Decreased thermal stability of red blood cell glu^{100}→gly superoxide dismutase from a family with amyotrophic lateral sclerosis

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Abstract Familial amyotrophic lateral sclerosis is a degenerative motor neuron disease associated in some cases with the presence of a mutant form of Cu/Zn superoxide dismutase. We have studied the stability of the gly^{100}→glu mutant in extracts of red cells obtained from members of a family with a history of the disease. Extracts containing the mutant had an average 68% of normal superoxide dismutase activity. On heating at 65°C, these extracts lost activity at twice the rate of extracts containing only the normal enzyme. Decreased heat stability was also evident on native polyacrylamide gel electrophoresis with activity staining. This showed selective loss of first the mutant homodimer and then the heterodimer of the enzyme. Decreased stability intracellularly could be a factor in motor neuron degeneration.

Key words: Familial amyotrophic lateral sclerosis; Superoxide dismutase; Motor neuron disease

1. Introduction

Familial amyotrophic lateral sclerosis (FALS) is a degenerative motor neuron disease that is inherited as an autosomal dominant trait [1]. Approximately 20% of cases have mutations in the gene coding for Cu/Zn superoxide dismutase (SOD) [2,3]. At least 12 different mutations that cause the disease are currently known, including glu^{100}→gly [4]. Studies with recombinant mutant SODs have shown that some but not others have a decreased specific activity [5–7]. Similarly, in red cells of affected individuals, SOD activity varies from 30% normal to within the normal range [2,8–11] and both active mutant homo- and heterodimers of a charge change variant can be detected [4]. These observations, in conjunction with the finding that transgenic mice expressing FALS mutant as well as their normal SOD develop motor neuron disease [12,13], are strong evidence that a gain in function rather than a loss of ability to dismutate superoxide is responsible for the disease. This additional function has not been identified.

Analysis of the positions of the mutations in relation to the 3-D structure suggest that they are critical for correct folding of the protein and that changes could decrease its stability [2]. This has not been tested directly. We have studied the stability of SOD in extracts of red cells obtained from members of a family with a history of FALS who carry the charge change glu^{100}→gly mutation. Activity measurements and native polyacrylamide gel electrophoresis (PAGE) have shown that the heat stability of their SOD is decreased with selective loss of the mutant protein.

2. Materials and methods

Details of the family are described elsewhere [4]. Of the eight members studied, one has developed ALS and five carry the mutation but are currently asymptomatic. The other two family members have normal SOD. Red cells were obtained from these family members and four other normal individuals. Lysates were prepared, haemoglobin concentrations measured by the Drabkins method, and extracts prepared by the standard procedure of adding ethanol and chloroform to precipitate the haemoglobin [14]. Extracts were diluted to contain uniformly the equivalent of 5 g/100 ml haemoglobin in 5.0 mM phosphate buffer, pH 7.4, and 20% ethanol (present as a result of the extraction procedure).

Extracts were incubated in covered tubes at 65°C. Samples were removed at intervals, assayed for SOD activity and subjected to PAGE. Activity was measured using the nitroblue tetrazolium method [14] except superoxide was generated with xanthine oxidase (0.006 U/ml) and hypoxanthine (50 mM). Inhibition data were best fitted to an exponential curve using SigmaPlot (Jandel Scientific) and volumes giving half maximum inhibition (by definition containing one unit) were calculated.

Native PAGE was performed on extracts following 3-fold concentration by lyophylization. The gels (4% acrylamide stacking gel, 8% acrylamide resolving gel) were run in 15.0 mM Tris, 11.5 mM glycine buffer, pH 9.1, at 70 V for 3 h, then stained for SOD activity using nitroblue tetrazolium [4].

3. Results

Individuals without the mutation (n = 6) had red cell SOD activities of 14,100 ± 2000 U/g haemoglobin. Activities in the six individuals with the mutation ranged from 7500 to 11,000 haemoglobin, with a mean of 68% normal.

