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One of the non-exchangeable nucleotides of the mitochondrial F_1-ATPase is bound at a β-subunit: evidence for a non-rotatory two-site catalytic mechanism

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Abstract

In active MF_1, one of the two non-exchangeable tightly bound adenine nucleotides is an ATP, while the other is an ADP. The respective sites are called the T-site and the D-site. The activity of the enzyme correlates linearly with the amount of bound ATP, ADP at the T-site being inhibitory. When MF_1 is stored at room temperature in 50% glycerol and 100 mM Tris-HCl (pH 7.3) after slow passage through a Sephadex column, the tightly bound ATP is slowly dephosphorylated to ADP which is subsequently released, without effect on activity. When enzyme with about one residual ADP left (at the D-site) was incubated at pH 7.3, after dilution of the glycerol, with 400 μM [^{14}C]ATP under varying conditions, the amount of tightly bound nucleotide triphosphate again correlated well with activity, the residual ADP being bound at the D-site. Optimal results were obtained when the incubation was performed in the presence of a regenerating system. Binding of 2-azido-ATP instead of ATP to the T-site as a triphosphate, as indicated by the specific activity of the enzyme, appeared to be optimal when the binding was performed at pH 6.4 in the absence of Mg^{2+} and with high concentrations of the nucleotide. Under such conditions, 3 mol 2-azido-AXP per mol F_1 remained tightly bound after ammonium sulfate precipitation and column centrifugation, in addition to about one residual ADP at the D-site. After a 2-min period of turnover with ATP/Mg^{2+} as substrate two mol 2-azido-AXP were left on the enzyme, of which one was bound at a β-site. These results show that one of the non-catalytic nucleotide binding sites that contain tightly bound nucleotides, is a β-site, in conflict with the requirements for a rotatory tri-site mechanism for ATP hydrolysis. This β-site can further be identified with the T-site. The validity of these conclusions for F_1 from other sources and for catalysis by membrane-bound enzyme is discussed.

Keywords: Rotatory catalysis; Dual-site catalysis; Mitochondrial ATP-synthase; 2-Azido-ATP; Tightly bound nucleotide; Covalent modification

Abbreviations: (M)F_1, soluble part of the (mitochondrial) ATP-synthase; F_0, membranous part of the ATP-synthase; FSBA, 5′-*p*-fluorosulfonylbenzoyladenosine; NbfCl, 4-chloro-7-nitrobenzofurazan; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl)-adenosine-5′ triphosphate; PEP, phospho*enol*pyruvate; PK, pyruvate kinase (EC 2.7.1.40); MES, 2-(*N*-morpholino)ethanesulfonic acid; 2-N_3-AXP, 2-azido-AXP; 2-*N*-AXP, 2-nitreno-AXP

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1. Introduction

Many data indicate or are interpreted as indicative for the participation of all three β-sites in a tri-site rotatory mechanism of catalysis by the F_1 part of all ATP-synthases [1–4], the γ-subunit rotating within the ring of α- and β-subunits. In the direction of ATP synthesis this rotation is induced by the proton translocation-coupled rotation of the ring of c-subunits in the F_0 and causes conformational changes in the nucleotide binding sites of the β-subunits, resulting in ATP synthesis. The reverse reaction occurs during ATP hydrolysis.

This view has gained even more weight by the finding that the F_1 is connected with the membranous F_0 part not only via the γ/ε-c connection, but also via a second stalk, connecting the α/β-part of F_1 via the OSCP (the δ-subunit in bacterial enzymes) with the b-subunits of the F_0 [5,6]. This newly described 'stator' is thought to allow the transmission of the rotation of the c-subunits relative to the a- and b-subunits, to the γ-subunit without inducing a concomitant motion of the α/β-moiety of the enzyme.

A rotatory mechanism implies that all three β-sites participate equally in catalysis and any finding that shows that the three β-sites are not equivalent and do not participate equally in catalysis, constitutes an argument against a rotatory mechanism. Such findings exist in our view and the idea of a rotatory mechanism should be rejected if these findings can be substantiated. Since, however, many features of a rotatory mechanism fit nicely with the available structural data, even Popper [7] would understand that only very rigorous evidence against rotation is effective in changing people's opinion.

Some data that we interpret as favouring participation of only two β-sites in multi-site catalysis, one β-site fulfilling a different role, are the following:

1. ligands that bind to catalytic sites, such as TNP-ATP [8] and fluoro-aluminium azido-ADP, bind only to two sites in mitochondrial F_1 [9,10];
2. dissociation-reconstitution experiments with Nbf-treated enzyme show that one specific β-subunit may contain the inhibitory ligand without affecting catalysis [11];
3. with 8-azido-adenine nucleotides, two non-catalytic α-sites and two catalytic β-sites can be largely modified while two adenine nucleotides remain tightly bound [12,13]. Also the data obtained with 2-azido-AXP analogues strongly indicate that in MF_1, two non-catalytic α-sites and two catalytic β-sites can bind a ligand molecule, while the enzyme still contains two tightly bound adenine nucleotides at the two sites with a non-exchangeable nucleotide ([14], A.F. Hartog and J.A. Berden, unpublished observations);
4. MF_1 can bind 5 mol of FSBA on α-subunits without loss of tightly bound nucleotides, leaving only one position at an α-site available for tightly bound AD(T)P, the other bound AD(T)P apparently being bound at a β-subunit [15];
5. with the enzyme from mammals [16], chloroplasts [17] and *Escherichia coli* [18], it has been shown that slow single-site catalysis by the first β-site is not or only slightly enhanced upon addition of high concentrations of ATP, which induce rapid catalysis at the other catalytic sites. The cases in which a large enhancement of the rate of catalysis at a single site is found upon addition of a high ATP concentration, either involve incomplete enzyme (lack of δ-subunit in *E. coli* [18]), or do not relate to the first β-site, but to the second. In the classical experiments of Grubmeyer et al. [19] and Cross et al. [20] the first β-site may be occupied with a tightly bound, not-exchanging nucleotide ([21] and Section 4).

