Cu$_2$Zn$_2$ Superoxide Dismutase Activity in an Air Dried Egyptian Mummy of the New Kingdom

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The structural intactness of many biopolymers in mummified tissues is well documented. By way of contrast no functional evidence of an isolated protein is known. The well defined air dried mummy of a deceased 16±2 year male teenager 1200 B.C. prompted us to search for the possible presence and function of Cu$_2$Zn$_2$ superoxide dismutase or remnants thereof. Using two different assay systems unequivocal proof of a specific Cu$_2$Zn$_2$ superoxide dismutase activity in brain extract was demonstrated. Fast protein liquid chromatography of the extract on Superose 12 revealed an approx. 5 kD Cu-containing polypeptide to which activity was assigned. It is assumed that during the ageing process considerable portions of the 31.3 kD homodimer were cleaved leaving this active core up to the present date. This would be the first case of the presence of an enzymically active component surviving the past 3000 years of mummification.

Introduction
Our knowledge on the intactness of mummified tissues has substantially increased. In a study on the air dried mummy of Nakht the deceased weaver of the kny-temple of User-khau-re or “Set-nakht” valuable data on the conservation status was obtained [1]. Nakht’s mummy of an 16±2 year male teenager was the most appropriate choice for autopsy. There exists a clear record of its discovery and excavation, and the text and the style of the coffin provide unusually clear evidence of its date i.e. the first half of the twelfth century B.C. The condition of the mummy was in accordance with Herodotus report that the least expensive form of mummification was that where no treatment at all was given and the body was simply wrapped in dry linen and allowed to desiccate in the peculiarly dry and stable Egyptian climate.

Nakht’s family was able to afford a fairly fine coffin because he did not receive the expensive process of mummification that included removal of the viscera, but had simply been washed prior to the wrapping in linen. Unlike extensive applications of natron, oils, resin or bitumen to the body this drying process ascertained a situation being close to lyophilization. The major portion of biopolymers was expected to remain in good condition. For example, red blood cells of indistinguishable shape to freshly prepared species were recorded in scanning electron micrographs [1, 2]. Comparative chromatography of protein extracts from freshly autopsied human skeletal muscle and the mummified skeletal muscle still revealed proteins in the 17000 Mr region. In sum this well defined mummy prompted us to search for the possible presence of an enzyme or remnants thereof which were expected to be still catalytically active.

Cu$_2$Zn$_2$ superoxide dismutase named after its enzymic function. It is ubiquitous in all tissues.

Experimental Part
In general most assays for superoxide dismutase activity measurements are based on coupled en-
zymic systems. During the enzyme catalysed oxidation of xanthine transiently formed superoxide radicals react with lucigenin and a strong chemiluminescence is observed [4]. This lucigenin amplified chemiluminescence is competitively inhibited by Cu$_{2}$Zn$_{2}$ superoxide dismutase. The concentration required to inhibit the xanthine dependent O$_2^-$ formation by 50% ($I_{50}$) was monitored by observing the photon emission in a luminometer. This powerful assay system prompted us to obtain unequivocal evidence whether or not Cu$_{2}$Zn$_{2}$ superoxide dismutase is still active in a mummified tissue.

A tissue sample from the extensively described and well preserved brain of Nakht (ROM I) taken at the time of the autopsy in 1974 [1], was stored prior to use in a desiccator in the presence of solid NaOH to ascertain complete dryness. Brain tissue was chosen attributable to its known high copper content. Some 80 mg were minced and suspended in phosphate balanced saline buffer over night.

**Results and Discussion**

After centrifugation the supernatant was assayed for enzymic measurements (Fig. 1). For comparative purposes dried rat brain was used. The chemiluminescence measurements revealed unequivocally the presence of Cu$_{2}$Zn$_{2}$ superoxide dismutase activity. The reaction pattern was identical to that of the extracted dried rat brain. A concentration dependency was noticed in Fig. 2. The addition of 0.1 mM cyanide known to react with the protein-bound copper completely abolished the initially observed activity. Of great importance was the phenomenon that no change of activity was seen in the presence of EDTA as this chelator is unable to reach the active centre of Cu$_{2}$Zn$_{2}$ superoxide dismutase under the given pH condition.

