RESEARCH COMMUNICATION

Leishmanial glycosomes contain superoxide dismutase

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In this work we report that superoxide dismutase is entrapped in a microbody-like organelle, the glycosome, present in *Leishmania* spp. Studies on the sensitivity of the enzyme to various inhibitors indicated that glycosomal superoxide dismutase is predominantly

of the Cu/Zn type. Localization of superoxide dismutase in glycosomes points to the major importance of this organelle in the survival of this pathogen, suggesting a new approach towards chemotherapy for leishmaniasis.

INTRODUCTION

Superoxide dismutase (SOD) is an enzymic scavenger of toxic oxygen radicals, which are responsible for cell damage [1]. The role of SOD in both eukaryotes and prokaryotes has been attributed its catalysis of the dismutation of superoxide radicals ($O_2^{\cdot-}$), producing molecular oxygen and hydrogen peroxide. The enzyme exists in three different forms [2–4]. Of these, Fe-SOD is the main form that has been demonstrated in parasitic protozoa, including *Leishmania* spp. [5], the causative agents for a wide spectrum of diseases ranging from cutaneous to visceral leishmaniasis [6,7]. *Plasmodium falciparum*, however, contains Mn-SOD [8]. Another isoform, which uses Cu/Zn as the cofactor, was found to be present in *Eimeria tenella* [9].

Cu/Zn-SOD has been reported to be localized in a microbody-like organelle, known as the peroxisome, prepared from rat liver [10,11]. Peroxisomes are known to contain enzymes taking part in fatty acid β -oxidation [12] and ether lipid biosynthesis [13]. In *Leishmania*, however, peroxisomes are not present [14]. On the other hand, a unique organelle, the glycosome, in which most of the glycolytic enzymes are compartmentalized, has been isolated from *Leishmania* species [15]. Glycosomes, which are also detected in the other members of the Trypanosomatidae [16], are known to be associated with ether lipid biosynthesis, *de novo* pyrimidine biosynthesis and fatty acid β -oxidation [17], suggesting that this particular organelle may be considered as one of the members of the microbody family that includes peroxisomes and glyoxysomes [18]. In this paper we report that the glycosomes of *Leishmania* spp. contain an endogeneous SOD that is similar to the peroxisomal enzyme with regard to the cofactor requirement.

MATERIALS AND METHODS

Materials

All biochemicals were purchased from Sigma Chemical Co. Culture media was obtained from Gibco.

Parasite

Leishmania donovani strain MHOM/IN78/UR6, of Indian origin, was maintained in solid medium [19] at 22–25 °C. Promastigotes from the late exponential phase were harvested from growth cultures and washed three times with buffer containing 10 mM Tes, pH 7.5, 1 mM EDTA, 0.2 mM leupeptin and 0.4 mM phenylmethanesulphonyl fluoride.

Subcellular fractionation

Glycosomes were isolated from the light mitochondrial fraction (5250 g supernatant, prepared by differential centrifugation), which was subjected to equilibrium density gradient centrifugation in a linear sucrose gradient (30–60 %, v/v) as described [20]. The gradient was prepared after layering equal volumes of 57 %, 50 %, 44 % and 33 % (v/v) sucrose in the buffer used to wash the cells. A 1 ml portion of 60 % sucrose solution was used for cushioning. Centrifugation was conducted at 4 °C for 160 min in an L7-55 Beckman ultracentrifuge (190 000 g_{av}) using a Ti-70 rotor. After centrifugation, the gradient was unloaded with a hypodermic needle attached to a peristaltic pump and the refractive index of each fraction was read in a Reichert digital refractometer.

Enzyme assay

SOD activity was determined on the basis of ability of this enzyme to decrease pyrogallol autoxidation [21]. Hexokinase [22], acid phosphatase [23], mitochondrial ATPase [24] and glucose-6-phosphate dehydrogenase [25] activities were assayed as described previously.

Protein determination

Protein was estimated using Folin–Ciocalteu's reagent [26] after precipitation with deoxycholate and trichloroacetic acid [27].

