SIZE AND SHAPE TRANSFORMATIONS CORRELATED WITH OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA

II. Structural Changes in Mitochondrial Membrane Fragments

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ABSTRACT

It has been demonstrated that the nature of the physical change in mitochondrial membrane fragments associated with the action of the respiratory enzymes is likely one of shape or symmetry rather than size. The findings suggest that in the state of decreased scattering the macromolecules may be present in an extended physical state. Conditions favorable for phosphorylation may give rise to a folding or contraction of the molecular complex to a more symmetrical structure. Since earlier studies have shown that there is a compulsory relationship between the integrity of systems operative in oxidative phosphorylation and scattering changes, experiments of this type may lead to values for the minimal size of a phosphorylating unit, which at present is estimated to be 2.1×10^6 from light-scattering studies.

The close correlation of oxidative phosphorylation with swelling-shrinkage states of mitochondria prompted an earlier study of structural change in mitochondrial membrane fragments (1). Membrane fragments prepared by disrupting mitochondria with digitonin (2) were found to change their light-scattering state in response to changes in the activity of the oxidative phosphorylation process (1). These results provided strong support for the assumption that the origin and mechanism of "active" volume changes of intact mitochondria reside in the macromolecular system of enzymes in the membrane. However, a better understanding of the structural changes at the macromolecular level and the way in which mechanochemical coupling can cause changes in volume in intact, osmotically sensitive mitochondria is desirable.

As shown in the previous article (3), scattering changes are accompanied by volume and associated viscosity changes in intact mitochondria. No evidence for volume change has been obtained, however, under various conditions of light-scattering or structural state in membrane fragments (1). In the present article, a more detailed examination has been made of the physical changes which occur in mitochondrial membrane fragments incubated under conditions of oxidative phosphorylation. Studies of the viscosity and angular dependence of light scattering in fragments suggest that shape changes accompany the metabolic states being studied. The results are discussed in terms of a possible contractile mechanism for this type of structural change in a membrane. A preliminary report of this research has appeared (4).

METHODS

Preparations and test conditions. Rat liver mitochondrial fragments were prepared by the method of Devlin and Lehninger (2), who employed digitonin as the disrupting agent. Water-washed mitochondria were prepared by centrifuging the mitochondrial suspension prepared in 0.25 M sucrose at 10,000 *g* for 10 minutes, and resuspending the pellet in 20 times its volume in water. The resuspended material was centrifuged at 10,000 *g* for 10 minutes and the pellet discarded. The supernatant was recentrifuged at 20,000 *g* for 20 minutes and the resulting pellet resuspended in 10 times its volume of water. The test conditions employed were similar to those previously described (1). An aliquot of either the water-washed mitochondria or mitochondrial fragments was combined with a reaction medium consisting of 0.05 M tris(hydroxymethyl)aminomethane buffer (tris) at pH 7.5 containing 0.005 M $MgCl_2$. Other additions are indicated for individual experiments.

Changes due to light scattering were measured by the absorbancy or turbidometric technique, employing a non-specific wave length (5, 6) or, in the case of angular scattering data, the Brice Phoenix Light-Scattering Photometer. Light-scattering data were plotted according to Zimm (7).

RESULTS

Light-Scattering Changes of Water-Washed Mitochondria

Some requirements for light-scattering changes in water-washed mitochondrial suspensions are illustrated in Fig. 1. Light-scattering increases were initiated in the suspension by addition of an oxidizable substrate (*e.g.* succinate) and an adenine nucleotide (ADP as shown or ATP) to the mitochondrial suspension in tris-Mg^{++} medium. The increased scattering change (measured as a change in optical density at 500 mμ using a 1-cm cuvette) continued for a period of about 5 minutes and then seemed to reach a steady state. At the point indicated in the figure, the oxygen of the suspension was exhausted as determined polarigraphically (oxygen electrodes inserted into the reaction mixture in the reference cuvette of the Cary spectrophotometer). Following the exhaustion of oxygen, there was a change in turbidity of the preparation. This is due to the presence of respiratory pigments which upon anaerobiosis become reduced, producing an artifact in the absorbancy changes recorded at 500 mμ. While