5 nM of intact Cu$_{2}$Zn$_{2}$ superoxide dismutase are required to cause 50% inhibition in the above assay system and is defined as one unit. Taking into account this Cu-concentration 40 units were present in one ml extract that contained 330 nM Cu. In other words 61% of the extracted copper (200 nM) could be assigned to Cu$_{2}$Zn$_{2}$ superoxide dismutase activity.

One µM Cu, a threefold higher Cu-concentration, was monitored in the extract of freshly dried rat brain. 32% of this copper was assigned to Cu$_{2}$Zn$_{2}$ superoxide dismutase activity. Unlike the mummy brain extract considerable portions of low Mr Cu-metallothioneins are normally found in fresh tissue extracts [5].

As in the liver the major source for Cu-storage in the brain is metallothionein. Upon mumification this Cu–S-rich protein is oxidized and/or polymerized [6] and not available for extraction. Nevertheless, the total concentration of SOD-active copper is substantially higher in extracts from
freshly prepared brain compared to that of the extracted mummified tissue.

Due to the pronounced activity in this highly sensitive chemiluminescence assay Cu,Zn-superoxide dismutase activity was monitored in a second independent way using the well established nitro blue tetrazolium assay [7], which requires approx. one order of magnitude higher Cu-concentrations. 40 nM Cu from fresh and intact Cu,Zn-superoxide dismutase are required to cause 50% inhibition. Again 66% of the copper present in the extract of the mummified tissue proved to exert this enzymic activity i.e. 5 SOD units were present in one ml of extract [8]. It should be emphasized that also in this assay the observed activity survived mM concentrations of EDTA.

A major objection against the observed activity could be raised. Secondary microbial infections could have been the cause for the successful detection of this enzymic activity. This point was seriously dealt with in control experiments to search for a wide range population of microorganisms [9]. 100 mg of tissue was extracted in phosphate balanced saline buffer and subcultured in a brain heart infusion boullion. Incubations at 25 °C were performed in broth or agar media including Sabouraud-glucose, Columbia agar with 5% sheep blood and brain heart infusion chocolate agar. Both aerobic and anaerobic incubations for up to 16 days showed no detectable sign of a fungal or bacterial population. Thus, the observed superoxide dismutase activity must have survived a 3000 year period.

The EDTA resistance in either enzymic assay strongly supports a genuine Cu,Zn-superoxide dismutase activity. Obviously, the active centre is still protected by a considerable portion of the protein moiety. Low molecular mass copper complexes of peptides or amino acids are enzymically active [10]. The catalytic activity, however, declines completely by Cu-chelation in the presence of EDTA [11].

It was attempted to characterize the Cu,Zn-superoxide dismuting component in more detail. The centrifuged extract was separated by fast protein liquid chromatography (FPLC) on Superose 12.

In accordance with the protein analyses on the mummified muscle tissues considerable portions were eluted at Mₐ near 17000 but no activity was measured in these fractions. All of the Cu,Zn-superoxide dismutase activity was confined to the major fraction in the 5000 Mₐ region. It is concluded that the 31 300 Mₐ protein has been progressively degraded to a 5000 Mₐ unit in the course of the ageing process. Much to our surprise this remnant remained catalytically active. To our knowledge this would be the first case of an enzymically active component surviving a 3000 year old drying process.

It is important to realize that mummification employing natron, resin, oils and especially bitumen cause deterioration of enzymic activities. Muscle tissue samples taken from several mummies of the New Kingdom or ptolemaic age pretreated in this manner showed considerable less or even no activity at all. Due to the reaction of the resin or bitumen most likely the intact tissue proteins were converted into non-catalytically active polymers.

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