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Assay of Mitochondrial Membrane-Bound Enzyme Activities in the Presence of Triton X-100

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Mitochondrial ATPase and cytochrome c oxidase activities are not severely affected by Triton X-100 concentrations between 0.1 and 2.0 % (w/v). The former is solubilized by the effect of the detergent, while the latter is not. Succinate:cytochrome c reductase and rotenone-sensitive NADH:cytochrome c reductase activities are destroyed even at low detergent concentrations. Succinate:coenzyme Q oxidoreductase is affected by the surfactant in a more complex way, so that selective solubilization of some subunit(s) could be involved.

Detergents have become important tools for the study of cell membrane structure. This is specially true with respect to the so-called reconstitution techniques (6, 10), in which specific integral membrane proteins have to be solubilized, purified and reassembled into a simple system. This process often requires sophisticated techniques involving detergents.

A great variety of soluble amphiphiles has been used for membrane solubilization. Some of them, such as sodium dodecylsulfate, induce drastic conformational changes and loss of biological activity of the proteins, and are therefore not suitable for reconstitution techniques. Bile salts and non-ionic detergents have, in general, milder effects. Triton X-100 (polyoxyethyleneglycol (9, 10) *p-t*-octylphenol; Rohm and Haas) belongs to the latter group. It does not usually function as a protein denaturant, and, among the various detergents whose membrane-solubilizing effect has been studied in detail, Triton X-100 appears to be the most generally applicable for the solubilization of integral membrane proteins (4).

The solubilization of mitochondrial membranes by detergents has not been systematically studied up to now, probably due to their complicated architecture. In a previous paper (2) we have explored the overall patterns of mitochondrial membrane solubilization by Triton X-100. As a further step in the understanding of this complex process, the present paper

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deals with the effects of Triton X-100 on the activity of several membrane-bound mitochondrial enzymes, and the extent to which some of them can be solubilized.

Materials and Methods

Male Wistar rats weighing approximately 200 g were used in all the experiments. Livers were homogenized in 0.25 M sucrose and mitochondria isolated according to the method of HOGEBOOM (5). Isolated mitochondria were subjected to osmotic rupture following the method of PARSONS *et al.* (9). Mitoplasts were obtained using PARSONS «low speed pellet» as starting material, according to SANTIAGO *et al.* (12). Inner mitochondrial membranes were then purified by sucrose density gradient centrifugation (17) and finally washed in a 0.25 M sucrose, 0.02 M HCl-Tris, pH 7.4 buffer, and sedimented at 150,000 xg_{av} for 60 min at 4° in a Beckman L3-50 ultracentrifuge. The purity of the membrane preparations was checked by marker enzymes.

The pellet of purified inner mitochondrial membranes was resuspended in the same sucrose buffer to a final concentration of about 4 mg protein/ml. Aliquots of this suspension were treated with the required amounts of Triton X-100 in order to obtain final detergent concentrations ranging from 0.1 to 2.0 % (w/v) and incubated at 20° for 30 min. When required, the incubated membrane suspensions were centrifuged at 150,000 xg_{av} for 60 min at 4°.

Proteins were determined in the presence of Triton X-100 by the method of LOWRY *et al.* (8) as modified by WANG and SMITH (19). The following enzyme activities were determined according to techniques already published in the literature: F_1-ATPase (11), succinate: cytochrome c reductase (18), cytochrome c oxidase (16), rotenone-sensitive NADH: cytochrome c reductase (3) and succinate: coenzyme Q oxido-reductase (21).

Results

The specific activity of ATPase was measured in mitochondrial inner membrane supensions containing Triton X-100 at concentrations varying between 0.1 and 2.0 % (w/v). The variation in specific activity is shown in figure 1 *a*. After an initial increase, the ATPase specific activity decreases gradually as the Triton X-100 concentration increases, up to 1 %; higher detergent concentrations tend to restore the activity, such that the native value is restored at a Triton X-100 concentration of 2 %.

Cytochrome c oxidase behaviour is similar to ATPase in that small detergent concentrations (0.1 % in the case of the ATPase; up to 0.25 % for cytochrome c oxidase) increase its specific activity in membranes, while higher concentrations tend to decrease this value (fig. 1 *b*). On the other hand, succinate: coenzyme Q oxido-reductase (fig. 1 *c*) appears to be very sensitive to small detergent concentrations, and about 80 % of the specific activity is lost after treatment with 0.1 % Triton X-100. However, higher detergent concentrations lead to restoration of the specific activity, this restoration being complete at 2 % Triton X-100 concentration. In this respect this enzyme is similar to ATPase.