All these data indicate to us that the first β-site is different from the other two, in agreement with the early interpretation of electron micrographs [22]. Also, the establishment of the presence of a second stalk between F_1 and F_0, consisting of OSCP (the δ-subunit in *E. coli*) connected with the b-subunits of F_0, in fact strengthens this interpretation of basic asymmetry, since it implies that one α/β-couple is permanently linked with the OSCP (δ) and this link is a separate feature of only one α/β-couple, discriminating it from the other two. Covalent linkage of the δ-subunit with an α-subunit in the *E. coli* enzyme does indeed not affect ATP hydrolysis [23]. We speculate that this one α/β-couple contains the two tightly bound nucleotides in MF_1 and does not participate directly in rapid multi-site catalysis.

In order to confirm or disprove our conclusions from the above data for the mechanism of catalysis,

we have to design experiments that can discriminate unequivocally between the two existing models for a catalytic mechanism. An essential difference between the two models is the localisation of the two tightly bound, non-exchangeable nucleotides present in isolated MF_1. For rotational catalysis it is essential that both are bound at α-sites (all three β-sites are participating in multi-site catalysis), while our model for a two-site mechanism of catalysis implies that only one of the non-exchangeable nucleotides is bound at an α-site, the other being bound at a β-site. A previous attempt to localise the tightly bound nucleotides, using dissociation–reconstitution experiments [24] did not provide conclusive results. In the present approach, using 2-azido-AT(D)P as suitable analogue for AT(D)P, we have used glycerol treatment to obtain nucleotide replacement and have found that one non-exchanging nucleotide is bound at a β-site. This result confirms our previous conclusions and contradicts a rotatory mechanism of catalysis.

2. Materials and methods

2.1. Preparation of F_1

F_1-ATPase was isolated from bovine hearts according to the method of Knowles and Penefsky [25] 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 stored enzyme F_1-ATPase preparations were made in TES-buffer (pH 7.3), containing three bound adenine nucleotides, as described previously [26]. TES-buffer contains 50 mM Tris, 4 mM EDTA and 150 mM sucrose brought to pH 7.3 with HCl. In the last step of this procedure (dilution of F_1 to 2 mg protein/ml), dependent on the experiment, sometimes 10 mM Mg^{2+} was added. The preparations had a specific activity of 120–150 μmol ATP/min/mg.

2.2. Preparation of nucleotide-depleted F_1

To prepare F_1 with only one bound adenine nucleotide the procedure of Garrett and Penefsky for nucleotide depletion [27] was largely followed, but HCl instead of H_2SO_4 was used to lower the pH to 7.3 (TEG-50 medium, containing 100 mM Tris-HCl (pH 7.3), 4 mM EDTA and 50% glycerol). In the eluate from the Sephadex G-50 medium column, A_{280}, A_{260}, ATP, ADP, protein and ATPase activity of each fraction were measured. The protein determination [28] correlated well with the A_{280}, 1 mg protein/ml corresponding with $A_{280} = 0.540$. Fractions with a certain concentration of F_1 were pooled. The F_1 still contained two or three bound nucleotides (mol/mol), but the nucleotides were slowly released upon further storage at room temperature in the same medium. At chosen time points free nucleotides were removed by repeating the slow passage through a Sephadex G-50 column until about one bound nucleotide per F_1 remained.

2.3. Incubation procedures

The F_1 preparation in TEG-50 (2.9 mg/ml, pH 7.3) containing ±1 mol bound ADP was incubated with labelled ATP or 2-azido-ATP and diluted with four parts TES-buffer (pH 7.3) or MES-buffer (pH 6.0, final pH 6.4) to obtain a 10% glycerol solution. The final concentration of nucleotide varied between 400 and 800 μM. The MES buffer contained 50 mM MES, 4 mM EDTA and 150 mM sucrose, brought to pH 6.0 with NaOH solution. After 1 h incubation at room temperature the protein was precipitated with one volume saturated ammonium sulfate solution and stored for 10 min at 0°C. The precipitate was solubilised in TES-buffer (1–2 mg/ml) and after one extra ammonium sulfate precipitation step twice passed through a centrifugation column (Sephadex coarse G-50 in TES buffer, pH 7.3).

Incubations under turnover conditions were performed by the sequential addition of labelled ATP or 2-azido-ATP, PEP (final concentration 20 mM) and 5 μl PK, followed by a 5-fold dilution with buffer to lower the glycerol concentration to 10%. Turnover was started by addition of 10 mM Mg^{2+} (from a 1-M stock solution). After 1 min of turnover the protein was precipitated with ammonium sulfate and further handled as above. A 2-min chase was performed with part of the labelled preparation, adding 1 mM ATP/10 mM Mg^{2+} as substrate, followed by a fast column centrifugation step.

2.4. UV-Illumination

The photolabelling was performed with a penray UV lamp (UV-Products) at a 1-cm distance above the protein sample that was shielded with a thin glassplate from the low-wavelength UV in order to protect the protein and eliminate evaporation of the sample. The illumination-time was between 2 and 5 min, depending on protein concentration. No protein damage by the illumination was observed.

