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Characteristics of the non-exchangeable nucleotide binding sites of mitochondrial F_1 revealed by dissociation and reconstitution with 2-azido-ATP

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The dissociation of mitochondrial F_1-ATPase with 3 M LiCl at 0°C, followed by reconstitution, has been analysed. FPLC over a gel filtration column in the dissociation buffer revealed the presence of two protein moieties, an $\alpha_3\gamma\delta\epsilon$ complex and single β-subunits. When the dissociation and chromatography is performed at pH 6.2, the former protein moiety still contains some adenine nucleotides. Reconstitution of the dissociated complex is not possible any more after FPLC, probably due to the loss of residual adenine nucleotides. After a single column centrifugation step one nucleotide per F_1 still remains bound. For reconstitution, additional ATP, or a suitable analog, is required. 2-Azido-ATP, but not 8-azido-ATP or ITP, can replace ATP during the reconstitution. F_1, reconstituted in the presence of 2-azido-ATP, contains three tightly bound nucleotides, similar to freshly isolated F_1, of which in this case one is an adenine nucleotide and two are azido-adenine nucleotides. One of the latter can be rapidly exchanged and is bound to a catalytic site. Covalent binding (at a β-subunit) of the other tightly bound 2-azido-ATP by ultraviolet illumination does not result in inhibition of the enzyme. Digestion of F_1 with trypsin, followed by HPLC, showed that the label is not bound to the fragment containing Tyr-368, nor to the fragment containing Tyr-345. This result was confirmed by CNBr digestion, followed by SDS-urea PAGE. We conclude that during dissociation of F_1 one tightly bound nucleotide (ADP) remains bound at an α/β interface site and that for reconstitution binding of ATP to a (non-catalytic) β-site is required. The conformation of this site differs from that of the two catalytic β-sites.

Introduction

To study the mechanism of the mitochondrial ATP-synthase the water-soluble catalytic moiety of the enzyme (MF_1), catalyzing ATP hydrolysis, can be isolated and investigated separately. The enzyme consists of five different subunits with the stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$ [1,2]. Each molecule can bind six adenine nucleotides [3,4]. To obtain insight into the mechanism of the process of ATP hydrolysis (and ATP synthesis by the enzyme in its membrane-bound form), we have focused our attention on the role and properties of the six nucleotide binding sites. Both we and others have used chemical modification, especially photo-affinity labeling, to study the properties of these sites (for review, see Ref. 5). To avoid confusion in the terminology, we will divide the sites into β-sites and α/β- or 'interface' sites when the location on the enzyme is considered, and 'catalytic' or 'non-catalytic' sites when the function is considered. The differentiation between β- and interface sites is based on the labeling with 8-azido-adenine nucleotides [6]. In practice it means that β-sites are not automatically catalytic sites, although it may be considered as an established fact that the catalytic sites are β-sites [7].

One of the techniques we have used in the past to study the involvement of the nucleotide binding sites in catalysis, regulation or maintenance of structure, was dissociation-reconstitution [8] in analogy with the studies of Wang [9,10]. Our studies with Nbf-treated F_1 showed, in agreement with our earlier conclusions from labeling experiments [6,11], that only two β-sites are involved in catalysis and that the third β-site is not directly involved in multi-site catalysis. The data obtained a few years later by Miwa et al. [12], using

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Abbreviations: MF_1, isolated mitochondrial coupling factor 1; SDS, sodium dodecyl sulphate; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; TEA, triethylamine; TDAB, tetradecyltrimethylammonium bromide; TFA, trifluoroacetic acid; CNBr, cyanogen bromide.

genetically modified TF_1 for the reconstitution experiments, confirmed our conclusions. In a recent paper [13] we have discussed these data in more detail and have concluded that indeed only two β-sites are directly involved in catalysis. A further conclusion was that the two tightly bound, non-exchangeable nucleotides, present in isolated F_1, are bound to both an α/β interface site and a β-site, most likely in one α-β pair.

To obtain additional and more direct proof for these conclusions, two types of analysis have to be made. The first type concerns the location (and function) of the two tightly bound, non-exchangeable nucleotides; the second concerns the function of the two postulated exchangeable non-catalytic α/β interface sites. In the present paper we will restrict ourselves to the first problem: the location and function of the two non-exchangeable tightly bound nucleotides.

Although the presence of tightly bound nucleotides had already been discovered by the mid-seventies [14] and the number of these tightly bound nucleotides was soon established [15,16], it took some time before it was clear that not all three of these nucleotides were non-catalytic, but that one nucleotide is always bound very tightly at a catalytic site [17,18]. The question then remained as to whether the two residual non-exchangeable nucleotides are both located at non-catalytic interface sites (as most authors seem to assume, see Refs. 19–22) or at both a non-catalytic interface site and a non-catalytic β-site [13]. In the latter case, one β-site is not directly involved in multi-site catalysis.

In a previous paper [13] we have explained why we think that, in fact, two different preparations of F_1 are studied with differences in tightly bound nucleotides. In the present manuscript we will only refer to data obtained with the same F_1 preparation as ours, i.e., the preparations isolated according to the procedure described by Knowles and Penefsky [23]. These preparations, after removal of loosely bound nucleotides in the presence of EDTA, contain only three tightly bound nucleotides, of which one is catalytic. The other two nucleotides consist approximately of one ADP and one ATP [6].

In the present paper it is shown that after dissociation of F_1 in the presence of LiCl, schematically represented in Fig. 1, one nucleotide remains bound to the non-β part of the molecule (an α/β interface site) and that, for reconstitution, binding of ATP or an appropriate analogue to a second site is required. This site is not catalytically active, but is very likely to be a β-site. The conformation of this site differs, however, from the conformation of the other β-sites, resulting in a different labeling pattern upon covalent binding of labeled 2-nitreno-ATP.

Materials and Methods

Characterization of the F_1 preparation

Mitochondrial F_1 was isolated from bovine-heart mitochondria according to the method of Knowles and Penefsky [23]. The preparation was stored in liquid nitrogen in a medium containing 10 mM Tris-HCl buffer (pH 7.5), 4 mM ATP, 250 mM sucrose and 4 mM EDTA. Before use the enzyme was precipitated with ammonium sulphate (50% saturation) at 0°C and solubilized in medium A (50 mM Hepes/NaOH buffer, 25% glycerol and 2 mM EDTA, pH 6.2 or 7.0). The solution was centrifuged twice through a Sephadex G-50 coarse column [24], equilibrated with medium A. The nucleotide content of the preparation was 2.9 ± 0.2 mol/mol F_1.