Two other enzyme activities, characteristic of the mitochondrial inner membrane, were investigated, succinate: cytochrome c reductase and rotenone-sensitive NADH: cytochrome c reductase (fig. 1 *d* and *e*). Both activities were practically destroyed by small amounts of detergent, and none of them could be restored by further increases in Triton X-100 concentrations.

All the enzyme activities under consideration are presumably due to integral proteins, embedded in the mitochondrial inner membrane lipid bilayer. The possibility that some of them could be solubilized by the detergent was explored by centrifuging the detergent-treated mem-

brane suspensions and assaying the supernatants for the corresponding enzyme. The various enzyme activities of the supernatants are shown in figure 2 as a function of detergent concentration.

In the case of ATPase (fig. 2 *a*), the enzyme activity of the supernatants nearly follows a linear relationship to the Triton X-100 concentration, and the values are of the same order of magnitude as those of the membrane suspension. Cytochrome c oxidase (fig. 2 *b*) shows a similar, almost linear increase, but the activities in the supernatants are only a small fraction of those in the membrane suspensions.

A different situation can be observed in the case of succinate:coenzyme Q oxido-reductase (fig. 2 *c*), since the specific activities in the supernatants are much higher (up to 14-fold) than in the corresponding detergent-treated membrane suspensions. The activities in the supernatants increase with increasing detergent concentrations up to a 0.5 % Triton X-100 (w/v), and decrease with further additions of detergent.

Finally, only negligible amounts of succinate:cytochrome c reductase and rotenone-sensitive NADH:cytochrome c reductase are recovered in the supernatants (fig. 2 *d* and *e*).

Discussion

The enzyme activities under consideration differ considerably by their complexity and degree of organization within the membrane. The mitochondrial ATPase complex shows several enzyme activities that can be separately assayed (15). The one that has been studied here, namely the ATP phosphohydrolase activity, is

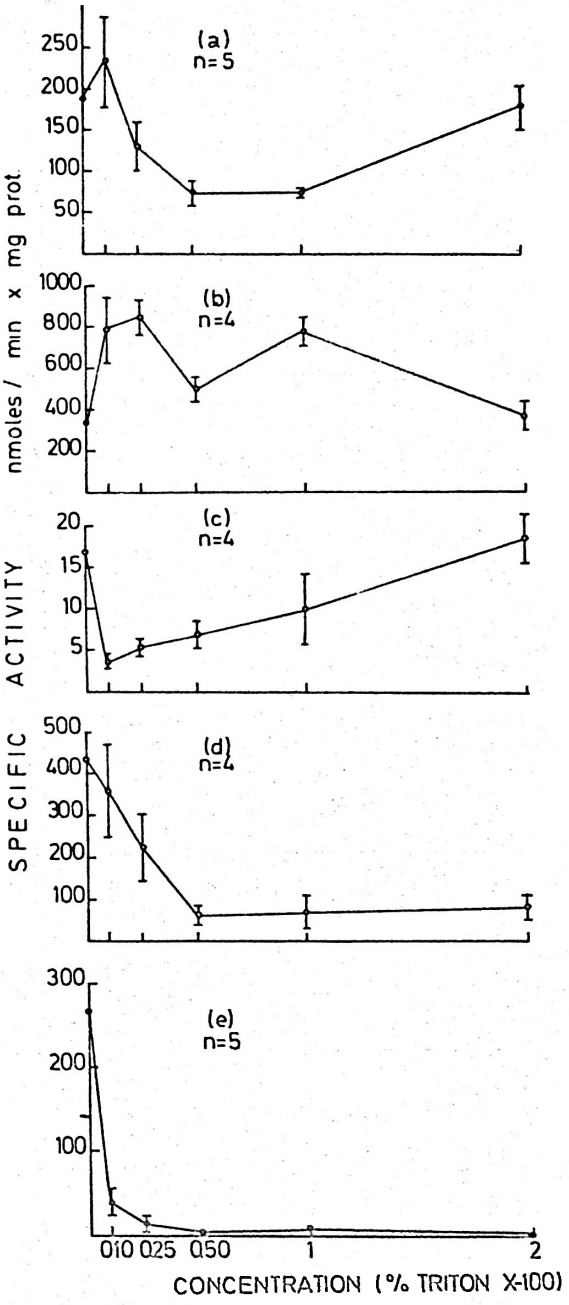

Fig. 1. *Variation of specific enzyme activity in mitochondrial inner-membrane suspensions after incubation with Triton X-100 at various concentrations.*