2.5. Assays for ATPase and ITPase activity

ATPase activities were measured with an ATP regenerating system as described [26]. The assay mix for the determination of the ITPase activity contained 10 U additional pyruvate kinase per assay (2 ml). Inhibition due to illumination was determined in relation to the activity of control samples treated in the same way, but not illuminated. The effect of illumination in the absence of any azido compound was measured separately.

2.6. Determination of protein and nucleotide content of F_1

Protein concentrations were measured with the Bio-Rad assay [28], with bovine serum albumin as standard. Bound nucleotides (ATP and ADP) were determined luminometrically as described by Van Dongen and Berden [29], but using a new Bio-orbit 1250 luminometer.

2.7. HPLC chromatography

Tryptic digests of F_1 labelled with 2-nitreno-[α-^{32}P]AXP were prepared as described by Xue et al. [30], but without acid precipitation. Trypsin was added to the F_1 solution in a 1:20 ratio (w/w) and incubated for 24 h at 37°C.

Ion-exchange HPLC was performed as described [30]. Reversed phase HPLC was performed either with fractions from the ion-exchange column, or directly with the trypsin digest. The samples were applied to 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) with a flow of 0.6 ml/min. The two LKB 2150 HPLC pumps, with a gradient mixer, were operated by a LKB 2151 controller. Absorbance was measured at 215 and 260 nm with a Pharmacia/LKB VWM 2141 detector. Radioactivity in the fractions was detected as Cerenkov radiation with the LKB/Wallac liquid scintillation counter.

2.8. Miscellaneous

TPCK-treated trypsin was obtained from Sigma. Enzymes for activity measurements were obtained from Boehringer, [^{32}P]H_3PO_4 and [^{14}C]ATP from Amersham. The 2-azido-[α-^{32}P]ATP was prepared as described before [25]. All used chemicals were of analytical grade.

3. Results

3.1. Preparations of F_1 and level of bound nucleotides

MF_1, isolated according to the procedure introduced by Knowles and Penefsky [25], can be prepared in two forms, containing either three or four bound nucleotides [31]. The enzyme with four nucleotides is obtained when the purified enzyme, stored in ATP-EDTA medium, is precipitated with ammonium sulfate, followed by passage through two filtration columns, in the presence of Mg^{2+}. In the absence of Mg^{2+}, only three bound nucleotides remain attached to the enzyme. The fourth nucleotide is an ADP that is bound at a non-catalytic α-site and whose binding affinity is strongly Mg^{2+}-dependent [31–33]. We will call this site the medium-affinity α-site.

Of the three nucleotides whose presence does not depend on Mg^{2+} [27,34], one is bound at a catalytic site and is easily replaced with an adenine nucleotide from the medium or removed from the enzyme when turnover is initiated with a substrate that does not bind tightly, such as GTP, ITP or CTP [31,35], or when the enzyme is treated with a high concentration of pyrophosphate [15].

The two other tightly bound nucleotides remain bound during catalysis and generally consist of one ADP and one ATP [12,15,36]. The two binding sites involved have been called the D-site and T-site, respectively. These sites are clearly non-catalytic and if

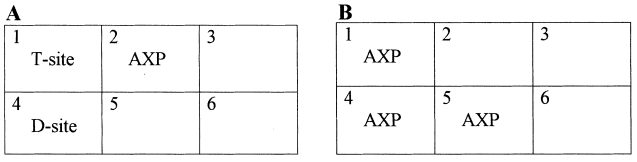

Fig. 1. Schematic representation of the nucleotide binding sites of MF_1 with high affinity. The localisation of the three tightly bound nucleotides is schematically shown, either according to our model for dual-site catalysis (A) or as envisaged by Kironde and Cross [32] and required for tri-site rotational catalysis (B). The upper row represents the three β-sites, the lower row the three α-sites, each numbered in order of decreasing affinity. In both models, one tightly bound nucleotide is catalytic and the difference between the two models is essentially the localisation of the two tightly bound not-exchanging nucleotides. In model A, the site of the tightly bound ATP (the T-site) is a β-site, while the D-site, containing tightly bound ADP, is an α-site. In model B, the sites for the tightly bound not-exchanging nucleotides are both α-sites, not differentiated between T- and D-sites. The medium-affinity non-catalytic site is site 5 in model A, but site 6 in model B. Model B does not account for a low-affinity non-catalytic site (site 6 in A) and all three β-sites are catalytically active.

all three β-sites participate in multi-site catalysis, both sites have to be α-sites, as is assumed by Kironde and Cross [32,33]. If, however, the enzyme contains a low-affinity non-catalytic regulatory α-site [13,37] in addition to the medium-affinity α-site [31–33], one of the two sites with a tightly bound nucleotide has to be a non-catalytically active β-site.

Fig. 1 gives a schematic representation of the binding sites for tightly bound nucleotides, both as we interpret the available data and as is postulated by Kironde and Cross [32,33] and required for a rotatory mechanism of catalysis.

In previous experiments [24], we have shown that upon dissociation of MF_1 into separate β-subunits and a $\alpha_3\gamma\delta\varepsilon$-moiety, one ADP molecule remains bound at the $\alpha_3\gamma\delta\varepsilon$-moiety. This suggests that the tightly bound ADP in the undissociated enzyme is bound at an α-site. This preliminary assumption also fits with the fact that in enzyme preparations that contain only bound ADP and nearly no ATP, such as isolated in the laboratory of Allison [38], all ADP is bound at α-sites. The crucial question, then, is at what type of site the tightly bound ATP is bound. Is this T-site also an α-site, as is required for a rotatory mechanism of catalysis, or is it a β-site?