Dissociation and reconstitution procedure

F_1 in medium A (5 mg/ml) is cooled on ice and after 5 min one volume of a cold solution of 6 M LiCl (or 0.33 volume of 12 M LiCl) in medium A is added. The solution is vortexed, put on ice for a specific time, usually 120 s, and sequentially desalted over a Penefsky column (at 0°C), which is pre-equilibrated (via centrifugation) with medium A. When the dissociation was

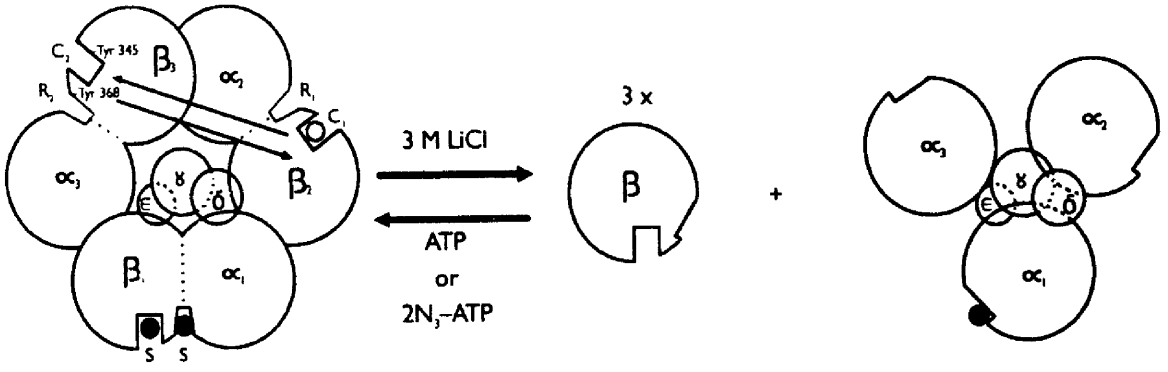

Fig. 1. Schematic representation of the dissociation and reconstitution process of F_1-ATPase. S, structural adenine nucleotide binding site; C, catalytic site; R, regulatory site; ●, tightly bound non-exchangeable adenine nucleotide; ○, tightly bound exchangeable adenine nucleotide. The regulatory sites bind ADP with higher affinity than ATP, while the catalytic sites bind ATP with higher affinity than ADP.

performed in the presence of nucleotides (250 or 500 μM), the latter were added 30 min before dissociation was started. Nucleotides, tested for their effect on the level of reconstitution, were present in the tube into which the desalted protein was centrifuged by column centrifugation. Samples for measurement of ATPase activity were taken both before the column centrifugation step (the dissociated preparation) and 10 min after the column step (the reconstituted sample).

Synthesis of 2-N_3-[α-^{32}P]AD(T)P

The non-radioactive compounds 2-azido-adenosine, 2-azido-AMP, 2-azido-ADP and 2-azido-ATP were synthesized as reported previously [11]. The synthesis of the labeled compounds was largely performed as described by Boulay et al. [25], with some minor modifications.

For the synthesis of 2-N_3-[α-^{32}P]AMP, according to the method described by Symons [26], siliconised glassware was used, which improved the yield significantly. The coupling of pyrophosphate to 2-N_3-[α-^{32}P]AMP was performed according to the procedure of Hoard and Ott [27]. The product was purified on a Sephadex DEAE A-25 column, using a TEA-HCO_3 gradient. From the product (2-N_3-[α-^{32}P]ATP) the 2-N_3-[α-^{32}P]ADP was prepared via dephosphorylation with hexokinase, Mg^{2+} and glucose. All labeled 2-azido-nucleotides were used as a TEA-salt solution in water. The specific radioactivity varied between 500 and 750 dpm/pmol.

Photolabeling

In the present study photolabeling was carried out with a Penray lamp (UV-Products), positioned 1 cm above the sample. The sample was covered with a thin glass plate to avoid damage of protein by low-wavelength ultraviolet-light and change in protein concentration by some evaporation. Samples were illuminated twice for 5 min, with a dark period in between of 20 min. Control experiments showed that illumination caused between 0 and 5% loss of ATPase activity.

