ν, 9.43 GHz; T, 100 K.

corresponding to 5% of the wild-type enzyme would have been detectable.

Double integration of the copper ESR spectra carried out on purified recombinant hSODwt (Fig. 2) indicates that the wild-type protein has about 80% of the copper expected on a 2 mol copper/mol dimer basis as detectable by ESR, and that the spectrum has the rhombic line shape typical of the metal bound to a fully functioning active site. Upon titration with copper, hSODwt was able to bind the copper complement to reach full metallation of the active site. Further copper addition caused the appearance of a spurious copper ESR signal, most likely due to metal binding to non-specific copper sites on the protein surface. On the other hand, purified H46R contained only about 5% copper, as evaluated by double integration of the ESR spectrum, and its shape significantly differed from that of the wild-type Cu_2Zn_2SOD enzyme. The spin hamiltonian parameters and the line shape of the ESR spectrum of the H46R mutant are in part characteristic of a copper bound in a tetragonal geometry, suggesting that Arg^{46} is not able to coordinate the copper, restoring the pentacoordinate geometry typical of the native copper site. The copper ESR signal for H46R increases in intensity upon titration with copper without the ap-

Table 1
Complementation test of *E. coli* QC779 (*sodAsodB*) strain by transformation with pKH or pKH46R

	72 h	96 h	120 h
pKK233-2	0	0	6.9 ± 3.2
pKH	105.7 ± 35	105.7 ± 35	105.7 ± 35
pKH46R	5.6 ± 3.3	64.3 ± 16.1	102.8 ± 13.7

Cells were grown at 30°C on M9 plates containing 10^{-5} M $CuSO_4$ and 10^{-9} M paraquat. The indicated values represent the percentage of cells able to produce visible colonies with respect to a control grown on LB plates and are the average of six independent experiments. It is worth noting that the size of the colonies containing plasmid pKH46R were small and highly variable, while all the colonies containing plasmid pKH were of comparable dimension.

pearance of new copper signals, and a fully metallated (2 copper/dimer) protein can be obtained by the addition of a small copper excess. However, the fully reconstituted purified H46R mutant displays only 1–2% of the SOD activity shown by the same amount of fully reconstituted hSODwt, as assayed by the pyrogallol method.

Stability of H46R was not significantly impaired, as judged by our ability to express and purify this protein from *E. coli* cells with the same yield as the wild-type, employing the same procedure which involves a heating step at 60°C for 30 min [10].

4. Discussion

H46R is the only FALS-typical mutation in the active site of CuZnSOD described so far. All other mutations most probably affect the correct β-barrel fold or the dimer contact of the protein [2], resulting either in a lower stability or in a less-efficient binding of metals, or both. Formation of a wild-type/mutant heterodimer could also affect overall residual activity in heterozygous cells.

In this work we have demonstrated that: (i) mutant H46R has a dramatically reduced SOD activity, although its stability appears unaltered by the mutation; (ii) that the typical coordination of copper in a tetrahedrally distorted square planar geometry is an essential requisite for SOD activity. In fact, a dramatic decrease in activity has also been observed in the case of the natural variant H48Y from *Haemophilus influenzae* [17], suggesting that integrity of imidazolate copper ligands is necessary for the function of this enzyme.

Heterozygous FALS patients carrying the H46R mutation have been reported to possess a residual SOD activity around 80% of unaffected controls in red blood lysates, apparently in line with the mildness of Japanese ALS [3], suggesting that severity of the disease is directly related to the level of SOD activity in patients. However, our results show a nearly complete inactivation of hSODwt by substitution of the His^{46} residue with Arg. Some doubt has been cast on the interpretation of blood SOD1 levels in these patients [6] since control individuals show a significant variability of this value.

It has been suggested [4,18,19] that mutations in *SOD1* may confer on cell a novel, toxic enzyme activity. If this is the case, severity of the disease would not be proportional to the decrease of SOD activity but most probably would be related to the extent of efficient gain of the new function. A low efficiency of H46R mutant protein in performing the postulated novel function would then explain the mildness of the Japanese FALS phenotype better than a moderate decrease of SOD activity. Whether this novel function lies in the ability of mutated SOD to generate peroxynitrite [18] or in a different activity remains to be clarified.

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References

[1] Rosen, D.R., et al. (1993) Nature 362, 59–62.
[2] Deng, H.-X., et al. (1993) Science 261, 1047–1051.
[3] Ogasawara, M., Matsubara, Y., Narisawa, K., Aoki, M., Nakamura, S., Itoyama, Y. and Abe, K. (1993) Nature Genet. 5, 323–324.
[4] Esteban, J., Rosen, D.R., Bowling, A.C., Sapp, P., McKenna-Yasek, D., O'Regan, J.P., Beal, M.F., Horvitz, H.R. and Brown, jr, R.H. (1994) Hum. Mol. Gen. 3, 997–998.
[5] Bannister, J.V., Bannister, W.H. and Rotilio, G. (1987) CRC Crit. Rev. Biochem. 22, 111–180.
[6] Borchelt, D.R., Lee, M.K., Slunt, H.S., Guarnieri, M., Xu, Z.-S., Wong, P.C., Brown, R.H. jr., Price, D.L., Sisodia, S.S. and Cleveland, D.W. (1994) Proc. Natl. Acad. Sci. USA 91, 8292–8296.
[7] Battistoni, A., Carrì, M.T., Steinkühler, C. and Rotilio, G. (1993) FEBS Lett. 322, 6–9.
[8] Sherman, L., Dafni, N., Lieman-Hurwitz, J. and Groner, Y. (1983) Proc. Natl. Acad. Sci. USA 80, 5465–5469.
[9] Kunkel, T.A., Bebenek, K. and McClary, J. (1990) Methods Enzymol. 204, 125–139.
[10] Polticelli, F., Battistoni, A., Bottaro, G., Carrì, M.T., O'Neill, P., Desideri, A. and Rotilio, G. (1994) FEBS Lett. 352, 65–68.
[11] Carlioz, A. and Touati, D. (1986) EMBO J. 5, 623–630.
[12] Laemmli, U.K. (1970) Nature 227, 680–685.
[13] Beauchamp, C. and Fridovich, I. (1971) Anal. Biochem. 44, 276–287.
[14] Marklund, S. and Marklund, G. (1974) Eur. J. Biochem. 47, 469–474.
[15] Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[16] Natvig, D.O., Imlay, K., Touati, D. and Hallewell, R.A. (1987) J. Biol. Chem. 262, 14697–14701.
[17] Kroll, J.S., Langford, P.R. and Loynds, B.M. (1991) J.Bacteriol. 173, 7449–7457.
[18] Beckman, J.S., Carson, M., Smith, C.D. and Koppenol, W.H. (1993) Nature 364, 584.
[19] Gurney, M.E., et al. (1994) Science, 264, 1772–1775.