3.2. Tightly bound ATP is required for full activity

We have found a clear relationship between the amount of tightly bound ATP and the specific activity of MF_1, with slight variations in slope depending on the exact conditions of buffer and pretreatment.

In Fig. 2, such a relationship is shown for various enzyme preparations in TES buffer (Tris-EDTA-sucrose, pH 7.3). Data points are used that were obtained with enzyme preparations containing either two or three bound nucleotides: all preparations contained two non-exchangeable nucleotides and the high-affinity catalytic site was either occupied with ADP (in F_1 with three bound nucleotides) or empty (in F_1 with two bound nucleotides), as a result of treatment of the enzyme with pyrophosphate [15]. The activity of the enzyme preparations seems to be linearly correlated with the amount of bound ATP, reaching a maximal value at 1 ATP/F_1. The data suggest that at zero ATP still some activity is left. This result implies that occupation of the relevant site (the T-site) with ADP results in an enzyme with low activity, providing an explanation for the phenomenon of hysteretic inhibition (see Section 4).

3.3. Slow hydrolysis and replacement of tightly bound ATP

When an enzyme preparation containing three bound nucleotides, of which one ATP, is incubated

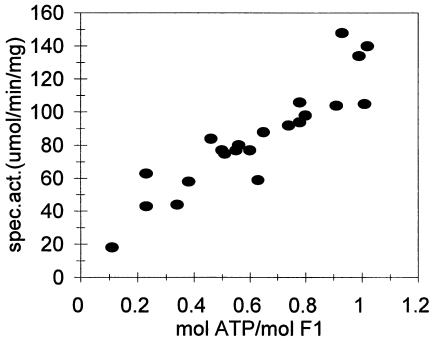

Fig. 2. Relationship between tightly bound ATP and catalytic activity of F_1. The ATP content of various preparations of MF_1 with two or three tightly bound nucleotides in TES medium was determined and plotted against their ATPase activity. In the preparations with two bound nucleotides, the tight catalytic site was not occupied due to treatment with pyrophosphate [15].

at room temperature in the absence of adenine nucleotides, the ATP is slowly (in about 24 h) converted to ADP, indicative for the catalytic nature of the binding site. Measurement of free ADP showed that the formed ADP dissociates from the enzyme and only upon prolonged incubation also the ADP from the D-site is lost. During this process, the ATPase activity declines proportional with bound ATP. Inactive enzyme cannot be activated again with e.g. glycerol, so loss of bound nucleotides results in denaturation of the enzyme. At pH 6.2 and 7.3, the same processes occur, at slightly different rates (not shown).

According to Garrett and Penefsky [27] the tightly bound nucleotides can also be removed from F_1 without inactivation by slow passage of the enzyme through a Sephadex column in the presence of 50% glycerol and 100 mM Tris-H_2SO_4. When, however, the slow passage through a Sephadex column in 50% glycerol is performed in the presence of 100 mM Tris-HCl in stead of Tris-H_2SO_4, the enzyme still contains between two and three bound nucleotides after passage through the column. Upon further storage at room temperature in the same medium, the bound ATP slowly hydrolyses and dissociates. This process takes several weeks as is shown in Fig. 3. The specific activity, however, remains constant (130 μmol phosphate/min/mg in the experiment of Fig. 3) during the whole period, since in 50% glycerol the enzyme maintains its active conformation, independent of bound nucleotides. The formed ADP slowly dissociates from the enzyme and removal of free ADP is required to obtain F_1 with only one ADP, at the D-site.

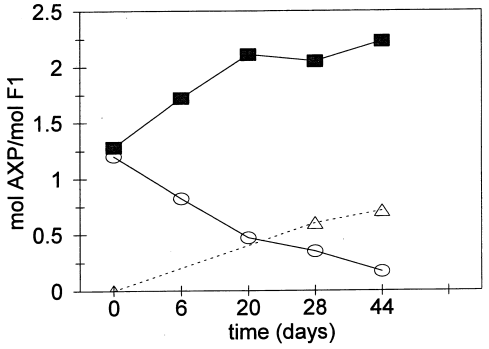

Fig. 3. Loss of bound nucleotides from F_1 during incubation in 50% glycerol. MF_1 with three bound nucleotides was slowly passed through a column in TEG-50 medium and then incubated for more than a month in the same medium at room temperature. The specific activity remained constant at 130 μmol/min/mg protein. ○, ATP; △, free ADP; ■, total ADP.

When all ATP is hydrolysed and the formed ADP has been removed by column centrifugation, the ADP at the D-site remains largely bound for several weeks.

