OSMOTIC PROPERTIES OF SONICATED MITOCHONDRIAL FRAGMENTS

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1. Introduction

Kagawa and Racker [1] have shown that the reconstitution of a preparation capable of energy conservation is accompanied by the presence of vesicular structures in the electron micrographs. Furthermore the reconstituted preparation is largely impermeable to inuline and ferritine. The data have been taken to support the hypothesis that energy coupling is associated with the development of electrical forces [2–6]. In contrast with this conclusion it has been shown recently by Massari and Azzone [7] that energy conservation occurs in damaged mitochondria, having pores of 14 Å of equivalent radius.

In the present study we have examined the accessibility of the water space of sonicated mitochondrial fragments to hydrophilic molecules of increasing molecular weight. The data here reported indicate that sonication induces a large increase of permeability of the mitochondrial membrane to hydrophilic and charged species. It appears that no relationship exists between capacity for energy conservation and function of the membrane as an osmotic barrier.

2. Methods

Mitochondria prepared from rat liver according to standard procedures were used throughout. Sonication was carried out after diluting the mitochondria in two standard media: i) for the EDTA particles, in 30 mM KCl, 2 mM EDTA pH 8.5; ii) for the Mg^{2+}-particles, in 30 mM KCl, 1 mM ATP, 2 mM succinate, 10 mM $MgCl_2$ and 10 mM Tris pH 8.5. In some cases 30 mM KCl was replaced with 0.1 M sucrose. The final concentration of the mitochondrial protein in the sonication medium was about 25 mg/ml. The sonication was carried out with a Branson apparatus at 5 A; 1 ml samples were taken from the sonication medium, either before treatment or after various sonication times as described in the figures, and directly added to a standard medium containing 30 mM KCl, 5 mM $MgCl_2$, 10 mM Hepes pH 7.0, 1 mM ATP and 2 mM succinate. Final volume 2.5 ml. Each tube contained also either ^3H_2O or the ^{14}C isotopes of 60,000 Dextrane, 15,000 Dextrane or inuline. All isotopes were obtained from New England Nuclear. Isotope dilution was obtained by adding 0.2% of cold Dextrane or inuline to the hot substrate in each tube. The samples were immediately centrifuged at 150,000 *g* for 45 min. After centrifugation the samples were carefully drained, dried with filter paper and weighed. This permitted a comparison for each tube of the gravimetric with the isotope procedure. The isotope data were in general about 15% lower than the gravimetric data although there was a very good correspondence between the two procedures in each series of samples. The data reported in the figures were calculated on the basis of the isotopic determination of the total pellet water. The determination of the number of counts in the pellet and in the supernatant were made by liquid scintillation counting after dissolving approximately equal volumes of the two in 10 ml of Istagel (Packard Instruments). The counts were corrected by reference to an internal standard. The protein determination was made with the biuret

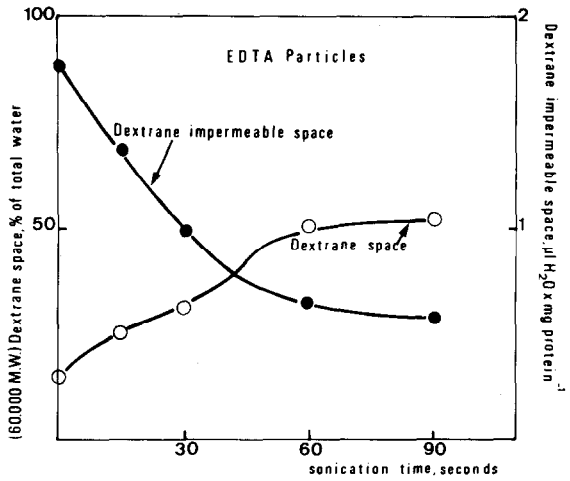

Fig. 1. Effect of sonication on 60,000 Dextrane permeable and impermeable spaces. Experimental conditions in this and other figures are explained in the Methods. The amount of protein in each tube was 33 mg.

