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Modification of membrane-bound F_1 by *p*-fluorosulfonylbenzoyl-5′-adenosine: sites of binding and effect on activity

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Abstract

Bovine heart submitochondrial particles (smp) were incubated with *p*-fluorosulfonylbenzoyl-5′-adenosine (FSBA) in order to study the binding of this ligand and its effect on ATP synthesis and ATP hydrolysis in smp and to compare the results with those obtained with isolated F_1. The binding was measured with the ^{14}C-labeled compound. ATP hydrolysis was in all cases as much inhibited as succinate-driven ATP synthesis and ITP hydrolysis was more inhibited than ATP hydrolysis. The binding experiments show that modification of three nucleotide binding sites results in nearly complete inhibition of ATPase activity. In the presence of pyrophosphate up to 6 mol [^{14}C]SBA/mol F_1 can be bound. FSBA preferentially modifies amino acids of the α-subunits but also β-subunits are modified. It is concluded that modification of both subunits results in inhibition of activity. The results are very well comparable with the results obtained with isolated F_1, which indicates that our preparation of F_1 is a good model for F_1 in the intact system. Furthermore it is concluded that each α-subunit of F_1 in smp, just like in the isolated form, contains two pockets where adenosine moieties can bind, one located above the P-loop, modifying α-Tyr-244 and α-Tyr-300 and the other one located below the P-loop where also the adenosine moiety of AD(T)P binds, modifying β-Tyr-368.

Keywords: Submitochondrial particle; ATP synthase; *p*-Fluorosulfonylbenzoyl-5′-adenosine; Non-catalytic binding site; ITP hydrolysis; ATP hydrolysis; ATP synthesis; ATP/ADP carrier

1. Introduction

The mitochondrial ATPsynthase is composed of two parts, F_1 and F_0. The F_1-part consists of five different subunits with a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ [1,2]. Disconnected from the F_0-part, it only catalyses ATP hydrolysis. The membrane-bound enzyme, F_0F_1, catalyses both the hydrolysis of ATP and its synthesis as the last step of oxidative phosphorylation. The enzyme contains six nucleotide binding sites, located on the α- and β-subunits of F_1 [3]. The catalytic sites are on the β-subunits, the α-subunits contain only non-catalytic sites.

Since the protein structure of MF_1 has been resolved at high resolution, the structure and environment of the catalytic and non-catalytic nucleotide

Abbreviations: FSBA, *p*-fluorosulfonylbenzoyl-5′-adenosine; SBA, covalently bound FSBA; smp, submitochondrial particles; MF_1, mitochondrial F_1; SHK, sucrose/Hepes/KOH; TDAB, tetradecyltrimethylammonium bromide; NAP_3-$2N_3$ADP, 3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}-2-azidoadenosine-5′-diphosphate; AAC, ATP/ADP carrier

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binding sites are known [4]. This knowledge has already proven to be very useful for the interpretation and design of functional studies. The structure itself, however, is not sufficient to derive a catalytic mechanism in which the function of each nucleotide binding site is described. Ligand-binding studies remain appropriate to define the role of each nucleotide binding site, whether catalytic or non-catalytic.

One example of such a ligand is the analogue 5′-*p*-fluorosulfonylbenzoyl adenosine (FSBA) which induces inhibition by binding exclusively to non-catalytic nucleotide binding sites of F_1-ATPase. According to the data of Bullough and Allison ATP hydrolysis will be completely inhibited when 3 mol SBA are bound per mol F_1. The ligand was reported to be covalently bound to amino acids of the β-subunits, Tyr-368 and His-427 (at high or low pH, respectively) [5]. For the pig heart enzyme the binding of 5–6 mol SBA per mol F_1 has been reported [6]. The latter authors explained the high level of binding by assuming that both catalytic and non-catalytic nucleotide binding sites were modified. Recently our group confirmed the possible binding of 5 mol FSBA per mol F_1, of which 3 mol were bound to α-subunits and 2 to β-subunits [7]. Modification of both α- and β-subunits was shown to be inhibitory, since ITPase activity was almost fully inhibited with just one nucleotide binding site occupied and that nucleotide was partially bound to β-subunits and partially to α-subunits. It was further shown, using protein digestion, purification of peptides and sequencing, that SBA was bound at specific amino acids, namely β-Tyr-368, α-Tyr-244 and probably α-Tyr-300 [7].

Up to now, nearly all experiments with FSBA have been performed with F_1 and different results have been obtained with different preparations. F_1 is considered to be a model for the intact ATPsynthase complex. Submitochondrial particles (smp) contain the whole enzyme, F_0F_1-ATPsynthase, together with the electron transport chain and are capable of performing oxidative phosphorylation. Therefore it is useful to study this intact system to investigate which type of F_1 is most similar to F_1 in smp, the Allison type or ours. The present study reports on the effects of FSBA on ATP hydrolysis and on ATP synthesis by smp and describes binding studies performed with this adenosine analogue. It is shown that the results obtained by our group with isolated F_1 are very well comparable with the results obtained with smp. It is also shown that the relative effects of binding of FSBA to non-catalytic sites on ATP synthesis and ATP hydrolysis are the same.

