INHIBITION BY DEXTRAN SULFATE OF 2,4-DINITROPHENOL-ACTIVATED ADENOSINE TRIPHOSPHATASE IN ISOLATED RAT-LIVER MITOCHONDRIA

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Received April 21, 1970

Small amounts of dextran sulfate, when added to isolated rat-liver mitochondria in a sucrose medium, caused a significant inhibition of the 2,4-dinitrophenol-activated ATPase. This dextran sulfate inhibition was competitive with ATP. The polyanion produced a similar inhibition of the ATPases activated either by other uncouplers or by treatments that induced an energy-requiring cation translocation. The ATPases activated by detergents, osmotic shock, or freezing-and-thawing were free from the polyanion effect. The dextran sulfate effect was largely prevented or reversed by raising the ionic strength or pH of the reaction medium. These findings are compatible with a concept that the polyanion interacts electrostatically with positively-charged loci on the mitochondrial membranes, resulting in a decrease in the ATP penetration across the mitochondrial membranes.

Much effort has been exerted in elucidating the details of the action of the mitochondrial adenosine triphosphatases (ATPases), especially of that induced by 2,4-dinitrophenol (DNP), because of its close association with the energy conservation mechanism in oxidative phosphorylation. When assayed in conventional *in vitro* systems, however, this activity in intact mitochondria depends not only on the energy conservation reactions, but also on the penetration of the substrate to the site of the reaction, most likely the inner aspect of the inner mitochondrial membrane (1). Differences in modes of the inhibitory effect between atractyloside and oligomycin have been explained by assuming that the former inhibits the penetration of ATP across the inner mitochondrial membrane while the latter inhibits the energy conservation process *per se* (2). A large body of evidence obtained from studies with atractyloside has led to the postulate that a special transport carrier for ADP and ATP exists in the inner mitochondrial membrane (1).

During the course of studies on the interactions between polyelectrolytes and mitochondrial membranes, we found that polyanions affected various functions of isolated rat-liver mitochondria (3). Because the polyanion effects were relatively specific to the mitochondrial reactions involving adenine nucleotides (3), elucidation of the precise nature of the polyanion effects in relation to the mitochondrial utilization of the adenine nucleotides appeared of importance.

The present communication describes experiments which demonstrate

that mitochondrial integrity is required for the inhibition by dextran sulfate of the 2,4-dinitrophenol-activated adenosine triphosphatase (DNP-activated ATPase). The kinetics of the inhibition, the electrostatic nature of the interaction between the dextran sulfate and the mitochondria, and the probable site of the polyanion action, suggest the presence in the outer and/or inner mitochondrial membranes of a positively charged portions that facilitate the ATP penetration.

Methods. Rat-liver mitochondria were prepared by the procedure of Rasmussen and Ogata (4).

Dextran sulfates were a generous gift of Dr. T. Numasawa (Research Lab. of Meito Sangyo, Nagoya). Two different kinds of dextran sulfates (the one with the approximate molecular weight of 5×10^3, and the other of 3.3×10^5), containing approximately 18% of sulphur in weight, were supplied as sodium salts. They were dissolved in water and neutralized to pH 7.5 with Tris. The other reagents were of the highest purity available from various commercial sources.

The ATPase activity was measured according to the method of Amons et al (5) with minor modifications. The basic reaction mixture contained 250 mM Sucrose, 6 mM ATP (disodium salt brought to pH 7.5 with Tris), 0.5 mM EDTA (free acid brought to pH 7.5 with Tris), and 0.1 mM DNP. The other additions are described in the legends to the tables and figures. The reaction was started at 25° C by adding the mitochondria to 3 ml of the reaction medium to a final concentration of about 1 mg protein per ml. At appropriate times it was stopped with trichloroacetic acid to 5%. Inorganic phosphate was measured in duplicate by the method of Sumner (6). Preliminary experiments showed that the presence of the amount of the polyanion employed in the present study did not interfere with the inorganic phosphate measurement. Mitochondrial protein was measured with the biuret reaction (7).

Results. As shown in Figure 1, a relatively small concentration (1.2 mg per ml) of the dextran sulfate of low molecular weight produced a significant inhibition of the DNP-activated ATPase. The inhibition was apparent at the initial reading (4 minutes) and continued throughout the observation (20 minutes). In the following experiments, therefore, the inhibition was evaluated from the results of a 16-minute or 20-minute incubation. Similar inhibition was produced with dextran sulfate of high molecular weight.

