Reversibility of the thermal denaturation of yeast superoxide dismutase

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1. INTRODUCTION

Superoxide dismutase, which consists of two identical subunits, catalyzes the dismutation of the superoxide anion to hydrogen peroxide and oxygen and is present in nearly all aerobic cells [1]. Superoxide dismutase from the cytosol of eucaryotic cells contains copper and zinc [2]. The complete amino acid sequences of the enzymes from bovine erythrocytes [3] and from the yeast *Saccharomyces cerevisiae* [4,5] are known and show 55% homology. This amino acid sequence homology leads one to expect considerable structural homology. High resolution proton nuclear magnetic resonance spectra have been interpreted as corroborating this expectation [6].

Although the amino acid sequences are quite similar, there are some radical substitutions. A number of substitutions of non-polar amino acids by charged ones occur in each enzyme. In particular, the substitution of Ile-94 and Val-98 in the bovine enzyme by Lys-96 and Lys-100 in the yeast enzyme [5] might be expected to produce local alterations which might lead to a general change in conformation. Substitutions of this sort, for example, might account for the greater stability of the bovine enzyme to urea denaturation [7].

This study demonstrates large differences in the thermal denaturation behavior and subunit exchange of the bovine and yeast superoxide dismutases, which must be attributable to differences in both intramolecular (within subunits) and intermolecular (between subunits) interactions. Differential scanning calorimetry (DSC) is shown to be a sensitive technique for demonstrating conformational differences between similar proteins.

2. MATERIALS AND METHODS

Superoxide dismutases from bovine erythrocytes (isolated by Dr J.V. Bannister, Malta) and *Saccharomyces cerevisiae* (Pharmacia and Carlsberg) were gifts of Dr A. Petkau (Pinewa, Manitoba) and were stored lyophilizied at −20°C until used.

Superoxide dismutase activity was determined using the reduction of nitroblue tetrazolium with the xanthine/xanthine oxidase system essentially as in [8].

The thermal denaturation was followed by differential scanning calorimetry using a Perkin-Elmer DSC-2. A heating rate of 2.5°C/min was used exclusively and the calorimetric enthalpy (ΔH_{cal}) was calculated from the area under the denaturation peak. The superoxide dismutase was dissolved in phosphate-buffered saline (pH 7.4) at 24 mg/ml and ~70 μl of this solution used for each heating curve.

The electrophoretic mobility of the superoxide dismutases was modified by addition of 2,4,6-trinitrobenzene sulfonic acid to free amino groups, as described for proteins in [9]. Briefly, 8.25 mg superoxide dismutase was added to 4.5 ml 0.5 M sodium borate and 0.1 M NaCl (pH 8.25). Then 0.5 ml 70 mM trinitrobenzene sulfonic acid in the same solution was added. The reaction mixture was maintained at 30°C and followed spectrophotometrically at $\lambda = 367$ nm [9]. The reaction was stopped by lowering the pH to 5.5 after the in-

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crease in absorption reached a maximum (~2 h). The protein solution was dialyzed for 24 h against 50 mM ammonium acetate (pH 5.5) and then lyophilized.

Polyacrylamide disc-gel electrophoresis was performed as in [10], except that no glycerol or SDS was added to the buffers. The stacking gels were 5% acrylamide (pH 6.8) and the separating gels either 7.5% or 10% acrylamide (pH 8.9). Bromocresol green was used as a tracking dye and the gels were stained with Coomassie blue as in [11].

Amino acid analysis of histidine, arginine and lysine was done using a Beckman model 120B amino acid analyzer.

3. RESULTS AND DISCUSSION

The heat capacity change during the thermal denaturation of yeast superoxide dismutase (Pharmacia) is shown in fig.1 and the thermodynamic parameters obtained from these scans are given in table 1. The denaturation temperature (T_m) is 361 K, as determined using a heating rate of 2.5°C/min and $\Delta H_{cal.}$ was measured as 9.04 and 8.26 cal./g for the enzyme from Pharmacia and Carlsberg, respectively. These values of $\Delta H_{cal.}$ were not corrected for the enthalpy of aggregation, which if appreciable would decrease the observed enthalpy of denaturation. Multiple heating scans of the same sample were made and it can be seen that the denaturation is partially reversible and

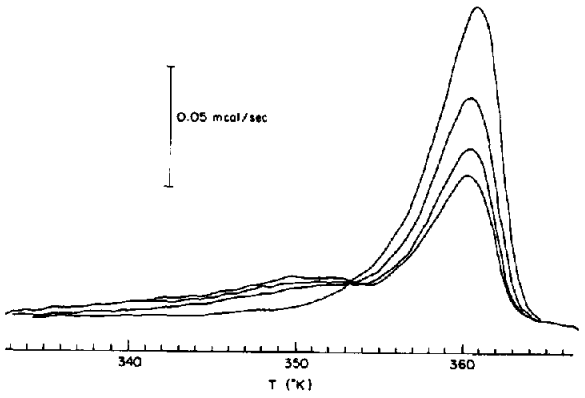

Fig.1. DSC scans of the thermal denaturation of 1.91 mg yeast superoxide dismutase (Pharmacia) at 24 mg/ml. The peaks of decreasing height at T_m = 361 K correspond to multiple heating scans of the same sample.

may be completely so if the superoxide dismutase was in dilute solution. In comparison, bovine superoxide dismutase at the same concentration undergoes an irreversible denaturation at 377 K with a calorimetric enthalpy of ~7.18 cal./g as determined by DSC (denaturation profile not shown). Dismutase activity of the bovine enzyme is also irreversibly lost by heating [12].

The degree of reversibility can be determined from the area under each peak at T_m = 361 K, assuming that the enthalpy does not change after the first denaturation, and 65–80% of the peak returns after each heating. There is a concomitant increase in the denaturation peak at 350 K, which contains ~50% of the remaining enthalpy. Thus there seem to be 3 different yeast superoxide dismutase species after heating: the native form (T_m = 361 K); one denaturing at 350 K; and an irreversibly denatured form. The irreversible denaturation may be due to aggregation, since the enzyme solution becomes cloudy after heating. Similar results were observed with yeast superoxide dismutase obtained from Carlsberg and are also summarized in table 1.

The appearance of a new species denaturing at 350 K after heating is quite interesting. The conformation of this new species is somewhat more thermally sensitive than the native enzyme and it may be an intermediate of refolding to the more stable, native state.

The enzyme activity was measured after heating and these data are given in table 2. Unheated yeast superoxide dismutase gave an activity of 5950 units/mg at room temperature. After heating for 10 min at 78°C (351 K) an activity of 3010 units/mg was obtained. A rather mild heating treatment was used to minimize aggregation. Incubation for 1 h at 37°C following heating gave an activity of 4310 units/mg. Thus there is further renaturation to the active form with incubation. An additional heating further reduced activity, again followed by an increase in activity with incubation at 37°C. Thus renaturation appears to proceed over a long period and to produce an active enzyme, as implied by the DSC studies.

In addition to differences in T_m, $\Delta H_{cal.}$ and the reversibility of denaturation, the yeast superoxide dismutase was found to exchange subunits under physiological conditions in which there was no subunit exchange by the bovine enzyme. This can