2. Materials and methods

2.1. Biological preparations

Heavy bovine heart mitochondria were isolated essentially according to Smith's procedure 3 [8]. After passing the meat through a meat grinder the tissue was homogenized using a Waring blendor equipped with a 5 l beaker. Four liter of 0.25 M sucrose, 5 mM succinate and 10 mM Hepes-KOH, pH 7.8 (buffer A) was added to approximately 1 kg of minced meat and homogenized at high speed for 60 s. An equal volume of buffer A was added and the pH was adjusted to 7.4 with 6 M KOH. After a slow centrifugation step the mitochondria were collected by centrifugation for 30 min at $11\,000 \times g$. The pellet was resuspended and homogenized in 0.25 M sucrose and 10 mM Tris-HCl, pH 7.5. The heavy mitochondria were separated from the light mitochondria by centrifugation for 15 min at $32\,000 \times g$. The heavy mitochondria were, after another washing step, suspended in 0.25 M sucrose, 2 mM ATP, 10 mM $MgCl_2$ and 10 mM Hepes-KOH, pH 7.5. Samples were frozen in liquid nitrogen and stored at -80°C.

Submitochondrial particles (MgMnATP) were prepared from this suspension essentially according to Hansen and Smith [9]. Mitochondria were suspended at a protein concentration of 20 mg/ml in a buffer which contained 0.25 M sucrose, 1 mM succinate, 5 mM $MgCl_2$, 10 mM $MnCl_2$,1 mM ATP, 2 μM cytochrome *c*, 1 mg/ml BSA and 10 mM Hepes-KOH pH 7.6. After saturation of the suspension with Argon the mitochondria were sonicated for four periods of 45 s with intermediate cooling periods of 60 s with a Branson sonifier at 70% output. During the sonication the suspension was kept at 0–4°C and flushed with Helium. Submitochondrial particles (smp) were separated from the mitochondria by centrifugation at $32\,000 \times g$ for 7 min. The supernatant, wich contains the smp, was incubated for half an hour at 30°C in the presence of 5 mM potassium-malonate to activate succinate:Q oxidoreductase. Af-

terwards the smp were pelleted at 150 000 × *g* for 25 min and washed twice with 0.25 M sucrose, 10 mM Hepes-KOH pH 7.5 (SHK). Three per cent (v/v) glycerol was added and smp were stored at about 30 mg/ml in liquid nitrogen.

2.2. F_1 content of the submitochondrial particles

The concentration of F_1 in the smp preparations was determined by measuring the concentration of the bc_1 complex via the concentration of the antimycin-binding sites, titrating smp with antimycin and measuring the uncoupled respiration with a Clark oxygen electrode. In smp preparations the concentration of bc_1 complex is identical with the concentration of F_1 [10]. In the smp preparations used the concentration of F_1 was usually 0.45 nmol/mg protein.

Protein concentrations were determined with the Bio-Rad protein assay which is based on the method of Bradford [11].

2.3. Assays

ATP hydrolysis was measured as described by Herweijer et al. [12] with the exception that $KHCO_3$ was used in stead of $NaHCO_3$.

ITP hydrolysis was measured in the same medium as used for ATP hydrolysis with the exception that 2.5 mM ITP was used in stead of 5 mM ATP and with ten times the amount of PK.

ATP synthesis was assayed as in Herweijer et al. [12] with the modification that smp were not incubated in an 1.4 ml but in an 2.0 ml oxygraph vessel.

TDAB gel electrophoresis was performed according to Penin et al. [13], with the adjustments described by Fellous et al. [14].

2.4. Incubation with FSBA

Smp (5 mg/ml) in SHK pH 7.5 were incubated at room temperature with 0.8 mM FSBA for up to several hours. After a certain time-period the incubation was stopped by centrifugation of smp through a Sephadex-G50 coarse column [15], equilibrated with SHK, removing unbound FSBA. One centrifuge column was sufficient to stop the incubation. The eluate was stored on ice till subsequent analysis. No additional modification occured by FSBA after storage of samples at 0°C

2.5. Determination of bound [^{14}C]SBA

Binding experiments with smp (5 mg/ml in SHK, pH 7.5) were performed by incubating smp with [^{14}C]FSBA (17 000 dpm/nmol) under the same conditions as used with the non-radioactive analogue. Incubations were stopped by the addition of DTT and free label was removed by a centrifuge column. The amount of bound label was determined, as follows: TDAB gel electrophoresis was performed with the radioactively labeled smp. The α/β regions of the gels were cut into slices after staining and destaining. The radioactivity was extracted from the slices by incubating them with 1.5 ml 50% Solvable (NEN Dupont) overnight at 50°C. After neutralization of the samples with 150 μl acetic acid, 9 ml scintillation cocktail (Packard Scintillator 299) was added and radioactivity was counted in a LKB 1214 Rackbeta liquid scintillation counter.