The extent of the inhibition depended not only on the concentration of the dextran sulfate, but also on that of ATP. The inhibition was

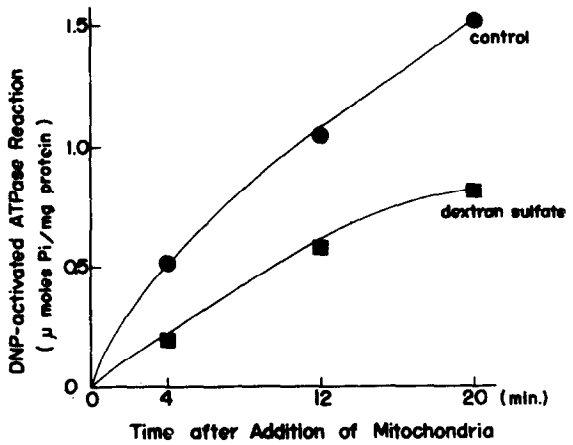

Figure 1. Time Course of the DNP-activated ATPase Reaction.
The mitochondria (3.34 mg protein) were added to 3 ml of the basic medium described in the text.
In one set of experiments, dextran sulfate (m.w. of 5×10^3) was added to the final concentral of 1.2 mg/ml.

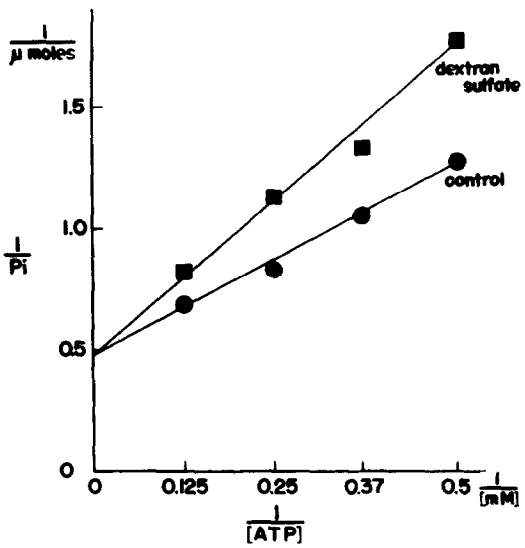

Figure 2. Effect of Dextran Sulfate on the Lineweaver-Burke Plot for ATP on the DNP-activated ATPase Reaction.
The reaction medium contained 250 mM sucrose, 0.1 mM EDTA, 0.1 mM DNP, 1.57 mg of mitochondrial protein per ml, and either 2, 2.7, 4, or 8 mM Na_2-ATP (brought to pH 7.5 with Tris). Adequate amount of NaCl was also added to attain the final Na^+ concentration of 18 mM in every reaction medium. In one set of the experiments, the media further contained 1 mg per ml of the dextran sulfate of low molecular weight. The reciprocal of the amount of inorganic phosphate released during the 16-minute incubation is plotted on the ordinate.

apparently competitive between the dextran sulfate and ATP on the active site(s) of the DNP-activated ATPase (Figure 2). Dextran sulfate caused a slight stimulation of the basal mitochondrial ATPase in the medium lacking DNP : 0.20 ± 0.06 in the absence, and 0.43 ± 0.08 (mean ± S.E., n= 4) μ moles Pi/16 min/mg protein, in the presence of 1.0 mg dextran sulfate/mg mitochondrial protein.

The ATPases activated in the intact mitochondria with the other classical uncouplers (Dicoumarol, and carbonyl cyanide m-chlorophenyl hydrazone), or those activated by the energy-requiring ion translocation (Ca^{++}translocation, and gramicidin-induced alkali metal translocation) were also inhibited by dextran sulfate (Table 1). On the other hand, the ATPases of the mitochondria activated by treatments which deteriorated the membrane integrity were not influenced by this anion (Table 1).

Table 1

Effect of Dextran Sulfate on Mitochondrial ATPases Activated by Various Treatments

	Treatment	ATPases (μ moles of Pi/16 min/ mg protein)	
		without Dextran Sulfate	with Dextran Sulfate
A	Cl-CCP (5×10^{-6} M)	0.364	0.242
B	Dicoumarol (1×10^{-5} M)	0.845	0.469
C	$CaCl_2$ (1×10^{-3} M)	1.320	0.795
D	Gramicidin (0.3 μg/ml)	0.989	0.917*
E	Triton-X (1 mg/ml)	1.770	2.060
F	osmotic shock	0.899	1.004
G	Freezing and thawing	0.717	0.754
H	deoxycholate (1 mg/ml)	1.230	1.536

Dinitrophenol was excluded from the media and dextran sulfate of low molecular weight was added to 1 mg per ml when indicated. EDTA was omitted in experiment C. $MgCl_2$ was added to 10 mM in experiments E, F, G, and H (this concentration of $MgCl_2$ did not prevent the dextran sulfate effect on the DNP-activated ATPase). In experiment D, 11 mM LiCl was included in the basic medium. In experiment F, mitochondria were preincubated in 1 mM Tris phosphate (pH 6.9) solution for 15 min at 0°C. In experiment G, the mitochondria as suspended in 0.37 M sucrose were once frozen at -20°C and then thawed to the room temperature prior to the use.
* This difference, thought small, was constantly observed in several repeated experiments.