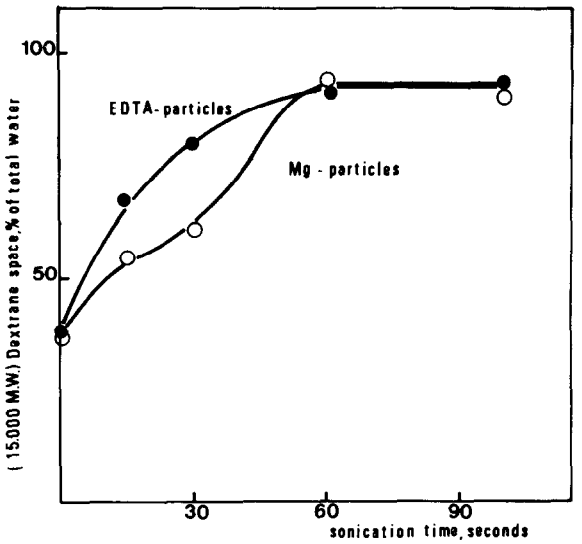

Fig. 2. Effect of sonication on the accessibility of mitochondrial water to 15,000 Dextrane. The amount of protein in each tube was 20 mg. The total amount of water in the pellet was: a) for the EDTA particles 18.9, 15.3, 15.0, 15.3 and 16.4 µl, and b) for the Mg particles, 28.4, 16.1, 16.1, 19.4 and 17.2 µl at 0, 15, 30, 60, and 90 sec of sonication, respectively.

procedure. After sonication the centrifugation involved the separation of soluble and membranous protein components. The determination of the particles space in µl × mg protein^{-1} therefore required an additional protein determination on the supernatant. At short sonication times, the absence of fractionation step implied that the suspension was a mixture of unbroken and broken mitochondria. It was therefore impossible to decide whether the changes observed were due to an increase in the:
a) number of permeable *vs* intact mitochondria, or
b) degree of permeability of each mitochondrion.

3. Results

The EDTA and Mg particles used in the present study were regularly tested for energy coupling in respect to a number of parameters such as ANS fluorescence, metachromatic shifts etc. Both preparations were found highly capable of energy coupling. However the Mg-particles were more efficient in respect to oxidative phosphorylation.

Fig. 1 shows the increase of 60,000 Dextrane space occurring during sonication. The Dextrane space was about 15% of the total water in the intact mitochondria and was increased to about 55% after 90 sec of sonication. On the other hand the Dextrane impermeable space, which includes in intact mitochondria both the space within the inner membrane and the space between the inner and outer and membrane, decreased from 1.75 to 0.5 µl × mg protein^{-1}. This last figure indicates that in the EDTA particles there is a space which is not accessible to a large molecular weight solute such as 60,000 Dextrane.

For obvious reasons, the surface being equal, the total inner volume of the submitochondrial particles must be smaller than the inner volume of the intact mitochondria from which they are derived. This might in principle explain the decrease of the 60,000 Dextrane impermeable space. The explanation however does not hold for the results of figs. 2–4 where the increase of accessible space is found to be dependent on the molecular weight of the solute.

Fig. 2 shows the effect of sonication on the penetration of 15,000 Dextrane. The Dextrane space was about 36% of the total water in the intact mitochondria and increased to about 92% after 90 sec of sonication. The increase of accessibility to 15,000 Dextrane was greater for the EDTA particles than for the Mg particles. After 90 sec of sonication there was only an

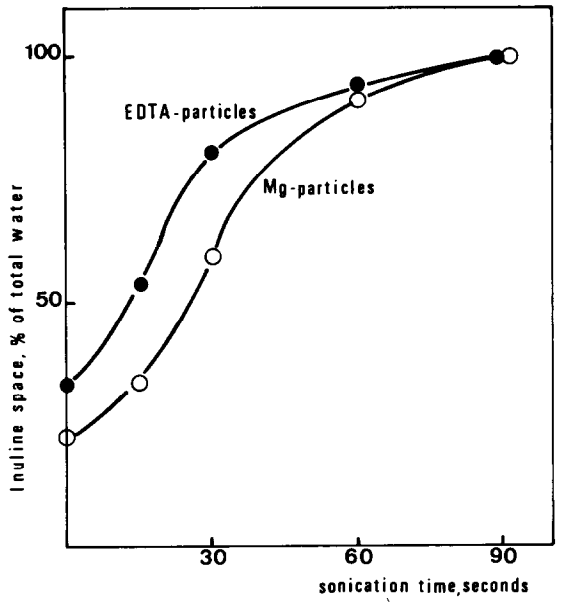

Fig. 3. Effect of sonication on the accessibility of mitochondrial water to inuline. The amount of protein per tube was 33 mg for the EDTA and 24 mg for the Mg particles. The total amount of water in the pellet was, for the EDTA particles, 59.2, 37.5, 28.0, 25.2 and 23.3 µl and for the Mg particles 45.2, 30.0, 24.7, 16.5, and 14.3 µl after 0,15, 30, 60, and 90 sec of sonication, respectively.

