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FSBA modifies both α- and β-subunits of F_1 specifically and can be bound together with AXP at the same α-subunit

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Abstract

Binding of 1 mole 5'-fluorosulfonylbenzoyladenosine (FSBA) per mol F_1 induces about 50% inhibition of ATPase activity and 80% inhibition of ITPase activity. The binding of additional ligand results in a further inhibition of both activities. Maximally 5 mol/mol F_1, causing complete inhibition of activity, can be bound. Using radioactive FSBA more label is found on α-subunits than on β-subunits under the usual buffer conditions. The modified amino acids are α-Tyr300, α-Tyr244 and β-Tyr368. Binding of FSBA, at least up to 3 mol/mol F_1, does not result in loss of bound ADP, whether the starting enzyme contains 2, 3 or 4 bound nucleotides. Added adenine nucleotides compete with FSBA only for binding that results in modification of β-subunits, shifting the α/β ratio of bound label to higher values. It is concluded that the α-subunits contain two hydrophobic pockets for the binding of nucleoside moieties, with a different orientation relative to the P-loop. One pocket contains α-Tyr244 and α-Tyr300, the other β-Tyr368. Since, however, in the binding of adenine nucleotide di- or triphosphates the P-loop is involved, only one of these ligands can bind per subunit. The previously not understood binding characteristics of several substrate analogues have now become interpretable on the assumption that also the structurally homologous β-subunits contain 2 pockets where nucleoside moieties can bind. The kinetic effects of FSBA binding indicate that the first FSBA binds at the regulatory site that has a high affinity for ADP and pyrophosphate. Binding of pyrophosphate at this high-affinity regulatory site increases the V_{max} of the enzyme, while binding at a second regulatory site, a low-affinity site, increases the rate of binding of FSBA with a factor of about 3. Binding of bicarbonate at this latter site is responsible for the disappearance of the apparent negative cooperativity of the ATPase activity.

Keywords: F_1-ATPase; Non-catalytic site; Adenosine binding pocket; Regulatory site; Anion binding site

1. Introduction

The publication of the protein structure of the mitochondrial F_1-ATPase at 2.8 Å resolution [1] has provided a very useful framework for the interpretation of data obtained from ligand-binding and kinetic studies and thereby stimulated the research on the mechanism of the enzyme. For the role of each of the

Abbreviations: FSBA, 5'-p-fluorosulfonylbenzoyladenosine; Ap_4A, 5',5'-diadenosinetetraphosphate; NAP_3-ADP, 3'-O-{3-[N-(4-azido-2-nitrophenyl)amino]propionyl}-ADP; NbfCl, 4-chloro-7-nitrobenzofurazan; TDAB, tetradecyltrimethylammonium bromide; DTT, dithiothreitol; PP_i, pyrophosphate; DMSO, dimethyl sulfoxide.

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6 nucleotide-binding sites of the enzyme [2] in the catalytic mechanism, the structural data obtained for one specific conformation do not provide the necessary information and ligand-binding studies in combination with kinetic studies remain appropriate to define the role of the various nucleotide binding sites, both catalytic and non-catalytic, as well as their mutual interactions. The authors of Ref. [1] have put emphasis on the structural possibility of rotation of the α/β moieties relative to the γ-subunit and interpreted this as support for a rotational model of catalysis by the intact native system. The data from studies on the role of the nucleotide binding sites, performed mainly with the isolated soluble F_1-ATPase, have been interpreted differently, either as evidence for an alternating two-site model [3–6], or as indicative of a three-site rotary model [7,8]. Our own conclusion, that in isolated mitochondrial F_1 only two β-sites are performing multi-site catalysis, the third β-site containing a non-exchangeable nucleotide [9], implies that the α-β moieties do not rotate relative to the γ-subunit. This property of the isolated enzyme may well be the consequence of the disconnection of F_1 from the membrane-embedded part of the enzyme, but we do not really know the mechanism of the intact system in such detail.

Various types of ligand have been used to identify the role of both α- and β-sites in the catalytic process of ATP hydrolysis by F_1. To study the regulatory sites the use of the analogue 5′-p-fluorosulfonylbenzoyladenosine (FSBA or $FSO_2PhCOAdo$) seems quite appropriate and it has been used to study adenine nucleotide binding sites in several systems [10–12]. The data obtained with isolated F_1 originate largely from the group of Allison, who studied the binding of FSBA to isolated bovine heart F_1 and its inhibitory properties very thoroughly [13–15]. We have previously shown [9] that the preparation of F_1 used by this group is significantly different from the preparations used by us and others. The differences involve both kinetic properties (hysteretic inhibition) and nucleotide content. We were specifically interested in the effect of nucleotide content on the binding and inhibitory properties of FSBA. Since FSBA is reported to bind, in the case of F_1-ATPase, to regulatory nucleotide binding sites only, we also wanted to compare the effects of FSBA with those of the azido adenine nucleotide analogues, used by us previously [3,6,16], and those of activating anions, in order to characterize in more detail the interaction between the regulatory sites and the catalytic sites. Another argument for studying the binding of FSBA to our preparation of F_1 was the fact that the binding of FSBA has been reported to induce removal of adenine nucleotides from the regulatory sites located at the α-subunits [15]. (Since the regulatory sites at the interfaces between α- and β-subunits are in essence located at the α-subunits [1], we will call them α-sites, although a covalently modifying probe may be attached to an amino acid of the β-subunit, as has been reported for most of the adenine nucleotide analogues, including FSBA.) According to our model of F_1, one non-catalytic tightly bound adenine nucleotide is located at a (merely potentially catalytic) site on a β-subunit, and the prediction was that this nucleotide should not be removed on binding of FSBA to the three regulatory sites on the α-subunits.

The resulting data confirm the existence of substantial differences between our F_1 preparations and those of the group of Allison and indicate the presence of two pockets in the α-subunits where nucleosides can bind. Additional data obtained with NbfCl, FSBI and 8-azido-ATP suggest a similar double pocket in the β-subunits. Our data furthermore provide an explanation for the results obtained by Vogel and Cross [17] with the Ap_xA compounds: the original interpretation of the authors (one adenosine at a catalytic site and one at a non-catalytic site) has become impossible on the basis of the structural data from Abrahams et al. [1], but we can now conclude that both adenosine moieties of a suitable Ap_xA, such as Ap_4A, can bind at the same α-subunit.

Some of the data have been reported at a meeting [18].

2. Materials and methods

2.1. The preparations of F_1

F_1-ATPase was isolated from bovine hearts according to the method of Knowles and Penefsky [19] and stored in liquid nitrogen in 10 mM Tris-HCl (pH 7.5), 250 mM sucrose, 4 mM ATP and 4 mM EDTA. From the isolated F_1-ATPase, stored in liquid nitrogen, preparations were made containing three or four bound adenine nucleotides. F_1 samples containing

three tightly-bound nucleotides, were prepared as described previously [16]. After the last step of this procedure (dilution of F_1 to 2 mg protein ml^{-1}), dependent on the experiment, Mg^{2+} or EDTA was added to a concentration of 4 mM from a 1 M $MgCl_2$ or a 500 mM EDTA pH 7.5 solution, respectively. These preparations had a specific activity of 125–165 μmol ATP min^{-1} mg^{-1}.