Table 2
Influence of Various Chloride Salts on the Dextran Sulfate Effect in the DNP-activated ATPase.

	Chloride Salt	ATPase (μ moles of Pi/20 min/mg protein)	
		without Dextran Sulfate	with Dextran Sulfate (1.2 mg/ml)
A	KCl	4.70	5.03
B	RbCl	4.82	4.96
C	NaCl	5.01	4.70
D	NH_4Cl	4.90	3.31
E	LiCl	4.92	3.18

Instead of 250 mM sucrose, 50 mM chloride salt and 80 mM sucrose were employed in the basic medium. In the absence of chloride salt, this concentration of dextran sulfate (1.2 mg/ml) caused the inhibition of the DNP-activated ATPase by approximately 45%.

In view of the strong electrostatic nature of the dextran sulfate and of the likely attribute as polyelectrolytes of the mitochondrial membranes ——possible targets of the dextran sulfate action——, physicochemical characteristics of the interactions between these two kinds of polyelectrolytes were examined. As shown in Table 2, dextran sulfate

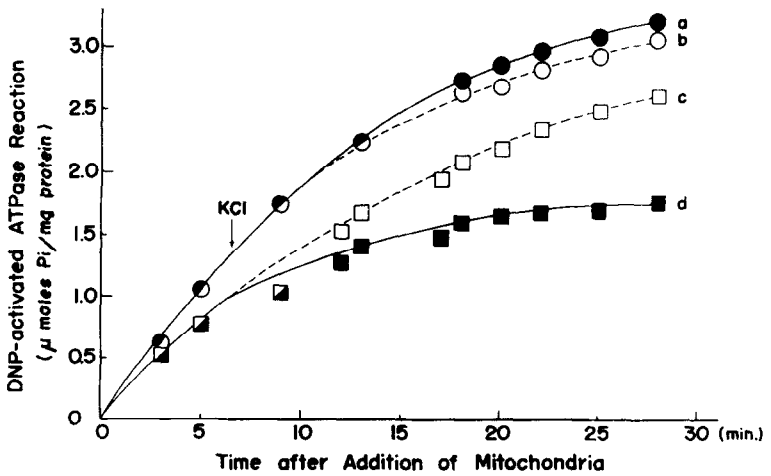

Figure 3. Effects of KCl Addition on the DNP-induced ATPase in the Presence of Absence of Dextran Sulfate.
In experiments c, and d, the basic medium was supplemented with dextran sulfate (1 mg per ml). At the time indicated by the arrow an appropriate amount of 3 M KCl was added to the media of experiments b, and c, to 50 mM. The final mitochondrial concentration was 1.31 mg protein per ml.

exerted less remarkable inhibition of the DNP-activated ATPase in the medium, the ionic strength of which were raised by replacing sucrose with iso-osmolar amount of a variety of chloride salts of alkali metals or of ammonium. The reversal of the dextran sulfate inhibition was also demonstrated in the experiment (Figure 3), where a later addition of KCl to the sucrose medium caused a significant release of the inhibition by dextran sulfate of the DNP-activated ATPase.

The inhibitory effect of dextran sulfate was also found pH dependent. More marked inhibition was produced when H^+ concentration of the reaction medium was higher (Addition of 0.3 mg of dextran sulfate per mg mitochondrial protein led to the inhibition by 73% at pH 6.9 and to that by 46% at pH 7.5).

Discussion. The inhibition of the DNP-activated ATPase by dextran sulfate was unique in that it required the structural integrity of the membranes. Also the kinetics of inhibition were competitive with ATP. These characteristics are similar to those of atractyloside but dissimilar to those of oligomycin (2). The inhibition was exerted not only on the activity of the DNP-activated ATPase, but on that of any mitochondrial ATPases in which supply of ATP through a membrane transport system was necessary. This suggests a selective inhibition of ATP permeation across the membrane. It is possible, however, that the dextran sulfate effect was a consequence of the action on the structures outside of the inner mitochondrial membrane, because similar inhibition was produced with dextran sulfate apparently too large (molecular weight : 3.3×10^5) to penetrate the outer mitochondrial membrane.

The influence of alterations in the physico-chemical properties of the reaction medium on the dextran sulfate effect was also striking. The inhibition was either prevented or reversed by increasing ionic strength or pH of the reaction medium. This was in line with the previous observations that the dextran sulfate effects on mitochondrial respiration were prevented by the same treatments (8). These phenomena might be explained if one assumes that dextran sulfate reacted as a potent polyanion with cationic sites on the mitochondrial membranes, and that this cationic site was also a part of the mechanism involved in ATP translocation across this membrane.

We wish to thank Dr. Yawara Yoshitoshi for his continuous interest and advice during this investigation, and Dr. Howard Rasmussen for his advice and criticism in the preparation of the manuscript.

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