3.4. Rebinding of ATP at the T-site

At the stage of the incubation at which about one ADP was still tightly bound (at the D-site), we incubated the enzyme with adenine nucleotides under varying conditions, concomitant with a 4-fold dilution of the glycerol. Incubation with ADP resulted in a (hysteretically) inhibited enzyme (not shown), in

Table 1
Incubations of glycerol-treated F_1(1 ADP) with [^{14}C]ATP at pH 7.3

Remaining endogenous ADP	mol [^{14}C]AXP/mol F_1				Specific activity (μmol/min/mg)		mol ATP/mol F_1	
	+EDTA		Mg^{2+}+PEP/PK PEP/PK		Before chase	After chase	Before chase	After chase
	Before chase	After chase	Before chase	After chase				
0.82	2.31	0.67			43	74	0.23	0.32
1.03	2.92	0.59[a]			68	54[a]	0.32	0.13[a]
0.91			2.39	1.52	95	125	0.74	0.77
0.55			2.40	1.36[a]	99	92[a]	0.84	0.69[a]

After incubation in 50% glycerol till all bound ATP had been lost, F_1 was incubated with 400 μM [^{14}C]ATP as described in Section 2, in the absence or presence of a regenerating system. After ammonium sulfate precipitation and two column centrifugation steps, nucleotide content, bound radioactivity and ATPase activity were determined. After a 2-min chase with ATP bound radioactivity and bound ATP were determined after one column centrifugation step.
[a]Chase with ITP instead of ATP.

agreement with the results reported by Jault and Allison [42] for fully nucleotide-depleted enzyme. Also in agreement with the data of Jault and Allison, binding of ATP in the presence of EDTA appeared not to be very efficient and required a high concentration. The results of two such experiments are shown in Table 1. The enzyme preparation was incubated first with 2 mM [^{14}C]ATP, 5-fold diluted with buffer (resulting in a solution with 10% glycerol and 0.4 mM ATP) and further incubated as described in Section 2. After two column centrifugation steps [43], the one originally bound ADP was still present together with between two and three additionally bound radioactive nucleotides, mainly ADP. The resulting enzyme had a relatively low specific activity. After a chase with cold ATP, followed by ammonium sulfate precipitation and two column centrifugation steps, about 0.6 mol of label per mol of enzyme were left in addition to the ADP at the D-site. The amount of total bound ATP and the specific activity had increased (see Table 1). This result indicates that not only the nucleotide at the tight catalytic site was exchanged, but that also some new ATP was bound at the T-site. When the chase was performed with ITP both bound ATP and activity decreased.

To obtain a more active enzyme with more bound ATP, a similar incubation was performed, but now Mg^{2+} and the components of a regenerating system with PEP and PK were present as well. After ammonium sulfate precipitation and two column centrifugation steps we obtained a preparation with a high specific activity, containing 0.9 residual originally bound ADP (at the tight D-site), 0.74 labelled ATP and 1.65 labelled ADP (mol/mol F_1). About one labelled ADP was removed by a 2-min chase with cold ATP/Mg^{2+} (see Table 1) and of the residual label about half was due to ATP and about half to ADP. All radioactivity had to be bound at non-catalytic and not well-exchangeable sites and since the activity corresponds quite well with the level of bound ATP (see next paragraph), all ATP is very likely bound at the original ATP-containing site, the T-site. Since about 1.5 radioactive nucleotide is bound to the enzyme and the D-site is still largely filled with its original ADP, we have to assume that the radioactive ADP is at least partly bound at the medium-affinity Mg^{2+}-dependent non-catalytic α-site.

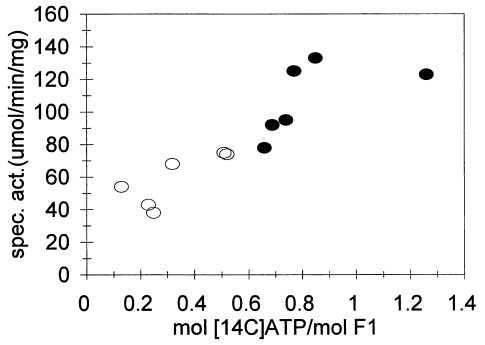

Fig. 4. Relationship between bound ATP and ATPase activity after incubation of glycerol-treated F_1 with ATP. MF_1 in TEG-50 medium with about one bound ADP left on the enzyme at the D-site, was incubated with [^{14}C]ATP after dilution of the solution to 10% glycerol. Bound [^{14}C]ATP, bound nucleotides and activity were determined at various time points ○, incubations in 4 mM EDTA; ●, incubations with turnover in the presence of PEP, PK and Mg^{2+}.

Table 1 also shows the results of an additional experiment in which the chase is not performed with ATP, but with ITP. It can be seen that upon the chase some bound ATP is lost and the specific activity is slightly decreased.

Fig. 4 shows the relationship between bound ATP and specific activity for enzyme preparations which first have been treated with 50% glycerol till all ATP had been lost (as in Fig. 3) and then incubated with ATP concomitant with dilution of the glycerol, with or without Mg^{2+}, with or without a regenerating system. The newly bound ATP has the same effect as the bound ATP in preparations that were not treated with glycerol: the specific activity of the enzyme parallels the level of bound ATP.

3.5. Binding of 2-azido-ATP at a non-catalytic β-site

The binding sites for the tightly bound non-exchangeable nucleotides are quite specific for adenine nucleotides in the anti-configuration. While 8-azido-AXP does not bind to the sites that contain the tightly bound nucleotides [29], the 2-azido-adenine nucleotides do and 2-azido-AXP can indeed be used as a suitable analogue of AXP [29,44]. They are also suitable for the characterisation of the binding sites, since upon illumination the formed nitreno-

compound only forms a covalent bond with specific amino acids. Is the binding site a β-site, then the 2-nitreno-AXP is linked to β-Tyr-345 [44]; is the binding site an α-site, then the 2-nitreno-AXP is linked to β-Tyr-368 ([45]; cf. [46]).

The previous experiments, therefore, in which labelled ATP was incubated with glycerol-treated enzyme that was free from ATP, but still contained ADP at the D-site, were repeated, using 400 μM 2-azido-[α-^{32}P]ATP instead of [^{14}C]ATP. The regenerating system was also included. The results are reported in Table 2.