2.6. Analysis of [^{14}C]SBA modified-peptides

Digests of the labeled smp were made by incubating them with trypsin at a protein ratio of 10:1 (smp:trypsin) for 24 h at 37°C. The obtained peptides were separated on HPLC equipped with a C4-Vydac RP column and eluted with a linear gradient of 0–70% B (90% acetonitril, 0.1% TFA, 9.9% water) in A (0.1% TFA, 99.9% water). The column was operated with two LKB 2150 HPLC pumps with a gradient mixer, operated by a LKB 2151 controller. The flow was 1.1 ml/min and each fraction contained 1.1 ml. Protein was detected at 215 nm and ligand at 260 nm with a Pharmacia/LKB VWM 2141 detector. Radioactivity in the collected fractions was determined by liquid scintillation counting. After rechromatography of the labeled fractions or further purification on the Pharmacia SMART system with a C2/C18-RP-column the radioactive fractions were lyophilised. Sequence analysis was performed with a Procise 494 protein micro sequencer from Applied Biosystems.

2.7. Miscellaneous

FSBA and trypsin were obtained from Sigma. [^{14}C]FSBA, with the ^{14}C at position 8 of the adenine ring, was purchased from NEN-Dupont. Enzymes used for activity measurements were from Boehringer. All used chemicals were of analytical grade.

3. Results

3.1. Inhibition of ATPsynthase by FSBA

When smp were incubated with FSBA not all activities of the ATPsynthase were affected to the same extent. After 1 h of incubation of smp at room temperature with 0.8 mM FSBA the ATPase activity was inhibited for 45% and after 2 h 65% inhibition was reached. Longer incubation times did not give much additional inhibition. Fig. 1 shows the time-course of inhibition of ATP hydrolysis and ITP hydrolysis. ITPase activity was more inhibited than ATPase activity. When ATP hydrolysis was inhibited for 50%, ITP hydrolysis was inhibited for about 70% (Fig. 2a). Also ATP synthesis was measured and this was as much inhibited as ATP hydrolysis as can be seen in Fig. 2b. ATP synthesis was measured with succinate in stead of the commonly used NADH as substrate for respiration, because NADH dehydrogenase appeared to be strongly inhibited by FSBA.

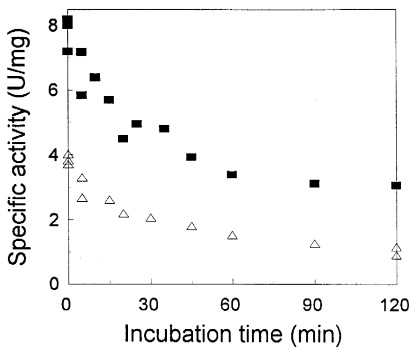

Fig. 1. Time course of ATPase and ITPase activity after addition of FSBA. Smp (5 mg/ml in SHK pH 7.5) were incubated at room temperature with 0.8 mM FSBA for 1–120 min. Samples were taken at intervals and were, after column centrifugation, stored on ice. Afterwards ATP hydrolysis (■) and ITP hydrolysis (△) were measured as described in Section 2. Activities are presented as U per mg particle protein.

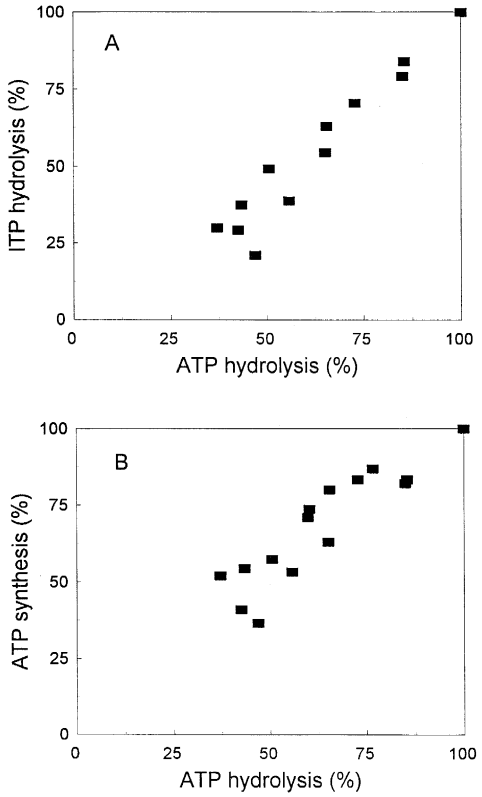

Fig. 2. Inhibition of enzyme activities by FSBA. Submitochondrial particles were incubated at room temperature with 0.8 mM FSBA for 0–120 min. Samples were taken at intervals and stored on ice. Afterwards succinate-driven ATP synthesis, ATP hydrolysis and ITP hydrolysis were measured as described in Section 2. In A ATP hydrolysis activity is plotted against ITP hydrolysis activity, in B ATP hydrolysis activity is plotted against ATP synthesis activity.

