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TIGHTLY-BOUND ATP AND ADP IN RECONSTITUTED SUBMITOCHONDRIAL PARTICLES

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SUMMARY: Soluble beef-heart mitochondrial ATPase (F_1) which was depleted of tightly bound nucleotide was reconstituted with depleted submitochondrial particles and oligomycin-sensitivity conferring protein. A correlation was noted between the recovery of energy-transduction capability and the reloading of tightly bound nucleotide. Reconstituted membrane-bound F_1 contained both ATP and ADP tightly bound; the total (ATP and ADP) was tentatively calculated to be around 3.6 moles per mole membrane-bound F_1.

INTRODUCTION:

We have recently found that trypsin-treatment and gel filtration at high ionic strength deplete soluble mitochondrial ATPase (F_1) of tightly bound nucleotide (ATP and ADP) and diminish the energy-coupling of the F_1 as measured in a reconstituted system (1). Native F_1 prepared in our laboratory contained zero ATP and around 1.8 moles of tightly bound ADP per mole of F_1 (1). It was of interest therefore to ascertain what the nucleotide content of reconstituted F_1 was, and to find out whether the loss of energy coupling capability could be reversed under suitable conditions.

MATERIALS AND METHODS:

Reconstitution of beef-heart F_1 with ASU[1] particles and OSCP[1] from beef-heart was done as previously described (1). Trypsin-treatment, gel-

[1] Abbreviations: ASU-particles are depleted submitochondrial particles prepared from beef-heart mitochondria by sonication at high pH in the presence of NH_3 and EDTA, gel filtration through Sephadex G-50 and extraction with 2M urea at 0°; OSCP, oligomycin-sensitivity conferring protein.

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filtration in high ionic strength sulfate-containing buffer, measurement of energy-coupling by following reversed electron-transfer from succinate to NAD driven by ATP, and analysis of tightly bound ATP and ADP by fluorometric enzyme assays have all been described (1). In the latter procedure, where reconstituted ASU particles were being analysed, phenol red was used to allow titration of the acid extract to constant end-point and 50 mM potassium phosphate-6N KOH was used for neutralisation to prevent adsorption of nucleotide to the $KClO_4$ precipitate (2). Other details of experiments are described under "Results and Discussion".

RESULTS AND DISCUSSION:

Reconstitution of trypsin-treated F_1 and native F_1 with OSCP and ASU-particles is shown in Table 1. It is seen that the reconstituted membrane-bound native enzyme contained approximately equal amounts of tightly bound ATP and ADP. If the assumption is made that on rebinding the native F_1 neither loses nor gains tightly bound ADP then the total amount of ADP tightly bound would be $\sim$ 1.8 moles/mole (as in the native soluble enzyme) and the amount of ATP tightly bound in the reconstituted enzyme would be very similar, for a total of around 3.6 moles of tightly bound nucleotide/mole F_1. Similar numbers were obtained by Harris, Radda and Slater (3) who studied the tightly bound nucleotide content of beef-heart sub-mitochondrial particles. It is also seen that reconstituted trypsin-F_1 does not have energy coupling ability, nor does it "reload" tightly bound ATP or ADP.

Table II shows the reconstitution of "depleted F_1" with ASU-particles and OSCP. "Depleted F_1" is F_1 treated by gel filtration in 50 mM Tris SO_4, 1 mM EDTA, 60 mM K_2SO_4, pH 8.0 ("sulfate buffer") which is depleted of tightly bound nucleotide (1). Several points emerge from consideration of the data. First it should be noted that the S.D. for our fluorometric assays of nucleotides is around 0.3 nmole nucleotide per mg reconstituted particle and

TABLE 1

Reconstitution of Trypsin-treated F_1 with ASU-Particles and OSCP.[a]

F_1 Sample	Tightly bound Nucleotide (nmole/mg reconstituted particle)[b]			Energy transduction (nmoles NADH formed/ min/mg)
	ATP	ADP	(ATP + ADP)	
Native F_1	1.26 ± 0.25(11)	1.31 ± 0.31(11)	2.57	24.8 ± 7.3(11)
Trypsin-F_1[c]	0.34 ± 0.23(5)	Zero(5)	0.34	Zero(5)

a Reconstitution was performed essentially as described (1). 6 mg ASU-particles were reconstituted with 0.4 mg OSCP and 2.4 mg F_1 in 6.0 ml buffer containing 60 mM Tris SO_4, 8 mM K^+ succinate, 6 mM $MgCl_2$ and 1 mM ATP pH 7.5.

b Reconstituted particles were washed 4 times by centrifugation and resuspension in 0.25M sucrose -10 mM Tris acetate - 2 mM EDTA pH 7.5. The results are corrected for small amounts of residual ATP and ADP in ASU plus OSCP alone. Results are expressed as mean ± S.D. with number of observations in brackets.

c F_1 was treated for 5 min. at 25° with 10 μg trypsin/mg F_1 as described in (1) This enzyme had enhanced ATPase activity, was stable in aqueous buffer and retained membrane-binding capacity equal to the native enzyme.

therefore values in this range are not significant. Thus some totals are omitted in the table. Comparing lines 1 and 2 of Table II it is seen that native F_1 reconstituted in the presence or absence of ATP regains coupling activity and that when ATP is present during reconstitution, tightly bound ATP is taken up. The time-course of this ATP uptake could not be established since no rapid "stop" procedure was available.

