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Covalent modification of the non-catalytic sites of the H^+-ATPase from chloroplasts with 2-azido-[α-^{32}P]ATP and its effect on ATP synthesis and ATP hydrolysis

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Abstract

Incubation of the isolated H^+-ATPase from chloroplasts, CF_0F_1, with 2-azido-[α-^{32}P]ATP leads to the binding of this nucleotide to different sites. These sites were identified after removal of free nucleotides, UV-irradiation and trypsin treatment by separation of the tryptic peptides by ion exchange chromatography. The nitreno-AMP, nitreno-ADP and nitreno-ATP peptides were further separated on a reversed phase column, the main fractions were subjected to amino acid sequence analysis and the derivatized tyrosines were used to distinguish between catalytic (β-Tyr362) and non-catalytic (β-Tyr385) sites. Several incubation procedures were developed which allow a selective occupation of each of the three non-catalytic sites. The non-catalytic site with the highest dissociation constant (site 6) becomes half maximally filled at 50 μM 2-azido-[α-^{32}P]ATP, that with the intermediate dissociation constant (site 5) at 2 μM. The ATP at the site with the lowest dissociation constant had to be hydrolyzed first to ADP before a replacement by 2-azido-[α-^{32}P]ATP was possible. CF_0F_1 with non-covalently bound 2-azido-[α-^{32}P]ATP and after covalent derivatization was reconstituted into liposomes and the rates of ATP synthesis as well as ATP hydrolysis were measured after energization of the proteoliposomes by $\Delta pH/\Delta\varphi$. Non-covalent binding of 2-azido-ATP to any of the three non-catalytic sites does not influence ATP synthesis and ATP hydrolysis, whereas covalent derivatization of any of the three sites inhibits both, the degree being proportional to the degree of derivatization. Extrapolation to complete inhibition indicates that derivatization of one site (either 4 or 5 or 6) is sufficient to block completely multi-site catalysis. The rates of ATP synthesis and ATP hydrolysis were measured as a function of the ADP and ATP concentration from uni-site to multi-site conditions with covalently derivatized and non-derivatized CF_0F_1. Uni-site ATP synthesis and ATP hydrolysis were not inhibited by covalent derivatization of any of the non-catalytic sites, whereas multi-site catalysis is inhibited. These results indicate that multi-site catalysis requires some flexibility between β- and α-subunits which is abolished by covalent derivatization of β-Tyr385 with a 2-nitreno-adenine nucleotide. Conformational changes connected with energy transduction between the F_0-part and the F_1-part are either not required for uni-site ATP synthesis or they are not impaired by the derivatization of any of the three β-Tyr385. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: H^+-ATPase; CF_0F_1; Nucleotide binding; Uni-site catalysis; 2-Azido-nucleotide; Non-catalytic site

Abbreviations: EDTA, ethylenediaminetetraacetic acid; CF_0F_1, proton translocating H^+-ATPase from chloroplasts; HPLC, high pressure liquid chromatography; AXP, AMP or ADP or ATP; TF_0F_1, proton translocating H^+-ATPase from the thermophilic bacterium PS3; CF_1, EF_1, MF_1, hydrophilic parts of the H^+-ATPases from chloroplasts, *Escherichia coli* and mitochondria

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1. Introduction

Membrane-bound F-type H^+-ATPases catalyze ATP synthesis and ATP hydrolysis coupled to transmembrane proton transport in bacteria, mitochondria and chloroplasts [1]. They consist of two domains, a membrane-integrated F_0-part involved in proton transport, and a hydrophilic F_1-part containing nucleotide and phosphate binding sites. The F_1-parts contain six nucleotide binding sites and X-ray analysis of the mitochondrial F_1-part showed that three are located on α-subunits, and three on the β-subunits [2]. Studies in different groups have been carried out to distinguish between catalytic and non-catalytic sites (for review see [3,4]). One criterion used for this distinction was the rapid exchange of nucleotides bound at catalytic sites during turnover of the enzyme, whereas those at non-catalytic sites remain bound or exchange so slowly that they are not competent for catalysis. Using this approach, it has been shown with 2-azido-[α-^{32}P]AD(T)P that two different β-tyrosines were covalently derivatized after UV-irradiation, when either catalytic or non-catalytic sites were occupied [5–7]. For CF_1 these tyrosines are β-Tyr362 (catalytic) and β-Tyr385 (non-catalytic). This result was fully understood only after elucidation of the structure of MF_1 near atomic resolution, β-Tyr362 being located in the binding site on the β-subunit and β-Tyr385 being located on a stretch of the β-subunit which extends into the nucleotide binding site of the neighboring α-subunit (CF_1 numbering).

The effect of covalent derivatization of different sites by 2-nitreno-nucleotides on catalysis was investigated mainly with the experimentally more amenable F_1-parts from mitochondria, chloroplast and bacteria [5,7–11]. Corresponding investigations with the holoenzymes have been carried out with submitochondria [12,13], with TF_0F_1 (proton translocating H^+-ATPase from the thermophilic bacterium PS3) [14,15] and recently with CF_0F_1 (proton translocating H^+-ATPase from chloroplasts) reconstituted into liposomes [16,17]. Stimulated by the interest in the binding change mechanism and its prediction of the effect of blocking of one catalytic site on the turnover of the enzyme, mainly the effect of derivatization of catalytic sites was investigated.

There are only few reports on the effect of occupation of non-catalytic sites on catalysis and the results are controversial. In heat-treated CF_1, three non-catalytic sites with different properties were identified, occupation of one site by ADP has no effect on ATP hydrolysis, whereas another site must be occupied by ATP in order to observe ATP hydrolysis [18]. In EF_1 all three non-catalytic sites show the same binding affinity for ATP and for ADP and their occupancy is not required for ATP hydrolysis [19]. For MF_1, ATP hydrolysis without ATP bound at a non-catalytic site was reported [20]. Partial inhibition of ATP hydrolysis was observed when 2-azido-ADP (or ADP) was bound to the medium-affinity non-catalytic site [21,22]. After coreconstitution of TF_0F_1 and bacteriorhodopsin into liposomes, the light-induced rate of ATP synthesis was increased when ATP was bound at a non-catalytic site [14].

Purified CF_0F_1 can be reconstituted into liposomes and, after energization of the proteoliposomes by $\Delta pH/\Delta\varphi$ jump, the rate of ATP synthesis is almost as high as in thylakoid membranes [23]. Recently, we have used this system to occupy selectively the different catalytic sites and investigated the effect of covalent derivatization of each site on the rate of ATP synthesis and ATP hydrolysis from uni-site to multi-site conditions. It was found that derivatization of site 1 blocks uni-site and multi-site catalysis, whereas blocking of either catalytic site 2 or 3 blocks only multi-site catalysis [16,17]. The role of the non-catalytic nucleotide binding sites and their occupation in the reaction cycle is not known yet. Therefore, in this work we developed procedures which allow a selective occupation of each of the three non-catalytic sites in isolated inactive CF_0F_1. To distinguish the three sites on the α-subunits they are called 4, 5 and 6 in order of decreasing affinity to ATP. The effect of covalent derivatization of each site on ATP synthesis and ATP hydrolysis was investigated.

We used CF_0F_1 for these investigations because this enzyme has several advantages. First, the effect of covalent derivatization on the complete reaction cycle can be studied. Second, the isolated CF_0F_1 is catalytically inactive. Therefore, enzyme species with well-defined nucleotide occupation patterns can be prepared, and this distribution does not change significantly during the handling of the enzyme. Third,

the rate of ATP synthesis is used for assaying the catalytic activity, i.e. we measure only enzymes which are functionally reconstituted into the membrane. For interpretation of the data, we have to assume that the reconstitution efficiency is the same for derivatized and non-derivatized CF_0F_1. Forth, both uni-site and multi-site catalysis can easily be measured.

2. Materials and methods

2.1. Purification of CF_0F_1

The H^+-ATPase from chloroplasts was isolated and purified as described by Fromme et al. [24], except that no nucleotides were present in the sucrose-density gradient centrifugation. The enzyme was obtained in a solution containing 300 g/l sucrose, 30 mM Tris (tris(hydroxymethyl)aminomethane)–succinate, pH 6.5, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 2 g/l Triton X-100 (Sigma) and 1 g/l asolectin (Fluka) with a protein concentration of about 2–5 g/l. It was rapidly frozen and stored in liquid nitrogen. Protein concentration was measured with the Bio-Rad protein assay using bovine serum albumin as a standard. A molecular mass of 550 kDa for the CF_0F_1 was assumed. After three consecutive passages through Sephadex G-50 centrifugation columns [25] equilibrated in buffer A (20 mM Na-succinate, 20 mM Na-Tricine (*N*-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]-glycin), pH 8.0, 80 mM NaCl), the enzyme contains approximately two bound ATP and one bound ADP per CF_0F_1. All the incubations described in this work were carried out at room temperature in buffer A supplemented with either EDTA or $MgCl_2$ and 2-azido-[α-^{32}P]ATP.

2.2. Synthesis of 2-azido-[α-^{32}P]ATP

The synthesis of 2-azido-[α-^{32}P]ATP was performed as described by Boulay et al. [26] with modifications introduced by Hartog et al. [27]. The specific activity after synthesis was 900–1000 dpm/pmol. The 2-azido-[α-^{32}P]ATP contained maximally 0.5% 2-azido-[α-^{32}P]ADP.

2.3. Incubation procedures, photoaffinity labeling and identification of the modified sites

Incubation with 2-azido-[α-^{32}P]ATP, photoaffinity labeling and identification of the modified binding site was carried out as described in Possmayer et al. [16]. The reconstitution of CF_0F_1 into liposomes was performed as described by Possmayer and Gräber [28]. The lipid concentration of the proteoliposome suspension was 10 mM. The concentration of CF_0F_1 was 80 nM for measurements under multi-site conditions, and 800 nM for measurements under uni- and bi-site conditions. After reconstitution the enzyme was reduced by incubation with 50 mM dithiothreitol for 2 h at room temperature. ATP synthesis and ATP hydrolysis were measured after a $\Delta pH/\Delta\varphi$ jump as described in Possmayer et al. [16].

3. Results

3.1. Experimental protocol

After isolation and purification of CF_0F_1, free and loosely bound nucleotides were removed by three consecutive passages through centrifugation columns. After this treatment, the enzyme contains about one ADP and two ATPs per CF_0F_1, the ADP being bound at a β-subunit and the two ATP molecules at α-subunits [16]. The experimental protocol for measuring the effect of covalent derivatization was the same as described in detail by Possmayer et al. [16]. In short, the nucleotide binding sites were specifically occupied with 2-azido-[α-^{32}P]-ATP using different incubation procedures. After incubation, free nucleotides were removed by three consecutive centrifugation columns, and protein concentration, bound 2-azido-[α-^{32}P]nucleotides and endogenous nucleotides were measured. Then, a part of the enzyme preparation with the bound 2-azido-[α-^{32}P]ATP was irradiated with UV-light for covalent modification and digested by trypsin. The labeled peptides were separated by high pressure liquid chromatography (HPLC) and the amino acid sequences of the labeled peptides were determined. In parallel, the irradiated and, separately, the non-irra-

diated enzymes were reconstituted into liposomes. Again, the endogenous nucleotide- and 2-azido-[α-^{32}P]ATP content were determined. Finally, the rates of ATP synthesis and ATP hydrolysis were measured as a function of the ADP or ATP concentration from uni-site to multi-site conditions. The same measurements were carried out with the non-irradiated part of CF_0F_1, which contained non-covalently bound 2-azido-[α-^{32}P]ATP. Additionally, the release of bound 2-azido-[α-^{32}P]ATP was measured after energization in the presence as well as in the absence of a catalytic turnover.

As a control, isolated CF_0F_1 without incubation with 2-azido-[α-^{32}P]ATP was either irradiated with UV-light or not irradiated. Again, both enzymes were reconstituted separately into liposomes and the nucleotide content, ATP synthesis and ATP hydrolysis were measured. As a second control, isolated CF_0F_1 was incubated with ATP in the same way as with 2-azido-[α-^{32}P]ATP and then reconstituted into liposomes. None of these treatments changes the results of the activity measurements.