Following incubation at 65°C, the normal red cell extracts progressively showed a small decrease in SOD activity with time (Fig. 1). Loss of activity over 30 min was twice as fast in extracts that contained the SOD mutant. The difference was highly significant ($P<0.0001$). The temperature of 65°C was selected because it caused only slight loss of normal SOD. At 70°C there was greater loss of activity, which was again about twice as fast in the extracts containing the mutant.

Superoxide dismutase exists as a dimer. The glu^{100}→gly charge change allows electrophoretic detection of heterodimers as well as homodimers of the normal and mutant proteins [4]. The effect of heating the extracts on the electrophoretic pattern is illustrated for one normal and one carrier of the mutation in Fig. 2. As observed previously [4], at zero time the carrier had less activity in the mutant homodimer band than in the normal homodimer band. Heating gave selective loss of both abnormal bands. Complete loss of the mutant homodimer occurred within 45 min, followed by loss of the heterodimer. Heating did

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Abbreviations: FALS, familial amyotrophic lateral scelerosis; SOD, superoxide dismutase; PAGE, polyacrylamide gel electrophoresis.

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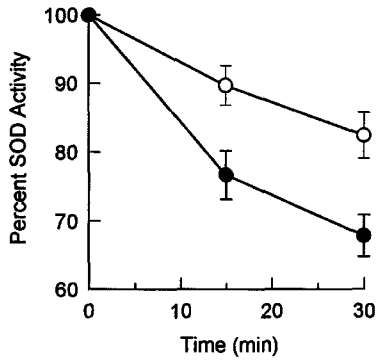

Fig. 1. Loss of SOD on heating red cell extracts from individuals with (●) and without (○) the glu^{100}→gly mutant. Lysastes were prepared as in Section 2, heated at 65°C then analysed for SOD activity. Results shown are mean ± S.D. for six individuals in each group, with each sample analysed on two separate occasions. Significance was assessed by two-way repeated ANOVA.

not affect the electrophoretic pattern of the normal homodimer.

Altered stability of the mutant SOD was also apparent when red cell lysates were heated prior to extraction. Lysates containing 10 g/100 ml haemoglobin that were heated at 65, 67, 69 and 71°C for 1 h, then subjected to native PAGE and activity staining. Loss of the mutant homodimer occurred at 67°C with loss of the heterodimer at 71°C (result not shown).

4. Discussion

In this study we have demonstrated that the glu^{100}→gly SOD mutant that causes FALS is less stable than normal. Total SOD activity was lost at twice the normal rates when extracts of red cells from heterozygotes for the mutant enzyme were heated, and there was selective loss of electrophoretic bands containing the mutant enzyme. Whether the protein actually precipitates from solution or loses activity on heating cannot conclusively be established from our studies.

SOD stability was examined in extracts that contained 20% ethanol. By decreasing the polarity of the solvent, it is possible that the ethanol could have effected our results by causing some unfolding of the protein with reduction in its stability. However, this effect was minor, as there was also selective loss of the mutant from red cell lysates at only a slightly higher temperature than for the extracts. Although the temperature used to demonstrate heat instability in vitro was rather high, this could be regarded as accelerating changes that occur more slowly under physiological conditions. An analogy can be drawn with the unstable haemoglobin mutants, which precipitate at 50–60°C and form inclusions (Heinz bodies) within circulating red cells [15]. Alternatively, conformational changes in SOD mutants, reflected by a decrease in heat stability, could have structural or functional implications unrelated to protein precipitation.

SOD mutations that give rise to FALS have been predicted to result in conformational changes in the β barrel at the dimer interface of the enzyme that could reduce its structural integrity [2]. Our findings demonstrate that this is indeed the case with the glu^{100}→gly mutant.

Other studies with recombinant mutant SODs have indicated that ala^{4}→val causes loss of activity but that specific activities of several other mutants are normal [12]. Our electrophoretic evidence with the glu^{100}→gly presented here, and reported in detail elsewhere [4], demonstrates that less active mutant than normal enzyme is present in the cell extracts before heating. This observation, and the fact that the total SOD activities in affected individuals are on average below normal, could indicate that some loss of the mutant dimer, due to instability, had already occurred. Our results are complemented by the observation [5] that the FALS gly^{37}→arg mutant is turned over more rapidly than normal, when transfected into COS-1 cells or into a lymphocyte line, raising the possibility of this mutant also having decreased stability.