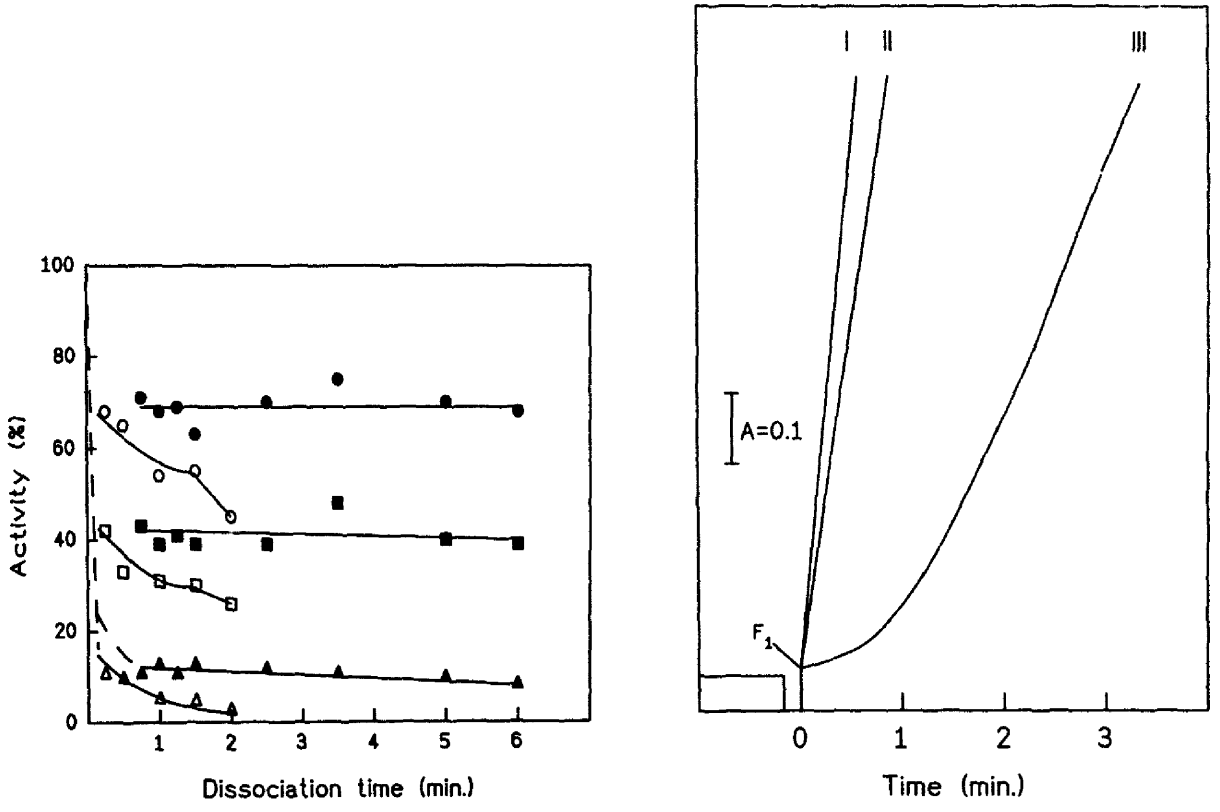

Fig. 2. (A) Effect of incubation time with LiCl on reconstitution of ATPase activity. Open symbols, incubation at pH 7.0; closed symbols, incubation at pH 6.2. 100% activity corresponds to the hydrolysis of 150 μmol ATP/min/mg protein. ▲ and △, activity immediately after incubation with LiCl; ■ and □, activity after reconstitution without added ATP; ● and ○, activity after reconstitution with 250 μM ATP. (B) Rate of ATP hydrolysis during the stages of the dissociation/reconstitution procedure. ATPase activity of 5 μg F_1 was measured by following the oxidation of NADH in an ATP-regenerating system at 30°C. Trace I, F_1 in medium A (pH 7.0) containing 250 μM ATP, before addition of LiCl; trace II, idem, after dissociation and reconstitution with 250 μM ATP; trace III, idem, 90 s after addition of LiCl. For the values of the specific activity given in A, the linear phase of this trace was used.

Miscellaneous

Protein concentrations were measured according to Bradford [28], using the Bio-Rad protein assay kit.

ATPase activities were determined spectrophotometrically with a regenerating system, as described previously [29].

The specific activity of the preparations used was around 150 μmol of ATP hydrolysed min^{-1} mg^{-1} protein.

The concentration of nucleotides was measured via the determination of luminescence with the luciferin/luciferase method as described earlier [6].

Urea-SDS gels were run according to Swank and Munkres [30] and TDAB gels according to Fellous et al. [31]. Peptide digestion with CNBr was performed in formic acid as described in Refs. 32 and 33 and trypsin digestion was carried out as described in Ref. 20, but without succinylation (the relevant peptide fragments from the β-subunit (R 20 and R 21) do not contain lysine).

FPLC of dissociated and reconstituted F_1 was performed in medium A (pH 6.2 or 7.0), using a Superose 12-column. Absorption was measured at 280 nm.

HPLC purification of trypsin-digested preparations was performed in two steps. The first step included a Partisil 10 SAX ion-exchange column, eluted with a gradient of 0.01 M NaH_2PO_4 (pH 4) till 0.4 M NaH_2PO_4 (pH 3) in a 29:71 mixture with acetonitril (Ref. 34, gradient A to B). The flow rate was 1.25 ml/min and absorption was measured both at 215 and 260 nm. Fractions of 1.25 ml were collected and assayed for radio-activity by measuring Cerenkov radiation with an LKB counter.

The second step included a reverse-phase Vydac C-4 column, eluted-with a gradient of 0 to 90% acetonitril in water, containing, additionally, 0.1% TFA (Ref. 34, gradient A' to B'). The flow rate was 1.1 ml/min. The absorption at 215 and 260 nm were determined and fractions of 1.1 ml were collected and assayed for radioactivity.

The Protein Assay kit was obtained from Bio-Rad and the luciferin/luciferase kit from LKB. ^{32}P-labeled phosphoric acid was obtained from Dupont-New England Nuclear and ^{14}C-labeled ATP from Amersham. The Superose 12 column and all Sephadex were bought from Pharmacia. The Partisil 10 SAX column came from Whatman and the Vydac C-4 column from Hewlett Packard.

Results

Optimalization of the dissociation-reconstitution procedure

The conditions for dissociation and reconstitution of F_1 can vary widely with respect to pH, dissociation time, nucleotide concentration, type of nucleotide, concentration of LiCl and concentration of glycerol. The last two parameters are kept the same as those used previously [8,9]. Fig. 2A shows two experiments: one at pH 7.0, the pH used by Wang [9,10] and Nieboer et al. [8], with a dissociation time of maximally 120 s and one at pH 6.2, a pH value used by Verschoor [35], with a dissociation time of maximally 360 s. The concentration of ATP was 250 μM, both during the dissociation and during the reconstitution. This latter value was chosen, since titrations with ATP had demonstrated that higher concentrations of ATP did not increase the level of reconstitution further (experiments not shown). The protein concentration was about 5 mg/ml, since, especially at pH 7.0 or above, one of the primary dissociation products dissociates further during the LiCl treatment, when the concentration of protein is lower, resulting in a low level of reconstitution. The experiment shows that, at pH 7.0, the inactivation, calculated from the maximal activity reached during the assay (Fig. 2B), is more complete than at pH 6.2, but also, that the level of reconstitution decreases considerably with longer times of incubation with LiCl. At pH 6.2 both the level of inactivation (dissociation) by the LiCl treatment and the level of the subsequent reconstitution do not change with longer incubation times. The finding, that a lower activity in the assay of the dissociated preparation (measured 2 min after the start of the assay) is accompanied by a lower level of final reconstitution, suggests that the time-dependent increase of the ATPase activity during the assay of dissociated preparations is due to reconstitution during the assay. The starting activity in the assay is close to zero under both conditions of incubation with LiCl that are used in this experiment, suggesting complete dissociation (Fig. 2B), in agreement with our earlier data [8]. The values for activity, given in Fig. 2A, are the values for the activity that is reached after 2 min, including, therefore, the reconstitution in the assay mixture, due to the dilution of the LiCl and the presence of 5 mM ATP. The low level of the reconstitution in the assay mix shows the importance of the protein concentration for reconstitution.