Preparations with four bound nucleotides [20] were obtained by adding Mg^{2+} at a concentration of 10 mM to the stored F_1. After 10 min one ammonium sulphate precipitation was performed by adding an equal volume of saturated ammonium sulphate (pH 7.0–7.5). After 10 min on ice the precipitate was spun down for 2 min at $10000 \times g$. The pellet was dissolved in 50 mM Tris-HCl (pH 7.5), 150 mM sucrose and 4 mM Mg^{2+} (TMS buffer). Residual salt and loosely bound nucleotides were removed by filtrating the sample twice by column centrifugation as described by Penefsky [21]. Columns of 1×5 cm, containing Sephadex G-50 coarse equilibrated in the above-mentioned buffer, were centrifuged for 1 min at 2000 rpm in a Homef LC-30 table centrifuge. These preparations were diluted to 2 mg protein ml^{-1}. The specific activity of these preparations was 100–120 μmol ATP min^{-1} mg^{-1}.

2.2. Incubation conditions

Four incubation media were used for the binding of FSBA: TEG medium (50 mM Tris-HCl (pH 7.5), 4 mM EDTA and 10% glycerol), TMG-medium (4 mM $MgCl_2$ instead of EDTA), TES-medium, containing 50 mM Tris-HCl (pH 7.5), 4 mM EDTA and 150 mM sucrose, and TMS-medium (4 mM $MgCl_2$ instead of EDTA). The standard concentration of FSBA was 0.8 mM (added from a 20-mM stock solution in DMSO), the time of incubation varied between 2 and 4 h and the temperature was 20°C. When [^{14}C]FSBA was used, the incubation was stopped either by addition of cold FSBA, followed by a column centrifugation step, or by addition of 50 mM DTT and a column centrifugation step after 30 min.

2.3. Assays for ATPase and ITPase activity

ATPase activities were measured with an ATP regenerating system as described in Ref. [16]. The assay mix for the determination of the ITPase activity contained 10 U additional pyruvate kinase per assay (2 ml). Inhibitions were calculated against control samples treated in the same way as the samples incubated with FSBA, except that no FSBA was added.

2.4. Determination of protein and nucleotide content of F_1

Protein concentrations were measured with the Bio-Rad assay [22], with bovine serum albumin as standard. Bound nucleotides (ATP and ADP) were determined luminometrically as described by Van Dongen [23]. The only difference was that a new luminometer Bio-orbit 1250 was used.

2.5. TDAB gel electrophoresis, determination of bound [^{14}C]FSBA

TDAB gel electrophoresis of F_1 samples with covalently bound [^{14}C]FSBA was performed as described by Penin et al. [24] with the adjustments of Fellous et al. [25]. After staining with Coomassie, the gels where scanned with an Bio-Rad model 1650 scanning densitometer. The Coomassie-stained gels were sliced by cutting out the stained bands and protein was extracted from these slices with 1.5 ml 50% Solvable (NEN Dupont), during 3–4 h at 50°C. After this period 500 μl 0.5 N HCl and 10 ml scintillation cocktail (Packard Scintillator 299) were added and after several times thorough mixing, radioactivity was measured in the LKB 1214 Rackbeta liquid scintillation counter.

2.6. HPLC reverse-phase chromatography, amino acid sequencing and electro-spray mass spectrometry (ESMS)

Tryptic digests of F_1 labeled with [^{14}C]SBA were prepared as described [26], but without acid precipitation. Trypsin was added to the F_1 solution in the used medium (ratio 1:20) and incubated for 24 h at 37°C. The peptides were brought on a Vydac C4 reverse-phase column, eluted with a linear gradient of 0.1% trifluoroacetic acid (eluant A) and 0.1% trifluoroacetic acid, 90% acetonitrile (eluant B). The two LKB 2150 HPLC pumps, with a gradient mixer, were operated by an LKB 2151 controller. Protein was

detected at 215 nm and ligand at 260 nm with a Pharmacia/LKB VWM 2141 detector. Radioactivity in the fractions was detected by liquid scintillation counting. After rechromatography of the labeled fractions with an extended gradient or further purification with the Pharmacia SMART system equipped 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, electro-spray mass spectrometry with a Fisons Platform ESMS.

2.7. Miscellaneous

FSBA and trypsin were obtained from Sigma, as was also Ap_4A. [^{14}C]FSBA was purchased from NEN-Dupont. Enzymes for activity measurements were from Boehringer. All used chemicals were of analytical grade.

3. Results

3.1. Choice of incubation conditions and effect of FSBA on the ATPase activity

The inhibition by FSBA of the ATPase activity of F_1 proceeds very slowly. At increasing pH the rate of inhibition increases, but at pH 8.0 FSBA is unstable and degrades in time (not shown, see also Ref. [27]). At pH 7.5 the compound is not significantly hydrolysed during 4 h, so this pH was chosen as the standard condition under which modification of mainly β-Tyr368 was expected [14]. The rate also increases with increasing concentrations of FSBA up till about 1–2 mM. As standard condition we used 0.8 mM FSBA, similar to the concentration used by Bullough and Allison [14]. The presence of Mg^{2+} has no significant influence on the time course of the inhibition, nor the number of bound nucleotides (3 or 4). The inhibition reaches about 35% after 1 h and after 2 h at 20°C 45–50% inhibition is obtained. The ITPase activity, however, is inhibited for 80–85% after 2 h (Fig. 1).

3.2. Binding of FSBA and effect of nucleotide content

The binding of [^{14}C]FSBA to F_1 in relation to the residual ATPase and ITPase activity is shown in Fig. 2 for preparations of $F_1(3)$ in TEG or TMS buffer. It can be seen that the binding of the first molecule of FSBA causes about 50% inhibition of the ATPase activity, while the binding of more than 3 molecules is required for complete inhibition. The breaks in the curve indicate that the first FSBA molecule binds at a much higher rate than the second and this again at a higher rate than the third, in agreement with the time curve. That nearly all F_1 molecules have bound one

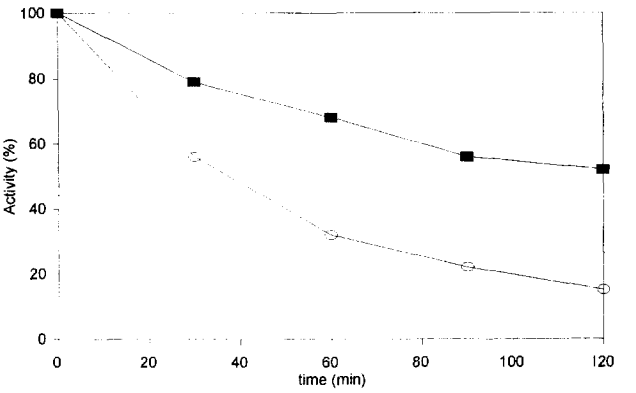

Fig. 1. Inactivation of F_1 by FSBA. F_1 with 4 bound nucleotides was incubated at a concentration of 2 mg/ml in TEG buffer (pH 7.5) with 0.8 mM FSBA at 20°C for various times. The incubations were stopped by addition of 50 mM dithiothreitol, followed after 30 min by a column centrifugation step. ■, ATPase activity; ○, ITPase activity.