3.2. Binding of FSBA to submitochondrial particles

To study the binding of the inhibitor to smp [^{14}C]FSBA was used. After incubation for 2 h with 0.8 mM [^{14}C]FSBA the smp were brought on polyacrylamide TDAB gel for electrophoresis. After running and subsequent drying of the gel an autoradiogram was made. Fig. 3 clearly demonstrates labeling of both the α- and β-subunits in isolated F_1 (lane 1) as well as in membrane-bound F_1 (lane 3). Lane 3 shows that besides the α- and β-subunits of F_1 also the ATP/ADP carrier (band at 30 kDa) was labeled in smp. Lane 2 represents the isolated complex I of the mitochondrial respiratory chain. No specifically labeled band was found despite the inhibitory effect of FSBA on NADH-dehydrogenase activity. In the

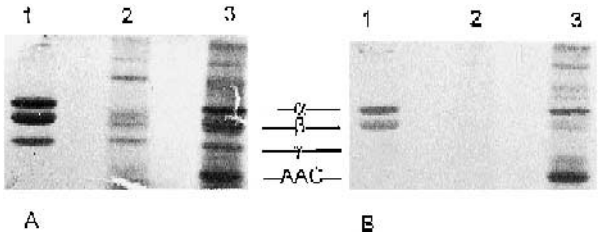

Fig. 3. TDAB gel electrophoresis of isolated F_1 (lane 1), isolated mitochondrial NADH dehydrogenase (lane 2) and smp (lane 3) after incubation for 2 h with 0.8 mM [^{14}C]FSBA. Panel A shows the the region with the relevant bands of the dried gel after staining with Coomassie Brilliant Blue and panel B shows the autoradiogram. α, α-subunit; β, β-subunit; γ, γ-subunit; AAC, ATP/ADP carrier.

region of the α- en β- subunits no additional radioactivity was detected, so gel electrophoresis could be used for determination of the binding stoichiometry. This was done by slicing the bands of interest out of the gel, extracting the protein with Solvable and counting the radioactivity in a liquid scintillation counter.

Incubation of smp with different concentrations of [^{14}C]FSBA for 60 min revealed that in that time-period the maximal binding is 1.8 mol SBA/mol F_1 (Fig. 4a). Half-maximal binding occurs at a concentration of 0.34 mM FSBA. This K_D value is not completely accurate (see Discussion), since no correction is made for the change in the occupation of the sites during the process of covalent attachment. From Fig. 4b two apparent K_D values can be derived which are 0.28 mM for the α-site and 0.88 mM for the β-site. Higher levels of covalent binding will be reached at each concentration when the incubation time is increased. Fig. 4b also shows that after 60 min of incubation α-subunits were more occupied with label than β-subunits at all tested concentrations of ligand. The binding to α-subunits is apparently saturated at lower concentrations of ligand than the binding to β-subunits.

After 1 h of incubation with 0.8 mM [^{14}C]FSBA about 1 mol [^{14}C]SBA per mol F_1 was bound as seen in Fig. 5. After 2 h of incubation with the label approximately 2 mol [^{14}C]SBA was bound per mol F_1. At that point the ATPase activity was reduced to 40% (not shown).

From Fig. 6 the conclusion can be drawn that, after extrapolation, three nucleotide binding sites must contain [^{14}C]SBA to obtain nearly full inhibition (black squares). The ratio of modification of α- and β-subunits was 2:1 at almost complete inactivation, but was higher at the initial stage of the incubation. This indicates that the label first preferentially binds to α-subunits and that only after a certain time period also β-subunits become modified by the ligand. Fig. 7 represents the relation between binding of [^{14}C]FSBA and inhibition of ATP hydrolysis, separated in binding to α-subunits (Fig. 7a) and binding to β-subunits (Fig. 7b). These pictures show that only when approximately 0.5 mol [^{14}C]SBA per F_1 is bound at α-subunits, also the β-subunits will bind ligand. ATP hydrolysis is 30% inhibited at that point so it is evident that the binding to α-subunits causes a decrease of activity, as well as the binding to the β-subunits. This is in agreement with the results obtained by our group with isolated F_1 [7], but in