In the two experiments shown, the residual bound ADP had decreased till about 0.6 mol/mol and this means that 0.4 mol 2-azido-AXP per mol F_1 could bind at this tight α-site during the incubation. After two ammonium sulfate precipitations and two column centrifugation steps about 2 mol of 2-azido-AXP per mol F_1 remained bound, of which 0.28 mol 2-azido-ATP. The activity of the enzyme was only 20–30 μmol/min/mg in agreement with the low level of bound 2-azido-ATP. This result may be due to the inefficiency of the regenerating system with 2-azido-ADP as substrate.

After a chase of 2 min with Mg^{2+}-ATP the enzyme showed increased activity and, in agreement with this increased activity, some bound ATP. Of the residual bound 2-azido-AXP (1.0–1.1 mol/mol F_1) 0.24 mol/mol was still 2-azido-ATP, indicating that the one exchanged nucleotide was mainly 2-azido-ADP.

The localisation of the bound 2-azido-AXP was determined as described in Section 2, using illumination, trypsin digestion and reversed phase HPLC. The efficiency of covalent binding upon illumination was 30% in all cases, at difference with the more common level of more than 50%. Illumination before the chase resulted in the covalent binding of 0.6 and

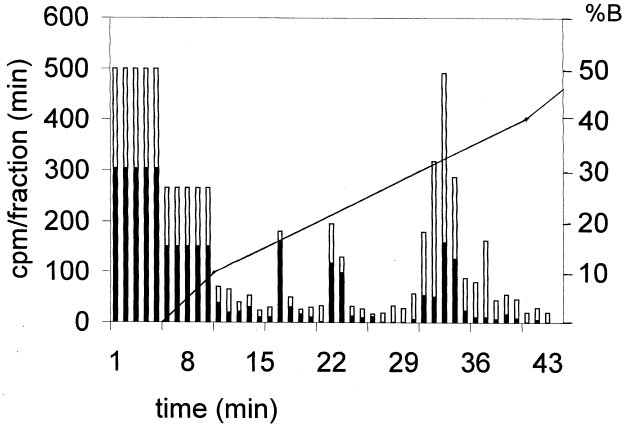

Fig. 5. HPLC analysis of trypsin-digested F_1 (incubation at pH 7.3) containing covalently bound 2-nitreno-AXP. Enzyme with about 1 bound ADP was incubated with 400 μM 2-azido-[α-^{32}P]ATP and a regenerating system at pH 7.3. Loosely bound and free ligand were removed by two ammonium sulfate precipitations and two column centrifugation steps. The samples were illuminated before or after a 2-min chase with ATP/Mg^{2+}. Further data are given in Table 2, second row. The radioactivity profiles of the eluate from the HPLC vydac-C4 RP column are shown. One-minute fractions were collected and counted for radioactivity. Open columns, the sample was UV-illuminated before a 2 min chase with ATP/Mg^{2+}; black columns, the sample was illuminated after a 2-min chase. The radioactivity in fractions 16/17 originates from the β-peptide Ile-362–Arg-372, in fractions 22/23 from the β-peptide Ile-357–Arg-372 and in fractions 31/34 the β-peptide Ala-338–Arg-356 [26].

Table 2
Incubation and photolabelling of glycerol-treated F_1 (1 ADP) with 2-N_3-[α-^{32}P]ATP

Remaining endogenous ADP	mol 2-N_3-[α-^{32}P]AXP/mol F_1		Specific activity (μmol/min/mg)	
	Before chase	After chase	Before chase	After chase
(pH 7.3+Mg^{2+}+PEP/PK)				
0.55	2.02	1.12	31	40
0.66	1.76	0.99	20	60
(pH 6.4, EDTA)				
0.81	3.27	2.33	71	110

After incubation in 50% glycerol till all bound ATP had been lost, F_1 was incubated with 400 μM 2-azido-[α-^{32}P]ATP at pH 7.3 in the presence of a regenerating system or with 800 μM 2-azido-[α-^{32}P]ATP at pH 6.4 in the presence of EDTA. Bound nucleotides, radioactivity and ATPase activity were determined after ammonium sulfate precipitation and two column centrifugation steps. Illumination was performed before or after a 2-min chase with ATP/Mg^{2+}.

0.53 mol 2-nitreno-AXP, respectively, for the two experiments reported in the upper half of Table 2. After trypsin treatment, the digests were separated on a reversed-phase HPLC column. The radioactivity profile of the eluate from the second experiment is shown in Fig. 5. It shows that two-third of the label is not bound to the column because it was not covalently bound to the protein and that of the covalently bound ligand 69% was bound at β-sites (fractions 30–35) and 31% at α-sites (fractions 17–18 and 22–23). This 9:4 ratio of the covalently bound ligand implies that in total 1.21 mol 2-azido-AXP/mol F_1 were bound at β-sites and 0.55 at α-sites. The 1.21 mol/mol at β-sites have to be distributed over two sites, one of them being the high-affinity catalytic site. For the first experiment a similar calculation gives 1.4 mol/mol distributed over the two β-sites.

After the chase with ATP, 0.99 mol 2-azido-AXP/mol F_1 were still bound in the second experiment. After illumination of the sample, inducing 30% inhibition, 0.3 mol/mol were covalently bound (30% efficiency) and the HPLC analysis (Fig. 5, closed bars) shows that half of it was bound at α-sites and half at β-sites. The effect of the chase is the specific removal of the ligand from β-sites (fractions 31–35). After the chase, then, 0.5 mol was bound at an α-site and 0.5 mol at a β-site. On one β-site, then, the bound nucleotide does not exchange during catalysis.