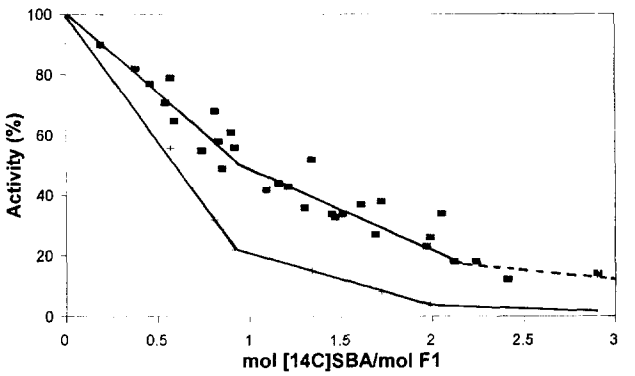

Fig. 2. Inhibition of F_1 by covalently bound [^{14}C]SBA. F_1 (2 mg/ml), containing 3 mol bound nucleotides/mol F_1, was incubated for various times with 0.8 mM [^{14}C]FSBA in either TMS or TEG buffer at pH 7.5. The TEG buffer was with or without 100 mM PPi. Bound radioactivity was determined from TDAB gels after a chase with cold FSBA, followed by 2 column centrifugation steps. The labeled bands of α- and β-subunits were excised and the radioactivity determined. ■, ATPase activity; +, ITPase activity

Table 1
Analysis of the binding of [^{14}C]FSBA to F_1 containing four bound nucleotides

F_1(4) time (min)	ATP (mol/mol)	ADP (mol/mol)	ADP + ATP (mol/mol)	% inh ATPase	SBA/F_1 (mol/mol)	ratio α/β
0	0.64	3.38	4.02	0	0	–
30	0.50	3.05	3.55	35	0.59	3.54
60	0.71	2.75	3.46	51	0.85	3.35
90	0.70	2.63	3.33	57	1.21	2.55
120	0.79	2.43	3.22	66	1.45	2.43
120*	0.78	2.86	3.64	66	1.51	2.46
240	0.78	2.39	3.17	77	1.97	1.88

F_1(4) was prepared as described in Section 2 and incubated at a concentration of 2 mg/ml in TMS (pH 7.5) at 20°C in the presence of 0.8 mM [^{14}C]FSBA. The incubation was stopped by addition of cold FSBA, followed by 3 column centrifugation steps. In the sample marked * the incubation was stopped with 50 mM dithiothreitol, followed by 1 column centrifugation step after 30 min. Bound adenine nucleotides were determined via bioluminescence [23], bound [^{14}C]SBA from TDAB gels.

FSBA before a substantial level of binding of the second FSBA molecule takes place, is also clear from an inspection of the ITPase activity: after the binding of one FSBA/F_1 the ITPase is largely inhibited, so most enzyme molecules (at least 80%) have to contain a molecule of ligand. The data on the ITPase activity are in agreement with the results of the group of Allison [28].

Allison has reported that FSBA binds at the non-catalytic sites in the position where also AD(T)P is bound. We did not, however, see an effect of the nucleotide content of F_1 on the inhibition with FSBA (with F_1(3) and F_1(4) the rate of inhibition was very similar) and also the amount of bound adenine nucleotides did not change upon incubation of the enzyme with FSBA, whether we started with F_1(3) or with F_1(4) (Tables 1 and 2). With F_1(4) a slight decrease of bound adenine nucleotides is measured, but this decrease also occurs in the absence of FSBA, since the fourth nucleotide dissociates off very slowly [16,20,29]. According to our earlier conclusions about the subunit localization of the bound adenine nucleotides (see Ref. [20] and Fig. 3), two α-sites in F_1(4) are occupied by ADP. In the experiment with F_1(4), described in Table 1, two FSBA molecules were bound per mol F_1 after an incubation of 4 h. The conclusion must be, then, either that ADP and FSBA can bind together to one α-subunit or that both FSBA molecules bind at the residual free α-subunit (site 6 in the scheme of Fig. 3). In either case one α-subunit has to contain two pockets for an adenosine moiety. If, on the other hand, the proposal of

Table 2
Effect of PPi and FSBA on nucleotide content of F_1

Inc. conditions (pH = 7.5)	Inc. time (min)	ATP/F_1 (mol/mol)	ADP/F_1 (mol/mol)	AXP/F_1 (mol/mol)
TEG	0	1.09	1.84	2.93
TEG	120	1.12	1.89	3.01
TEG + FSBA	120	0.99	1.65	2.64
TEG + 100 mM PPi	120	1.42	0.80	2.22
TEG + 100 mM PPi + FSBA	120	1.45	0.46	1.91
TMG	0	1.45	2.37	3.82
TMG	120	1.12	2.62	3.74
TMG + FSBA	120	1.18	2.27	3.43
TMG + 100 mM PPi	120	1.26	0.62	1.87
TMG + 100 mM PPi + FSBA	120	1.44	0.53	1.97

F_1(3) in TEG buffer and F_1(4) in TMG buffer were incubated at pH 7.5 for 2 h with 0.8 mm FSBA. The incubations were stopped by column centrifugation to remove free nucleotides and the bulk of the PPi and FSBA. Protein and bound nucleotides were determined as described in Section 2.

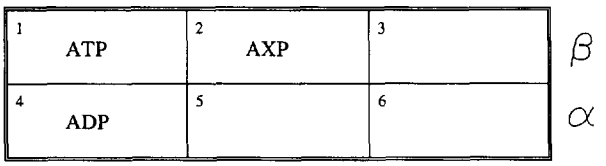

Fig. 3. Schematic representation of the nucleotide binding sites of F_1. The upper row represents the nucleotide binding sites on the β-subunits, the lower row the binding sites on the α-subunits. 1 and 4, sites containing tightly bound, non-exchangeable adenine nucleotides; 2, high-affinity catalytic site; 3, low-affinity catalytic site; 5, regulatory site with high affinity for ADP in the presence of Mg^{2+}; 6, regulatory site with low affinity for ATP.

Cross for the occupancy of nucleotide binding sites in $F_1(4)$ is assumed to be correct and all three α-sites contain ADP [30], both FSBA molecules are bound at a site that already contains ADP.

3.3. Effect of pyrophosphate on the inhibition of F_1-ATPase by FSBA

In a recent paper [31] Jault et al. have shown that the formerly reported [32] stimulatory effect of pyrophosphate on the binding of FSBA is not real: FSBA just abolished the activity-stimulating effect of pyrophosphate, but the binding of FSBA was not changed. The stimulatory effect of pyrophosphate on the ATPase activity [31] of nucleotide-depleted F_1 was already maximal at a concentration of 1 mM. Under our conditions (our F_1 preparation and the presence of bicarbonate in the assay medium), the stimulatory effect of 1 mM pyrophosphate on the ATPase activity is very low, maximally about 15–20%. This concentration of pyrophosphate also has no effect on the binding of FSBA, nor on the nucleotide content of F_1. At much higher concentrations of pyrophosphate, however, the binding of FSBA is much faster than in its absence, and nearly full inhibition is reached after a much shorter incubation period. After 30 min in the presence of 100 mM pyrophosphate and 0.8 mM FSBA 1.5 mol/mol F_1 is bound instead of 0.6 in the absence of pyrophosphate. When, however, more than one SBA/mol F_1 is bound, the relation between the level of binding and the level of residual activity is not changed by pyrophosphate (see below). Pyrophosphate accelerates the binding of FSBA and the stimulatory effect on the activity disappears upon binding of FSBA. So in a plot of inhibition versus occupation we may combine data obtained in the presence of pyrophosphate with those obtained in its absence, as has been done in Fig. 2. This figure is quite similar to the data presented by Allison's group with the difference that the latter authors only included binding to β-subunits [14].