3.2. Binding of 2-azido-[α-^{32}P]ATP to non-catalytic site 5 or 6

CF_0F_1 containing one bound ADP and two bound ATP was incubated with 2-azido-[α-^{32}P]ATP. After covalent derivatization, the enzyme was subjected to sodium dodecyl sulfate gel electrophoresis. Autoradiography of the gel revealed exclusive labeling of the β-subunit in accordance with earlier observations [29]. The protein was digested by trypsin and the resulting peptides were separated by HPLC. Amino

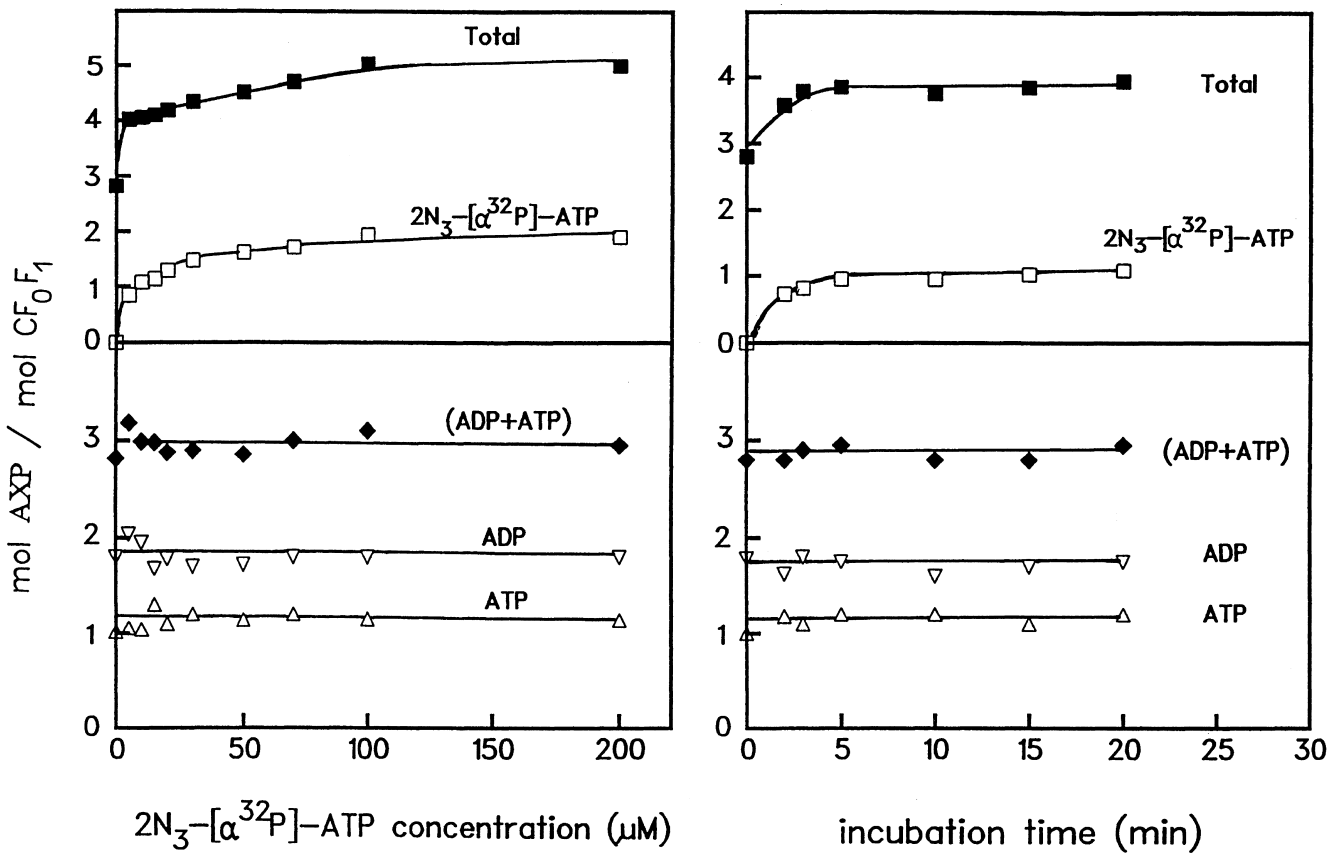

Fig. 1. Binding of 2-azido-[α-^{32}P]ATP to CF_0F_1 and endogenous nucleotide content. Isolated CF_0F_1 was pretreated in the following way: the enzyme (5 μM) was incubated with 2 mM EDTA and 100 μM ADP for 1.5 h. Free nucleotides were removed by three consecutive passages through centrifugation columns. Thereafter, the enzyme contained about two ADPs and one ATP bound. Left: the pretreated enzyme was subjected to a second incubation with different concentrations of 2-azido-[α-^{32}P]ATP in the presence of 2 mM $MgCl_2$ for 5 min. Right: incubation of the pretreated enzyme with 2 mM $MgCl_2$ and 20 μM 2-azido-[α-^{32}P]ATP for different times.

acid sequence analysis revealed that Tyr362 and Tyr385 were covalently derivatized, i.e. catalytic and non-catalytic binding sites were occupied during incubation. We tried a variety of incubation conditions, however, amino acid sequence analysis always indicated labeling of both catalytic and non-catalytic sites. During these extensive surveys, we found that CF_0F_1 which contained one ATP at a non-catalytic site and two ADPs at catalytic sites can be specifically labeled with 2-azido-[α-^{32}P]ATP. To obtain this occupation pattern, CF_0F_1 containing one ADP and two ATPs was incubated with 100 μM ADP in the presence of 2 mM EDTA for 1.5 h at room temperature. Thereafter, free and loosely bound nucleotides were removed by three consecutive passages through Sephadex G-50 centrifugation columns and bound nucleotides were determined. After this treatment, CF_0F_1 contains one ATP at a non-catalytic site and two ADPs at catalytic sites [16]. All the following incubations and measurements were carried out starting with CF_0F_1 with this nucleotide occupation pattern ('pretreated enzyme').

Pretreated CF_0F_1 was incubated for 5 min with different concentrations of 2-azido-[α-^{32}P]ATP in the presence of 2 mM $MgCl_2$ and free and loosely bound nucleotides were removed by column centrifugations. Fig. 1, left, shows the resulting binding of 2-azido-[α-^{32}P]ATP and the endogenous nucleotide content with increasing ligand concentration. The data indicate that one 2-azido-[α-^{32}P]ATP per CF_0F_1 is bound at a 10–20 μM concentration of the ligand, whereas binding of a second molecule of 2-azido-[α-^{32}P]ATP requires concentrations above 100 μM. The endogenous nucleotide content did not change during these incubations. This treatment of CF_0F_1 leading to occupation of two sites is shown schematically in Fig. 2 and is called procedure 6 (procedures 1–5 are used for binding to catalytic sites, Possmayer et al., [16,17]).

In order to occupy only one binding site, pretreated CF_0F_1 was incubated for different times with 20 μM 2-azido-[α-^{32}P]ATP in the presence of 2 mM $MgCl_2$. The result is shown in Fig. 1, right, and indicates that, at incubation times longer than 2 min, one 2-azido-[α-^{32}P]ATP is bound per CF_0F_1. This result does not change with increasing incubation time. Endogenous nucleotides, as before, did not change during these conditions. This treatment

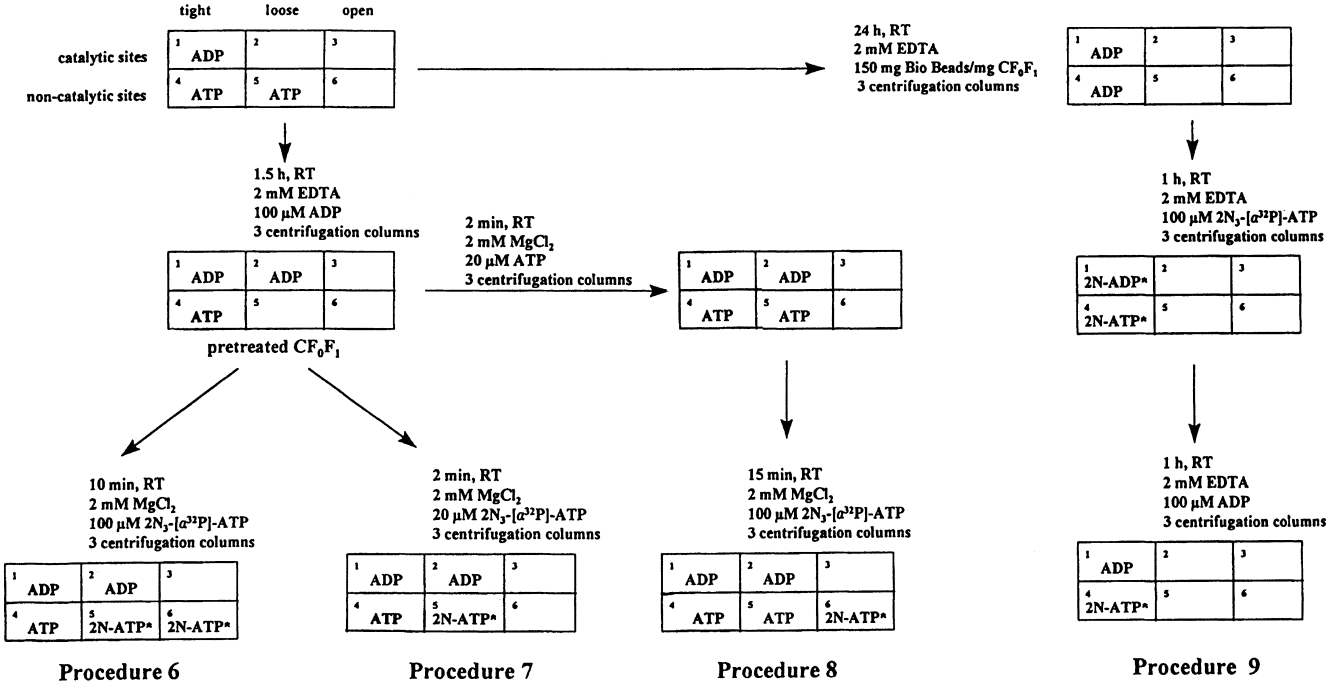

Fig. 2. Scheme of the procedures for specific labeling of the different non-catalytic sites. For didactic reasons the resulting nucleotide occupation patterns are already shown in this scheme, although the arguments for this assignment are given in Section 4.

Table 1
Characterization of the isolated CF_0F_1 and of CF_0F_1 reconstituted into liposomes and analysis of the covalently bound nucleotides after UV-irradiation

Incubation conditions	Isolated CF_0F_1				CF_0F_1 reconstituted into liposomes				Analysis of irradiated CF_0F_1			
	ATP_{bound}	ADP_{bound}	ADP_{free}	2-N_3-[α-^{32}P]AXP	ATP_{bound}	ADP_{bound}	ADP_{free}	2-N_3-[α-^{32}P]AXP	covalently labeled CF_0F		AXP labeled peptide (%)	labeled Tyr
Buffer A, 30 min	1.75	1.3	0.015	0	0.70	1.45	0.45	0	–		–	–
Pretreatment	1.1	2.0	0.01	0	nd	nd	nd	nd				
Procedure 6	1.1	1.9	0.05	2.0	1.0	1.5	0.5	2.0	1.0	-ADP	< 15%	Y-385
										-ATP	85%	Y-385
Procedure 7	1.0	1.9	0.03	1.0	0.9	1.6	0.4	0.9	0.5	-ADP	< 20%	Y-385
										-ATP	> 80%	Y-385
Procedure 8	1.9	2.0	0.07	1.1	1.2	1.9	0.6	1.1	0.5	-ADP	< 10%	Y-385
										-ATP	> 90%	Y-385
Procedure 9												
EDTA treatment	0	1.9	0	0								
2-N_3-[α-^{32}P]ATP treatment	0.02	0.3	0	2.1								
ADP treatment	0.03	1.0	0.02	1.0	0	1.0	0.03	1.0	0.5	-ADP	30%	Y-362
										-ATP	70%	Y-385

Data are given in mol nucleotide per mol CF_0F_1. No free ATP was detected under each condition. The % of AXP labeled peptides was given as percent of radioactivity of the sample applied on the ion exchange column. Incubations were carried out at room temperature. After each incubation free nucleotides were removed by three consecutive passages of the CF_0F_1 solution through centrifugation columns. The data represent averages from three experiments (initial condition (buffer A) average of 12 preparations). The standard deviation was less than 10% for all data shown. nd, not determined.

leads to the occupation of a binding site with a half maximal occupation at approximately 2 μM. It is shown schematically in Fig. 2 and is called procedure 7.

To occupy only the site with the higher dissociation constant with 2-azido-[α-^{32}P]ATP, the pretreated enzyme was first incubated with 20 μM ATP in the presence of 2 mM $MgCl_2$, resulting in the additional binding of 1 mol ATP per mol of enzyme. This preparation, containing four bound nucleotides (two ADPs and two ATPs), was then incubated for 15 min with 100 μM 2-azido-[α-^{32}P]ATP in the presence of 2 mM $MgCl_2$. After removal of free and loosely bound nucleotides by three consecutive passages through centrifugation columns, the enzyme contained four adenine nucleotides and one 2-azido-[α-^{32}P]ATP. This treatment is shown schematically in Fig. 2 as procedure 8.

