ENERGY TRANSFER FACTOR A.D (ATP SYNTHETASE) AS A COMPLEX Pi-ATP EXCHANGE ENZYME AND ITS STIMULATION BY PHOSPHOLIPIDS.[1]

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Received February 10, 1972

SUMMARY

The soluble energy transfer factor A.D (ATP synthetase), which displays oligomycin-sensitive Pi-ATP exchange, was compared with insoluble oligomycin-sensitive ATPase (OS-ATPase) by sodium dodecyl sulfate gel electrophoresis. Both entities have most bands in common, including the dicyclohexylcarbodiimide binding protein, but Factor A.D has two additional bands, which may be related to the Pi-ATP exchange capability lacking in OS-ATPase. Pi-ATP exchange activity of Factor A.D was inhibited by dicyclohexylcarbodiimide, but stimulated 11-fold by treatment with phospholipids.

Recently it was shown that a soluble energy transfer factor A.D, as isolated by Sani and coworkers (1,2) from bovine heart mitochondria, exerts a Mg^{2+} dependent Pi-ATP[2] exchange activity which is inhibited by oligomycin and p-chloromercuriphenylsulfonate (3,4). Hence, Sanadi et al. (3) propose the term "ATP synthetase" for Factor A.D.

Another oligomycin- and mercurial-sensitive factor isolated from bovine heart mitochondria is the oligomycin-sensitive ATPase (OS-ATPase), described by Tzagoloff and coworkers (5-7). However, this factor lacks the Pi-ATP exchange activity of Factor A.D. Therefore, it seemed of interest to compare their behaviour in SDS-gel electrophoresis.

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[1] This work was supported in part by the Netherlands Organization for Basic Research (ZWO).

[2] Abbreviations used are: Pi for inorganic phosphate, DCCD for N,N'-dicyclohexylcarbodiimide, OS-ATPase for oligomycin-sensitive ATPase complex isolated from bovine heart mitochondria by the procedure of Tzagoloff et al.(5-7), SDS for sodium dodecyl sulfate, and DTT for dithiothreitol.

Recently, we established that OS-ATPase is inhibited by dicyclohexylcarbodiimide (DCCD), and we are able to identify by means of SDS-gel electrophoresis a 13,000-14,000 molecular weight protein as the binding site for this inhibitor (8). Hence, we also investigated the effect of DCCD on Factor A.D.

Furthermore, Sanadi et al.(4) reported that the rate of Pi-ATP exchange by Factor A.D, which was quite low and variable, could be enhanced by the addition of submitochondrial particles depleted in this factor. Considering the membranous nature of these particles, we decided to test the effect of addition of phospholipids on the Pi-ATP exchange of this factor.

MATERIALS AND METHODS

Energy transfer factor A.D and OS-ATPase were prepared as previously described (1,9,5-7), except that 0.1 mM DTT was present in the preparation of Factor A.D from the pH 5.6 fractionation on and its final step (150 mM phosphate DEAE-cellulose elute) was made 0.5 M in sucrose and 1 mM in DTT for protection of its activity at -70° (10). Both preparations were stored at this temperature before use. Phospholipid treatment was carried out on Factor A.D essentially as described by Kagawa and Racker (10) and this preparation was assayed for Pi-ATP exchange activity immediately afterward. Pi-ATP exchange activity was measured according to the first procedure of Conover et al.(11) in the presence of 1 mM DTT. The protein containing aliquot of 0.2 ml contained 25 mM Tris-sulfate, pH 7.5, + 1 mM DTT (4) and no Mg^{2+} unless otherwise indicated. Specific radioactivity was 10^5 cpm per μmole phosphate. Further conditions of the assay (protein concentration, temperature, period) are specified under Results. The reaction was terminated by addition of 0.1 ml 50% trichloroacetic acid and phosphate extraction carried out on 0.1 ml of the deproteinized supernatant. Radioactivity was determined in 1 ml of the water phase in a Nuclear-Chicago Model 1043 gas flow counter, and the cpm value corrected for a blank containing no Factor A.D in the assay. All values for Pi-ATP exchange activity represent means of simul-