Pyrophosphate has been shown to bind to both catalytic and non-catalytic sites [4,33,34] and Jault et al. [31] show the presence of 3 mol of pyrophosphate per mol F_1 when nucleotide-depleted F_1 is incubated with 1 mM pyrophosphate. Since the activity of our enzyme is not increased by this concentration of pyrophosphate and also the nucleotide content is not changed, we may assume that the three sites that bind pyrophosphate, at these concentrations in the nucleotide-free preparation of Jault et al., contain adenine nucleotides in our preparations. Upon incubation with 100 mM pyrophosphate, however, the nucleotide content decreases to 2 mol/mol F_1 (Table 2). The initial level of nucleotides (3 or 4) does not affect this value. From the scheme in Fig. 3 it is clear that the nucleotides at sites 2 and 5 (5 is occupied only in $F_1(4)$) are removed. The removal of ADP from the catalytic site is clear from the initial kinetics of the ATPase reaction (not shown): the lag phase at the start, after the addition of ATP, has disappeared due to the removal of ADP from the catalytic site [35]. The treatment with 100 mM pyrophosphate results in the stimulation of the ATPase activity. The k_{off} of pyrophosphate at the relevant binding site is so low that the ligand remains bound during the assay of the ATPase activity, just like ADP when bound at the high-affinity regulatory site (site 5 in our model, site 6 in the model of Cross [30]). At a concentration of 100 mM the pyrophosphate apparently replaces the ADP at this site, but instead of inducing inhibition [2], it induces an activation. It is likely that, during the incubation with FSBA at 100 mM pyrophosphate, sites 3 and 6 are also occupied with pyrophosphate, but these sites will lose the ligand after column centrifugation and/or dilution in the assay mix. The stimulation of the covalent binding of FSBA by pyrophosphate largely disappears after a column centrifugation step, so the increased rate of modification by FSBA is due to binding of pyrophosphate at an additional low-affinity site (site 6 in the model), while stimulation of ATPase activity is due to binding at site 5. Since 100 mM pyrophosphate does not

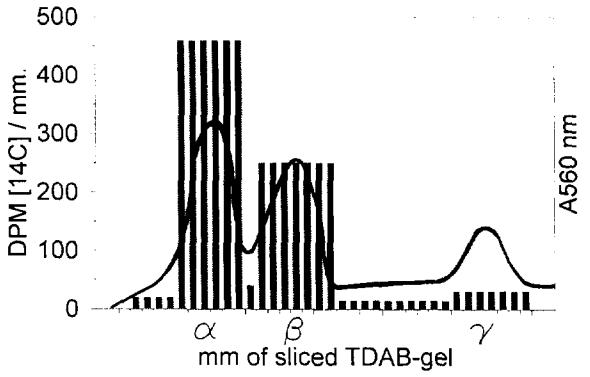

Fig. 4. Determination of the distribution of bound radioactivity over α- and β-subunits. Incubations of F_1 with 0.8 mM [^{14}C]FSBA were stopped by the addition of cold FSBA, followed by repeated column centrifugation. The samples were subjected to TDAB gel electrophoresis and the gel was stained with Coomassie brilliant Blue. After scanning with a densitometer the gel was sliced and the radioactivity of single or multiple slices measured as described in Section 2.

remove the nucleotides from sites 1 and 4, and at least 4 FSBA molecules bind covalently in the presence of pyrophosphate (see below), we have to conclude that probably both sites 5 and 6 can bind two mol of FSBA, while site 4 may also be able to bind one FSBA in addition to the tightly bound ADP. According to the model of Cross [30], sites 4 and 5 contain the two non-exchangeable adenine nucleotides, and in that model the specific binding of 4 mol of FSBA at non-catalytic sites is not possible without concomitant binding of FSBA and ADP. It may be noted that in the model of Cross no low-affinity regulatory site exists.

3.4. Localisation of bound SBA

After incubation of F_1 with FSBA TDAB gel electrophoresis (Fig. 4) showed that both α- and β-subunits were labeled. Depending on the type of incubation medium and the length of the incubation, the α- versus β-ratio varies between 3.5 and 1. This result is principally not much different from the results of Allison's group [14], but this group dismissed the binding to α-subunits as aspecific and concluded that only binding to β-subunits was specific and inhibitory. When, however, one SBA was bound in our experiments, the ITPase activity was more than 80% inhibited, so at least 80% of the bound SBA was inhibitory. When one SBA was bound, the α/β-ratio of the labeling was minimally 1.5 and we have to conclude from this that the modification of the α-subunits is as specific and as inhibitory as that of the β-subunits. This conclusion is further strengthened by gel electrophoresis of samples incubated with FSBA in medium with sucrose and Mg^{2+} (TMS buffer). In this case the α-subunits contained much more label than the β-subunits. The α/β-ratio varied from 3.5 in the initial phase of the incubation till about 2 after 2 h (Table 1). The importance of the α-modification will also be shown in the competition experiments with AXP and other nucleotides (see below).

For the determination of the sites of modification we wanted to be sure that no hydrolysis of bound ligand occurred, so we avoided acid precipitation. Preliminary experiments had shown that much label disappeared after an acid precipitation step. After one or two column centrifugation steps the FSBA-treated enzyme was digested with trypsin for a very long time (24 h) in order to make more cuts than the normal ones (behind Lys and Arg), since under normal conditions some very large α-fragments are ob-

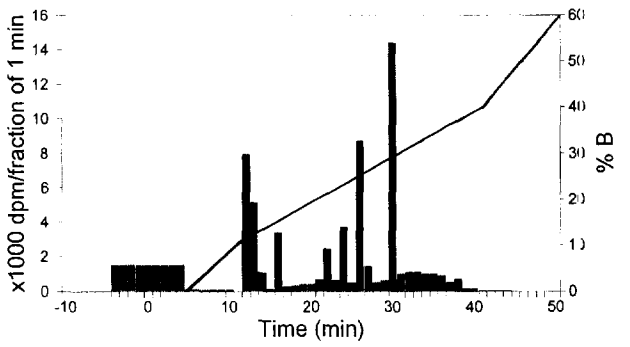

Fig. 5. HPLC of [^{14}C]SBA-labeled peptides. F_1, containing 3 mol nucleotides/mol F_1, was incubated at a concentration of 2 mg/ml for 2 h at 20°C in TMS buffer (pH 7.5) containing 0.8 mM [^{14}C]FSBA. The incubation was stopped by addition of 50 mM dithiothreitol, followed after 30 min by column centrifugation. After trypsin treatment (1 mg/20 mg F_1 for 24 h at 30°C) the sample was put on a C4-Vydac RP-HPLC column and eluted with a gradient of 0–70% B (90% acetonitril, 0.1% TFA, 9.9% water). The flow was 1.1 ml/min and fractions of 1.1 ml were collected. The absorbance at 215 and 260 nm was followed (not shown) and the radioactivity in each fraction was determined by liquid scintillation counting. The total radioactivity of bound SBA (45000 dpm, 1.73 mol SBA/mol F_1) was determined separately.

tained. The disadvantage of this procedure is that not all fragments can be predicted.

The separation of the various trypsin-treated preparations on reverse-phase HPLC resulted in a very reproducible pattern of label distribution (Fig. 5). All fractions of the reverse-phase chromatography contained several peptides and the fractions that possibly contained protein-bound radioactivity (16, 22, 24, 26, 30) were rechromatographed with a more extended gradient. The two fractions with the highest radioactivity (fractions 26 and 30) both appeared to contain two different labeled fragments, named A and B. Amino acid sequencing of all fractions showed that still several peptides were present in each fraction, making the analysis quite difficult. But since the amount of applied label was known, the peptides with the wrong intensity could be disregarded and in most cases the labeled amino acid could be detected as the absence in the sequence of a tyrosin. The labeled peptides appeared to be the peptides containing α-Tyr300 (in fraction 26A), α-Tyr244 (in fraction 30A) and β-Tyr368 (in fractions 30B and 26B). Labeling of α-Tyr244 is only seen when Mg^{2+} was present during the incubation. We also identified with ESMS in a fraction with a low amount of label, eluting just after 30B and therefore called 30C, a peptide that could not be identified by sequence analysis. This peptide appeared to be the same peptide as found in fraction 30A, containing the modified α-Tyr244, but with a blocked N-terminus. Cyclisation of the N-terminal glutamine by deamination explained the results of both the sequencing and ESMS. In agreement with the original distribution of the label over α- and β-subunits, the two labeled α-fragments contained twice as much label as the β-fragments. Because all experiments were performed at pH 7.5, no β-His427 was found. The fractions 16, 22 and 24 contained label that could be identified as originating from FSBA itself, without protein. The label in fractions 12–14 was also not protein-bound, as was verified in control experiments (not shown).