The CF_0F_1 treated according to procedure 7 contained 1.0 bound 2-azido-[α-^{32}P]ATP and three residual endogenous nucleotides (see Table 1 and Fig. 1). This enzyme was then irradiated with UV-light for up to 60 s. After each irradiation time, the protein was denatured and the amount of covalently bound 2-azido-[α-^{32}P]AXP was measured (Fig. 3, top). The data indicate that maximally 50% of the bound 2-azido-[α-^{32}P]ATP can be covalently bound under these conditions. In order to measure the effect of covalent modification on ATP synthesis activity, the enzyme was reconstituted into liposomes after each irradiation time, and, after a $\Delta pH/\Delta\varphi$ jump, the rate of ATP synthesis was measured with 100 μM ADP (Fig. 3, center). In order to distinguish the effect of covalent modification of the binding sites from the potential harmful effect of UV-irradiation, the enzyme was treated according to procedure 7, but the 2-azido-[α-^{32}P]ATP was substituted by ATP. This enzyme was irradiated in the same way, reconstituted into liposomes and the rate of ATP synthesis was measured. Up to an irradiation time of 30 s, the rate remains constant and a small decrease is observed after 40 s irradiation (Fig. 3, center). Fig. 3, bottom, shows the rate of ATP synthesis at different levels of covalently bound 2-azido-[α-^{32}P]ATP. Extrapolation of the curve indicates complete inhibition, when one 2-azido-[α-^{32}P]ATP per CF_0F_1 is covalently bound.

CF_0F_1 treated according to procedure 8 contained 1.0 bound 2-azido-[α-^{32}P]ATP and four residual endogenous nucleotides (see Table 1 and Fig. 1). This enzyme was treated in the same way as described above and the corresponding measurements are shown in Fig. 3, right panels. The data indicate that again maximally 50% of the bound 2-azido-[α-^{32}P]ATP is covalently bound leading to a 50% inhibition of ATP synthesis. Extrapolation of ATP synthesis as a function of the covalently bound 2-azido-[α-^{32}P]ATP indicates complete inhibition when one azido-nucleotide per CF_0F_1 is bound.

3.3. Localization of the bound 2-nitreno-ATP

In order to identify the site where the 2-azido-[α-^{32}P]ATP is bound, the enzyme was irradiated by UV-light, treated by trypsin and the resulting peptides were separated by HPLC. Fig. 4 shows the results of these separations. The bars represent the radioactivity of the collected fractions. The solid lines indicate the concentration of eluent B. The left panels show the results obtained with CF_0F_1 treated according to procedure 7, right panels those obtained with procedure 8. For the ion exchange chromatography, the expected regions for nitreno-AMP, nitreno-ADP and nitreno-ATP containing peptides are indicated (top panels). The labeled peptides eluted mainly as ATP peptides in the fractions around 45 min for both treatments. ADP-labeled peptides represent less than 15% of the total radioactivity. No AMP-labeled peptides were observed and, presumably, this indicates that also the nitreno-ADP is bound at a non-catalytic site. The amino acid sequence analysis of this peptide confirms this assumption.

The nitreno-ATP labeled peptides (fraction 45 from ion exchange chromatography) were further analyzed by reversed phase HPLC. The results are shown in Fig. 4, bottom. The fractions eluting at 18 min (left, procedure 7) and 17 min (right, procedure 8) were used for amino acid sequencing. For both procedures, the sequence was identified as ^{379}IVGEEH-EIAQR^{390}, being part of the β-subunit. The empty position is that of Tyr385, this being the modified amino acid. According to Xue et al. [6] this peptide is part of the non-catalytic binding site. The site with the lowest affinity for ATP occupied by procedure 8 is called site 6, that occupied by proce-

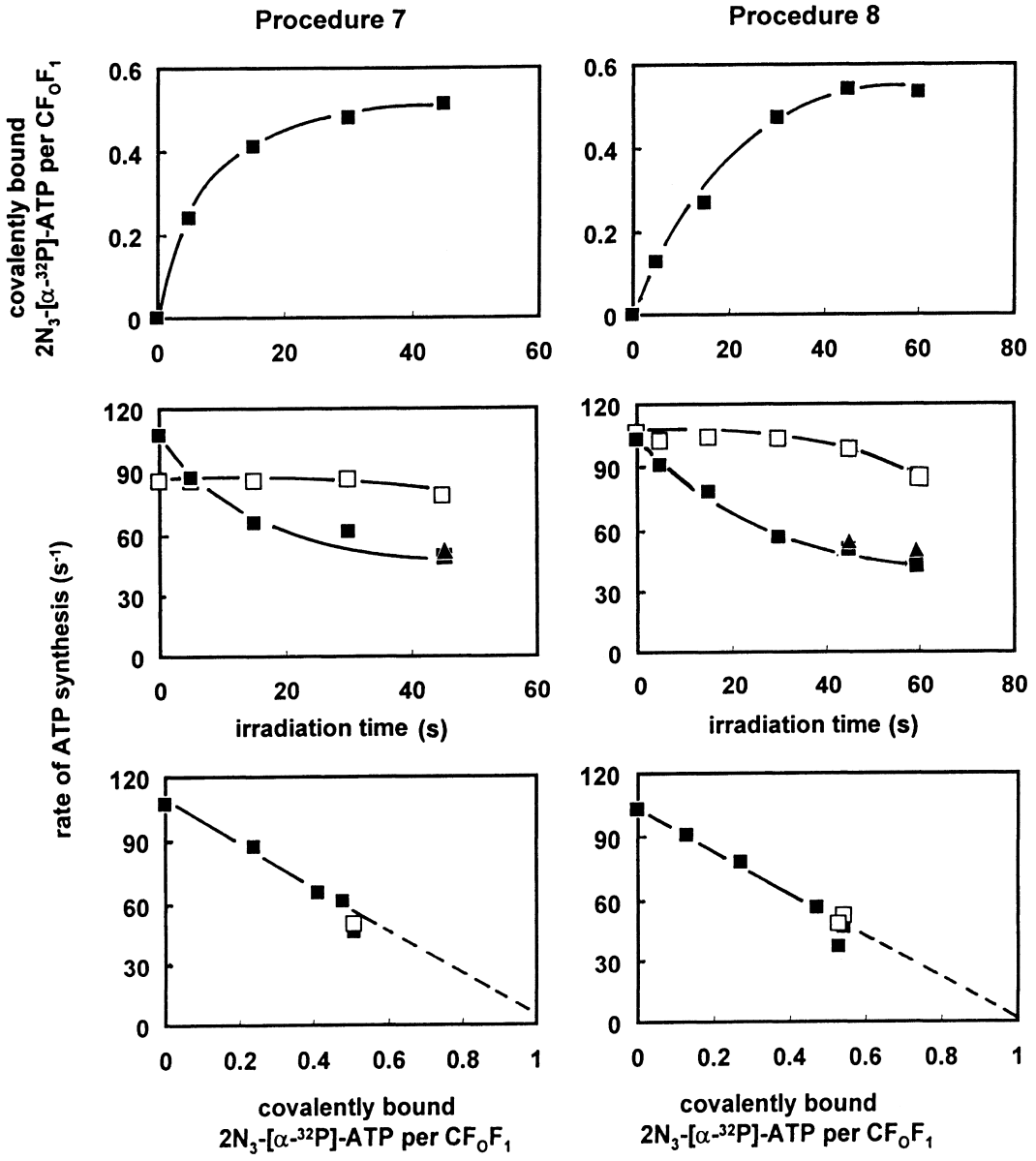

Fig. 3. Effect of UV-irradiation on the extent of covalent derivatization of CF_0F_1 with 2-azido-[α-^{32}P]ATP and on the rate of ATP synthesis. CF_0F_1 (5 μM) was treated according to procedure 7 (left panels) or procedure 8 (right panels). The enzyme was then irradiated by UV-light for different times. Top panels: CF_0F_1 was denatured and the amount of covalently bound 2-azido-[α-^{32}P]AXP was determined as a function of irradiation time. Center panels: after irradiation, the enzyme was reconstituted into liposomes and the rate of ATP synthesis was measured after a $\Delta pH/\Delta\varphi$ jump as a function of irradiation time (filled squares). As a control, the rates are shown for irradiated CF_0F_1 treated with ATP instead of 2-azido-[α-^{32}P]ATP in the same way (open squares). The solid triangles indicate the rates corrected for the UV-effect on CF_0F_1. Bottom panels: the rate of ATP synthesis is shown as a function of covalently bound 2-azido-[α-^{32}P]AXP (data are from top and center panels). The filled squares are the measured data, the open squares are data corrected for the UV-effect on CF_0F_1.

dure 7 is called site 5. Schematically, the different occupation patterns are depicted already in Fig. 2, although the reasons for this assignment are first given in Section 4.

3.4. Effect of covalent modification of non-catalytic site 5 or 6 on enzyme activity

When the enzymes with one non-catalytic site

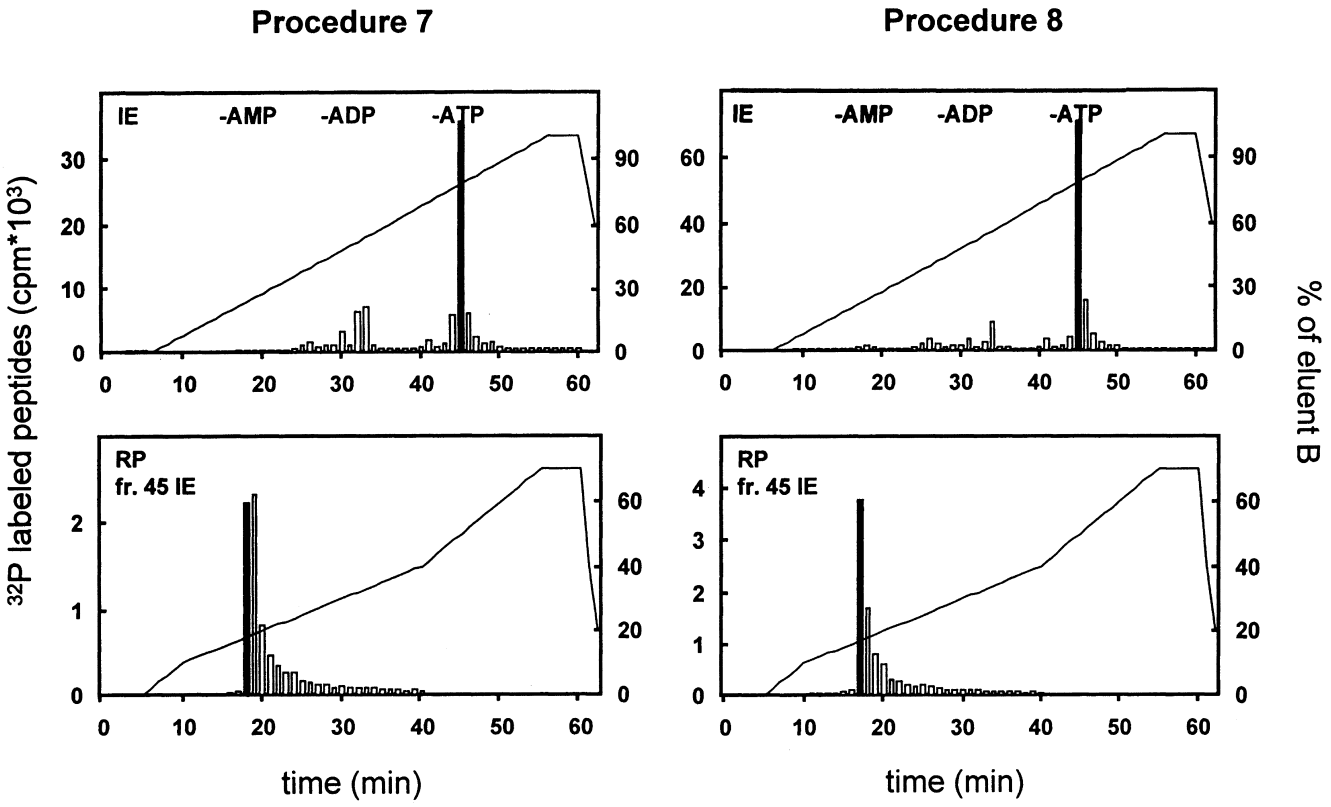

Fig. 4. HPLC elution profiles of photolabeled CF_0F_1 after trypsin treatment. CF_0F_1 (5 μM) was incubated as described in procedure 7 (left panels) or procedure 8 (right panels). After UV-irradiation photolabeled CF_0F_1 was treated with trypsin and the resultant labeled peptides were separated by HPLC. The radioactivity of the collected fractions is represented by bars. The elution gradients in percent of eluent B are superimposed (right hand scale). The upper panels show the separation into the labeled nitreno-AMP, nitreno-ADP and nitreno-ATP peptides with the ion exchange (IE) column. The bottom panels represent the result of the separation of nitreno-ATP-modified peptides (fractions 45 from the ion exchange chromatography) by reversed phase chromatography. Amino acid sequence analysis was carried out with the fractions 18 of both nitreno-ATP-modified enzymes.