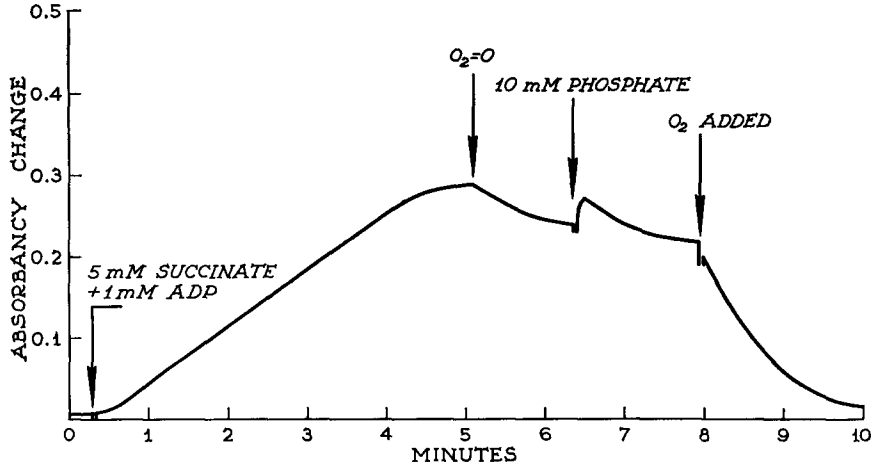

FIGURE 1 Demonstration of respiration-dependent changes in light scattering by water-washed rat liver mitochondria. Water-washed mitochondria were prepared and tested in air-saturated tris-$MgCl_2$ medium as described in the section on Methods. A time recording of the absorbancy changes under the conditions described in the text was made in a Cary model 14 recording spectrophotometer. A suspension of water-washed mitochondria in reaction mixture (approximately 2 mg protein per ml) was added to the reference and experimental cuvettes; this gave an initial absorbancy (or optical density) difference between the two samples which was almost negligible. Explanation of the additions made (to the experimental cuvette only) are given in the text.

this arrangement results in an artifact in the scattering tracing, it (*a*) serves to point out that the absorbancy changes due to oxidation-reduction of the cytochrome pigments are considerably smaller than those due to scattering, and (*b*) serves as a convenient indicator for the time at which the preparation exhausts its oxygen. The addition of 10 mM phosphate gave no appreciable reversal of the light-scattering decrease, although it may be noted that the addition of phosphate must have led to a trace introduction of oxygen, for there was a momentary reversal of the optical change due to pigment oxidation-reduction. When, however, oxygen was added to the system by rapidly bubbling 100 per cent oxygen into the reaction mixture, it is seen that this promoted a rapid and complete reversal of the light-scattering change.

This experiment demonstrates a quantitative reversal of light scattering in water-washed mitochondria that is associated with a respiration-dependent mechanism. Since water-washed mitochondria have lost their ability to respond osmotically to reagents such as sucrose (8), it can be concluded that these respiration-dependent scattering changes are a manifestation of structural changes in the membranes. These findings also provide independent support for the validity of respiration-dependent scattering changes studied in digitonin fragments (1), for these preparations rule out a possible involvement of digitonin in light-scattering changes observed in the earlier study.

The requirements for scattering changes in water-washed mitochondria and digitonin fragments have been found to be remarkably similar. Table I summarizes the requirements for such changes identified for both water-washed mitochondria and digitonin fragments. For scattering increases, the reactants of the respiratory chain such as substrates and oxygen must be present.

TABLE I

Summary of Requirements for Light-Scattering Changes in Water-Washed or Fragmented Rat Liver Mitochondria

Light-scattered changes		
Increases	Decreases	Inhibition
Electron transport	*Oxidation of respiratory chain*	*Inhibition of electron transport*
O_2	Exhaustion of substrate	Anaerobiosis
Substrate, *e.g.* succinate or β-hydroxybutyrate	Inhibition of electron transport at dehydrogenase level (*e.g.* malonate block of succinate respiration)	Antimycin A, cyanide, or azide
		Amytal (for β-hydroxybutyrate, but not succinate, respiration)
Adenine nucleotides	*Phosphate acceptors*	
ADP or ATP	Inorganic phosphate or arsenate	
Ions	*Uncoupling agents*	
Mg^{++} or Mn^{++}	*e.g.*, 2,4-dinitrophenol, pentachlorophenol, or Dicumarol	
	Ions	
	Ca^{++}, Cd^{++}	

In addition, the presence of adenine nucleotides such as ADP or ATP and a cation such as magnesium or manganese is needed. Increased scattering can be reversed by complete oxidation of the respiratory chain, as may occur after exhaustion of substrate. Oxidation of the chain can also be brought about by inhibition of electron transport at the dehydrogenase level, as in the malonate block of succinate respiration. Appropriate concentrations of phosphate or arsenate also reverse scattering, as do certain uncoupling agents and ions. Scattering increases brought about by the conditions in column 1 can be halted by inhibition of electron transport either by exhaustion of oxygen or by reagents which interrupt electrons

in the middle of the chain. Thus there is considerable similarity between the conditions which will effect light-scattering changes in intact mitochondria (*cf.* 1–4) and in osmotically insensitive mitochondrial membrane preparations.