Since in the reported experiments the specific activity was rather low, it cannot be excluded that part of the enzyme molecules were not active and that the nucleotide at the tight catalytic site of these molecules did not exchange. Searching for conditions under which an enzyme with higher activity is obtained after incubation with 2-azido-ATP, we found that binding of 2-azido-ATP is strongly improved at lower pH values (see also [24]). The incubation was therefore repeated at pH 6.4 in the absence of a regenerating system and in the presence of EDTA (MES buffer) and an increased concentration (800 μM) of 2-azido-[α-^{32}P]ATP. After ammonium sulfate precipitation, the column centrifugation steps were performed at pH 7.3 (TES buffer). The data in Table 2, lower row, show that now a 'normal' specific activity was obtained, indicating substantial binding of 2-azido-ATP at the T-site (not measured). In total, more than 3 mol 2-azido-AXP per mol of F_1 were bound in addition to the 0.8 mol of ADP that were still bound at the D-site of the enzyme. It is clear that at pH 6.4, 2-azido-AXP is not removed from the medium-affinity non-catalytic α-site by ammonium sulfate precipitation in the presence of EDTA, since in total 4 nucleotides remained bound. After a 2-min chase with ATP, about one 2-azido-adenine nucleotide was lost (from the tight catalytic site) and 3 bound nucleotides remained, of which more than two were 2-azido-AXP. With three bound nucleotides after a chase all non-catalytic high-affin-

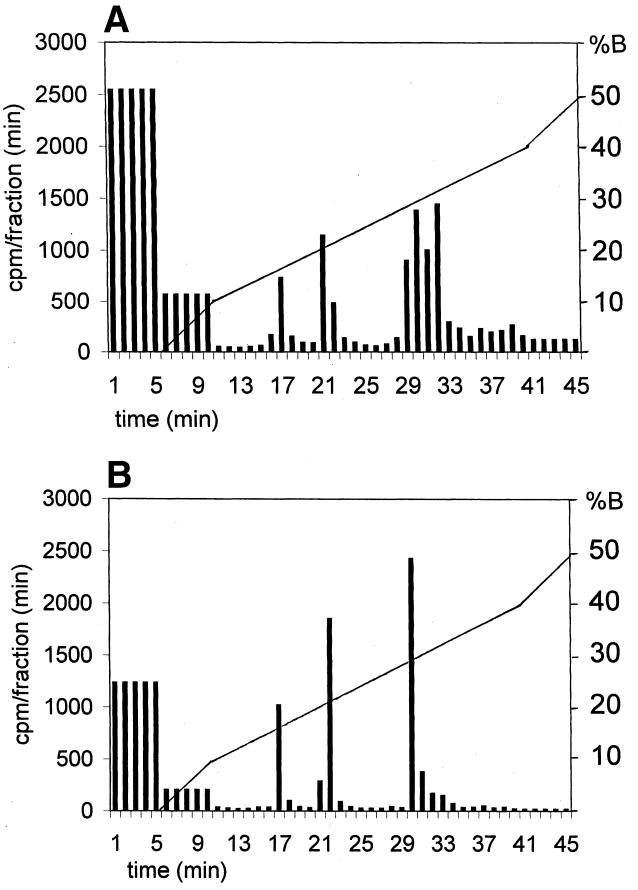

Fig. 6. HPLC analysis of trypsin-digested F_1 (incubation at pH 6.4) containing covalently bound 2-nitreno-AXP. Enzyme with about 1 bound ADP was incubated with 800 μM 2-azido-[α-^{32}P]ATP at pH 6.4. Loosely bound and free ligand were removed by two ammonium sulfate precipitations and two column centrifugation steps. Samples were UV-illuminated either before (A) or after (B) a 2-min chase with ATP/Mg^{2+}. Further data are given in Table 2, last row. The radioactivity profile of the eluate from the HPLC vydac-C4 RP column is shown. One-minute fractions were collected and counted for radioactivity.

ity sites are occupied and the question now is whether one of them, the T-site, is a β-site.

Illumination of the enzyme containing slightly more than 3 bound 2-azido-AXP and 0.8 residual bound ADP resulted in the covalent binding of 0.9 mol 2-nitreno-AXP per mol of enzyme (again 30% efficiency) and about 80% inhibition. Subsequently the enzyme was digested with trypsin and reversed phase HPLC of the resulting peptides showed that of the covalently bound 2-nitreno-AXP 65% was bound at β-sites and 35% at α-sites (Fig. 6A), indicating that before the illumination about two 2-azido-nucleotides were bound at β-sites and one at α-sites, in addition to the residual ADP.

After treatment of the enzyme preparation for 2 min with ATP and Mg^{2+} just more than 2 mol of 2-azido-AXP per mol of enzyme were retained. After illumination and trypsin digestion the digest was fractionated on reversed phase HPLC and now the elution pattern of the radioactivity showed that 48% of the covalently bound 2-nitreno-AXP was bound at β-sites (Fig. 6B). The non-covalently bound ligand appears in the void volume. It accounts for 65–70% of total radioactivity. The two peaks of radioactivity in fraction 17 and fractions 21/22 represent the β-Tyr-368 peptides Ile-362–Arg-372 and Ile-357–Arg-372, respectively. The peak of radioactivity eluting in the fractions 30/31 contains the β-peptide Ala-338–Arg-356, the β-Tyr-345 peptide [26,44,45]. One may conclude, then, that some 2-azido-AXP was bound at the D-site, that about one nucleotide per enzyme molecule was bound at the medium-affinity non-catalytic α-site and nearly one nucleotide at a non-catalytically active β-site. This has to be the T-site. After the chase, some ATP was also found to be bound after column centrifugation and this may be related with the increased activity.