RESULTS AND DISCUSSION

Hexokinase, which is one of the key enzymes of the glycolytic pathway, predominantly occurs in the lower part of the gradient at a buoyant density of 1.21–1.23 g/ml (Figure 1), where the glycosome fraction is also reported to be separated [14]. We were curious to investigate the presence of SOD in these fractions for several reasons. First, there is no convincing data for the subcellular localization of this enzyme. Secondly, several functions of that are attributed to peroxisomes in mammals are found to be associated with glycosomes in protozoan parasites and other closely related organisms [28], but a possible similarity between these two microbodies in defence mechanisms against oxygen toxicity has not yet been explored. Thirdly, leishmanial SOD has been postulated to be a possible chemotherapeutic target [5]. Therefore proper knowledge of the subcellular distribution and the topogenic signal for the organellar internaliza-

Abbreviation used: SOD, superoxide dismutase.

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Figure 1 Subcellular distribution of hexokinase (a) and SOD (b) activities

A post-mitochondrial fraction was subjected to density gradient centrifugation on sucrose (see the Materials and methods section). Enzymic activities were measured in the unloaded fractions. The ordinate denotes the percentage of total activity/percentage of total protein.

Figure 2 Subcellular distribution of acid phosphatase

A post-mitochondrial fraction was unloaded after being subjected to sucrose density gradient centrifugation (see Materials and methods section). Each fraction was assayed for enzymic activity. Data are means for triplicate experiments.

tion of this enzyme may give a lead towards a possible therapeutic approach to the control of leishmaniasis.

To our knowledge, this is the first report to establish the

Figure 3 Subcellular distribution of mitochondrial ATPase

Activity was measured in each unloaded fraction after sucrose density gradient centrifugation. Other particulars are same as described for Figure 2.

Figure 4 Subcellular distribution of glucose-6-phosphate dehydrogenase

Activity was measured in each fraction unloaded after sucrose density gradient centrifugation. Other particulars are same as described for Figure 3.

glycosomal occurrence of SOD in *Leishmania* spp. Moreover, SOD in this pathogen is known to contain Fe as a cofactor [5], whereas glycosomal SOD was found to be of the Cu/Zn type, as shown by cyanide inhibition [29]. SOD activity in the glycosomal fraction (fraction 2 of Figure 1) was inhibited by 0.3 mM NaCN up to a maximum of $61 \pm 9\%$ ($n = 4$). Complete inhibition of this enzyme was observed when 1 mM NaCN was used. Thus the SOD activity present in the glycosomal fraction is of the Cu/Zn type. This finding was further confirmed by the observation that chloroform/ethanol (3:5, v/v; in a 0.5 ml sample), which inactivates both Mn- and Fe-SOD but not the Cu/Zn enzyme, [30], was unable to alter basal SOD activity in glycosomes.

Possible contamination of glycosomes by other subcellular organelles was investigated by assaying marker enzymes. Figures 2, 3 and 4 show that the activities of lysosomal acid phosphatase, mitochondrial ATPase and cytosolic glucose-6-phosphate dehydrogenase were very low in the first four fractions. Moreover, the possibility of mitochondrial SOD contamination of glycosomal fractions may be ruled out, as this particular organelle-bound enzyme generally contains Mn as cofactor [31]. Lysosomal

contamination, on the other hand, must be considered because Cu/Zn-SOD is usually present in this organelle [32]. When calculated on the basis of acid phosphatase activities in fractions 2 and 5, we have observed that lysosomal Cu/Zn-SOD activity in the glycosomal fraction contributes up to a maximum of 20% of total SOD activity. The percentage recovery of enzymic activities ranged between 87 and 105%.

The SOD activity in each fraction unloaded after sucrose density gradient centrifugation was assayed with or without 1 mM cyanide. Enzyme activity was totally inhibited by 1 mM NaCN in fractions 1–3, whereas in fractions 4 and 5 the percentage inhibition was 92.4 ± 2.8 and 38.6 ± 1.6 %, respectively (means $\pm$ S.D.). However, 1 mM cyanide did not inhibit significantly SOD activity in fractions 6–10. This shows that Cu/Zn-SOD activity was predominant in the glycosomal fractions separated through the sucrose density gradient as described. During the inhibition studies, equal amounts of NaCN as used for assaying the enzyme were added to the control mixture, to take account of any effect of this inhibitor on the assay system.