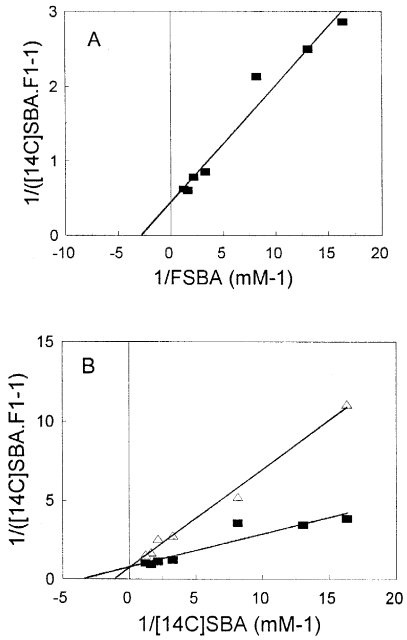

Fig. 4. Concentration dependence of the binding of [^{14}C]FSBA to the F_1-part of ATPsynthase in smp. Smp were incubated for 60 min with 0–0.8 mM [^{14}C]FSBA. Afterwards the binding stoichiometry was determined as described in Section 2. In A the reciprocal [^{14}C]FSBA concentration is plotted against the reciprocal binding to F_1. In B the reciprocal [^{14}C]FSBA concentration is plotted against the reciprocal concentration of bound ligand to F_1, separated in binding to α-subunits (■) and binding to β-subunits (△).

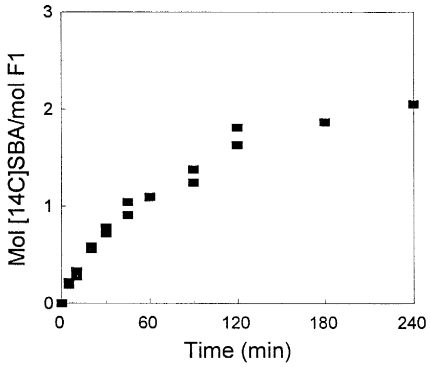

Fig. 5. Time dependence of the binding of [^{14}C]FSBA to the F_1-part of ATPsynthase in smp. Smp were incubated with [^{14}C]FSBA for 0–240 min. Samples were taken at intervals and were, after column centrifugation, stored on ice. Afterwards the binding stoichiometry was determined as described in Section 2.

contradiction with the conclusions of the group of Allison [5].

3.3. Effect of pyrophosphate on the binding of FSBA

Jault and Allison [16] found that low concentrations of pyrophosphate increase the inhibition induced by FSBA in isolated F_1. In a more recent paper [17] they showed that pyrophosphate stimulates the control activity and this increased inhibitory effect of FSBA was not due to an increased rate of binding but

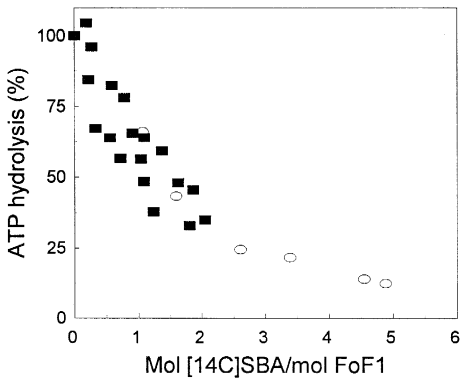

Fig. 6. Relation between binding of [^{14}C]FSBA and ATP hydrolysis. Submitochondrial particles were incubated with 0.8 mM [^{14}C]FSBA with (○) or without (■) 100 mM pyrophosphate in the incubation buffer. Samples were taken at intervals and were, after column centrifugation, stored on ice. Afterwards the binding of [^{14}C]FSBA to smp and ATP hydrolysis were measured as described in Section 2. After 9 h of incubation in the presence of pyrophosphate 5.5 mol/mol F_1 was bound.

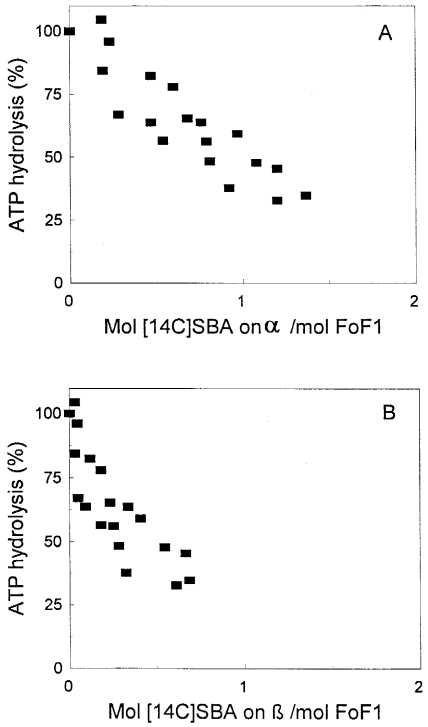

Fig. 7. Relation between the binding of [^{14}C]FSBA to α- and β-subunits and ATP hydrolysis. The same procedure was followed as indicated in the legend to Fig. 6. In A the amount of [^{14}C]SBA per F_1 bound to α-subunits, in B the amount of [^{14}C]SBA per F_1 bound to β-subunits is shown.