Analysis of dissociation products

To investigate the process of dissociation we analysed dissociated samples with FPLC, using a gel filtration column. Not only marker proteins, but also native F_1 and dissociated/reconstituted F_1, were run as controls.

Fig. 3 shows an experiment performed at pH 6.2. We have seen in Fig. 2A that the dissociated sample remains quite stable at this pH, as far as reconstitutive capability is concerned. This also holds for the condition that no added ATP is present during the dissociation step.

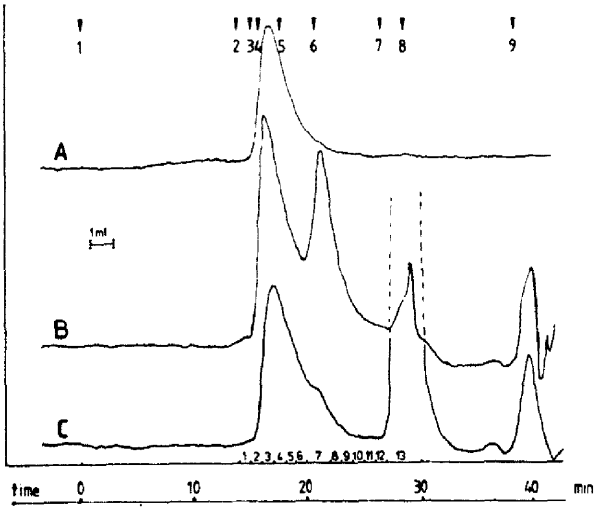

Fig. 3. FPLC elution profile (measured at 280 nm) of F_1 in medium A, eluted from a Superose 12 column at pH 6.2. Between 0.4 and 0.5 mg protein was loaded onto the column and eluted at a flow rate of 0.5 ml/min. (1), Time of loading the column; (2), elution of void volume (6.8 ml or 13.6 min); (3–9), position of the elution peaks of the following markers: catalase (240 kDa, 3), aldolase (158 kDa, 4), dimeric BSA (135 kDa, 5), monomeric BSA (68 kDa, 6), monomeric catalase (60 kDa, 6), aldolase (40 kDa, 7) ATP/ADP [8] and cytochrome *c* (12 kDa, 9). Trace A, F_1, without added ATP and containing 3 mol tightly bound nucleotides per mol of enzyme; trace B: F_1 dissociated for 3 min with LiCl in the absence of added nucleotides; Trace C, F_1 reconstituted in the presence of 250 μM ATP. The numbers on the abscissa correspond to the collected and analysed fractions.

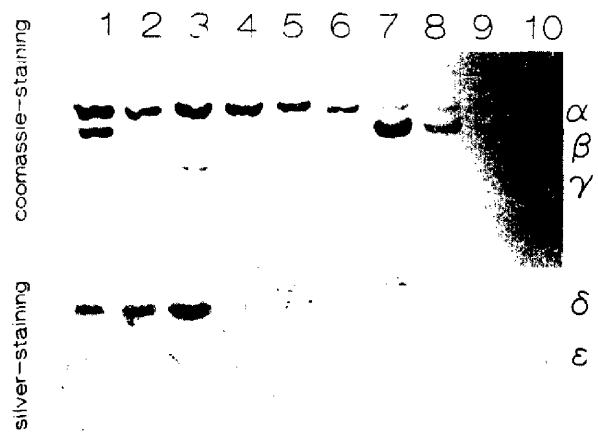

Fig. 4. Polyacrylamide TDAB-gel electrophoresis of FPLC fractions. 12% polyacrylamide gels were stained with Coomassie brillant blue and the region of the small subunits additionally with silver. Lane 1 contains F_1 after passing the FPLC column in medium A at pH 6.2 and lanes 2–10 contain the fractions 2–10 of the experiment shown in trace B of Fig. 3.

Isolated F_1 elutes at 16.5 min, which is quite surprising, since, according to the molecular weight markers, molecules of about 180 kDa should elute at this position. F_1 should elute closer to the void volume (14 min). Additionally, the F_1 peak shows some tailing, while all other polypeptides, e.g., the marker proteins, elute with a symmetrical peak. Both phenomena can be explained by a weak interaction of intact F_1 with the column material. The eluted enzyme has not lost any of the tightly bound nucleotides and the specific activity is even slightly increased by the gel filtration step (Table I).

Dissociated F_1 shows more elution peaks: at 29 min adenine nucleotides are eluted; and the protein elutes in two peaks, at 16.2 and at 21.2 min. The TDAB-gels of Fig. 4 show that the samples eluted at 16.2 min (lanes 2–4) contain the α, γ, δ and ϵ subunits, with an α/γ stoichiometry of 3/1. A complete stoicheiometry of $\alpha_3\gamma\delta\epsilon$ is in agreement with the apparent molecular weight of about 200 kDa. The virtual absence of β-subunits, and the lack of any ATPase activity in these fractions, confirm the completeness of the dissociation step. The fraction that elutes at 21.2 min (lane 7) mainly contains β-subunits and, according to the molecular weight markers, these subunits are not associated. Trace C finally shows reconstituted F_1. At 29 min a large peak of adenine nucleotides is seen, due to the ATP that is required for the reconstitution process. At 16.8 min reconstituted F_1 is eluted, while a shoulder of not-reconstituted β-subunits is still present at 20.8 min. The reconstituted F_1, eluted at 16.8 min, is highly active and both the specific activity and the nucleotide content are only 10–15% lower than those of the

TABLE I

Characterization of isolated and reconstituted F_1

F_1 was prepared as described in Materials and Methods and further purified with FPLC gel filtration (fractions 3, 4 and 5 of Fig. 3, trace A) or subjected to dissociation/reconstitution followed by FPLC gel filtration (fractions 3, 4 and 5 of Fig. 3, trace C).