Since the analysis of the sequence data was not completely unambiguous, all fractions were analyzed with electro-spray mass spectrometry as well (Table 3). When necessary the peptides were purified on the SMART system. In most cases our analysis proved to be correct since the predicted mass of the labeled fragments corresponded with the found mass peak(s) in the ESMS spectrum. An exception was obtained with fraction 26A: no peptide could be identified with ESMS. Also after further purification no specific peptide could be identified in the fraction with ra-

Table 3
Mass of SBA-modified peptides

HPLC fraction	Sequence	Calculated MS/MS + SBA	ESMS MH_z^{z+}	Labeled AA
26 A	EAYPGDVF-....	1553.6/1987.0	n.d.	αTyr300
26 B	IVGSEH-DVAR	1245.3/1678.7	420.8	βTyr368
			560.7	
			840.3	
			1678.9 [a]	
30 A	Q-LAPY	753.8/1187.2	594.8	αTyr244
			1187.3	
			1187.0 [a]	
30 B	IMDPNIVGSEH-DVAR	1816.0/2249.4	563.6	βTyr368
			751.0	
			1125.1	
			2249.4 [a]	
30 C	blocked Q-LAPY	736.8/1170.2	1170.4	αTyr244
			1169.4 [a]	

After purification of [^{14}C]SBA-containing peptides with reverse-phase chromatography, the samples were used for sequence analysis and additionally analysed with ESMS to verify the identification of the modified peptides. The modified amino acids, not seen in the sequence analysis (-), are all Tyrosines.

[a] Mass calculated from the found masses.

n.d. not detected.

dioactivity, leaving some uncertainty in the identification of α-Tyr300 as a modified amino acid.

The primary conclusion, then, has to be that in our F_1 FSBA binds at two positions, one being not detected in the preparations from Allison's laboratory. In both positions non-catalytic sites are modified. The extra binding site may explain why we see no effect on bound nucleotides, while Allison does. We have also not observed any clear competition of added ADP with the inhibition by FSBA (see below).

3.5. Competition between FSBA and Ap_4A, ADP and ATP

Vogel and Cross [17] have shown that diadenosine tetraphosphate (Ap_4A) inhibits F_1 partly by tight binding of one mol/mol F_1. Their data clearly show that a non-catalytic site is occupied and a combined binding to a non-catalytic and a catalytic site, as proposed by the authors, can be excluded on the basis of the reported structure of F_1 [1]. The results can nicely be interpreted, however, on the basis of our finding that the α-subunits contain two pockets where nucleoside moieties can bind, each at opposite sides of the P-loop. The site of binding clearly is site 5 (according to our nomenclature, Fig. 3), the slowly exchangeable non-catalytic site with high affinity for ADP and analogues in the anti-configuration.

If we now assume that the first FSBA molecule binds at this same site 5, but initially not in the pocket where the adenosine moiety of ADP binds, but in the other one, modifying α-Tyr244 or α-Tyr300, then the Ap_4A is expected to compete with FSBA for binding at this latter site, while ADP does not. The experiment of Fig. 6 shows that the inhibition by FSBA is indeed hardly influenced by the presence of ADP, but the same holds true for AP_4A. It can be seen, however, that in the presence of both ADP and Ap_4A, the α/β labeling ratio is high (Table 4). This implies that the binding of FSBA largely occurs at the pocket of α-Tyr300 and α-Tyr244, probably both at site 5 and site 6 (at 1 mM ADP or Ap_4A site 6 will also contain ligand). So both ADP and Ap_4A induce only minor competition

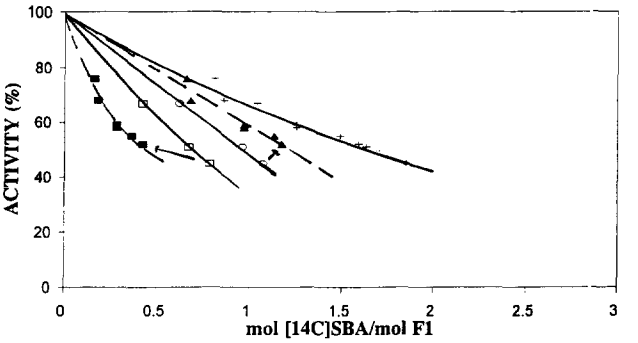

Fig. 6. Effect of ADP and Ap_4A on the labeling of F_1 with [^{14}C]FSBA. F_1 (2 mg/ml), containing 3 mol adenine nucleotides per mol F_1, was incubated for various times with 0.6 mM [^{14}C]FSBA in TMS buffer. ADP and Ap_4A, when present, had a concentration of 1 mM. The incubations were stopped with 50 mM dithiothreitol, followed by column centrifugation. Samples were put on a TDAB gel and [^{14}C] in the bands of the α- and β-subunits was determined after excision of the stained bands and solubilization. The ATPase activity was measured before the column centrifugation step. +, total [^{14}C]SBA/F_1 (all samples); ○, α-bound [^{14}C]SBA/F_1 in the absence of ADP or Ap_4A. □, β-bound [^{14}C]SBA/F_1 in the absence of ADP or Ap_4A; ▲, α-bound [^{14}C]SBA/F_1 in the presence of ADP or Ap_4A; ■, β-bound [^{14}C]SBA/F_1 in the presence of ADP or Ap_4A. Arrows indicate the change in modification of α- and β-subunits, respectively, by ADP.

Table 4
Effect of adenine nucleotides on binding of [^{14}C]FSBA and distribution of bound radioactivity

F_1 prep.	% inh. of ATPase	SBA/3α	SBA/3β	SBA/F_1	ratio α/β
F_1 (3)	55	1.07	0.78	1.85	1.37
F_1 + 1 mM ADP	45	1.13	0.36	1.49	3.11
F_1 + 1 mM ATP	45	1.35	0.40	1.75	3.36
F_1 + 50 μM Ap_4A	48	1.03	0.53	1.56	1.96
F_1 + 1 mM Ap_4A	43	1.17	0.42	1.59	2.79

F_1, containing three bound nucleotides, was incubated at a concentration of 2 mg/ml in TMG buffer (pH 7.5) for 200 min at 20°C in the presence of 0.6 mM [^{14}C]FSBA. In the incubation with ATP $MgCl_2$ was replaced by EDTA. The incubations were stopped by addition of 50 mM DTT, followed after 30 min by column centrifugation. The amount of bound [^{14}C]SBA and the distribution of radioactivity were determined from TDAB gels.

with FSBA, but the bound FSBA is now mainly located in a position in which the α-subunit is modified. The presence of ATP (in EDTA buffer) also gives similar results.

Since we see a difference in modification, but little difference in inhibition, it is obvious that modification of both α- and β-subunits is correlated with inhibition of the ATPase activity.

A further analysis of the different modifications by FSBA is given in Fig. 7. Because the extent of the relative inhibition of the ATPase activity by FSBA is different under different conditions, we plotted (Fig. 7A) the specific activity and not the relative activity versus the total binding of FSBA. The conditions were $F_1(3)$ in TES or TEG buffer in the presence or absence of 100 mM PPi. The experiment shows that after binding of 1–1.5 FSBA/F_1 the differences in specific activity largely disappear, so already the first FSBA removes the effects of the buffer and pyrophosphate. Fig. 7B,C further shows that each of the three α-subunits can be modified, while only 2 β-subunits can be modified. The highest level of binding (after 3 h incubation) is obtained in the presence of pyrophosphate *plus* sucrose.