Volume Changes in Mitochondrial Fragments

The type of swelling and shrinking which accompanies the normal function of the respiratory chain in intact phosphorylating mitochondria is estimated to result in volume changes of about 20 to 40 per cent (3, 9). The magnitude of the mitochondrial volume changes was calibrated by correlating osmotically induced volume changes and absorbancy changes with direct determinations of volume found by the mitocrit method of Jackson and Pace (10, 11). However, similar measurements of volume changes accompanying scattering changes in water-washed mitochondria or digitonin fragments have thus far been negative. With mitochondrial fragments, the experiments were conducted by distributing samples maintained, in the state of either decreased or increased scattering, in capillary tubes and centrifuging at high speed (105,000 *g* for 60 minutes) in the preparative ultracentrifuge. The centrifugal force used was the same as that employed in the original preparation of the digitonin fragments. No change was detected even with a concentration of solids of 45 per cent (error of method 0.5 per cent). Parallel studies of time recordings of scattering show that fragments could be maintained in their respective states of scattering for considerable periods of time. However, the possibility exists that during centrifugation of the fragments in capillary tubes the scattering states may have changed. Though these experiments do not rule out a volume change even under conditions in which light scattering of digitonin fragments was maintained in scattering states differing by 35 per cent, they do indicate that if volume changes occur, they are not detectable.

Viscosity Studies with Mitochondrial Fragments

The use of viscosity measurements seemed promising as an empirical technique for following changes in size or shape of mitochondrial fragments. In the case of digitonin fragments, if viscosity changes are observed, one cannot be sure whether or not a change in outflow time through a capillary tube reflects a change in size or shape in macromolecules. Fig. 2 shows a plot of the corrected outflow time through a capillary tube (relative viscosity) as a function of the concentration of protein in digitonin fragments maintained in the state of decreased scattering (substrate and adenine nucleotide are absent). It is clear that an increase in outflow time accompanies the increase in protein concentration. Fig. 3 shows a plot of the reduced viscosity (relative viscosity per unit protein concentration) as a function of concentration for such data. The results suggest that this system behaves as a Newtonian solution,

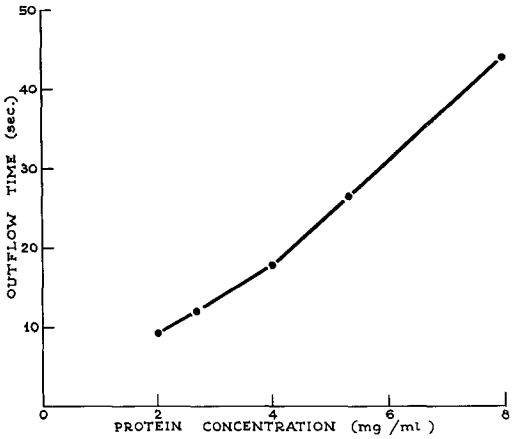

FIGURE 2 Viscosity as a function of concentration of digitonin fragments maintained in the state of decreased light scattering. Measurements were made in a Cannon-Ubbeholde type viscometer (viscometer constant, 0.01392 centistoke/sec.) at 20°C. The outflow time of the reaction mixture (*te*) containing different concentrations of mitochondrial membrane fragments was corrected for the outflow time of the reaction mixture only (*tc*); (*te* − *tc*) was plotted against concentration.

i.e., no concentration-dependent effects such as aggregation are occurring. Experiments were therefore devised to determine the viscosity during different scattering states in digitonin fragments. Decreased scattering was maintained in a system lacking substrates and adenine nucleotides (*cf.* Fig. 1). Increased scattering was brought about by the addition of these reagents. The results shown in Fig. 4 indicate that in the concentration range examined, increased scattering is associated with a decrease of viscosity. It may be noted from the plot of per cent viscosity decrease against concentration that the extent of the change is dependent on concentration, since at lower concentrations

there were greater percentage increases. In contrast, the viscosity change was independent of concentration when digitonin fragments were in the state of decreased scattering. These results suggest that either size or shape changes occur under conditions where the scattering change associated with phosphorylation is expected.