	Protein conc. (mg/ml)	Spec. act. (μmol/min/mg)	Bound nucleotides (mol/mol F_1)		
			ADP	ATP	total
F_1 in buffer A	2.2	149	1.70	1.22	2.9
F_1 in buffer A after FPLC (Fig. 3, trace A)	0.24	174	2.08	1.02	3.1
F_1 in buffer A after dissociation/reconstitution and FPLC (Fig. 3, trace C)	0.116	149	1.79	0.85	2.6

purified, isolated F_1 (Table I). This difference is fully explained by the presence of some non-reconstituted $\alpha_3\gamma\delta\epsilon$ in the F_1 fraction (equivalent to the amount of free β-subunits visible in the elution profile).

Similar experiments were carried out at pH 7.0 (not shown). In the trace of F_1, dissociated at this pH value, the band of the $\alpha_3\gamma\delta\epsilon$ complex was very broad, suggestive of the presence of the complex in various aggregation states, with different molecular weights. Also, when the time of incubation with LiCl was increased, the $\alpha_3\gamma\delta\epsilon$ complex appeared to become more aggregated, with the peak maximum appearing close to the position of the void volume (results not shown). The trace of F_1 dissociated and reconstituted at pH 7.0 shows not only a shoulder of residual, free β-subunits (as is also seen in Fig. 3C), but also a shoulder of aggregated $\alpha_3\gamma\delta\epsilon$ complex. These data suggest that aggregation of the $\alpha_3\gamma\delta\epsilon$ complex accompanies the decrease of the reconstitutive capability of the dissociated enzyme at pH 7.0.

After dissociation of F_1 (containing 3 mol of adenine nucleotides per mole F_1) in LiCl at pH 6.2, in the absence of added ATP, one mole of adenine nucleotides remains bound per mole of enzyme, after a column centrifugation step (Table II).

Although, during chromatography over an FPLC column, part of the bound nucleotides dissociates from the protein, part of them is still retained. In the experiment shown in Fig. 3, trace B, the nucleotide content of the eluted fractions was measured. The results of this analysis are shown in Fig. 5. Since the background of nucleotides is low in fraction 1 (just before the $\alpha_3\gamma\delta\epsilon$ complex elutes), but high in fractions 8–10 (in between the β-subunit and the nucleotide peak), one may conclude that the nucleotides present in fractions 8–10, and, also, most of those present in fractions 6 and 7 (in which the β-subunit is present), originate from dissociation of bound nucleotides from the $\alpha_3\gamma\delta\epsilon$ complex during the chromatography. After elution the $\alpha_3\gamma\delta\epsilon$ complex still contains about 0.3 mol of adenine nucleotides per mol of complex so that it is evident that the one adenine nucleotide per F_1, present in the dissociated F_1 preparation after column centrifugation,

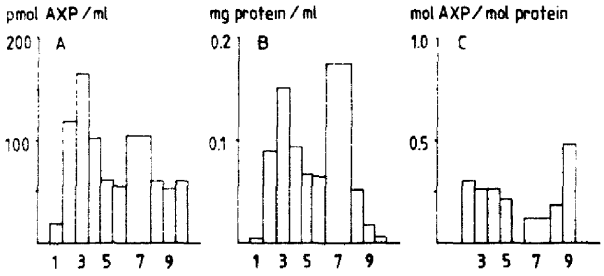

Fig. 5. Nucleotide and protein content of the fractions collected from the FPLC experiment shown in Fig. 3, trace B. For the determination of the stoicheiometry (panel C) it was assumed that the molecular weight of the protein in fractions 2–5 was $180 \cdot 10^3$ ($\alpha_3\gamma\delta\epsilon$) and that in fractions 7–9 $150 \cdot 10^3$ (3 β).

is bound to the $\alpha_3\gamma\delta\epsilon$ moiety and not to β-subunits. Because of the involvement of the α-subunit, this nucleotide has to be bound at an interface site in the intact enzyme and is probably closely linked with the structure of at least the $\alpha_3\gamma\delta\epsilon$ moiety of the complex (see Fig. 1).

Nucleotide binding and reconstitution

The bound nucleotide, retained at the $\alpha_3\gamma\delta\epsilon$ moiety after incubation of F_1 with LiCl in the presence of glycerol, is mainly ADP. The data in Table II indicate that, at pH 7.0, this nucleotide is more easily lost than at pH 6.2, in agreement with the finding that the dissociated preparation is more stable at pH 6.2 than at pH 7.0 (Fig. 2A). After removal of the LiCl and incubation with [^{14}C]ATP to obtain maximal reconstitution, the reconstituted preparation was precipitated with 50% saturated ammonium sulphate and the amount of additionally bound labeled nucleotides was determined. Table II shows that about 2 nucleotides are additionally bound, and one of these can be exchanged with unlabelled ATP during a short period of turnover. The reconstituted enzyme, therefore, is very similar to the isolated enzyme, containing, as it does three tightly bound nucleotides, of which one is bound to a catalytic site.

TABLE II

Bound nucleotides (mol / mol F_1) after dissociation / reconstitution with [^{14}C]ATP (A) or 2-N_3-[α-^{32}P]-ATP (B) followed by ammonium sulphate precipitation and two column centrifugations

Starting F_1 in buffer A contains three tightly bound nucleotides.

	Remaining non-exchangeable nucleotide	(A) [^{14}C]ATP		(B) 2-N_3-[α-^{32}P]ATP					
		before turnover	after turnover	before turnover	UV-illumination		after turnover	UV-illumination	
					covalent	% inh		covalent	% inh
pH 7.0	0.6	1.7	0.64	2.0	1.46	70	0.46	0.22	3
	0.7	2.0	0.70						
		2.1	1.1						
pH 6.2	1.2	1.9	0.73	2.3	1.0	60	0.80	0.20	6
		1.7	0.80	2.4	0.77	32	0.83	0.21	1

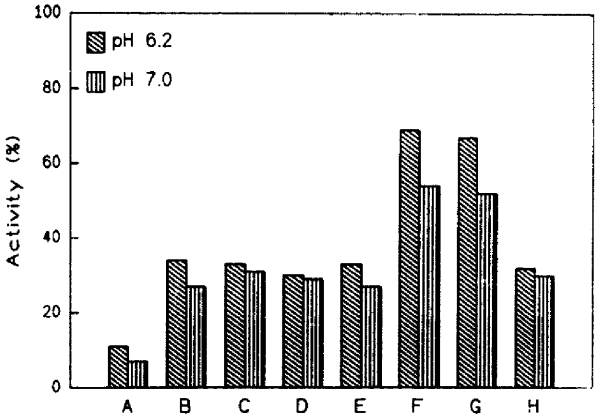

Fig. 6. Reconstitution efficiency of different nucleotides. A, activity measured directly after dissociation. The reconstitution occurring during the assay is included; B, activity after reconstitution without added nucleotides; C–H, activity after reconstitution with 0.5 mM ITP (C) 0.5 mM 8-azido-ATP (D), 1 mM pyrophosphate (E), 0.25 mM ATP (F), 0.25 mM 2-azido-ATP (G) or 0.5 mM ADP, 1 mM phosphate (H).