occupied by 2-azido-[α-^{32}P]ATP (either treated with procedure 7 or with procedure 8) were irradiated with UV-light for up to 45 s, covalent binding of about 0.5 mol nitreno-[α-^{32}P]ATP per mol CF_0F_1 was measured in both cases. The efficiency of covalent linkage, then, is similar to that found for catalytic site 3 but significantly lower than that for the catalytic sites 1 and 2 [16,17]. In order to characterize these occupied binding sites, we investigated the effect of their covalent derivatization on the catalytic properties of the enzyme. CF_0F_1 treated according to procedure 7 or procedure 8 was irradiated for 60 s to achieve maximal derivatization. The irradiated enzyme and, as a control, the non-irradiated enzyme were reconstituted into liposomes. ATP synthesis was measured after a $\Delta pH/\Delta\varphi$ jump with ADP concentrations ranging from 10 nM to 100 μM. The initial rates of ATP synthesis as a function of ADP concentration are depicted in Fig. 5. The left panels show results when site 5 is occupied (procedure 7). The right panels show the results when site 6 is occupied (procedure 8). At low ADP concentrations (uni-site conditions, top panels), no inhibition is observed. At medium APD concentrations (center panels) and high ADP concentrations (multi-site conditions, bottom panels) the rate of ATP synthesis is inhibited by covalent derivatization. Approximately 50% of the initially bound 2-azido-[α-^{32}P]ATP is covalently bound after irradiation in both cases and, in parallel, the rate of ATP synthesis decreased by 50% at high ADP concentrations.

In addition, the rate of ATP hydrolysis was measured with the irradiated and non-irradiated CF_0F_1. First, the reconstituted enzyme was activated by a

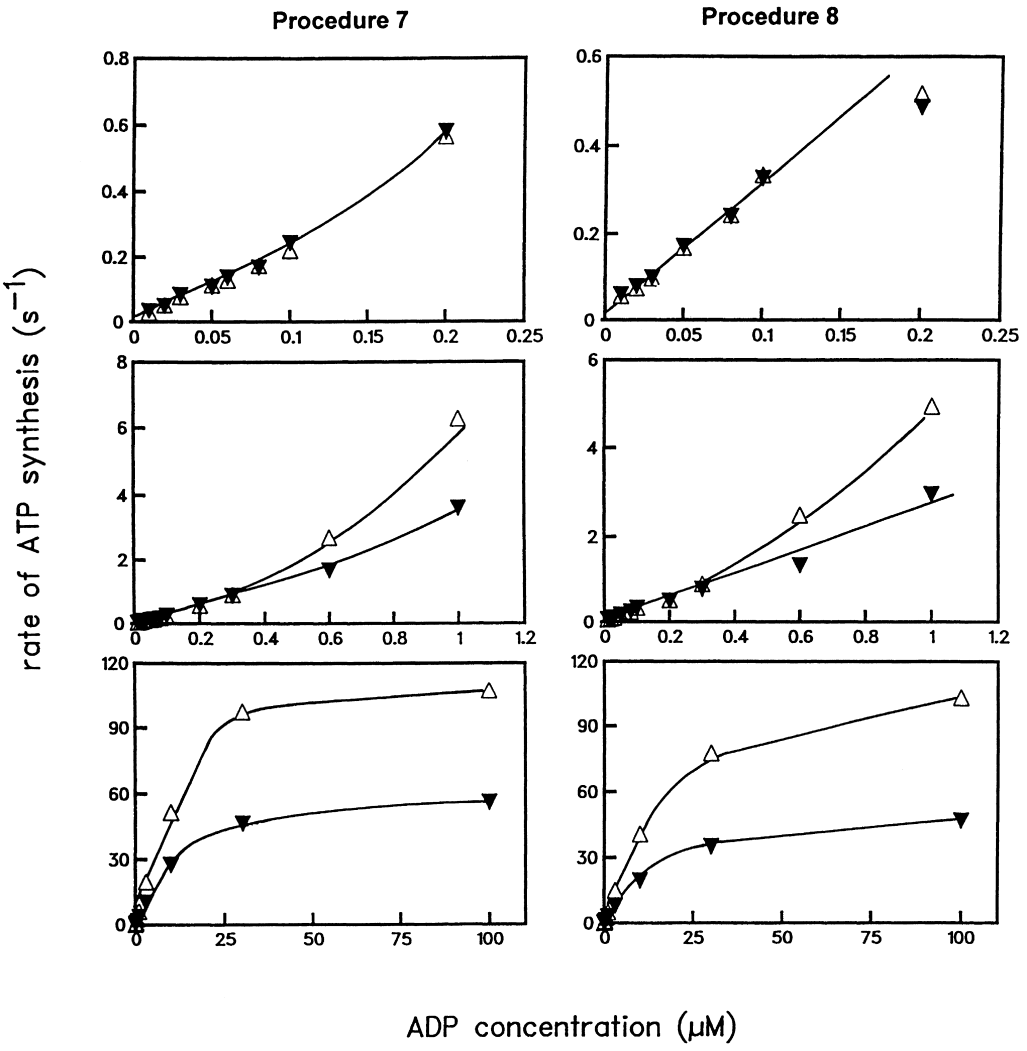

Fig. 5. Rate of ATP synthesis of CF_0F_1 with covalently bound and non-covalently bound 2-azido-[α-^{32}P]ATP at different ADP concentrations. CF_0F_1 (5 μM) was treated according to procedure 7 (left panels) or procedure 8 (right panels) and irradiated for 60 s with UV-light. After irradiation, CF_0F_1 contained covalently bound 0.5 mol 2-nitreno-[α-^{32}P]ATP per mol CF_0F_1 at site 5 (procedure 7) or 0.5 mol 2-nitreno-[α-^{32}P]ATP per mol CF_0F_1 at site 6 (procedure 8). CF_0F_1 with covalently bound (filled triangles) or non-covalently bound 2-azido-[α-^{32}P]ATP (open triangles) was reconstituted into liposomes and the rate of ATP synthesis was measured after a $\Delta pH/\Delta\varphi$ jump as a function of the ADP concentration. Top panels show the data under uni-site conditions (up to 200 nM ADP). Bottom panels show the data under multi-site conditions (up to 100 μM ADP). Center panels show the intermediate range. Open triangles represent data with non-covalently bound 2-azido-[α-^{32}P]ATP, filled triangles represent data with covalently bound 2-azido-[α-^{32}P]ATP.

$\Delta pH/\Delta\varphi$ jump and then the activated enzyme was injected into a buffer containing an uncoupler, the luciferin/luciferase assay and different ATP concentrations [16]. Fig. 6 shows the initial rates of ATP hydrolysis at low (uni-site conditions, top panels) and medium concentrations (bottom panels). At high ATP concentrations (1 mM, multi-site conditions), the rate was measured with [γ-^{32}P]ATP [30]. With the non-covalently labeled enzyme, the rate was 16 s^{-1}, with the covalently derivatized enzyme 8 s^{-1} (data not shown in Fig. 6). At low ATP concentrations (uni-site conditions), the rate of ATP hydrolysis was not inhibited, at medium and high concentrations, the rate decreased by approximately 50% after covalent derivatization. Correspondingly, 50% of the initially bound 2-azido-[α-^{32}P]ATP was cova-

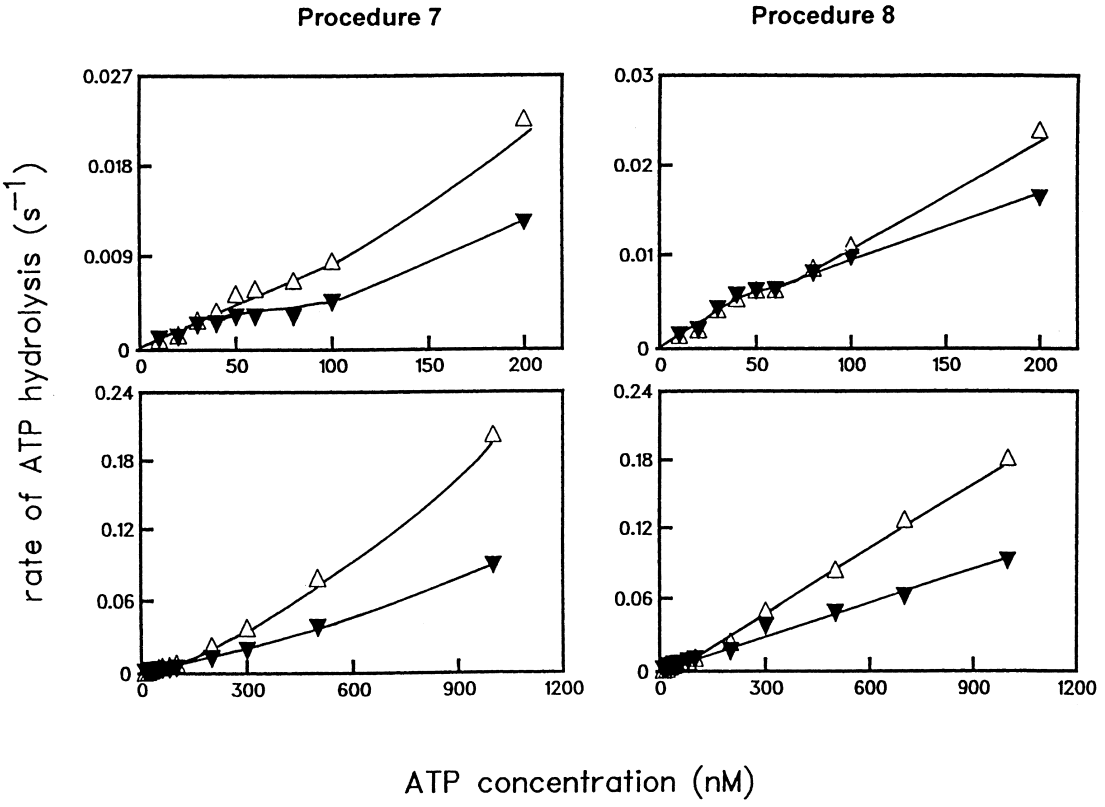

Fig. 6. Rate of ATP hydrolysis of CF_0F_1 with covalently bound and non-covalently bound 2-azido-[α-^{32}P]ATP at different ATP concentrations. CF_0F_1 (5 μM) was treated according to procedure 7 (left panels) or procedure 8 (right panels) and irradiated for 60 s with UV-light. After irradiation, CF_0F_1 contained covalently bound 0.5 mol 2-nitreno-[α-^{32}P]ATP per mol CF_0F_1 at site 5 (procedure 7) or 0.5 mol 2-nitreno-[α-^{32}P]ATP per mol CF_0F_1 at site 6 (procedure 8). CF_0F_1 was reconstituted into liposomes and the rate of ATP hydrolysis was measured after activation of the enzyme by a $\Delta pH/\Delta\varphi$ jump from uni-site (upper panels) to multi-site (lower panels) conditions. Open triangles represent data with non-covalently bound 2-azido-[α-^{32}P]ATP, filled triangles represent data with covalently bound 2-nitreno-[α-^{32}P]ATP.

lently bound after irradiation, i.e. at high concentrations the inhibition of the rate is proportional to the fraction of covalently derivatized enzyme. These data indicate that covalent labeling of non-catalytic sites 5 or 6 inhibits both multi-site ATP synthesis and ATP hydrolysis proportionally to the degree of derivatization. Uni-site ATP synthesis and ATP hydrolysis is, however, not inhibited.

3.5. *Binding of 2-azido-[α-^{32}P]ATP to sites 5 and 6 together and to site 4*

CF_0F_1 treated with procedure 7 or procedure 8 contains one bound 2-azido-[α-^{32}P]ATP at site 5 or site 6, respectively. In the next step, we investigated the effect of covalent derivatization when both sites are occupied by 2-azido-[α-^{32}P]ATP. In order to occupy both non-catalytic sites, pretreated CF_0F_1 containing one bound ATP and two bound ADP was incubated with 100 μM 2-azido-[α-^{32}P]ATP in the presence of 2 mM $MgCl_2$ for 10 min at room temperature and then free and loosely bound nucleotides were removed by three consecutive passages through centrifugation columns. This is called procedure 6 and the nucleotide occupation pattern is schematically shown in Fig. 2.