The aim of the present work was to investigate glycosomal function in cellular metabolism in *Leishmania* spp. The presence of the predominant cytosolic Fe-SOD activity probably did not allow earlier investigators to detect Cu/Zn-SOD of lower activity, unless the glycosomes were separated from the other organelles. It will be of interest to establish whether this newly detected glycosomal SOD plays an active role in the survival of this pathogen. If so, this would open up the possibility of anti-leishmanial drug design targeting this unique subcellular organelle, the glycosome.

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REFERENCES

- McCord, J. M., Keele, B. B., Jr. and Fridovich, I. (1971) Proc. Natl. Acad. Sci. U.S.A. **68**, 1024–1027
- Sawada, Y., Ohyama, T. and Yamazaki, I. (1972) Biochim. Biophys. Acta **268**, 2875–2880
- Vance, P. C., Keele, B. B., Jr. and Rajagopalan, K. V. (1972) J. Biol. Chem. **247**, 4782–4786
- Trant, N. L., Meshnick, S. R., Kitchener, K., Eaton, J. W. and Cerami, A. (1983) J. Biol. Chem. **258**, 125–130
- Meshnick, S. R. and Eaton, J. W. (1981) Biochem. Biophys. Res. Commun. **102**, 970–976
- Chang, K. P. and Bray, S. (eds.) (1985) Leishmaniasis, Elsevier Biochemical Press, Amsterdam
- Kahl, L. P. and McMohan-Pratt, D. (1987) J. Immunol. **138**, 1587–1595
- Ranz, A. and Meshnick, S. R. (1989) Exp. Parasitol. **69**, 125–128
- Michalski, W. P. and Prowse, S. J. (1991) Mol. Biochem. Parasitol. **47**, 189–196
- Keller, G. A., Warner, T. G., Steimer, K. S. and Hallegwell, R. A. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 7381–7385
- Dhaunsi, C. S., Gulati, S., Singh, A. K., Orak, J. K., Asayama, K. and Singh, I. (1992) J. Biol. Chem. **267**, 6870–6873
- Tolbert, N. E. (1981) Annu. Rev. Biochem. **50**, 133–157
- Hajra, A. K. and Bishop, J. E. (1982) Ann. N.Y. Acad. Sci. **386**, 170–182
- Oppenheimer, F. R. (1981) Annu. Rev. Microbiol. **41**, 127–151
- Hart, D. T. and Oppenheimer, F. R. (1984) Mol. Biochem. Parasitol. **13**, 159–72
- Michels, P. A. M. (1989) Exp. Parasitol. **69**, 310–315
- Oppenheimer, F. R. (1988) Trends Biochem. Sci. **13**, 255–260
- Kindle, H. and Lazarow, P. B. (1982) Ann. N.Y. Acad. Sci. **386**, in the press
- Ray, J. C. (1932) Ind. J. Med. Res. **20**, 355–357
- Oppenheimer, F. R., Markos, A. and Steiger, R. F. (1981) Mol. Biochem. Parasitol. **4**, 291–309
- Marklund, S. and Marklund, G. (1974) Eur. J. Biochem. **47**, 469–474
- Bergmeyer, H. U. (1974) Methods of Enzymatic Analysis, vol. 1, pp. 473–474, Academic Press, San Diego
- Oppenheimer, F. R., Borst, P. and Spits, H. (1977) Eur. J. Biochem. **76**, 21–28
- Pullman, M. E. and Penefsky, H. S. (1963) Methods Enzymol. **6**, 277–282
- Bergmeyer, H. U. (1974) Methods of Enzymatic Analysis, vol. 1, pp. 458–459, Academic Press, San Diego
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
- Bensadoun, A. and Weinstein, D. (1976) Anal. Biochem. **70**, 241–250
- Hammond, D. J., Gutteridge, W. E. and Oppenheimer, F. R. (1981) FEBS Lett. **128**, 27–29
- Crapo, J. D., McCord, J. M. and Fridovich, I. (1978) Methods Enzymol. **53**, 382–393
- Bannister, J. V. and Bannister, W. H. (1984) Methods Enzymol. **105**, 88–93
- Slot, J. W., Genze, H. J., Freeman, B. A. and Crapo, J. D. (1986) Lab. Invest. **55**, 363–371
- Change, L. Y., Slot, J. W., Genze, H. J. and Crapo, J. D. (1988) J. Cell Biol. **107**, 2169–2179