The experiment of Fig. 6 (lane B) shows that partial reconstitution can be obtained without addition of extra ATP during the reconstitution period. The ATP that is still present in the solution after the column centrifugation step in which the bulk of the LiCl and ATP is removed, is responsible for this reconstitution. In most experiments 250 μM ATP was present during the dissociation step (see Refs. 8 and 9), and, after the column centrifugation step, about 10 μM ATP was estimated to be still present. When the dissociation is performed in the absence of ATP, no significant reconstitution occurs without added nucleotides.

When, additionally, 500 μM ITP, 500 μM 8-azido-ATP or 1 mM pyrophosphate is added (Fig. 6, lanes C–E), the final level of reconstitution does not change. With 250 μM 2-azido-ATP, however, the same level of reconstitution is obtained as with 250 μM ATP. Finally ADP does also not result in any additional reconstitution. Apparently, a nucleotide in the anti-configuration is required for reconstitution, in agreement with earlier data which show that 2-azido-ATP, but not 8-azido-ATP, can be bound to the two non-exchangeable nucleotide binding sites [6]. The nucleotide, furthermore, has to be a triphosphate, in agreement with the fact that, of the two non-exchangeable nucleotides in isolated F_1, one is ADP and the other ATP [6].

When reconstitution is performed in the presence of 2-azido-[α-^{32}P]ATP, while also during the dissociation step 2-azido-[α-^{32}P]ATP was present, the tightly bound 2-azido-adenine nucleotides (2 mol/mol F_1) can be partially bound covalently by illumination. The residual presence of some not-reconstituted material, after the ammonium sulphate precipitation step, does not influence the results since it does not retain any bound labeled nucleotide. The illumination results in a significant inhibition of the ATPase activity (Table II). A chase with cold ATP before illumination removes not only the 2-azido-ATP bound at a catalytic β-site, but also part of the 2-azido-ATP bound at the originally 'non-exchangeable' site, especially at pH 7.0 (see Table II). After reconstitution a slow exchange at this site is apparently possible. Subsequent illumination induces a partial covalent binding of the residual bound 2-azido-ATP, not accompanied by a significant or proportional level of inhibition of ATPase activity. We may be ascertained, then, that this covalently labeled nucleotide binding site is the second tight nucleotide binding site, not directly involved in catalysis. TDAB-gel electrophoresis, followed by autoradiography, showed that the β-subunit was exclusively labeled (not shown). We are aware, however, that attachment of the nitreno-ATP to a β-subunit does not, in itself, rule out the possibility that an α/β-site is labeled.

Localisation of the second tightly bound nucleotide

Previous experiments have shown that covalent labeling of a catalytic site with 2-azido-ATP results in the binding of 2-nitreno-AT(D)P to, mainly, the Tyr-345 of a β-subunit [20,33,36], while labeling of an exchangeable non-catalytic site results in the modification of, mainly, Tyr-368 of a β-subunit [20,36]. This localisation of the exchangeable nucleotide binding sites has been confirmed by us (unpublished data). Boulay et al. [32] have reported the labeling of another region of the β-subunit, after long incubation of F_1 with 2-azido-ATP. This region was never identified, but labeling of a different fragment was also obtained when nucleotide-free F_1 was treated with 2-azido-ATP [37]. This site is apparently a site which only exchanges on a time-scale of hours and becomes available for labeling after removal of tightly bound non-exchangeable nucleotides. Since we have seen that one of the two tightly bound non-exchangeable nucleotides, bound at an α-β interface site, does not exchange, even during the whole dissociation/reconstitution procedure, the tight site, which is partially labeled with 2-nitreno-ATP in our experiment, could well be the same site as the site labeled by Boulay et al. [32] and Lunardi et al. [37]. Digestion of our labeled F_1 with CNBr or trypsin should reveal what the localisation of the label is.

To detect the localization of the covalently bound 2-azido-[α-^{32}P]ATP the labeled enzyme was digested with trypsin (a method used by others and ourselves to localize the site of labeling when catalytic sites or exchangeable non-catalytic sites are covalently occupied with 2-nitreno-AT(D)P). Using an HPLC ion-exchange column the (low amount of) peptides containing covalently bound adenine nucleotides were separated from most of the non-labeled peptides, since the radioactivity does not coincide with the main absorption peaks. The absorption profile may be compared

with that in Ref. 34, but the radioactivity profile is different. The radioactive fractions were pooled and were further analysed with a reverse-phase column. From different studies (unpublished data) we know that the fragment containing Tyr-345 (R 20) elutes from the latter column at 30% B′ and the fragment containing the Tyr-368 (R 21) elutes at 23% B′, while an additional cleavage, between Ile-362 and Val-363, results in a shorter labeled peptide, containing Tyr-368, which elutes at 18% B′. From the ion-exchange column not less than five labeled peaks are eluted (Fig. 7). The labeled material eluting at 23% B, contains -N-AMP, while the fragments eluting at 42, 48 and 53% B contain -N-ADP and the label eluting at 78% a represents bound -N-ATP. It should be mentioned that labeling of the exchangeable binding sites (catalytic and non-catalytic) results in elution peaks at different percentages of B. Chromatography of fractions II, III, IV and V over the reverse-phase column gave unexpected results. A large part of each fraction was not bound to the column (This is much more than we have encountered in other cases), and the rest mainly eluted at low concentrations of B′. Very little, or no, label eluted at 30% or at 18 and 23%, respectively. We have not been able to determine the amino acid sequence of