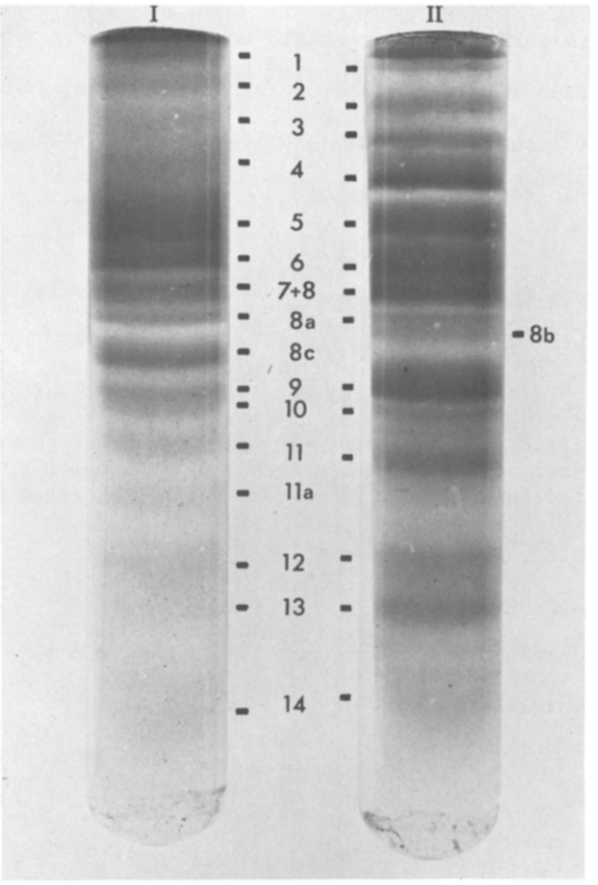

Fig. 2. Protein patterns of Factor A.D and OS-ATPase following SDS-gel electrophoresis. Gel I represents Factor A.D (200 µg) and gel II OS-ATPase (400 µg). Both preparations were run simultaneously. Individual protein bands are indicated by numbers in the same order as in a previous report (8). Bands 7 and 8, which were separated in previous runs (8), coincide in the present one.

OS-ATPase (gel II). Both preparations have many bands in common (band 1-8a, 9-11, and 12-14). They differ in that Factor A.D contains in addition bands 8c and 11a which are not observed in OS-ATPase, but lacks band 8b present in OS-ATPase. The most prominent additional band in Factor A.D is 8c (gel I of Fig. 2), which according to its electrophoretic mobility (0.441-0.457) relative to tracking dye travel (bromophenol blue; 16) may be the 33,500 mo-

taneous duplicate determinations. Protein was determined in Factor A.D according to Lowry et al.(12), in phospholipid-treated Factor A.D according to Hess and Lewin (Method C; 13), and in OS-ATPase according to Jacobs et al. (14) using bovine serum albumin as standard. SDS-gel electrophoresis was performed as previously described (8).

RESULTS

Energy transfer factor A.D showed Pi-ATP exchange activities at pH 7.4 of 3.4 nmoles x min^{-1} x mg $protein^{-1}$ at 30° and 4.6 nmoles x min^{-1} x mg $protein^{-1}$ at 37° (0.2 mg protein per assay; assay period 1½ hr). These values lie in the range reported by Fisher et al.(4). The exchange reaction is inhibited by DCCD as shown in Fig. 1, maximal inhibition (83-85%) being obtained at concentrations of the inhibitor ranging between 0.3 and 0.5 µg (1.4-2.4 nmoles) of DCCD per mg protein. A comparable concentration of DCCD (2 nmoles x mg $protein^{-1}$) abolished Pi-ATP exchange in bovine heart submitochondrial particles (15).

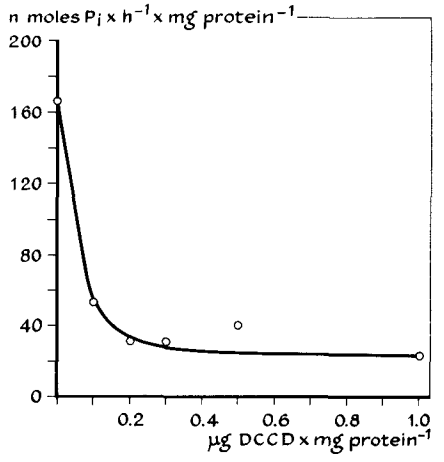

Fig. 1. Inhibition of Pi-ATP exchange of Factor A.D by DCCD. Factor A.D (200 µg) was preincubated for 2 min at 36° in the presence of DCCD (0.1-1.0 µg x mg $protein^{-1}$). The reaction proceeded for 2 hrs at 36°. DCCD was dissolved in ethanol, of which maximally 5 µl was added. A blank containing the same volume of ethanol but no DCCD showed no inhibition.

Fig. 2 shows the complex protein pattern of Factor A.D following SDS-gel electrophoresis (gel I) in comparison with the pattern obtained for

lecular weight component described by Sanadi et al.(9). Two other prominent bands (5 and 6 in gel I and II) probably are the Factor A and D band of molecular weight 57,000 and 53,000, respectively (2,4,9). Strikingly both preparations contain band 12 which has been identified as the DCCD binding protein (8).

TABLE I

Influence of Phospholipid Treatment on Pi-ATP Exchange Activity of Factor A.D

In experiment 1 75 µg Factor A.D was used; in experiment 3 the same amount of protein was preincubated in the presence of 2.5 µmoles $MgSO_4$; in experiment 2 phospholipid-treated Factor A.D (66 µg protein) was used; in experiment 4 phospholipid-treated Factor A.D (same amount of protein as in experiment 2) was preincubated in the presence of 2.5 µmoles $MgSO_4$. All samples were preincubated for 2 min at 38° and the Pi-ATP exchange run at the same temperature for 1 hr.