Angular Scattering Studies with Mitochondrial Fragments

The viscosity studies suggested that the nature of the physical change occurring in response to the

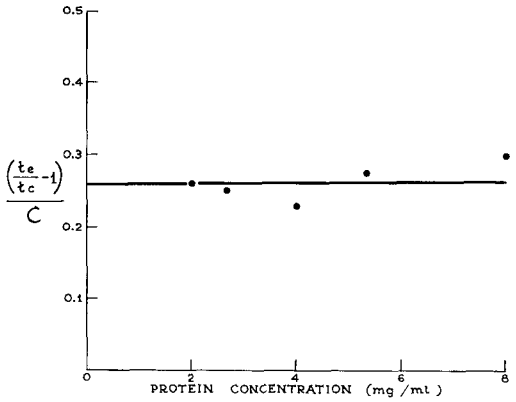

FIGURE 3 Reduced viscosity as a function of concentration of digitonin fragments maintained in the state of decreased light scattering. Conditions as in Fig. 2. The relative viscosity, $(te/tc) - 1$, was calculated from the outflow times of the solution (te) and the solvent (tc). Reduced viscosity (relative viscosity divided by concentration) was plotted against the concentration of digitonin fragments. Explanation in text.

action of the respiratory enzymes in digitonin fragments was one of either size or shape. It was therefore of interest to examine the full angular dependence of scattering of the mitochondrial fragments in order to distinguish which of these physical changes was occurring. Fig. 5 shows a typical Zimm plot (7) of reciprocal reduced intensity for digitonin fragments.[1] The control curve represents fragments in a system containing tris-magnesium medium. Scattering was increased in the experimental curve by addition of succinate and ATP. It is apparent that it is not possible to extrapolate the curves to a positive intercept. Both curves seem to converge near the lower angles and may have a common negative intercept, suggesting no change in size. It appears that the fragments must have been contaminated with amounts of high molecular weight scattering material sufficient to prevent extrapolation from either low or high angles to a positive intercept, which, if possible, would give a value for the reciprocal of

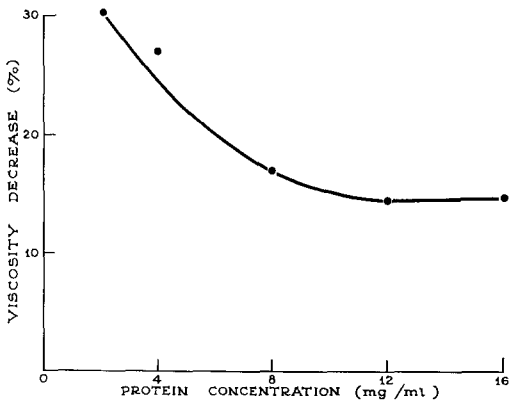

FIGURE 4 Viscosity changes under conditions of an increased light-scattering state of digitonin fragments induced by succinate and ATP. Various concentrations of digitonin fragments were incubated in tris-$MgCl_2$ medium in the absence (decreased scattering state) and in the presence (increased scattering state) of 5 mM succinate + 1 mM ATP. The corrected outflow time ($te - tc$) for the system without succinate + ATP was taken as 100 per cent. The per cent viscosity decrease was calculated from the shorter outflow time observed when succinate + ATP was present in the system, *viz.*:

$$\frac{(te - tc)_{\text{control}} \times 100}{(te - tc)_{\text{succinate + ATP.}}}$$

the weight average or twice the number average molecular weight, respectively.

[1] In the Zimm plot for treatment of angular scattering data, the ordinate is the reciprocal of the scattering intensity at each angle (corrected for solvent, reflection at the glass surfaces, etc.) and the abscissa is a sine function of the angle. Extrapolation of the data to zero angle gives an intercept on the ordinate which is the reciprocal of the weight average molecular weight (when extrapolation is from low angles) or ½ times the number average molecular weight (when extrapolation is from high angles), thus providing an indication of molecular size. The slope of the curves is a function of the shape of the macromolecules. If the slope or intercept was unchanged in the two metabolic states under study, then a shape or size change, respectively, would be unlikely.

Attempts were therefore made to prepare a suspension of fragments of smaller and more uniform size which still would retain the ability to change their scattering state. Ultracentrifugal experiments demonstrated that scattering changes of the type discussed here were distributed in fractions collected over a wide range.[2] These results agree with those of Siekevitz and Watson (12), who have shown by electron micrographs that the size of digitonin fragments varies over a range of some 100-fold. Fig. 6 shows a Zimm plot for a supernatant fraction obtained after centrifuging fragments at 25,000 *g* for 25 minutes. It can be seen that the magnitude of the change in scattering resulting from the addition of succinate and ATP is much reduced from that seen in the previous experiment. The decrease in scattering was, however, above experimental error and was reproducible in several experiments. Large scattering material apparently still contaminated the system, preventing the extrapolation to a positive intercept from low angles. Extrapolation to a positive intercept could be achieved, however, from high angles; the intercept in this case being ½ N (2 × number average molecular weight). These results indicate that the size of fragments still capable of scattering changes remains the same in the two states examined and that the number average molecular weight for this unit is of the order of 2.1×10^6.