Our interpretation of these experiments, as far as the binding of FSBA is concerned, is depicted in Fig. 8. Site 5 is the site where bound pyrophosphate enhances the ATPase activity and this activation is lost when one FSBA is bound. So this is also site 5, either the one or the other pocket. In total 5 mol FSBA can be bound to $F_1(3)$ or $F_1(2)$ without loss of bound nucleotides.

3.6. Kinetics of F_1 in the presence of bound SBA

Fig. 9 shows the effect of FSBA on the kinetics of the ATPase when the V_{max} is inhibited for about 50%. The ITPase activity of this same preparation is inhibited for 85%, indicating that nearly all F_1 molecules contain at least 1 FSBA/F_1. It is clear that

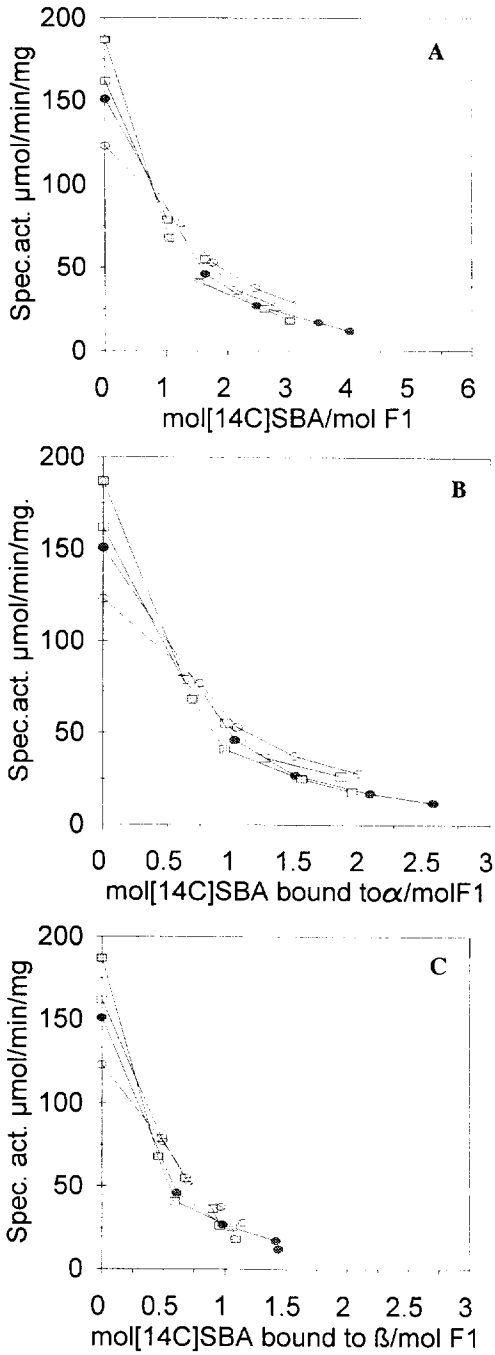

Fig. 7. Effect of modification of α- and β-subunits of F_1 by FSBA on the ATPase activity. F_1 (2 mg/ml) was incubated with 0.8 mM [^{14}C]FSBA under different conditions and samples were taken at various times. The incubations were stopped by addition of 50 mm dithiothreitol, followed by column centrifugation. ATPase activity and bound label were determined as described in Section 2. The distribution of label over α- and β-subunits was determined as in Fig. 4. The binding data are plotted as total F_1-bound label (A), label bound to α-subunits (B) and label bound to β-subunits (C). ●, incubation in TES buffer in the presence of 100 mM PPi; ■, incubation in TEG buffer in the presence of 100 mM PPi; ○, incubation in TES buffer; [□], incubation in TEG buffer.

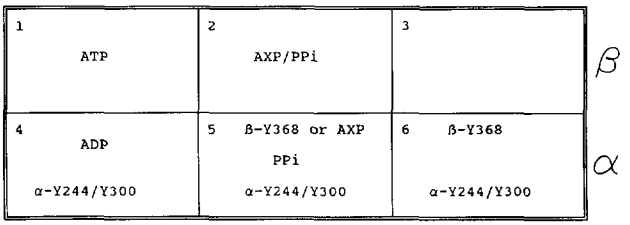

Fig. 8. Schematic representation of the FSBA binding sites on F_1. The nucleotide binding sites of the α- and β-subunits are represented as in Fig. 3. Only the α-subunits bind FSBA and each subunit contains 2 pockets for this ligand, modifying either β-Y368 or α-Y244/Y300. The pocket containing the β-Y368 can be occupied with FSBA only when no ADP is bound. PPi occupies only the P-loop and does not inhibit binding of FSBA in either pocket. After treatment of F_1 with PPi, sites 1 and 4 retain their bound adenine nucleotide and 5 sites are available for FSBA.

the effect is similar to the effect of the binding of 2-azido-ADP [16] or NAP_3-2-azido-ADP [20] to the slowly exchangeable non-catalytic site (site 5 in the scheme of Fig. 3): decrease of the V_{max} with about 50%, without a clear effect on the K_m values. However, the inhibition at low ATP concentrations is only 20% instead of 50% and this differential inhibition at high and low ATP concentrations can be interpreted as being the result of a decreased affinity of the regulatory low-affinity non-catalytic site for ATP (site 6 in the schemes of Figs. 3 and 8), resulting in a shift of the bending in the Lineweaver-Burk plot to a higher ATP concentration, according to the model for apparent cooperativity, proposed a long time ago [36,37], and more recently confirmed [6]. This model will be further explained in Section 4.

We have reported previously that the V_{max} of ITP hydrolysis is higher than that of ATP hydrolysis and the K_m values are also higher [20]. The differential effect of NAP_3-2-N_3-ADP at site 5 on ATPase and ITPase activity was explained as being due to the fact that ADP at site 5 decreased the dissociation of product from the catalytic site. For the ATP hydrolysis this decrease affects the rate of the total reaction since the dissociation of product is largely rate-controlling. For the ITPase, however, the dissociation of product is much faster, so a decrease of the dissociation rate does not significantly affect the rate of steady-state hydrolysis. Binding of one FSBA/F_1 has the opposite effect: ITP hydrolysis is much more inhibited by binding of one FSBA than the ATP hydrolysis. This can be explained when binding of FSBA decreases mainly the turnover and not the dissociation of product.

Incubation with 100 mM pyrophosphate (tight binding of pyrophosphate at site 5) stimulates the ATPase activity in the absence of bicarbonate by about 70% at high ATP concentration, while at lower ATP concentrations this stimulation is about 100% (Table 5). The K_m values from a Lineweaver-Burk plot hardly change.

Bicarbonate changes the K_m value as well as the V_{max}, linearizing the Lineweaver-Burk plot [38,39]. Bicarbonate and pyrophosphate have a largely additional effect on the ATPase activity, indicating that they activate by binding at different sites (Table 5).

Table 5
Effect of FSBA on the activation of ATPase activity by PPi and bicarbonate

10 mM HCO_3^-	1 mM ATP		10 μM ATP		SBA bound mol/molF_1
	−	+	−	+	
F_1	56	100%	49	100%	–
F_1 + 100 mM PPi	97	155	98	184	–
F_1 + FSBA 90'	30	37	43	55	±1.2
F_1-SBA + 100 mM PPi	42	51	–	–	±1.2
F_1 + 100 mM PPi + FSBA 90'	10.5	12	16	17	±2.9

F_1, containing 4 bound nucleotides, was incubated at a concentration of 2 mg/ml in TMS buffer (pH 7.5) at 20°C with 0.8 mM FSBA in the presence or absence of 100 mM PPi. The incubations were stopped by addition of 50 mM dithithreitol, followed after 30 min by a column centrifugation step. Part of the sample that was incubated with FSBA in the absence of PPi was afterwards incubated for 10 min with 100 mM PPi, followed by a column centrifugation step. The ATPase activities with 1 and 0.01 mM ATP as substrate were measured both in the presence and absence of 10 mM potassium bicarbonate. The activities of non-treated F_1 in the presence of bicarbonate is put as 100%. The amount of bound FSBA was estimated from similar incubations with radioactive ligand.