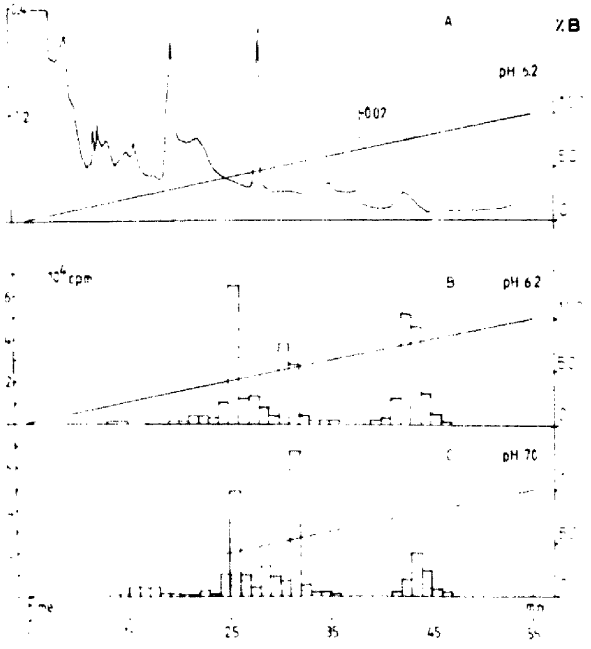

Fig. 7. Ion-exchange HPLC of tryptic digests of F_1. F_1 was dissociated and then reconstituted with 0.25 mM 2-azido-[α-^{32}P]ATP. The sample was illuminated under conditions of turnover with added ATP and digested with trypsin. Trace A, absorption at 215 nm of the eluate; the whole experiment was carried out at pH 6.2. Trace B, radioactivity profile of the eluate measured spectrophotometrically in trace A. Trace C, as trace B, but now for an experiment carried out at pH 7.0. For further details see Materials and Methods.

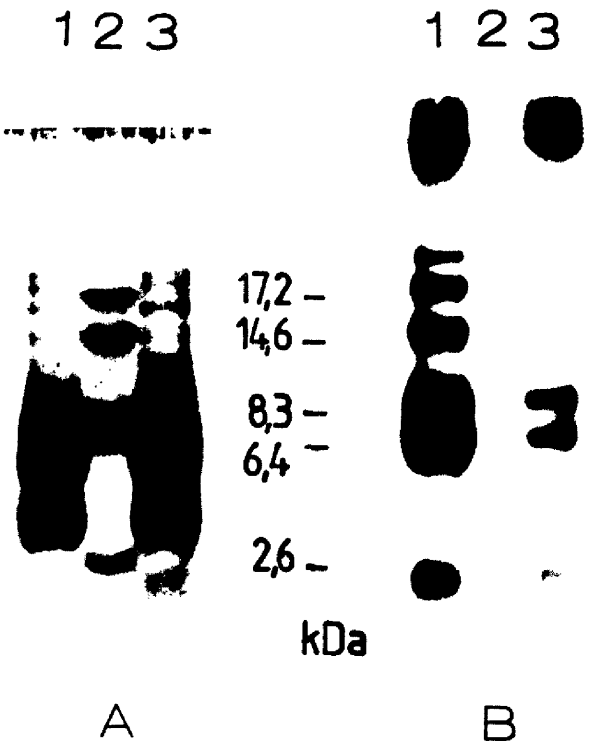

Fig. 8. Urea-SDS-polyacrylamide gel electrophoresis of CNBr digests of F_1 reconstituted with 250 μM 2-azido-[α-^{32}P]ATP and illuminated before (lane 1) or after (lane 3) the start of turnover with ATP. Panel (A) shows the gel after staining with Coomassie brilliant blue and panel (B) shows the autoradiogram. Lane 2 contains marker peptides with the indicated molecular weights.

the labeled peptides eluting from the column without binding or at low percentage B′ because of contaminations. This material probably consists of short peptide fragments or hydrolysed label [38].

Two preparations of F_1, one partly labeled with 2-azido-[^{32}P]ATP at the second tight binding site (as described in the previous section), and the other labeled at both this tight site and the tight catalytic site (illumination was performed without previous chase with cold ATP), were digested with CNBr and the fragments were separated on an SDS-urea gel. The results of the experiment, carried out at pH 6.2, are shown in Fig. 8 (see also Ref. 32). The effect of the chase is two-fold: (1) in the low-molecular weight region the label has largely disappeared, indicating that these labeled fragments originate from labeling of the catalytic site; and (2) the label at the top, however, is only slightly decreased, as expected for label originating from labeling of the non-exchangeable site. Because of the position of this label, the labeled peptide is probably aggregated. Once again, the only, but relevant, conclusion is that the label is bound at a fragment (fragments) not labeled under conditions of labeling exchangeable binding sites.

Discussion

On the basis of previous experiments (especially our own results with photo-affinity labeling with 8-azido-adenine nucleotides [6]), our dissociation/reconstitution experiments with Nbf-modified F_1 [8] and the reconstitution experiments of Miwa et al. [12], we have concluded in a recent paper [13] that F_1 contains only two sites that perform rapid catalysis. The 8-azido-adenine nucleotides seemed to be the best choice to discriminate between various nucleotide binding sites on F_1: binding to catalytic sites results in labeling of β-subunits, binding to regulatory sites results in about equal labeling of both α- and β-subunits and the non-exchangeable sites cannot be labeled at all with these nucleotides, even when the sites are freed from endogenous ATP and ADP [6]. Since, in the present study, however, we have focused our attention on the non-exchangeable sites, the 8-azido-adenine nucleotides could not be used to discriminate between a real β-site and an interface site. The 2-azido-adenine nucleotides are more suited to these studies since they prefer the same anti-configuration as the adenine nucleotides themselves [39] and can bind to the non-exchangeable sites [6]. In all cases known, however, they label the β-subunits nearly exclusively and β-sites and interface sites can only be differentiated on the basis of the specifically labeled amino acids. It should be noted, however, that the sites which we call β-sites (from the 8-azido labelling) are probably also close to the interface between α- and β- subunits, as is demonstrated via crosslinking with diazido-adenine nucleotides both for TF_1 [40] and MF_1 (unpublished results).