The CF_0F_1 treated according to procedure 6 contained 2.0 bound 2-azido-[α-^{32}P]ATP, one bound ATP and two bound ADP (see Fig. 1 and Table 1). This enzyme was then irradiated with UV-light for up to 45 s. After each irradiation time, the protein was denatured and the amount of covalently bound 2-azido-[α-^{32}P]AXP was measured (Fig. 7, top). The data indicate that one 2-nitreno-

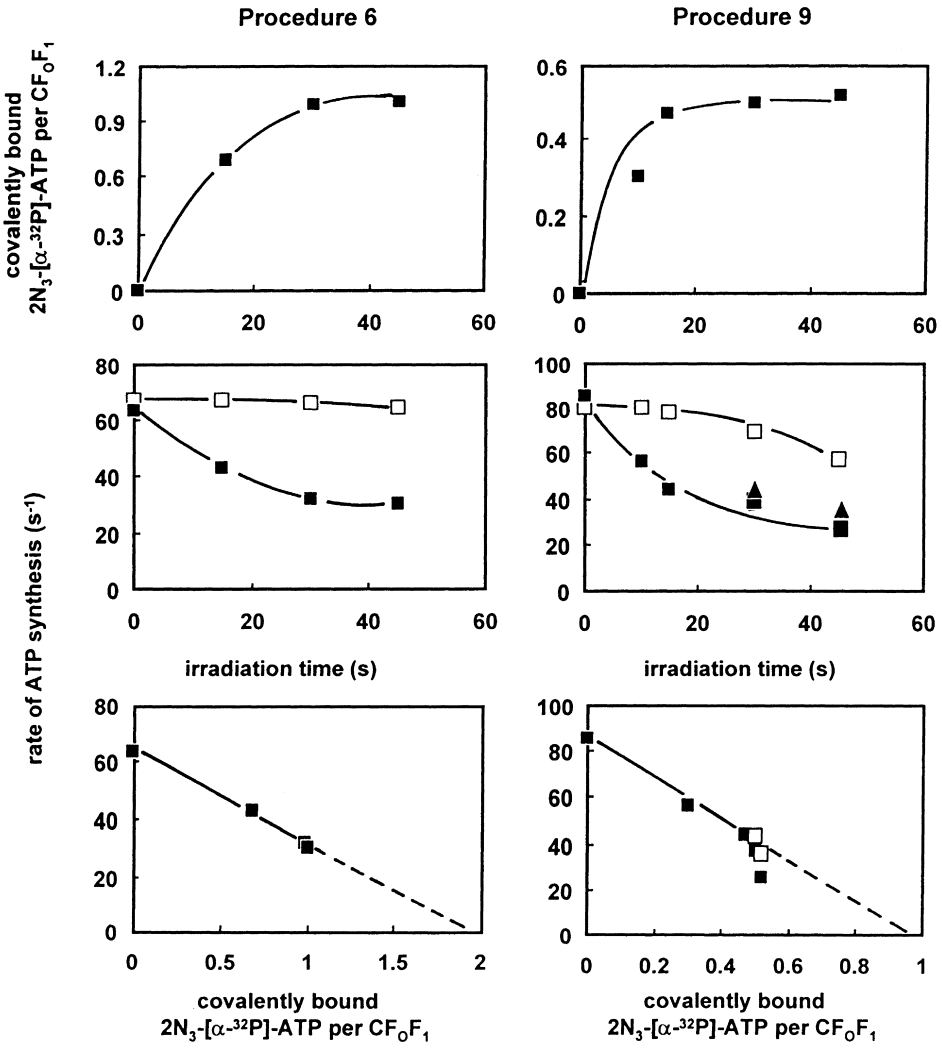

Fig. 7. Effect of UV-irradiation on the extent of covalent derivatization of CF_0F_1 with 2-azido-[α-^{32}P]ATP and on the rate of ATP synthesis. CF_0F_1 (5 μM) was treated according to procedure 6 (left panels) or procedure 9 (right panels). The enzyme was then irradiated by UV-light for different times. Top panels: CF_0F_1 was denatured and the amount of covalently bound 2-azido-[α-^{32}P]AXP was determined as a function of irradiation time. Center panels: after irradiation, the enzyme was reconstituted into liposomes and the rate of ATP synthesis was measured after a $\Delta pH/\Delta\varphi$ jump as a function of irradiation time (filled squares). As a control, the rates are shown for irradiated CF_0F_1 treated with ATP instead of 2-azido-[α-^{32}P]ATP in the same way (open squares). The solid triangles indicate the rates corrected for the UV-effect on CF_0F_1. Bottom panels: the rate of ATP synthesis is shown as a function of covalently bound 2-nitreno-[α-^{32}P]AXP (data are from top and center panels). The filled squares are the measured data, the open squares are data corrected for the UV-effect on CF_0F_1.

[α-^{32}P]ATP per CF_0F_1, i.e. maximally 50% of the bound 2-azido-[α-^{32}P]ATP, can be covalently bound under these conditions. In order to measure the effect of covalent modification on ATP synthesis activity, the enzyme was reconstituted into liposomes after each irradiation time, and, after a $\Delta pH/\Delta\varphi$ jump, the rate of ATP synthesis was measured with 100 μM ADP (Fig. 7, center). In order to distinguish the effect of covalent modification of the binding sites from the potential harmful effect of UV-irradiation, the enzyme was treated according to procedure 6, but the 2-azido-[α-^{32}P]ATP was substituted by ATP. This enzyme was irradiated in the same way, reconstituted into liposomes and the rate of ATP synthesis was measured. Up to an irradiation time of 45 s, the rate remains almost constant (Fig. 7,

center). Fig. 7, bottom, shows the rate of ATP synthesis at different levels of covalently bound 2-azido-[α-^{32}P]ATP. Extrapolation of the linear part of the curve indicates complete inhibition, when about two nucleotides per CF_0F_1 are covalently bound.

During all the treatments described up to now, one ATP remains tightly bound at the enzyme. This ATP, obviously, is most difficult to remove from the enzyme, i.e. its binding site has the lowest dissociation constant for ATP. Therefore, this non-catalytic binding site is called site 4. We tried to remove this ATP by a multitude of different treatments, however, either the ATP was not removed or the enzyme was denatured, i.e. it was found to be inactive in ATP synthesis after reconstitution into liposomes. During these surveys, we noticed that after storage of the isolated enzyme in buffer A for 48 h at room temperature one ATP was lost (presumably from site 5) and one ATP (on site 4) was slowly hydrolyzed to bound ADP. Based on this observation, the following treatment was developed which allows substitution of the bound ATP by 2-azido-[α-^{32}P]ATP. It is shown schematically as procedure 9 in Fig. 2. The isolated enzyme was incubated for 24 h at room temperature with 2 mM EDTA in the presence of 150 mg BioBeads per mg CF_0F_1. After removal of free and loosely bound nucleotides by three consecutive passages through centrifugation columns, the resulting enzyme contains usually two, sometimes less, bound ADP per CF_0F_1. It was then further incubated for 1 h with 100 μM 2-azido-[α-^{32}P]ATP and 2 mM EDTA. Again free and loosely bound nucleotides were removed by three consecutive passages through centrifugation columns. Thereafter, the enzyme contained two bound 2-azido-[α-^{32}P]AXP (see Table 1). After UV-irradiation and trypsin treatment the covalently derivatized peptides were analyzed by HPLC. Ion exchange chromatography revealed that approximately 50% eluted as nitreno-ATP peptide and 50% as nitreno-ADP peptide. Reversed phase HPLC analysis of the -ATP labeled peptides indicated that a non-catalytic site was derivatized, whereas the corresponding analysis of the ADP labeled peptides showed derivatization of a catalytic site. This observation indicates that the 2-nitreno-[α-^{32}P]ATP is located at a non-catalytic site, the 2-nitreno-[α-^{32}P]ADP on a catalytic site, the occupation pattern being shown in Fig. 2. To label only the non-catalytic site, the enzyme was then subjected to a third incubation for 1 h with 2 mM EDTA and 100 μM ADP, and free and loosely bound nucleotides were removed by three consecutive passages through centrifugation columns. Finally, the enzyme contained one 2-azido-[α-^{32}P]ATP and one ADP (see procedure in Fig. 2 and Table 1).

CF_0F_1 treated according to procedure 9 was then irradiated by UV-light and the amount of covalently bound 2-nitreno-ATP was measured (Fig. 7, right). The top panel shows that maximally 0.5 mol 2-nitreno-[α-^{32}P]ATP is bound per mol CF_0F_1, i.e. 50% of the non-covalently bound 2-azido-[α-^{32}P]ATP can be covalently bound. After each irradiation time the enzyme was reconstituted into liposomes and the rate of ATP synthesis was measured with 100 μM ADP. To correct enzyme inactivation by UV-irradiation in parallel experiments, the enzyme was treated according to procedure 9 but 2-azido-[α-^{32}P]ATP was substituted by ATP. These data are shown in Fig. 7, right, center panel. UV-irradiation leads to a significant decrease of the rate and the data obtained with 2-azido-[α-^{32}P]ATP were corrected correspondingly. The reason for the different sensitivities of CF_0F_1 prepared by procedure 6 or 9 is not known. Fig. 7, bottom, right, shows the rate of ATP synthesis as a function of covalently bound 2-azido-[α-^{32}P]ATP. Extrapolation of the linear part of the curve indicates complete inhibition, when one nucleotide per CF_0F_1 is bound.

3.6. Localization of the bound 2-nitreno-ATP

In order to identify the site where the 2-azido-[α-^{32}P]ATP is bound, the enzyme was irradiated by UV-light, treated by trypsin and the resulting peptides were separated by HPLC. Fig. 8 shows the results of these separations. The bars represent the radioactivity of the collected fractions. The solid lines indicate the concentration of eluent B. The left panels show the results obtained with CF_0F_1 treated according to procedure 6, right panels those obtained with procedure 9. For the ion exchange chromatography, the expected regions for nitreno-AMP, nitreno-ADP and nitreno-ATP containing peptides are indicated (top panels). After procedure 6, the labeled peptides eluted mainly as ATP peptides

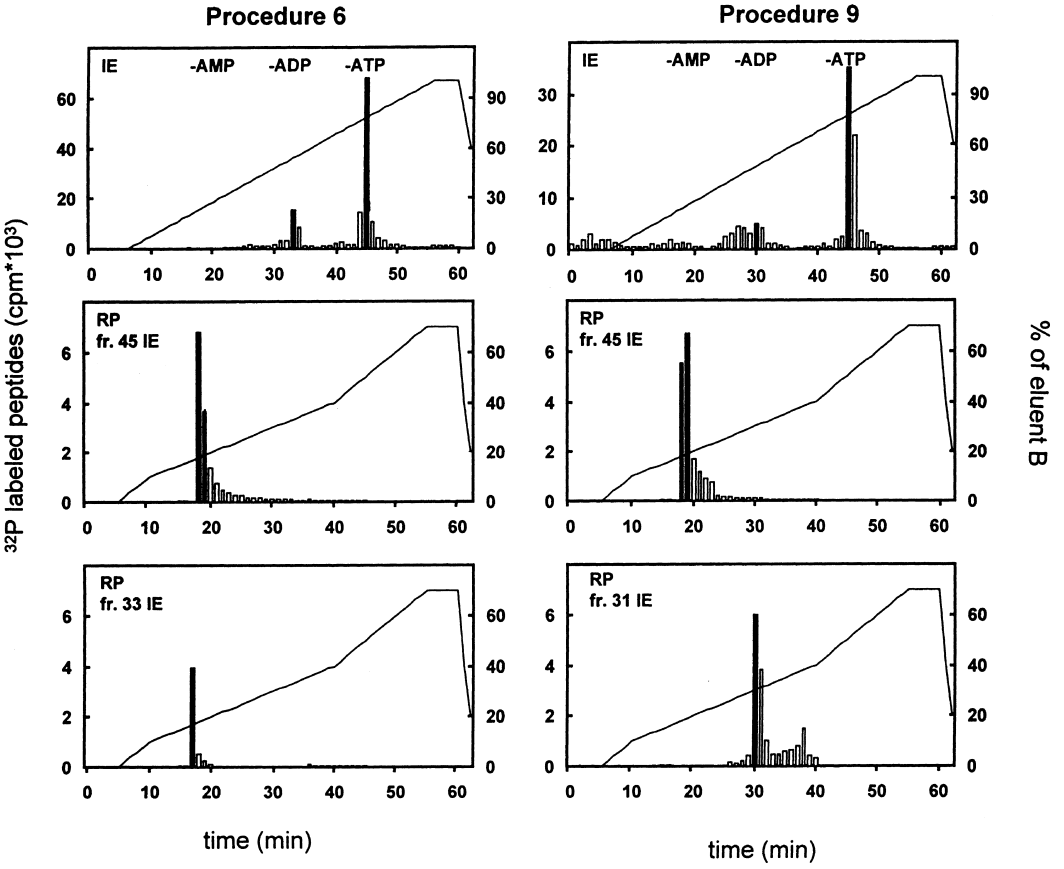

Fig. 8. HPLC elution profiles of photolabeled CF_0F_1 after trypsin treatment. CF_0F_1 (5 μM) was treated according to procedure 6 (left panels) or procedure 9 (right panels). After UV-irradiation, the photolabeled CF_0F_1 was trypsinized and the resultant labeled peptides were separated by HPLC. The radioactivity of the collected fractions is represented by bars. The elution gradients in percent of eluent B are superimposed (right hand scale). The upper panels show the separation of the labeled peptides with the ion exchange (IE) column into AMP, ADP and ATP peptides. Center panels represent the result of the separation of ATP-modified peptides (fractions 45 from IE) by reversed phase chromatography. Amino acid sequence analysis was carried out with the pooled fractions 18 and 19 of the reversed phase chromatography. Bottom panels represent the result of the separation of the ADP-modified peptides (fraction 33, left and fraction 30, right from IE) by reversed phase chromatography. Amino acid sequence analysis was carried out with fractions 17, left and fraction 30, right of the reversed phase chromatography.