to the fact that FSBA abolishes this stimulatory effect of pyrophosphate on ATP hydrolysis. In experiments with our F_1, containing endogenous nucleotides, only incubation in the presence of high concentrations of pyrophosphate resulted in a stimulation of the inactivation of the ATPase activity (measured in an assay system, in which bicarbonate is present as activating anion) of F_1 by FSBA [7]. This effect was not due to activation of the control activity, but an increased rate of binding. In order to obtain a higher level of binding, smp were, therefore, incubated with [^{14}C]FSBA in the presence of 100 mM pyrophosphate.

Also in smp the binding was strongly accelerated when incubation took place in the presence of 100 mM pyrophosphate: after 30 min 1.5 mol [^{14}C]SBA/mol F_0F_1 was bound in stead of 0.6 (not shown). The relation between binding and inactivation did not change in the presence of pyrophosphate (Fig. 6, white circles). So pyrophosphate enhances

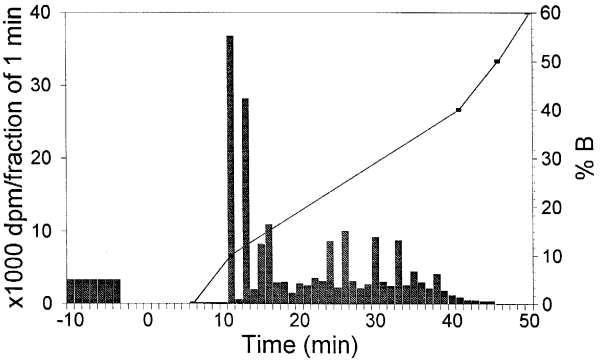

Fig. 8. Reversed phase HPLC of tryptic digests of smp. Smp were incubated with 0.8 mM [^{14}C]FSBA for 2 h and digested with trypsin. For further details see Section 2.6.

the binding of the label to F_1 in smp, especially binding to the β-subunits. More than 5 mol [^{14}C]SBA/mol F_1 could be bound under these circumstances, 2.7 mol being bound to the β-subunits and 2.8 mol being bound to the α-subunits (not shown).

3.4. Sites of modification by FSBA

Fig. 8 shows an experiment in which, after incubation of smp with [^{14}C]FSBA and digestion with trypsin, peptides were separated on reversed-phase HPLC. This resulted in a pattern of label distribution which is comparable with that of isolated F_1 (see [7] for details). Three main peaks (26, 30 and 33) have been further analysed and were further purified by HPLC or the Pharmacia Smart system to get a pure enough sample for amino acid sequencing. Fraction 26 gave the same radioactive fragments as obtained with F_1, which contained α-Tyr-300 and a small amount of β-Tyr-368. Fraction 30 also contained two major radioactivity peaks. One of them contained a fragment with modified β-Tyr-368, just like in F_1, and the other one eluted at the same position as the fragment from isolated F_1 which contained modified α-Tyr-244. From these analyses the conclusion can be drawn that in smp the same amino acids bind FSBA as in soluble F_1.

Fraction 33 was identified as being part of the ATP/ADP carrier. Sequencing showed the presence of the peptide containing amino acids 80–86, −FPTQAL. The first amino acid of this peptide was not detected, apparently since it was modified. This modified residue of the ATP/ADP carrier had to be Tyr-80. This is part of the second membrane-spanning α-helix of the carrier, according to the model of Klingenberg [18].

4. Discussion

The results obtained by incubation of smp with FSBA are comparable with those obtained with isolated F_1. In both systems 3 mol SBA are bound per mol F_1 at nearly complete inhibition. However, the K_D of 0.34 mM for FSBA derived from Fig. 4a is not the same as has been reported before. Di Pietro and coworkers found a K_D of 0.23 mM for FSBA measured in pig heart F_1 [6]. This difference might be due to the fact that they have determined it in F_1 while we have determined it in smp. There is also a difference in method of determination. Di Pietro and collegues have determined the K_D by measuring the first-order rate constant of the slow phase of inactivation at several concentrations of FSBA. K_D was then derived from the reciprocal plot of this rate constant against the reagent concentration as was described before by Wyatt and Colman in the case of pyruvate kinase [19]. In the present study we derived this value from the reciprocal plot of the concentration of FSBA against the concentration of bound FSBA after an incubation of 1 h. The procedure of Di Pietro and collegues is correct since the rate of the irreversible binding is proportional to the concentration of reversibly bound FSBA. We have in addition assumed that the level of binding after 1 h is proportional with the rate of binding at zero time and this introduces a small error, such that the found K_D value gives the lower limit. Furthermore, the measured K_D is an average of two values, since at saturating concentrations of the ligand more than one mol [^{14}C]SBA per mol of F_1 is bound after the period of incubation. The two separate apparent K_D values are 0.28 mM and 0.88 mM, and represent binding to an α-site and to a β-site, respectively. This difference in the two K_D values, the site that results in modification of α-subunits having a higher affinity for FSBA than the site resulting in modification of β-subunits, fits with our finding that in the initial stages of the incubations more label is bound to α-subunits than to β-subunits.