(*a*) ATPase; (*b*) cytochrome c oxidase; (*c*) succinate:coenzyme Q oxido-reductase; (*d*) succinate:cytochrome c reductase; (*e*) rotenone-sensitive NADH:cytochrome c reductase. Bars denote S.E.M. Number of experiences indicated in each case by *n*.

located in RACKER's F_1 fraction. This component of the ATPase complex can be readily separated from the membrane and assayed in a lipid-free, soluble form (7). Cytochrome c oxidase, on the other hand, cannot be completely delipidated without loss of enzyme activity, and therefore cannot be maintained in solution unless in the presence of detergents. However, it has been reconstituted in vesicles formed with well defined, externally added lipids (20).

The other three enzyme activities that have been studied are more complex in organization, and they have not yet been reconstituted. Succinate:Coenzyme Q oxido-reductase is GREEN's complex II. It contains the flavoprotein succinate dehydrogenase, which is also an iron-sulphur protein, coenzyme Q and citochrome b (14). The succinate:cytochrome c reductase activity requires the concerted operation of the so-called complexes II and III, and, in a similar way, the rotenone-sensitive NADH:citochrome c reductase corresponds to the co-ordinated action of complexes I and III.

The effect of Triton X-100 on these enzyme activities can be explained, at least partially, on the basis of their different complexities. ATPase activity is perhaps the least affected by the detergent. Moreover, the enzyme activities of the supernatants and membrane suspensions are of about the same order of magnitude. In the case of cytochrome c oxidase, that cannot be solubilized, the detergent does not greatly affect the activity of the membrane suspensions, but, when they are centrifuged, only a small fraction remains in the supernatant.

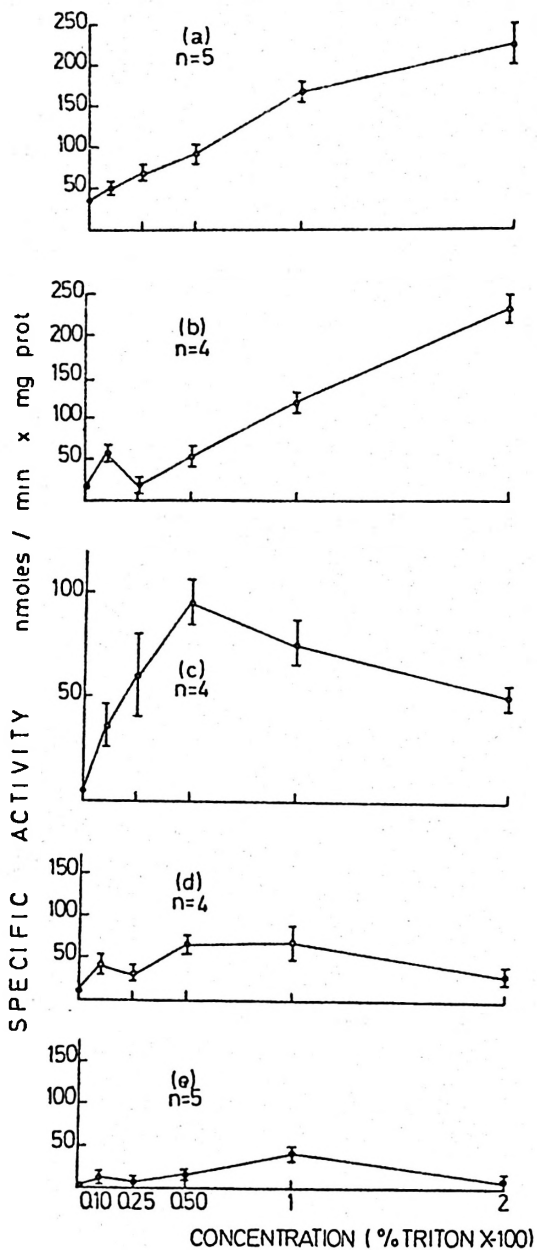

Fig. 2. *Specific activity of the soluble fraction (supernatants) after treatment of mitochondrial inner-membrane suspensions with Triton X-100 at different concentrations.* See text for experimental details. (*a*) ATPase; (*b*) cytochrome c oxidase; (*c*) succinate:coenzyme Q oxido-reductase; (*d*) succinate:cytochrome c reductase; (*e*) rotenone-sensitive NADH:cytochrome c reductase. Note that vertical scales are not necessarily the same as in figure 1. Bars denote S.E.M. Number of experiments is indicated in each case by *n*.