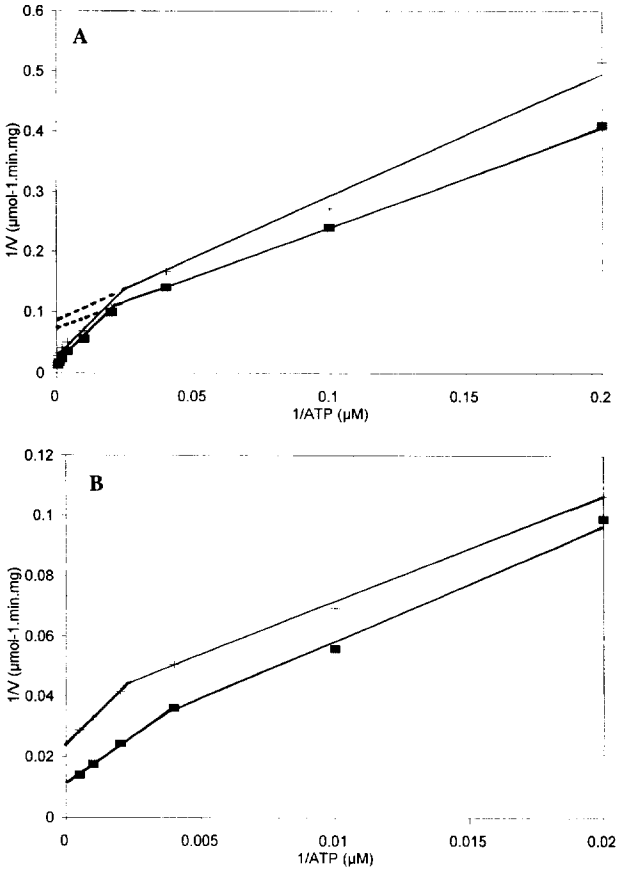

Fig. 9. Effect of bound FSBA on the kinetics of the ATPase activity of F_1. F_1 with three bound nucleotides was incubated at a concentration of 2 mg/ml with 0.8 mM FSBA in TEG buffer till about 50% inhibition of the V_{max} of the ATPase activity. The incubation was stopped as described in the legend to Fig. 1 and the ATPase activity at various substrate concentrations were measured in the absence of bicarbonate and plotted according to Lineweaver-Burk. In A the whole range of used substrate concentrations is covered, in B only the range above 50 μM. ■, control F_1; +, FSBA-treated F_1.

The activation by both bicarbonate and pyrophosphate decreases after binding of 1–1.5 mol FSBA and is absent when 2–3 mol FSBA/F_1 are bound (Table 5). The inhibitory effect of the first FSBA (in the absence of a stimulating anion) is larger at high ATP than at low ATP, indicating again that FSBA mainly affects the catalytic reaction of the enzyme. When 2 mol FSBA/mol of F_1 are bound, the turnover has been inhibited so much that it is completely rate-limiting and the addition of anions, that largely stimulate by increasing the dissociation of products [39], has little effect any more.

4. Discussion

The reaction of FSBA with our enzyme differs in several aspects from the reaction with the enzyme isolated by the group of Allison. Some results, however, are identical for both preparations:

(1) FSBA modifies only non-catalytic sites; (2) The binding of 1 FSBA inhibits the ITPase activity to a larger extent than the ATPase activity; (3) The modification of the β-subunit (at pH 7.5) is at Tyr368; (4) Binding of 1 FSBA/F_1 largely removes the activating effect of bound pyrophosphate.

Differing with the data reported by Allison and co-workers are the following results:

(1) Binding of FSBA does not induce loss of bound adenine nucleotides; (2) Binding of FSBA results in specific modification of both α- and β-subunits. The distribution of label, expressed as α/β ratio, varies from 1 to 3.5, depending on the conditions; (3) Modification of α-subunits is as specific and as inhibitory as modification of β-subunits; (4) ADP and ATP compete with FSBA only to a very limited extent. Their main effect is a shift of the modification to a higher α/β ratio; (5) The maximal number of bound SBA is 5 mol/mol F_1, three modifying an α-subunit and 2 a β-subunit.

The different aspects of the binding of FSBA reveal many characteristics of the mechanism of catalysis by F_1 and the role of the regulatory sites.

4.1. Two pockets for an adenosine moiety in both α- and β-subunits

Our data show clearly that only non-catalytic sites are modified and that our enzyme apparently binds FSBA in a region of the α-subunit (Tyr300 and Tyr244, the latter is seen only in the presence of Mg^{2+}) that is not seen in the crystal structure as a pocket for adenosine [1]. At the other hand, etheno-FSBA also modifies α-Tyr244 [40] and the fluorescence of Trp257 (in the enzyme from *Saccharomyces Pombe* [41]) is sensitive to binding of nucleotides. Although it remains possible that Allison and co-workers missed the specific labeling of α-Tyr244 as a consequence of the fact that with trypsin a very large fragment is obtained if not additional cuts are produced upon very long incubation), we may assume that indeed the additional binding pocket is not

present in the enzyme as used by Allison and co-workers. Allison and co-workers mainly use nucleotide-depleted enzyme that has been treated with 50% glycerol. The same holds for the enzyme used by Abrahams et al. [1]. We have concluded previously [9] that the glycerol-treated enzyme has different properties, such as a lower affinity of the tightly bound nucleotides. Since glycerol decreases the volume of proteins by extraction of water [42], it is quite possible that the pocket near α-Tyr 300 and α-Tyr244 disappears in 50% glycerol. It is worthwhile mentioning that the specific labeling of α-Tyr244 by etheno-FSBA is obtained with enzyme that was not treated with glycerol. The group of Gautheron also measured binding of 5–6 mol FSBA/mol F_1 in the absence of glycerol treatment [43]. The interpretation of these latter authors, however, that both catalytic and non-catalytic sites were modified, has to be corrected on the basis of our results.

The second pocket for the binding of adenosine analogues has to be located near the P-loop region. The Tyr300 is close to the Lys273 in the structure, and the equivalent of the latter in CF_1 (Lys266) is labeled with pyridoxalphosphate [44]. We also may assume that in Cross's enzyme (nucleotide-depleted and glycerol-treated F_1) both pockets of one α-subunit can be occupied with a tightly-binding Ap_4A, although in our enzyme Ap_4A apparently does not bind to two pockets at the same time. This latter can be concluded from the affinity of Ap_4A (which is lower than measured by Vogel and Cross [17]), and from the fact that it does not compete with FSBA for modification of the α-subunit and behaves exactly like ADP. The distance between the second pocket and the P-loop may be different in the two enzyme preparations. Further arguments for a location of the second pocket near the P-loop can be derived from the structural homology between α- and β-subunits. We know that 8-azido-ATP modifies β-Tyr311 when bound at a catalytic site, just as NbfCl. The crystal structure of F_1 does not provide the possibility of modification of β-Tyr311 by a nucleotide bound at the detected binding site (J. Walker, personal communication), and we may speculate that the absence of a detectable site near β-Tyr311 is also due to the effect of glycerol treatment. 8-azido-ATP certainly binds to the P-loop, since it inhibits further binding of nucleotides at the same subunit, so both pockets in the β-subunits are close to the P-loop. The presence of two pockets in the β-subunit, both connected with the P-loop, is also evident from the binding of azidonitrophenylpyrophosphate: modification of either Tyr345 or Tyr311 upon binding to the β-subunit has been reported [34]. Also the data obtained by Wu et al. [45] show that the second phosphate group of ADP is localized quite close to β-Tyr311, the site that is modified by NbfCl. Both α- and β-subunits of our enzyme, therefore, contain 2 pockets for adenosine residues, differently oriented towards the P-loop region and each subunit can bind two adenosine analogues like FSBA or one nucleotide di- or triphosphate *plus* one adenosine analogue. Only one nucleotide di- or triphosphate can be bound, however, because of the participation of the P-loop in the binding, although with two possible positions for the adenosine moiety.