in the fractions around 45 min. ADP-labeled peptides represent less than 15% of the total radioactivity. No AMP-labeled peptides were observed. The nitreno-ATP labeled peptides (fraction 45 from ion exchange chromatography) were further analyzed by reversed phase HPLC. The results are shown in Fig. 8, center. The fractions eluting at 18 and 19 min were pooled and used for amino acid sequencing. The sequence was identified as ^{379}IVGEEH-EIAQR^{390}, being part of the β-subunit. The empty position is that of Tyr385, this being the modified amino acid. According to Xue et al. [6], this peptide is part of the non-catalytic binding site. Also the nitreno-ADP labeled peptides (fractions 33 from ion exchange chromatography) were analyzed by reversed phase HPLC (Fig. 8, bottom). Amino acid sequence analysis of fraction 17 resulted in ^{379}IVGEEH-EIAQR^{390}, i.e. the nitreno-ADP is also located at a non-catalytic site.

After procedure 9, the labeled peptides eluted in the ion exchange chromatography as nitreno-ATP and nitreno-ADP peptides (Fig. 8, right). Both peptides were analyzed by reversed phase HPLC. The result for the nitreno-ATP peptide is shown in Fig. 8 right, center panel. Amino acid sequence analysis of the pooled fractions 18 and 19 gives the result ^{379}IVGEEH-EIAQR^{390} indicating labeling of a non-catalytic site. In the bottom panel, the result for

the nitreno-ADP peptide is shown. Amino acid sequence analysis of fraction 30 resulted in ^{360}GIPAVDPLDSTDT^{373}. According to Xue et al. [6], this peptide is part of a catalytic site. We conclude from these results that with procedure 9, mainly a non-catalytic site is occupied (70%), however, some label (30%) is found at a catalytic site and not exchanged with ADP in the last step of procedure 9.

3.7. *Effect of covalent modification of two non-catalytic sites (5 and 6) and non-catalytic site 9 on enzyme activity*

When the enzyme with two non-catalytic sites occupied by 2-azido-[α-^{32}P]ATP was irradiated with UV-light for up to 45 s, covalent binding of about 1 mol nitreno-[α-^{32}P]ATP per mol CF_0F_1 was

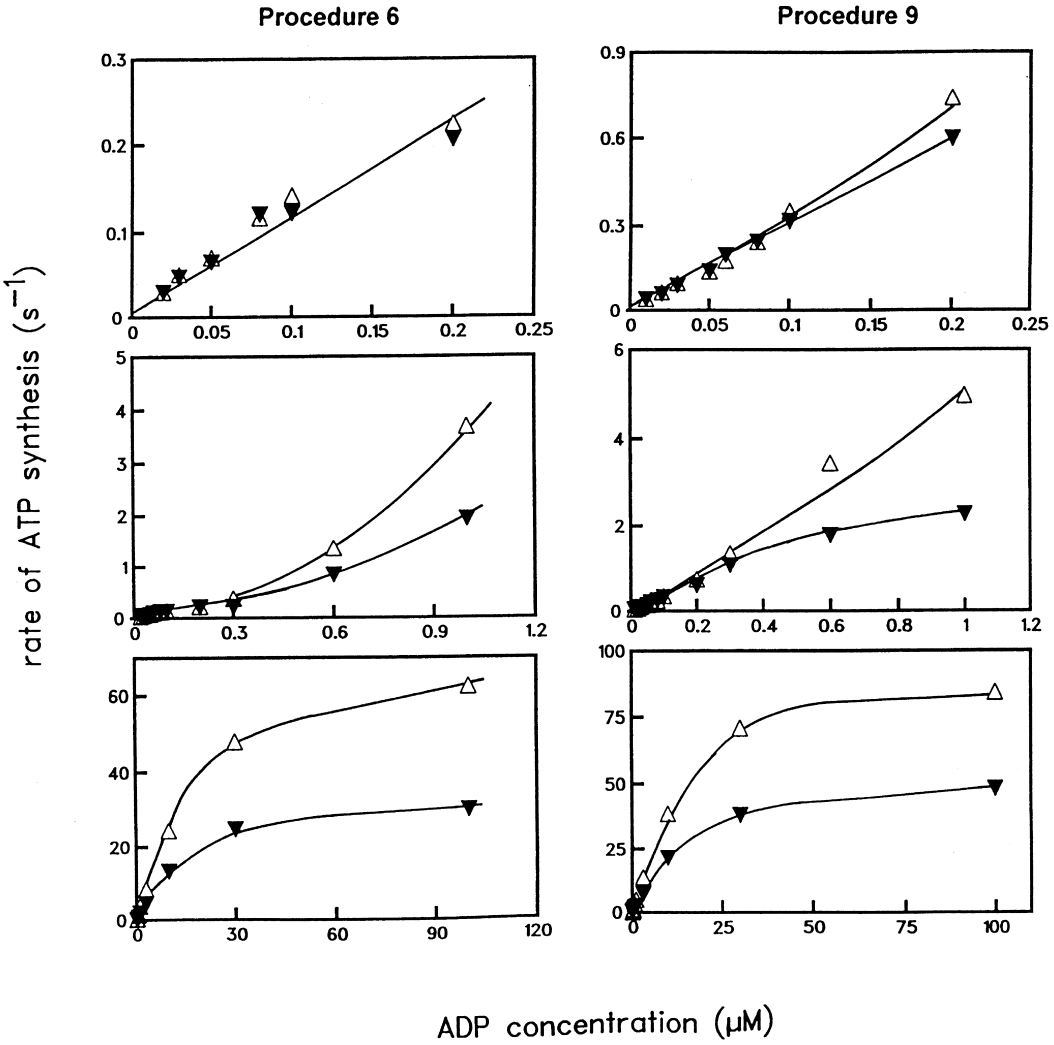

Fig. 9. Rate of ATP synthesis of CF_0F_1 with covalently bound and non-covalently bound 2-azido-[α-^{32}P]ATP at different ADP concentrations. CF_0F_1 (5 μM) was treated according to procedure 6 (left panels) or procedure 9 (right panels) and irradiated for 60 s with UV-light. After irradiation, CF_0F_1 contained covalently bound 1.0 mol 2-nitreno-[α-^{32}P]ATP per mol CF_0F_1 at the sites 5 and 6 (procedure 6) or 0.5 mol 2-nitreno-[α-^{32}P]ATP per mol CF_0F_1 at site 4 (procedure 9). CF_0F_1 with covalently bound (filled triangles) or non-covalently bound 2-azido-[α-^{32}P]ATP (open triangles) was reconstituted into liposomes and the rate of ATP synthesis was measured after a $\Delta pH/\Delta\varphi$ jump as a function of the ADP concentration. Top panels show the data under uni-site conditions (up to 200 nM ADP). Bottom panels show the data under multi-site conditions (up to 100 mM ADP). Center panels show the intermediate range. Open triangles represent data with non-covalently bound 2-azido-[α-^{32}P]ATP, filled triangles represent data with covalently bound 2-nitreno-[α-^{32}P]ATP.

measured. The enzyme treated with procedure 9 contained one 2-azido-[α-^{32}P]ATP and covalent binding of about 0.5 mol nitreno-[α-^{32}P]ATP per mol CF_0F_1 is observed, i.e. the efficiency of covalent linkage is similar for all non-catalytic sites.

In order to analyze the involvement of the occupied binding sites in the catalytic behavior of the enzyme, we investigated the catalytic properties after covalent labeling. CF_0F_1 treated according to procedure 6 or procedure 9 was irradiated for 60 s to achieve maximal derivatization. The irradiated enzyme and, as a control, the non-irradiated enzyme were reconstituted into liposomes. ATP synthesis was measured after a $\Delta pH/\Delta\varphi$ jump with ADP concentrations ranging from 10 nM to 100 μM. The initial rates of ATP synthesis as a function of ADP concentration are depicted in Fig. 9. Open triangles refer to non-irradiated enzymes, closed triangles to irradiated ones. The left panels show results when sites 5 and 6 are occupied (procedure 6). The right panels show the results when site 4 is occupied (procedure 9). For both occupation patterns at low ADP concentrations (uni-site conditions, top panels) no inhibition is observed. At medium ADP concentrations (center panels) and high ADP concentrations (multi-site conditions, bottom panels) the rate of ATP synthesis is inhibited by covalent derivatization. Approximately 50% of the initially bound 2-azido-[α-^{32}P]ATP is covalently bound after irradiation in both cases and, in parallel, the rate of ATP synthesis decreased by 50% at high ADP concentrations.

In addition, the rate of ATP hydrolysis was measured with the irradiated and non-irradiated CF_0F_1. First, the reconstituted enzyme was activated by a $\Delta pH/\Delta\varphi$ jump and then the activated enzyme was injected into a buffer containing an uncoupler, the

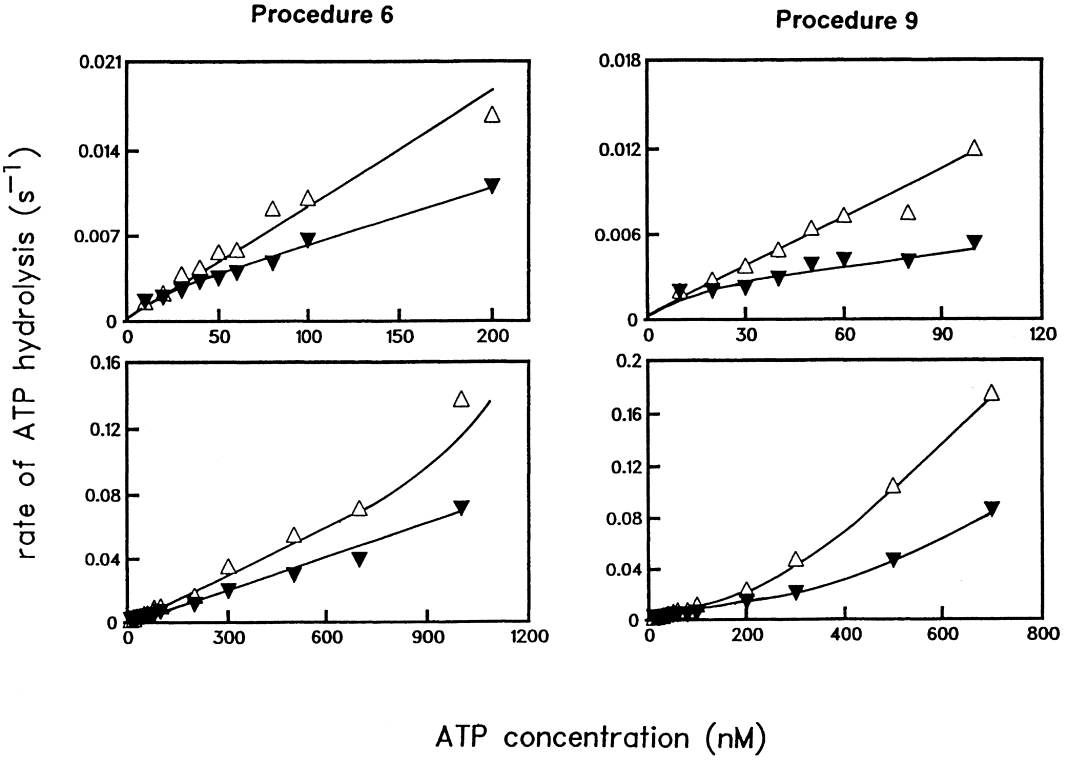

Fig. 10. Rate of ATP hydrolysis of CF_0F_1 with covalently bound and non-covalently bound 2-azido-[α-^{32}P]ATP at different ATP concentrations. CF_0F_1 (5 μM) was treated according to procedure 6 (left panels) or procedure 9 (right panels) and irradiated for 30 s with UV-light. After irradiation, CF_0F_1 contained covalently bound 1.0 mol 2-nitreno-[α-^{32}P]ATP per mol CF_0F_1 at the sites 5 and 6 (procedure 6) or 0.5 mol 2-nitreno-[α-^{32}P]ATP per mol CF_0F_1 at site 4 (procedure 9). CF_0F_1 was reconstituted into liposomes and the rate of ATP hydrolysis was measured after activation of the enzyme by a $\Delta pH/\Delta\varphi$ jump from uni-site (upper panels) to multi-site (lower panels) conditions. Open triangles represent data with non-covalently bound 2-azido-[α-^{32}P]ATP, filled triangles represent data with covalently bound 2-nitreno-[α-^{32}P]ATP.