The relation between inhibition of activity and binding is not a linear one, which means that the binding of the first molecule of FSBA to an enzyme molecule has relatively more effect on activity than binding of additional ones. Combination of the data of Fig. 2a and Fig. 6 leads to the conclusion that after binding of one SBA/F_1 the ITPase activity is inhibited for more than 70%. This implicates that most enzyme molecules have bound one SBA before a second molecule of ligand is bound.

ITP hydrolysis is more inhibited than ATP hydrolysis. This is in line with our results obtained with isolated F_1, but in contrast with the results obtained when the slowly exchangeable non-catalytic site of F_1 was modified by NAP_3-2-nitreno-ADP [20]. In the latter case the V_{max} of the ATPase activity was decreased but not that of the ITPase activity, of which the K_m was increased. It was concluded that the dissociation of product was affected by the binding of the ADP analogue and not the turnover at the catalytic site, the former being the rate-limiting step in the ATP hydrolysis, the latter being the rate-limiting step in ITP hydrolysis. If we assume that also in smp ATPase activity is mainly limited by the rate of dissociation of ADP, while ITPase activity is mainly limited by the turnover at the catalytic site, then the differential inhibition of ATP and ITP hydrolysis can be explained by an effect of FSBA on the turnover at the catalytic sites, just as proposed for isolated F_1 [7].

It is remarkable to find that more than 5 SBA/F_1, in the presence of pyrophosphate, can be bound to F_1 in smp, while only non-catalytic sites are modified. The identity of the modified amino acids and the fact that ATP hydrolysis is only partially inhibited by covalent binding of the ligand, indicate that no catalytic sites are modified by the analogue. It was reported before that (pig heart) F_1 is capable of binding 5–6 mol FSBA per mol F_1 [6] and the interpretation of the authors was that non-catalytic as well as catalytic sites were modified. It is clear that this interpretation cannot be correct. The only possible explanation is that there are two types of adenosine binding sites on each α-subunit and that FSBA can be located on both sites at the same time.

Pyrophosphate has been shown to bind to both catalytic and non-catalytic sites [21–23]. Our group has shown that upon incubation of soluble F_1 with 100 mM pyrophosphate the nucleotide content decreases to 2 mol/mol F_1 leaving only the two non-exchangeable nucleotides which are bound to an α- and a β-site [7]. Two out of three α-subunits are empty under those conditions and each can bind two molecules of SBA while the 5th molecule can be bound at the α-subunit which still contains 1 ADP. Apparently this ADP can be exchanged, partly, by the 6th FSBA in smp, resulting in two molecules of SBA bound per α-subunit, albeit after a very long incubation time (9 h), assuming that all of the more than five molecules are bound specifically. The amino acids of isolated F_1 which could be detected as the binding sites for SBA were β-Tyr-368, α-Tyr-300 and α-Tyr-244. The first two were also identified in the present study as to be modified by SBA, indicating that in smp FSBA binds to the same sites of F_1 as in the isolated form of the enzyme.

One of the amino acids involved in nucleotide binding at the non-catalytic site is β-Tyr-368 which is part of the adenosine-binding pocket [4]. This amino acid is also modified by 2-nitreno-ATP when bound at an α-subunit and lies below the P-loop, the phosphate-binding residues [24], while α-Tyr-244 and α-Tyr-300 lie above the P-loop. So it is possible that two molecules of FSBA are bound to one α-subunit, one above and one below the P-loop which itself is not modified by FSBA. In [7] it has been proposed that the reason why Abrahams et al. [4] have not seen a second binding pocket for adenosine and why the group of Allison [5] has not detected a second binding site, might be the treatment of the enzyme with glycerol to remove endogenous nucleotides. Since smp are also not treated with glycerol to remove endogenous nucleotides, the finding that also the α-subunits of F_1 in smp contain two binding pockets for FSBA, supports this proposal.

It should be noted that α-Tyr-244 has been reported to be modified with 5′-*p*-fluorosulfonylbenzoylethenoadenosine (FSBεA) in preparations of Allison's laboratory [25] which were not treated with glycerol.