Initial measurements of SOD activity in red cell lysate containing six different FALS mutants, including the glu^{100}→gly mutation, detected <50% of the normal activity [2]. This was taken to suggest that a low ability to dismutate superoxide could be responsible for the motor neuron degeneration. How-

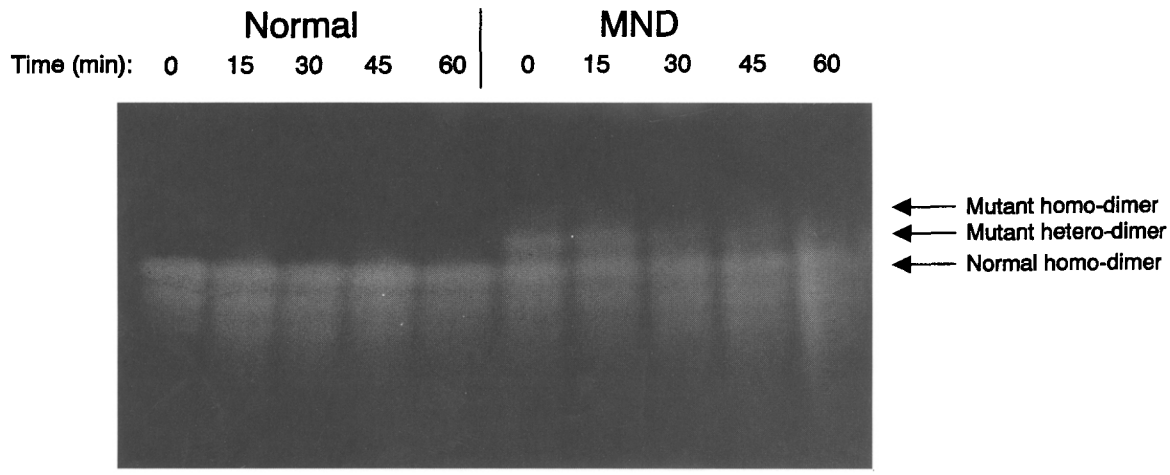

Fig. 2. SOD activity stain of native PAGE SOD extracts were prepared as outlined in Section 2 and concentrated 10-fold by lyophylization. The purified extracts were heated at 65°C for the times 0, 15, 30, 45 and 60 min as indicated. Heated SOD fractions were then separated on mini gels by native PAGE. The gels were run at 70 V for 3 h and then stained for SOD activity. The arrows indicate the mobility of the mutant homodimer, heterodimer and the normal dimer.

ever, mean activities of 65–80% have subsequently been measured for other mutants [10,11,16] and we have found activities with the $glu^{100} \rightarrow gly$ mutant to be ~80% of normal [4].

While it is not known how mutations in SOD give rise to FALS, available information favours a mechanism involving a gain in function. Our result suggests possible ways by which this might occur. Decreased heat stability indicates that the protein is more prone to denaturation and precipitation. The mutant dimer may be absent as observed with the $gly^{97} \rightarrow arg$ mutant [8], or present in decreased amounts as observed in the $glu^{100} \rightarrow gly$ mutant studied here, because it has failed to form initially or because once formed it has decreased stability.

A consistent histopathological observation in patients with ALS, whether or not it is caused by an SOD mutation, is the presence of neurofilament protein deposition in affected neurons [17]. The question remains as to whether this deposition is central to the pathogenesis of the disease or whether it is a result of other unidentified chemical changes. SOD has been identified as one component of Lewy body-like protein inclusions seen in spinal cords of patients with FALS [18]. One can speculate that the mutant SOD, due to its decreased stability, is having an aberrant effect on the protein processing pathway.

In conclusion, the evidence to date indicates that motor neuron degeneration caused by mutation to SOD is due to a change in the protein that is not simply a decrease in enzyme activity. Our findings that the heat stability of the $glu^{100} \rightarrow gly$ mutant is decreased adds weight to this argument and opens up further avenues of investigation that can be explored with purified mutant proteins.

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