luciferin/luciferase assay and different ATP concentrations [16]. Fig. 10 shows the initial rates of ATP hydrolysis at low (uni-site conditions, top panels) and medium concentrations (bottom panels). Left panels show the results when sites 5 and 6 are occupied (procedure 6), right panels when site 4 is occupied (procedure 9). At high ATP concentrations (1 mM, multi-site conditions), the rate was measured with [γ-^{32}P]ATP [30]. With the non-covalently labeled enzyme, the rate was 16 s^{-1}, with the covalently derivatized enzyme 8 s^{-1} (data not shown in Fig. 10). For both occupation patterns at low ATP concentrations (uni-site conditions), the rate of ATP hydrolysis is not inhibited, at medium and high concentrations, the rate decreases by approximately 50% after covalent derivatization. Correspondingly, 50% of the initially bound 2-azido-[α-^{32}P]ATP is covalently bound after irradiation, i.e. at high ATP concentrations the inhibition of the rate of ATP hydrolysis is proportional to the fraction of covalently derivatized enzyme. These data indicate that covalent labeling of non-catalytic sites 5 and 6 together and of site 4 inhibits both multi-site ATP synthesis and ATP hydrolysis proportionally to the degree of derivatization. Uni-site ATP synthesis and ATP hydrolysis are, however, not inhibited.

3.8. Release of non-covalently bound nucleotides by energization

CF_0F_1 treated with procedure 6 contained after reconstitution in liposomes about two, CF_0F_1 treated with procedures 7, 8 or 9 contained one non-covalently bound 2-azido-[α-^{32}P]ATP per enzyme (Table 1). We investigated the release of the radioactive 2-azido-nucleotide upon energization of the membrane by $\Delta pH/\Delta\varphi$ in the presence and in the absence of substrates. The data are shown in Table 2. In all cases, approximately 4% of the bound radioactive nucleotides are released in the absence of substrates and 6% in the presence of either ADP or ATP. During the time between energization and separation of the enzyme from the released nucleotides (5 s) CF_0F_1 carries out at least 500 turnovers in synthesis direction and about 80 turnovers in hydrolysis direction. If the radioactive nucleotide is located on a site not involved in the catalytic reaction cycle, no loss of the label is expected. Obviously, this was not the case; 4–6% of the enzymes lost their label.

When 2-azido-[α-^{32}P]ADP was bound at catalytic sites, approximately 20–25% of the nucleotides were released upon energization in the presence of substrates [16,17]. For the investigation of the effect of covalent derivatization on the enzyme kinetics (Figs. 5, 6, 9, 10) the enzymes were irradiated by UV-light immediately after preparation of the different occupation patterns (Fig. 2). Reconstitution was carried out with the covalently derivatized enzymes, so that no change of the occupation pattern can occur. However, when nucleotide release is to be measured, reconstitution must be carried out with the non-covalently bound 2-azido-[α-^{32}P]ATP and it might be

Table 2
Release of non-covalently bound 2-N_3-[α-^{32}P]AXP in $\Delta pH/\Delta\varphi$ jump

Incubation conditions	Occupied site	Labeled peptide from catalytic sites after reconstitution (%)	Non-covalently bound 2-N_3-[α-^{32}]AXP after reconstitution	2-N_3-[α-^{32}P]AXP released in $\Delta pH/\Delta\varphi$ jump without substrate	2-N_3-[α-^{32}P]AXP released in $\Delta pH/\Delta\varphi$ jump with substrate		Calculated release from catalytic site	
					hydrolysis 1 mM ATP	synthesis 100 μM ADP	without substrate	with substrate
Procedure 6	5+6	25	2.0	0.031	0.042	0.028	0.05	0.1
Procedure 7	5	60	1.05	0.062	0.102	0.093	0.06	0.12
Procedure 8	6	35	1.05	0.042	0.060	0.064	0.035	0.07
Procedure 9	4	50	1.0	0.043	0.062	0.059	0.05	0.1

CF_0F_1 (5 μM) was treated as described in the different procedures and then reconstituted into liposomes without UV-irradiation. Energization of the membrane leads to the release of the non-covalently bound 2-azido-[α-^{32}P]ADP. Free nucleotides were separated by rapid filtration from the liposomes 5 s after energization. Experiments were carried out in the absence of substrates or in the presence of substrates, either 1 mM ATP or 100 μM ADP. Data are given in mol nucleotide per mol CF_0F_1.

asked whether the initially prepared occupation pattern changes during reconstitution of the enzyme into liposomes. Therefore, after reconstitution, the proteoliposomes were irradiated with UV-light, treated with trypsin and the resulting peptides were analyzed by reversed phase HPLC. Peptides from the catalytic site elute in the reversed phase HPLC at 30 min, that from non-catalytic sites at 18 min (see Fig. 8). The fraction of labeled peptides from non-catalytic sites was calculated from the ratio of radioactivity in fractions 14–24 to total radioactivity applied to the column. For catalytic sites the radioactivity in the fractions 25–35 was used. The radioactivity in the void volume was assumed to refer to free nitreno-[α-^{32}P]-nucleotides. The resulting fractions of labeled peptides from catalytic sites are shown in Table 2. When only catalytic sites are occupied, energization of the proteoliposomes resulted in a release of the bound 2-azido-[α-^{32}P]ADP to an extent of 10%, in the absence and 20% in the presence of substrates [16,17]. Since we know the occupation of catalytic sites from the fraction of labeled catalytic peptides after reconstitution we can calculate the expected release, if it occurred only from catalytic sites.

The experimental data for the release of 2-azido-nucleotides after energization when CF_0F_1 was treated with procedures 6–9 are collected in Table 2. In addition, the release was calculated for the differently treated enzymes when it occurred only from catalytic sites. Comparison of the calculated data with the measured ones shows that the observed release of the labeled nucleotides can be explained by an exclusive release from the catalytic sites. We have to conclude, therefore, that energization of the membrane either in the presence or in the absence of substrates does not lead to release of bound nucleotides from non-catalytic sites.

4. Discussion

4.1. Occupation of the different nucleotide binding sites

Catalytic and non-catalytic sites are distinguished in this work by their different response to covalent labeling with 2-azido-[α-^{32}P]AD(T)P: labeling of β-Tyr362 indicates a catalytic site, labeling of β-Tyr385 a non-catalytic site [6]. With this approach, we have recently developed methods for selective occupation of the three different catalytic sites and investigated the effect of their covalent derivatization on ATP synthesis and ATP hydrolysis [16,17]. In this work we used the labeling of β-Tyr385 by 2-azido-[α-^{32}P]ATP as an indication for binding at a non-catalytic site and developed incubation procedures for selective occupation of these sites. The following characteristics of these sites were found: (1) non-covalent binding of 2-azido-[α-^{32}P]ATP to CF_0F_1 does not lead to inhibition of catalysis. (2) Incubation with 2-azido-[α-^{32}P]ADP in the presence as well as in absence of Mg^{2+} does not lead to a labeling of β-Tyr385, i.e. non-catalytic sites do not bind ADP in the concentration range used in this work. (3) Incubation of isolated CF_0F_1 with 2-azido-[α-^{32}P]ATP leads to labeling of β-Tyr362 and β-Tyr385. The covalently bound nucleotide at β-Tyr362 was predominantly ADP and some AMP, while the covalently bound nucleotide at the non-catalytic site (β-Tyr385) was mainly ATP and some ADP. We conclude from these results that isolated CF_0F_1 binds 2-azido-ADP selectively at catalytic sites, 2-azido-ATP binds to both catalytic and non-catalytic sites. When 2-azido-ATP is bound to non-catalytic sites, it is not hydrolyzed. When, however, 2-azido-ATP is bound to a catalytic site, it is hydrolyzed to 2-azido-ADP, so that, after covalent derivatization, only nitreno-ADP-modified β-Tyr362 is found.

The nucleotide binding sites have different properties. In order to distinguish the different catalytic sites, we have called them sites 1, 2 and 3 in order of decreasing affinity for ADP [16]. Correspondingly in this work, the three non-catalytic sites are called 4, 5 and 6 in order of decreasing affinity for ATP, i.e. site 4 has the lowest dissociation constant for ATP, site 6 has the highest and site 5 has an intermediate dissociation constant. The isolated CF_0F_1 used in this work contained one ADP bound at a catalytic site and two ATPs bound at non-catalytic sites per CF_0F_1. We assume that the binding properties of ADP and ATP are similar to those of the corresponding 2-azido-nucleotides described above. Based on this assumption and the nomenclature of the binding sites, we conclude that the ADP at the catalytic site is localized on site 1, the site with the lowest ADP dissociation constant. The ATP at non-cata-

lytic sites must be localized on sites 4 and 5, since these have the lowest ATP dissociation constants. These considerations lead to the distribution of bound nucleotides in isolated CF_0F_1 depicted in Fig. 2.

Trying to occupy selectively the different non-catalytic binding sites, we found no possibility to achieve this when the incubation was started with the isolated CF_0F_1. Incubation of isolated CF_0F_1 with ADP in the presence of EDTA and subsequent removal of free and loosely bound ADP resulted in CF_0F_1 where one ATP is bound at the non-catalytic site 4 and two ADPs are bound at the catalytic sites 1 and 2 [16]. With this pretreated CF_0F_1 it was possible to occupy selectively at low 2-azido-[α-^{32}P]ATP concentrations (20 μM, 2 min) one binding site (procedure 7 in Fig. 2). When the 2-azido-[α-^{32}P]ATP concentration is increased (100 μM, 10 min), two sites are occupied (procedure 6 in Fig. 2). In order to occupy selectively the binding site which required the higher 2-azido-[α-^{32}P]ATP concentration, the pretreated CF_0F_1 was first incubated with a low ATP concentration. This leads to binding of one ATP per CF_0F_1. A further incubation with 100 μM 2-azido-[α-^{32}P]ATP then resulted in the occupation of the low-affinity site (procedure 8 in Fig. 2).

HPLC analysis of the tryptic peptides shows that for procedures 6, 7 and 8 more than 80% of the peptides are recovered as nitreno-ATP peptides and 10–20% as nitreno-ADP peptides. Amino acid sequence analysis of both ATP and ADP peptides indicates labeling of β-Tyr385. Obviously, with procedures 6, 7 and 8 exclusively non-catalytic sites are labeled and a small fraction of the bound 2-nitreno-[α-^{32}P]ATP is hydrolyzed. Presumably, this hydrolysis does not occur at the native enzyme but during the trypsin treatment. When the 2-azido-[α-^{32}P]ATP used in this work is treated in the same way as the enzyme during trypsination (i.e. incubation with trypsin for 18 h at 37°C) we found that between 10 and 20% of the 2-azido-[α-^{32}P]ATP is hydrolyzed to 2-azido-[α-^{32}P]ADP. Since similar fractions of nitreno-ADP peptides were found after derivatization of the different non-catalytic sites, we assume that nitreno-ATP peptides are partially hydrolyzed during trypsination.