"Depleted F_1" redissolved in the sulfate buffer after gel filtration and ammonium sulfate precipitation (1) and reconstituted either in the pres-

TABLE II

Reconstitution of "depleted F_1" with ASU-particles and OSCP.[a]

F_1 Sample	Tightly bound Nucleotide (nmole/mg reconstituted particle)[b]			Energy transduction (nmoles NADH formed/ min/mg)
	ATP	ADP	(ATP + ADP)	
Native F_1	0.20 ± 0.24(4)	1.15 ± 0.3(4)	1.35	21.8 ± 8.1
Native F_1 + 1mM ATP	1.26 ± 0.25(11)	1.31 ± 0.31(11)	2.57	24.8 ± 7.3(11)
"Depleted F_1" (in sulfate buffer)	0.33(3)	0.17(3)	not significant	Zero
"Depleted F_1" (in sulfate buffer plus 1mM ATP)	0.62 ± 0.33(7)	0.23 ± 0.28(7)	0.85	1.47 ± 2.33(7)
"Depleted F_1" (in 50mM Tris SO_4-1mM EDTA plus 1mM ATP)	1.19 ± 0.41(8)	1.03 ± 0.48(8)	2.22	14.3 ± 4.85(8)
"Depleted F_1" (in 50 mM Tris SO_4-1 mM EDTA)	0.21 ± 0.27(5)[c] 1.04 ± 0.38(4)[d]	0.36 ± 0.38(5)[c] 1.30 ± 0.21(4)[d]	not significant 2.34	16.1 ± 3.77(5)

[a] Reconstitution was performed as in Table 1 except that the ATP was optional.

[b] Results are mean ± S.D. with number of observations in brackets. See Table 1 for experimental details. Results are corrected for small amounts of residual ATP and ADP in ASU particles plus OSCP.

[c] Nucleotide assays performed on reconstituted particles prior to coupling assay.

[d] Nucleotide assays performed on reconstituted particles after addition of 2 mM ATP which starts the assay.

ence or absence of ATP had low or zero energy-transduction activity and apparently "reloaded" very little or no tightly bound nucleotide (lines 3 and 4, Table II). "Depleted F_1" redissolved in 50 mM-Tris SO_4 - 1 mM EDTA pH 8.0 and reconstituted in the presence of ATP (line 5, Table II) did show energy-transduction activity and did "reload" tightly bound nucleotide. "Depleted F_1" redissolved in 50 mM Tris SO_4 - 1 mM EDTA in the absence of ATP also regained coupling activity (line 6). In this situation tightly bound nucleotide could not be taken up during the reconstitution (line 6) but it seemed that ATP and ADP were "reloaded" on addition of ATP which initiated the coupling assay (line 7). As noted above, the time course of this uptake was difficult to follow since the reconstituted particles have to be spun down for nucleotide analyses.

Our main conclusion from Table II is that the recovery of energy-

transduction capability in the particles is apparently correlated with the ability of the membrane-bound F_1 to take up tightly bound ATP and ADP. Also the data show that this system may provide a suitable vehicle for studying the tight nucleotide binding sites e.g. by reloading of analogues of ATP and/or ADP.

The behavior of the "depleted F_1" is not simple to interpret. We presume that redissolving in the sulfate buffer prevents some conformational change in the "depleted F_1" which allows recovery of energy-coupling and reloading of nucleotides in the membrane-bound enzyme (binding to ASU particles is not inhibited, ref. 1). In this regard two observations are perhaps relevant. Leimgruber (4) has shown that "depleted F_1" dissolved in the sulfate buffer has a sedimentation coefficient of 9.5 (S_{20_w}), significantly lower than that of native F_1 ($\sim$ 11.9 S). This conformation change may be reversed when the enzyme is dissolved in 50 mM Tris SO_4 - 1 mM EDTA. Secondly, if very low concentrations of oligomycin (0.1 µg per mg ASU particle[2]) are included in the reconstitution system used, substantial energy-transduction capability is seen even when "depleted F_1" dissolved in sulfate buffer is rebound to the ASU particles plus OSCP. These observations suggest that both the ionic strength of the medium in which the F_1 is dissolved and the membrane potential or "state of energisation" of the particles in the reconstitution system may influence the behavior of the system in regard to reloading of tightly bound nucleotide and energy-transduction.

Finally we wish to add that we have recently found that four-times washed preparations of both an oligomycin-sensitive ATPase (5) and sarcoplasmic reticulum (6) (the latter kindly given to us by Drs. Terry Scott and Adil Shamoo) were also found to contain tightly bound nucleotide.

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[2] We have noted that addition of small amounts of oligomycin greatly improves the efficiency and reproducibility of this reconstitution system and that the reconstitution of energy coupling is still totally dependent on added F_1 and OSCP.

The former preparation contained insignificant amounts of ATP and 1.18 nmole ADP/mg protein; and the latter (R_1W of ref. 6) contained 2.71 nmole ATP and 2.59 nmole ADP per mg protein. These data further confirm the widespread occurrence of tightly bound nucleotide in membrane-bound ATPase preparations.

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