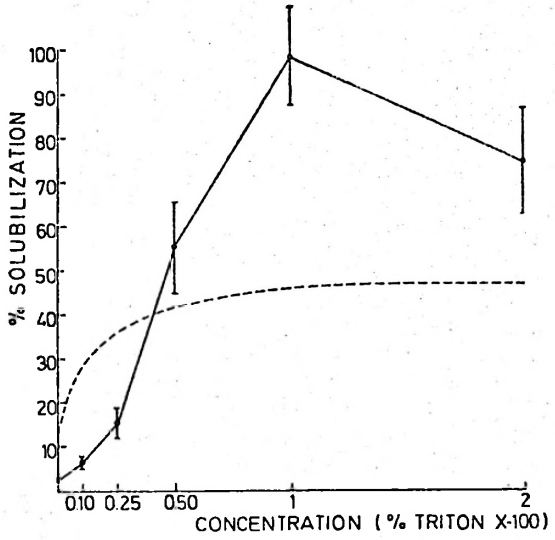

Fig. 3. *A comparison between the solubilization of total protein and the specific solubilization of ATPase activity.*

Solid line: Percent solubilization of ATPase by Triton X-100 as a function of detergent concentration. Dotted line: Percent solubilization of total proteins from mitochondrial inner membranes under the same experimental conditions. Bars denote S.E.M. Number of experiments for ATPase solubilization: 5. Data for total protein are redrawn from (2).

As expected from their very complex nature, the enzyme activities of succinate: cytochrome c reductase and rotenone: sensitive NADH: cytochrome c reductase in membrane suspensions are destroyed by small amounts of Triton X-100, and virtually no activity is recovered in the supernatants. This is probably due to physical disintegration of the different components of the complexes, rather than to enzyme inactivation as such.

Succinate: coenzyme Q oxido-reductase represents a special case. Small concentrations of detergent causes its activity to be lost in membrane suspensions. However, when these suspensions are centrifuged, the supernatants show a specific activity one order of magnitude higher than the corresponding suspensions. This implies an appreciable increase in actual enzyme activity. This phenomenon is difficult to explain: a possible hypothesis would be that, whilst in the membrane suspension, some catalytic subunit (5) of the complex would remain loosely bound to an inhibitory subunit, the latter being firmly linked to the bulk of non-solubilized material. The centrifugation would be able to break the weak bond between the catalytic and the inhibitory subunits. This would imply some sort of specific solubilization of certain mitochondrial inner membrane components by Triton X-100. Such specificity has been suggested by us previously (2).

When the activity of a particular enzyme is not seriously affected by the detergent, and furthermore can be recovered in the supernatant after centrifugation of the detergent-treated membrane suspensions, as is the case of ATPase, a study can be made of the fraction of enzyme solubilized as a function of detergent concentration. The corresponding plot is shown in figure 3, together with the pattern of total protein solubilization. It is remarkable that a considerable proportion of the enzyme is solubilized at Triton concentrations between 0.5 and 1 %; this solubilization is quite specific, since it is not accompanied by an overall protein solubilization. Another important fraction of the enzyme activity is solubilized at detergent concentrations between 0.25 and 0.50 %. It is noteworthy that, in this range of concentrations, most of the cardiolipin of the mitochondrial inner membrane is solubilized (I. G. GURTUBAY, unpublished). This would be in accord with the proposed specific interaction of cardiolipin and mitochondrial ATPase (1, 13).

Bilayer membranes are reconstituted when detergent is removed from solubilized membrane proteins in the presence of phospholipids (6, 10). The present

comunication intends to be a step towards the reconstitution of the mitochondrial membrane-bound enzyme activities solubilized by Triton X-100.

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Resumen

Las actividades enzimáticas ATPasa y citocromo c oxidasa de mitocondrias no resultan muy afectadas por el Triton X-100 a concentraciones de 0,1 a 2,0 (p/v). El detergente es capaz de solubilizar la primera, pero no la segunda de estas actividades. La succinato:citocromo c reductasa y la NADH:citocromo c reductasa sensible a la rotenona se destruyen incluso a concentraciones bajas de detergente. La succinato:coenzima Q oxidorreductasa se afecta de forma compleja, que pudiera implicar la solubilización selectiva de alguna(s) de sus subunidades.

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