One ATP per CF_0F_1 remains bound during all these treatments and, since ATP bound at catalytic sites is hydrolyzed rapidly to bound ADP, this ATP must be bound at a non-catalytic site. Obviously, this ATP is very tightly bound, i.e. it is located at the site with the lowest dissociation constant. According to our nomenclature this site is called site 4. The site with the intermediate dissociation constant which is occupied with procedure 7 is then site 5 and the site with the highest dissociation constant, occupied with procedure 8, is site 6.

To achieve a selective occupation of site 4, the ATP on this site was converted to ADP by treatment with EDTA at room temperature in the presence of BioBeads. This results in an enzyme containing two ADPs, one at catalytic site 1 and one at non-catalytic site 4. Incubation of the enzyme with this occupation pattern with 2-azido-[α-^{32}P]ATP resulted in CF_0F_1 with one bound 2-azido-[α-^{32}P]ADP and one bound 2-azido-[α-^{32}P]ATP. Since, at catalytic sites, bound ATP is always completely hydrolyzed to bound ADP whereas at a non-catalytic site only ATP is found [16], the 2-azido-[α-^{32}P]ADP must be located at a catalytic site and 2-azido-[α-^{32}P]ATP at a non-catalytic site. Per definition, the corresponding sites are site 1 for catalytic sites and site 4 for non-catalytic sites. During the incubation the two bound nucleotides apparently exchange and the exchange at site 4 can be explained by the fact that ADP is less tightly bound at this site then ATP. When, finally, the enzyme was incubated with ADP an exchange of most of the 2-azido-[α-^{32}P]ADP at catalytic site 1 occurred whereas the 2-azido-[α-^{32}P]ATP at site 4 remained bound. UV-irradiation and HPLC analysis of the tryptic peptides show that about 70% of the radioactivity is obtained as nitreno-ATP peptide, about 30% as nitreno-ADP peptide. Amino acid sequence analysis indicated that the nitreno-ATP was located exclusively at Tyr385, i.e. at a non-catalytic site; the nitreno-ADP at Tyr362, i.e. at a catalytic site. From these data we conclude that site 4 is occupied by 2-azido-[α-^{32}P]ATP, and that some 2-azido-[α-^{32}P]ADP remained on site 1, i.e. in the last step of procedure 9 a complete exchange of nucleotides at this site was not obtained. These considerations lead to the occupation patterns depicted in Fig. 2 for the different procedures, the analytical data are summarized in Table 1.

4.2. Differences between the non-catalytic nucleotide binding sites

Our results show clearly that the inactive isolated CF_0F_1 contains three non-catalytic sites with different nucleotide binding affinities. The procedures described here for selective occupation of these sites make use of these different ATP-binding affinities in non-activated CF_0F_1. During these treatments, no catalytic reaction, with a possible interchange of sites, takes place but, nevertheless, binding of a nucleotide at one site might influence the affinity of the nucleotide at another site.

The non-catalytic site 6 has the lowest affinity for ATP, i.e. the highest dissociation constant. Correspondingly, this site is usually not filled in the isolated CF_0F_1. We have not measured the dissociation constant exactly, however, the data from Fig. 1 show that half of this site is filled at a concentration of approximately 50 μM. Site 5 has a higher affinity showing half maximal occupation at 2 μM. Site 4 has a very high affinity for ATP. We have not found a procedure which allows the removal of this ATP without simultaneous denaturation of the enzyme although, for isolated CF_1, removal of all nucleotides was reported [31]. The dissociation constant of ATP from site 4 is very low, we estimate that it is below the nM range. Similarly, as reported for isolated CF_1 [32], it is possible with CF_0F_1 to hydrolyze the ATP bound at site 4 during a long incubation at room temperature. The ADP remains bound at this site, but upon incubation with 2-azido-[α-^{32}P]ATP it is replaced with the ATP analogue.

The rate constant for binding of 2-azido-[α-^{32}P]ATP to site 5, k_{on}, can be estimated from the initial rate of binding (see Fig. 1), resulting in $k_{on} = 240\ M^{-1}\ s^{-1}$. With the dissociation constant ($K_D \approx 2$ μM), we obtain for the rate constant for dissociation $k_{off} = 5 \cdot 10^{-4}\ s^{-1}$. The association rate constant is similar to that found for ATP binding to non-catalytic sites of EF_1 [19].

Our data show that there is no large change in binding affinity of site 5 by occupation of site 6 with 2-azido-[α-^{32}P]ATP: occupation of site 6 does not lead to the release of ATP from site 5 (or 4). The binding affinities at the non-catalytic sites are influenced by interaction between the α- and β-subunits. Different occupation of the catalytic sites leads to changes in the affinities at the non-catalytic sites and vice versa. For example, in the pretreatment of CF_0F_1 occupation of catalytic site 2 with ADP facilitates the release of ATP from non-catalytic site 5 (in the presence of EDTA). We have observed earlier that site 5 has to be empty in order to bind ADP tightly at the catalytic site 3.

The treatments of CF_0F_1 with procedures 6–9 generate different occupation patterns of the non-catalytic sites as shown schematically in Fig. 2, i.e. either all non-catalytic sites are occupied or site 6 is empty or site 5 and site 6 are empty. Without UV-irradiation, the rates of ATP synthesis and ATP hydrolysis observed with all four different occupation patterns are similar (ATP synthesis 60–100 s^{-1}, ATP hydrolysis 16–22 s^{-1}). Obviously, the occupation of non-catalytic binding sites 5 and/or 6 has no influence on the rate of catalysis. In accordance with this observation, it has been found that the occupation of the three non-catalytic sites of EF_1 has no effect on ATP hydrolysis, however, the dissociation constants of the three non-catalytic sites were identical in EF_1 ($K_D = 25$ μM) and also ADP binds to the non-catalytic sites [19]. In heat-activated CF_1, the three non-catalytic sites have different affinities for ATP, however, it was found that occupation of one non-catalytic site (site 6 in our nomenclature) is necessary for ATP hydrolysis [18].

Specific occupation of non-catalytic sites with a 2-azido-nucleotide or another suitable analogue in the anticonfiguration has been performed with the medium-affinity non-catalytic site of MF_1 and in this case the 2-azido-ADP induced a partial inhibition, just like ADP. Binding of ATP to the low-affinity non-catalytic site was proposed to lower the affinity of the catalytic sites for ATP [33].

A major issue is the question whether the differences between the three non-catalytic sites are permanent or whether the sites interchange during catalysis. The results reported in this paper do not discriminate between these two possibilities. However, when enzyme with 2-azido-ATP at site 6 is activated and performs ATP hydrolysis, the analogue is lost, while preliminary data indicate that this is not the case when the analogue is bound at site 4, suggesting a permanent asymmetry. Studies with Lucifer Yellow Vinyl Sulfone reported in the literature indicate the same: during preincubation one specific

α-subunit binds the ligand and during catalysis no other subunit acquires this property and after catalysis the original conformation is restored [34,35]. Richter et al. [35] even conclude from FRET measurements that during catalysis only two of the three catalytic sites randomize, indicating that also the catalytic sites are not equivalent.

4.3. Release of non-covalently bound 2-azido-[α-^{32}P]ATP during energization

Energization of proteoliposomes containing CF_0F_1 with non-covalently bound 2-azido-[α-^{32}P]ADP at any of the catalytic sites leads to a partial release of these nucleotides [16,17]. In this work, we observed upon energization a release of about 4–6% of non-covalently bound 2-azido-[α-^{32}P]ATP when non-catalytic sites are occupied. This effect was surprising since non-catalytic sites were not expected to be involved in catalysis. On the other hand, it is possible that energization leads to a change of the affinities of non-catalytic sites resulting in a release of bound nucleotides. For the kinetic experiments reported here, CF_0F_1 with the different occupation pattern (see Fig. 2) was irradiated immediately after preparation before it was reconstituted into liposomes and, therefore, the initial occupation pattern cannot change during reconstitution. The investigation of the release of the azido-nucleotides after energization requires that these are not covalently bound, and it is possible that the nucleotide content and/or the occupation pattern changes during reconstitution. UV-irradiation of CF_0F_1 after reconstitution and HPLC analysis of the tryptic peptides indicated that in fact the occupation pattern changes. After reconstitution, a part of the 2-azido-[α-^{32}P]ATP is lost from the non-catalytic site and found as 2-azido-[α-^{32}P]ADP at a catalytic site. The extent of this migration depends on the site being occupied and the duration of the reconstitution. Since energization leads to a release from catalytic sites and we know its extent, we can calculate the expected release, when the fraction of occupied catalytic sites is known. This calculation (see Table 2) shows that the observed release can be explained when it occurred only from catalytic sites. We conclude from this result that energization of the proteoliposomes does not lead to release of 2-azido-[α-^{32}P]ATP from non-catalytic sites. This implies that energization of the enzyme does not lead to a significant change of the dissociation constants of non-catalytic sites.

4.4. Covalent labeling and inhibition of catalysis

Non-covalent binding of 2-azido-[α-^{32}P]ATP to either site 4, 5 or 6, before reconstitution, did not affect the rate of ATP synthesis, whereas covalent binding of 2-nitreno-[α-^{32}P]ATP inhibited ATP synthesis proportional to the degree of labeling. When only one site, either 4, 5 or 6, is labeled, extrapolation indicates complete inhibition when one nucleotide is covalently bound per CF_0F_1 (Figs. 3 and 7). When sites 5 and 6 together are derivatized, extrapolation reveals that inhibition is complete when two nucleotides per enzyme are covalently bound (Fig. 7). The latter result is surprising since bound nitreno-radical, formed upon illumination, should have the same probability to react with β-Tyr385 or with water, independent of what happens at other sites. The efficiency of the formation of a covalent bond appears to be about 50% at all non-catalytic sites. When two sites are occupied simultaneously, this efficiency remains the same, since 1 mol 2-azido-[α-^{32}P]ATP becomes covalently linked when sites 5 and 6 together are occupied (see Table 1). Since one covalently bound nucleotide completely blocks multisite catalysis at this enzyme and some enzymes contain two bound nucleotides, a non-linear relation between activity and the amount of covalently bound nitreno-ATP is expected. This is obviously not observed. The most probable explanation seems to be that the covalent derivatization at one site induces a slight conformational change at the second site such that, upon formation of the nitreno-radical, in almost all cases a covalent bond with the tyrosine is formed. Correspondingly, in the enzyme molecules in which at the first site the nitreno-ATP has not reacted with Tyr385 but with water also the nitreno-ATP formed at the second site reacts almost exclusively with water. A similar observation was made when catalytic sites 1 and 2 are simultaneously derivatized [17].

The relation between inhibition and covalent derivatization with 2-nitreno-nucleotides has been extensively studied with the F_1-parts from different sour-

ces. In most cases, one covalently bound nucleotide was sufficient to inhibit ATP hydrolysis, however, in some cases two nucleotides were required for complete inhibition and not always simple correlations have been found. Derivatization of a catalytic site always induces complete inhibition, either of only multi-site catalysis or of both uni- and multi-site catalysis (see [17] for discussion). The effect of derivatization of a non-catalytic site seems to be organism-specific. For heat-activated CF_1, Melese et al.[36] report nearly full inhibition when mainly a non-catalytic site was derivatized with 2-nitreno-AXP. Similarly, when in TF_0F_1 one non-catalytic site was occupied with 2-nitreno-[α-^{32}P]ADP, the rate of ATP synthesis as a function of the derivatized enzyme extrapolated to full inhibition when one nitreno-ADP was bound per TF_0F_1 [15]. With the mitochondrial enzyme, MF_1, however, no inhibition by covalent modification of a non-catalytic site was reported [21,22,33].

Our data indicate that in CF_0F_1 covalent derivatization of any of the non-catalytic sites inhibits completely multi-site ATP synthesis and ATP hydrolysis. Since none of these sites is expected to participate directly in the catalytic reaction, we have to conclude that in the chloroplast ATP synthase the cooperative interactions that accompany multi-site catalysis not only include amino acids at the interphase between α- and β-subunits [37] but also the non-catalytic binding sites, in such a way that a covalent linkage of nitreno-ATP to β-Tyr385 in the α-site inhibits the conformational changes required for site–site interaction between the catalytic sites.

On the other hand, the covalent derivatization of β-Tyr385 at any of the α-sites does not inhibit uni-site catalysis. This implies first, that the activation of the enzyme is still possible and second, that the conformational changes involved in multi-site catalysis do not occur during uni-site catalysis, in line with other reports in the literature [38,39].

The transition between inhibited and non-inhibited rate occurs at 200 nM ADP for ATP synthesis, independent of the site that is derivatized. For ATP hydrolysis this transition occurs at 20 nM when site 4 or 5 is blocked and at 200 nM when site 6 is blocked. The reason for this difference is not clear.

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