Since with our enzyme ADP does not really compete with FSBA for binding to F_1 and we always see a α/β labelling ratio higher than 1, FSBA binds more easily at the pocket that is not preferred by ADP. Some modification of the adenosine is apparently required to switch preference of the adenosine moiety from the lower pocket to the upper pocket. FSBA is not a real analogue of ADP or ATP. Its binding properties are probably partly due to the sulfonylbenzoyl moiety which forms a sandwich structure with the adenosine moiety of the molecule [46]. A similar conclusion has to be drawn for the two pockets of the β-subunits: 8-azido-ATP prefers the newly detected pocket and 2-azido-ATP prefers the pocket described by Abrahams et al. [1]. Nucleotides in the anti-configuration apparently prefer the one pocket and the nucleotides in the syn-configuration the other one. Nbf binds in the same pocket where also the adenosine moiety of the 8-azido-analogues binds, but in the presence of fluoroaluminate 8-azido-ADP shifts its preferred localisation, now modifying Tyr345 [47].

4.2. *Which subunit is labeled first by FSBA?*

We have previously shown that site 5 binds ADP, 2-azido-ADP, NAP_3ADP and $2\text{-}N_3\text{-}NAP_3ADP$ with high affinity, inducing partial inhibition of the ATPase activity [16]. Site 6 has a much lower affinity for these nucleotides and is more easily modified by

8-azido-ATP (it is not known which amino acids are modified). The inhibition by ADP at site 5 is due to a decrease of V_{max}, without a significant change in cooperativity and K_m values [20]. Binding of 1 FSBA/F_1 causes about 50% inhibition, and the kinetics of Fig. 9 show that only the V_{max} is really changed, while the K_m values and the cooperativity are only slightly affected. Also incubation with high pyrophosphate, followed by column centrifugation or dilution in the assay mix, induces a similar change of the kinetics, although in that case the V_{max} is increased instead of decreased. Both ligands, therefore, seem to be bound at the same site, the high-affinity regulatory site for ADP, site 5 in the model. The disappearance of the effect of preincubation with pyrophosphate by 1–1.5 FSBA/mol F_1 (Table 5, see also Ref. [31]) confirms the proposal that the first FSBA binds to site 5.

4.3. Differential inhibition by FSBA of ATPase and ITPase activity

The binding of one FSBA per F_1 inhibits the maximal ATPase activity less than the maximal ITPase activity. This effect is at difference with the effect of ADP and analogues in the anticonfiguration: binding of one of these ligands affects the V_{max} of ATP hydrolysis much more than the V_{max} of ITP hydrolysis [20]. The cooperativity and the K_m values of the ATPase activity are very little affected. The conclusion was drawn previously [20] that binding of ADP or an analogue affected the rate of dissociation of the product. This step is supposed to be largely rate-limiting for the ATPase activity, but not for the ITPase activity. For the ITPase activity the turnover at the catalytic site is more rate-limiting, in agreement with the finding that the V_{max} of the ITPase is significantly higher than that of the ATPase activity. Binding of FSBA apparently has the opposite effect from that of ADP: it mainly decreases the turnover, not the dissociation of product. Therefore the ATPase activity is less affected than the ITPase activity.

4.4. The effect of FSBA on the stimulatory effects of anions

The effect of bicarbonate is quite different from that of pyrophosphate and both effects are largely additional. Bicarbonate changes not only the V_{max}, but also the cooperativity. Studies with the yeast enzyme led both Recktenwald and Hess and ourselves to the conclusion that F_1 contains a regulatory site that can bind various anions, apart from ATP and ADP, thereby modulating the K_m of the catalytic sites [36,37]. Bicarbonate and bisulphite induce the low-K_m mode, while sulphate and ATP induce the high-K_m mode. This effect of ATP is responsible for the fact that at low ATP concentrations (1–40 μM, below the K_d) the K_m is low, while at high ATP concentrations this site becomes occupied with ATP, resulting in a high K_m. The V_{max} is not influenced by binding of a ligand at this site. With the bovine enzyme, however, the V_{max} is also affected (increased) by bisulphite and bicarbonate. This additional effect can be interpreted as the consequence of a more rapid dissociation of product from the catalytic site, induced by these anions [38,39]. We have to assume, then, that in the absence of activating anions, the V_{max} of ATP hydrolysis by bovine F_1 is limited by the rate of dissociation of product, what should not be the case for the yeast enzyme. This agrees with the finding that the V_{max} of the yeast enzyme is about equal to the V_{max} of the bovine enzyme in the presence of an activating anion. The same holds for the ITP hydrolysis by the bovine enzyme: also in this case the V_{max} is larger and the activity is less stimulated by anions.

When bicarbonate is bound at this low-affinity regulatory site, site 6, it prevents the binding of ATP and the induction of the high K_m mode of the enzyme. The resulting linearization of the Lineweaver-Burk plot by bicarbonate is still largely present when 1 FSBA/F_1 is bound. The additional activating effect of bicarbonate (due to increased dissociation of product) is of course partly lost, since the decrease of the turnover at the catalytic site by FSBA has made the dissociation of product less rate-limiting.

When more than 2 FSBA/F_1 are bound, bicarbonate has lost nearly all effect (Table 5).

We propose, then, that the high-affinity site for pyrophosphate (site 5) is the first site of modification by FSBA in our preparations, followed by the low-affinity site (the bicarbonate site, site 6). It was shown that binding of pyrophosphate at site 6 (incubation in the presence of 100 mM pyrophosphate) increases the

rate of binding of FSBA. This implies a conformational change of the binding site of FSBA, in agreement with the fact that binding of a ligand to site 6 also affects the conformation of the catalytic sites. We have not investigated whether bicarbonate also increases the rate of binding of FSBA.

It is clear that site 4 also has a pocket available for binding of FSBA. In our experiments it is probably modified only in the presence of pyrophosphate and when both pockets of sites 5 and 6 are already largely occupied. Whether this modification contributes to the inhibition cannot be concluded. Since we have no indications that site 4 is involved in catalysis, not even in a regulatory role, it is likely that the inhibition seen during binding of FSBA is completely due to binding at sites 5 and 6.

The model of Jault et al. [31] explains negative cooperativity by entrapment of product MgADP at the catalytic sites at low ATP concentrations, which does not occur at low temperatures. Such a model, however, is a model for positive cooperativity, not for negative cooperativity. Our model states that ATP by binding to a non-catalytic site at increasing concentrations of ATP, increases the K_m of the catalytic sites. Not only was this model verified by the finding that upon covalent binding of 8-nitreno-AT(D)P at this non-catalytic site the high K_m value is also seen at low ATP concentrations, it also explains why Jault at al. find that at low temperatures, when the cooperativity has disappeared, the low K_m value is still present and not the high K_m value. The conclusion of Jault and Allison, however, that ATP regulates activity by binding at a non-catalytic site with low affinity [32], is in agreement with our findings. This conclusion, however, implies that only two sites are available for catalysis.

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