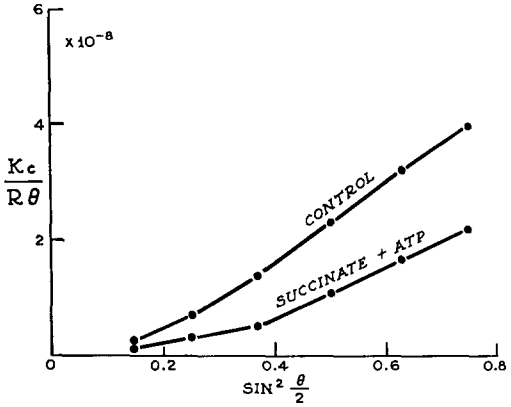

FIGURE 5 Reciprocal reduced intensity plot for digitonin fragments. Experimental conditions as in Fig. 4 with 4 mg/ml protein. Light-scattering intensity was measured at various angles in the Brice-Phoenix Light-Scattering Photometer in a cylindrical cell. After addition of succinate + ATP, the angular scattering was once again measured. The data are plotted according to Zimm (7) as discussed in the text.

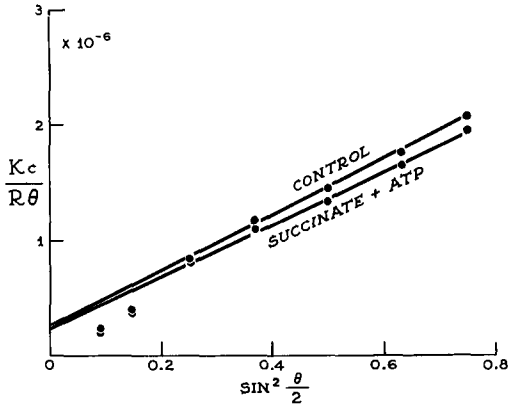

FIGURE 6 Reciprocal reduced intensity plot for digitonin fragments clarified by high speed centrifugation. Conditions as in Fig. 5 and as described in text.

DISCUSSION

The nature of the macromolecular change in mitochondrial membranes is of considerable interest in relation to the interpretation of its functional significance. An understanding of this problem is related to the question whether mitochondria contain a contractile protein. Does a metabolically driven change in mitochondrial membrane structure lead to an altered permeability of the membrane? If so, this might provide a mechanism for the regulation of cellular reactions by influencing the distribution of essential metabolites between different compartments in the cell. The findings suggest the presence of a component in mitochondrial membranes which is capable of undergoing reversible changes in structure depending on the state of the respiratory chain and associated phosphorylation enzymes. Since these changes occur in the absence of volume changes but are accompanied by viscosity changes, a difference in shape rather than size is suggested. Although the interpretation of angular light-scattering data is complicated, the preliminary findings suggest that a change in molecular size or aggregation does not accompany the

[2] The presence of a wide range of fragment sizes was initially observed in sedimentation velocity studies with the analytical ultracentrifuge carried out through the kindness of Dr. Verne Schumaker.

different light-scattering states. Perhaps it may be suggested, as has already been done for the contractile protein of muscle by Blum and Morales (13) and by Von Hippel *et al.* (14), that the macromolecular change is a manifestation of a conformational change from a compact to a less compact or more expanded state. If, as previously suggested (15–17) and as recent papers (18, 19) indicate, mitochondria truly contain a contractile protein, the results of the present investigation lend some general support to this concept in so far as the reversible configurational changes suggested by the light-scattering data might be a manifestation of contractility of the membrane.

The large variation in the particle size found in the course of centrifugation studies has imposed considerable limitations on the refinements of the physical studies with the fragment preparations. The smallest fragments obtained which still manifested reproducible differences in scattering states yielded a calculated number average molecular weight of 2.1×10^6 by extrapolation of the angular dependence of scattering from high angles. Assuming that there is a compulsory relation between the integrity of the phosphorylation apparatus and light-scattering changes of the membrane, then these findings suggest that a preliminary value for minimum molecular weight of a phosphorylating unit (phosphorylating particle) in this preparation is of this order of magnitude. Although further studies will be needed to establish the molecular size of phosphorylating particles, it may be noted that 2.1×10^6 compares favorably with the average value of 2.5×10^6 obtained by Green and coworkers (20) for the elementary particles prepared from heart mitochondria.

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