Exp.	Sample	Preincubation condition	Pi-ATP exchange (nmoles x min^{-1} x mg $protein^{-1}$)
1	Factor A.D	-	2.1
2	Phospholipid-treated Factor A.D	-	23.8
3	Factor A.D	+ $MgSO_4$	4.9
4	Phospholipid-treated Factor A.D	+ $MgSO_4$	21.8

Finally the influence of phospholipid treatment on the soluble ATP synthetase was tested. As shown in Table I phospholipid treatment of Factor A.D according to the method of Kagawa and Racker (10) enhanced its specific Pi-ATP exchange activity elevenfold. Presence of Mg^{2+} during preincubation of Factor A.D more than doubled specific Pi-ATP exchange activity (experiment nr. 3 in Table I) but had no influence on phospholipid-treated Factor A.D (experiment nr. 4 in Table I).

DISCUSSION

Several of our present findings may find their origin in the similar pathways followed by the OS-ATPase reaction and the Pi-ATP exchange process catalyzed by Factor A.D. Both processes are sensitive to oligomycin and

mercurials (4,5), and also to DCCD as shown in a previous (8) and the present paper. Both processes are thought to proceed via formation of an energy-rich phosphorylated intermediate symbolized by X~P (4,17,18), the latter reacting with another hypothetical carrier C=I under formation of X~C (4) or X~I (17,18) plus Pi. In the exchange process the above reactions proceed to equilibrium, whereas in the ATPase process energy is dissipated via hydrolysis of X~P (19) or X~I (20). Inhibition by oligomycin or DCCD is at the step involving reaction of X~P with I or H_2O (17-19,21).

Recently, we established the site of interaction of DCCD with OS-ATPase as a protein with molecular weight 13,000-14,000 in SDS-gel electrophoresis (8). Assuming identical sites of interaction of DCCD in the OS-ATPase process and the Pi-ATP exchange reaction, the DCCD binding protein should also be present in Factor A.D. A protein with the same electrophoretic mobility as DCCD binding protein in OS-ATPase (band 12 in gel II of Fig. 2) has indeed been observed by us in Factor A.D (gel I in Fig. 2). Sanadi et al.(9) did not observe this protein band in their preparations of Factor A.D, although they analyzed the factor at the same stage of purification via the same method of SDS-gel electrophoresis. This discrepancy may simply be due to the application of a smaller amount of protein to the gel by them (12 µg) than by us (200 µg). This would tend to make minor components undetectable in their case, explaining also the smaller number of protein bands (3 bands) observed by them compared to the number (16 bands) observed by us.

The striking similarity in the SDS-gel electrophoretic profile for Factor A.D and OS-ATPase is not surprising in view of the similarity of chemical pathways displayed by both factors. However, Factor A.D does show two protein bands (8c and 11a in gel I of Fig. 2) which are not observed in OS-ATPase (gel II in Fig. 2). A possible explanation for these missing bands may be derived from the following observations of Kagawa and Racker (10). They found that phospholipids could induce the latent Pi-ATP exchange activity

in depleted submitochondrial particles, but OS-ATPase did not show a phospholipid-induceable, latent Pi-ATP exchange activity. Most likely, this means that the energy transfer factor I is absent from OS-ATPase and present in the depleted submitochondrial particles and Factor A.D. Hence, bands 8c and/or 11a may represent factor I.

The enhancement of the Pi-ATP exchange activity of Factor A.D by treatment with phospholipids to about the same level as obtained by Kagawa and Racker (10) during phospholipid treatment of the depleted submitochondrial particles indicates a general requirement of phospholipids for this exchange activity. This in turn supports Sanadi's hypothesis (4) that interaction of Factor A.D with a membrane enhances its exchange activity. Further reconstitution of the depleted submitochondrial particles with their complementary energy transfer factors gives a further increase of the specific Pi-ATP exchange activity above the level obtained for Factor A.D in the presence of phospholipids (10). This supports Sanadi's second hypothesis (4) that Factor A.D in its present form lacks full supplementation with energy transfer factors necessary for maximal Pi-ATP exchange activity.

Our observation that the exchange activity of Factor A.D is stimulated by preincubation in the presence of Mg^{2+} may indicate a stabilization of the enzyme system by Mg^{2+}. We did not observe this effect for phospholipid-treated Factor A.D, indicating that here interaction with phospholipids gave sufficient stabilization by itself.

ACKNOWLEDGEMENT

The author wishes to thank Dr. D.R. Sanadi for providing him with preprints concerning his work on Factor A.D and for the donation of soybean phospholipids. Prof.Dr. S.L. Bonting is gratefully acknowledged for his help in the preparation of this manuscript.

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