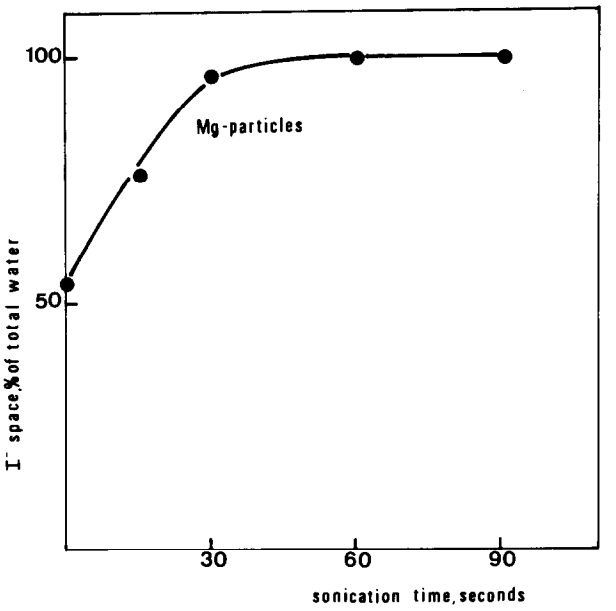

Fig. 4. The effect of sonication on the accessibility of mitochondrial water to iodide. The amount of protein per tube was 26 mg. The amount of water in the pellet was 38, 33.9, 26.2, 24.2 and 23.6 µl at 0, 15, 30, 60, and 90 sec of sonication, respectively. 2 mM KI was also present during centrifugation. Measurement of I^- was carried out with $^{131}I^-$.

8% fraction of fragment water which was not accessible to 15,000 Dextrane.

Fig. 3 shows the accessibility to inuline due to sonication in EDTA and Mg-ATP. In both media 100% of the water was accessible to inuline at the end of the sonication. Again, the increase of accessibility was greater for the EDTA particles, where usually it reached completion within 60 sec. A similar pattern for the effect of sonication was also observed in respect to the increase of accessibility for I^- (fig. 4). It may be noted by comparing the relative accessibilities at identical sonication times, that the extent of penetration decreased in the order I^-, inuline, 15,000 Dextrane and 60,000 Dextrane. This corresponds to a series of molecules of increasing radii.

4. Discussion

A key question for the hypotheses of energy transduction is the role of membrane organization i.e. whether a membrane is required for osmotic or for structural purpose [8].

The osmotic role of the membrane is taken to be supported by the reconstitution experiments of Kagawa and Racker [1]. A main objection against the electron microscopical evidence is that this technique does not answer the question as to whether what looks like a membrane does indeed act as a membrane in respect to the osmotic parameters. The impermeability to ferritine is in substantial agreement with the observation reported here that about 50% of the fragment water is not accessible to 60,000 Dextrane. An apparent contradiction on the other hand concerns the impermeability to inuline of Kagawa and Racker [1] preparation as compared to the high permeability of our sonicated fragments to inuline and practically to 15,000 Dextrane. The two experimental systems are however very different. In the case of Kagawa and Racker [1] the random combination of membrane proteins with phospholipids during reconstitution may lead to a very irregular organization without physical continuity of the aqueous

pores across the membrane. On the other hand the native mitochondrial membrane has been shown to contain aqueous pores of 6 Å of equivalent radius [7]. This figure is similar to that of other plasma membranes and is presumably dependent on the architecture of the lipid–protein interactions in the membrane phase. An increased accessibility to hydrophilic molecules of molecular radius larger than 6 Å, as inuline and 15,000 Dextrane, implies the formation of very large aqueous pores. This is due to perturbation of the lipid–protein interactions, occurring to a very large extent after sonication, as indicated by recent NMR studies [9, 10].

From the comparison between phosphorylation and energy linked transhydrogenase activity Lee et al. [11] were led to the conclusion that the EDTA submitochondrial particles, although incapable of oxidative phosphorylation, were still capable of respiratory chain linked energy coupling. Since the particles used in the present study are capable of respiratory chain and ATP linked energy coupling although they possess a membrane which is permeable to high molecular weight solutes, we are led to the conclusion that energy conservation in fragments is not linked to the development of osmotic forces. This conclusion is in agreement with that reached at recently in regard to the properties of Ca^{2+}–P_i swollen mitochondria [7].

A much debated question is whether the sonicated fragments are in inside-out, (possess inverted polarity) in respect to intact mitochondria [8, 12–14]. A further conclusion from the present results is that, if the polarity of a membrane is expressed by the vector of the transport reaction for a given solute, the sonicated liver mitochondrial fragments exhibit no polarity for any solute having a molecular weight lower than 15,000.

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