The ratio of modification of α- and β-subunits was very high at the initial stages of the incubation. Also the incubations with different concentrations of FSBA resulted in binding of more label to α-subunits than binding to β-subunits. This difference in binding might be due to differences in affinity or to occupation with endogenous nucleotides. ADP is normally

bound with the adenosine moiety in the pocket below the P-loop, which contains the β-part of the non-catalytic binding site. It is tempting to speculate that when non-catalytic sites are occupied by ADP, FSBA can only be bound at the pocket above the P-loop where the amino acids α-Tyr-244 and α-Tyr-300 are situated. However, in the presence of pyrophosphate this is less important, because under those circumstances the nucleotide content decreases as stated above. Considering the fact that smp are able to bind more FSBA in the presence than in the absence of pyrophosphate, we may assume that also in smp nucleotides are removed upon incubation with pyrophosphate. The ratio of labeling of α- and β-subunits was indeed decreased by pyrophosphate, but was still higher than 1. So a combination of both explanations for the α to β binding ratio might be valid, depending on the occupation of the non-catalytic nucleotide binding sites.

Matsuni-Yagi has reported that ATP hydrolysis is inhibited to a greater extent than ATP synthesis activity when smp are incubated with certain inhibitors, including FSBA [26]. The explanation for this phenomenon, according to the author, was that ATP synthesis proceeds with two kinetic states, one with a high and one with a low K_m with an associated high and low turnover capacity for ATP synthesis, respectively. These states are capable of interconversion in dependence of the proton motive force. Such a conversion from low to high activity occurs after inactivation of part of the F_0F_1. The remaining uninhibited complexes will operate mainly in the high in stead of the low K_m mode, resulting in a higher rate of ATP synthesis per ATPsynthase.

In the present study ATP synthesis is as much inhibited as ATP hydrolysis. This is not easy to understand considering the above-mentioned data. The most logical explanation might be that the particle preparation used in our laboratory is already in the high K_m mode before any inhibition has taken place. The model, presented by Matsuno-Yagi, demands that every inhibitor of the ATPsynthase complex results in the same effect, regardless the nature of the inhibitor. However, this is not the case. Furthermore, in studies with chloroplast F_0F_1-ATPsynthase it was shown that blocking any of the 6 nucleotide binding sites separately with covalently bound 2-nitreno-AXP resulted in equal inhibition of multi-site ATP hydrolysis and synthesis activity [27]. There was a linear relationship between binding of the used analogue and inactivation of both activities. This was determined by reconstitution of the chloroplast enzyme in artificial membranes which made it possible to investigate the effect of modification of one specific site on ATP hydrolysis and synthesis.

The data on the chloroplast enzyme are in agreement with our own data, which means that the non-catalytic sites involved regulate both ATP hydrolysis and synthesis to the same extent. It might be concluded that the control of F_1 in both ATP hydrolysis and ATP synthesis is the same which means that they operate as each others reversal. This is a concept which is not commonly accepted. As stated above, the differential effects of FSBA on ATP hydrolysis and ITP hydrolysis activity can be explained by the fact that both activities have different rate-limiting steps, being dissociation of product and the catalysis itself, respectively. It was concluded that apparently FSBA, at first, influences mainly the catalysis and not the dissociation of product. This effect on catalysis might be achieved by affecting the conformational changes which occur during the cycle of catalysis. It is very well understandable that those conformational changes can be the same for both ATP hydrolysis and ATP synthesis. Influencing those changes by modification of non-catalytic sites with FSBA, then, results in equal inhibition of both ATP hydrolysis and ATP synthesis activity, which we found indeed. This process is independent of whether two or three catalytic sites are involved in either the hydrolysis or the synthesis reaction.

When catalytic sites were modified by 8-nitreno-ATP we found another result (not shown). In that case ATP synthesis was less inhibited than ATP hydrolysis, indicating that the ATPsynthase has an overcapacity for ATP synthesis which is dependent on the build-up of $\Delta\mu_H^+$. This phenomenon is less important in the present study since the sites which were modified were non-catalytic nucleotide binding sites which will never result in complete inhibition of activity. It was not possible to investigate the effect of FSBA on ATP synthesis in the presence of pyrophosphate, since that compound had a negative effect on the coupling between respiration and ATP synthesis.

The overall conclusions from this study are: (1) the

binding studies performed in phosphorylating smp gave the same results as our group found earlier with isolated F_1 [7], so this type of F_1 is more similar to F_1 in smp than for example the preparations used by the group of Allison; (2) just like in isolated F_1, the α-subunits in smp contain 2 pockets where adenosine moieties can bind; (3) ATP hydrolysis and ATP synthesis are equally affected by binding of FSBA to non-catalytic sites and there is no α-site directly involved in ATPsynthase catalysis.

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