Our dissociation experiments show that the parallel between loss of tightly bound nucleotides and dissociation of F_1 [41] is only partial. After dissociation of the enzyme with LiCl in a β-fraction and an $\alpha_3\gamma\delta\epsilon$ fraction (see also Refs. 35 and 42) one nucleotide remains bound to protein after centrifugation of the enzyme through a Sephadex column. Our data from the FPLC chromatography show that this residual binding is not very tight. It was noted that during chromatography a large part of the bound nucleotides is released from the protein and is eluted in the fractions between the $\alpha_3\gamma\delta\epsilon$ fraction and the main nucleotide fraction (Fig. 3). The binding moiety has to be the $\alpha_3\gamma\delta\epsilon$ complex and not the β-subunit. Since the β-subunits fully dissociate into single polypeptides, which have a relatively low affinity for adenine nucleotides (e.g., Refs. 43, 44), this result is not surprising. Verschoor [35] also showed that, under the conditions of his experiments, the α- and γ-subunits could be prevented from aggregation during column chromatography only by the presence of ATP in the elution buffer, while ATP was not required for elution of the β-subunits. These data clearly indicate that the one remaining nucleotide, bound at the $\alpha_3\gamma\delta\epsilon$-moiety, is important for the stability of this complex and that the loss of reconstitutive capability after FPLC chromatography is probably due to the dissociation of the residual nucleotide from the $\alpha_3\gamma\delta\epsilon$ complex. This localization indicates that the remaining nucleotide (mainly ADP, not shown) occupies, in the intact enzyme, an interface site and not a β-site. It also suggests that the dissociated nucleotide was connected with a β-subunit and that the loss of this nucleotide may induce the dissociation of the β-subunits into single polypeptides. At this point we may recall our earlier finding, that covalent binding of 2-N-ATP to a catalytic site prevents the dissociation of the β-subunits from the enzyme [8]. It may be noted, as well, that our conclusions about the dissociation process of F_1, as schematically depicted in Fig. 1, fit very well with the recent X-ray data reported by Bianchet et al. [45].

The finding, that the one site that retains the bound adenine nucleotide is an original interface site, narrows the original question about the localization of the two non-exchangeable nucleotides to the question of whether the second tight site is also an interface site (in which case the enzyme may contain three catalytic β-sites) or a β-site (in which case the enzyme contains only two catalytic β-sites). To find the answer to this question we specifically tried to label this second tight site. Our data show that reconstitution of dissociated F_1 requires the presence of nucleotides in the anti-configuration (8-azido-ATP or ITP are not active) and the nucleotide has to be a triphosphate (ADP is not active at all). This specificity for nucleotides in the anti-configuration, is not required for reconstitution of TF_1 [46], but TF_1 does not contain (or require) tightly bound nucleotides for preservation of the quarternary structure. The absolute specificity for the anti-configuration already indicates that the type of binding is different from that of the binding to the four exchangeable sites (which only show relative specificity) and that the relevant site still has the same properties as the non-exchangeable nucleotide binding sites in native F_1. The requirement for a triphosphate may be considered as an indication that, indeed, a β-site has to be occupied, since all known data indicate that the β-sites prefer triphosphates and the interface sites diphosphates. The reconstitution is a real reconstitution. The fraction of reconstituted F_1 that is eluted from the FPLC column has nearly the same specific activity as isolated F_1, with the difference being due to the presence of some residual not-reconstituted $\alpha_3\gamma\delta\epsilon$. The same holds for the number of tightly bound nucleotides.

Illumination of F_1, reconstituted in the presence of 2-azido-ATP, resulted in a 60–70% inhibition of the enzyme, although more than one nucleotide was covalently bound. It is clear that one of the covalently occupied sites has to be non-catalytic and after a chase

to remove 2-azido-ATP from any catalytic site, the residual 2-azido-ATP appears to be bound to a site which is not directly involved in catalysis: the inhibition upon illumination is virtually zero, certainly if one takes into account that illumination as such, usually causes some inhibition. The data in Table II indicate two phenomena: (1) that in reconstituted F_1 the second tightly bound nucleotide, certainly when it is 2-azido-ATP, is slowly exchangeable, especially at pH 7; (2) that the efficiency of the covalent labeling of this site with 2-nitreno-ATP seems to be lower than we have encountered with all the other sites on F_1 that we have investigated in the past. It could well be that in this case no reactive tyrosine is close to the azido-group and now other, less reactive, amino acids have to react with the formed nitrogen radical, causing, at the same time, a less specific labeling. The data of Table II also suggest a pH-dependence of the formation of a covalent link at this site. A labeling of serine or histidine may also explain the appearance of most of the label (after trypsin digestion) in the void volume of the (hydrophobic) reversed-phase column chromatography, indicative for hydrolysis of the nucleotide [38]. The residual label, eluting at low percentages of B', has to be bound to short fragments. The main conclusion, from the analysis of the labeled trypsin fragments, is that neither the Tyr-345, nor the Tyr-368, is labeled. In experiments in which exchangeable sites are labeled, we always find, in agreement with the literature [34], that one, or both, these tyrosines are labeled and only in the present experiment is this not the case. Also the digestion with CNBr indicates that another peptide (or peptides) is labeled than when exchangeable nucleotide binding sites are labeled. It should be mentioned that on gels of cyanogen bromide digests a labeled CB 10 fragment (containing Tyr-368 of the exchangeable non-catalytic site) is never seen (unpublished data), and the labeling of Tyr-368 is also not detected when the tryptic digestion is carried out after CNBr treatment [32,37]. The modified CB 10 fragment is probably very unstable in acid. From the CNBr digestion the possibility cannot be excluded, therefore, that a modified peptide has lost its label.

One should keep in mind that knowledge of the modified amino acids, when they are not very close to the Tyr-345 and the Tyr-368 of the β-subunit, do not give information about the principally β- or α/β-site character of the site as long as the structure of the two types of binding site is not really known.

Although the labeling data tell us only that the tight nucleotide binding site, involved in dissociation and reconstitution of F_1, is structurally different from both the catalytic sites and the exchangeable non-catalytic sites, the finding, that the binding is specific for the triphosphate adenine nucleotides and that the bound nucleotide is lost when the β-subunits dissociate from the enzyme and from each other, indicates a β-site character of the binding site. Finally, we may recall our former result [8] that covalent binding of 2-nitreno-ATP to a catalytic β-site prevents the dissociation of the 8-subunits from the enzyme in the presence of LiCl. Dissociation of F_1 occurs, apparently, only when all three β-sites are free of nucleotides.

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