4. Discussion

The role of the tightly bound nucleotides in F_1 has been an intriguing object of research from the moment they were discovered in the early seventies [27,34,47]. The discovery of tightly bound nucleotides was very helpful in the development of the idea that in the processes of ATP synthesis and hydrolysis, tight binding of the nucleotide was an intermediate step [48,49] and the binding change mechanism [1] was the final result. However, this mechanism explains the presence of only one tightly bound nucleotide, bound at a catalytic site. Harris and colleagues [50] proposed that the other tightly bound nucleotides were involved in ATP synthesis, being released from the enzyme under conditions of high energy. This proposal, however, could not be maintained as the measured release under high-energy conditions was much too slow to be part of the catalytic mechanism [51]. Also, in more recent years, rapid exchange of tightly bound nucleotides during ATP synthesis has never been observed. Using 2-azido-ADP as substrate for phosphorylation in submitochondrial particles the total exchange of bound nucleotides was the same as found when 2-azido-ATP was used as substrate for hydrolysis [52]. The conclusion, then, is that the two non-exchangeable nucleotides in MF_1 (but also in MF_0F_1, see [53]) are bound at sites that are not catalytically active and most authors assume, as a consequence, that these nucleotides are bound at α-sites. The third α-site is then the medium-affinity non-catalytic binding site that binds ADP quite tightly in the presence of Mg^{2+} [26,33] and no regulatory low-affinity α-site exists, despite many data in favour of such a site [14,37,54,55]. A low-affinity regulatory α-site can only exist when one of the two non-exchangeable high-affinity sites is located at a β-subunit.

The data presented in this paper show first that one of the two non-exchangeable sites has to contain ATP in order to have an active enzyme. Also, after removal of this ATP during incubation in 50% glycerol and binding of new ATP at the same site after dilution of the glycerol, the relationship between activity and bound ATP holds. Since the D-site is an α-site, this T-site is the candidate for a possible non-catalytically active β-site.

Second, bound ATP dissociates from the enzyme only after dephosphorylation, indicating that the binding site is a potentially catalytic site, a β-site. In the usual buffer system at room temperature, this hydrolysis of ATP, followed by dissociation of the formed ADP, results in inactivation and denaturation of the enzyme. In the presence of 50% glycerol, the hydrolysis and subsequent loss of ATP has no consequences for the activity, 50% glycerol being a medium in which the enzyme does not require bound

nucleotides for stabilisation of the active conformation.

Third, HPLC analysis shows that after incubation of enzyme that contains only ADP at the D-site, with 2-azido-ATP at pH 7.3 in the presence of a regenerating system, more than 1 mol 2-azido-AXP/mol F_1 is bound at β-sites whose occupation with ligand is resistant to ammonium sulfate precipitation and two column centrifugation steps. When the incubation is performed at pH 6.4 in the presence of EDTA, even 2 mol of 2-azido-AXP remain bound at β-sites after ammoniumsulfate precipitation and two column centrifugation steps, while only one catalytic site retains its bound nucleotide under these conditions.

Fourth and finally, after a chase of 2 min with ATP and Mg^{2+} in order to exchange the 2-azido-AXP at catalytic sites, half the residual 2-azido-AXP appears to be bound at a β-site. For the experiment at pH 7.3 this is about 0.5 mol/mol, and for the experiment at pH 6.4 this is nearly 1 mol/mol of enzyme. In this latter experiment, the medium-affinity non-catalytic α-site also retains bound 2-azido-AXP.

The straightforward conclusions from our experiments are: (1) one high-affinity nucleotide binding site in active MF_1 contains a tightly bound ATP that does not exchange with medium nucleotides during multi-site catalysis and the presence of ATP at this site is required for an optimal catalytic activity of the enzyme; and (2) one β-site in MF_1 retains its bound ligand during catalysis and does not participate in multi-site catalysis. The T-site, therefore, is a β-site. The consequence is that multi-site catalysis is performed by only two sites and not by three sites. In addition, rotational catalysis is not possible.

4.1. Hysteretic inhibition can now be explained

Literature data show that preparations that do not contain ATP after isolation, but only 2 mol ADP/mol F_1, are hysteretically inhibited after incubation with ADP [38–41]. After incubation these enzyme preparations contain three tightly bound non-exchanging nucleotides, just as the preparations isolated according to the procedure of Knowles and Penefsky and freed from loosely bound nucleotides in the presence of Mg2+ (see Section 1). Since the 2 mol of ADP in the isolated Allison preparation are bound at α-sites, the additional ADP will bind at the site where in the Knowles and Penefsky preparation ATP is bound, the T-site. But ADP at this site is inhibitory, resulting in an enzyme with low activity upon proper binding of the ADP-Mg^{2+} [42,43]. Without incubation with ADP, the substrate ATP will bind to the T-site and the enzyme shows proper activity.

4.2. Comparison with previous data in the literature

Jault and Allison [42] have reported studies with nucleotide-depleted F_1 and 2-azido-AXP in which they detected only minor labelling of β-sites. They found that 2-azido-ADP showed the highest affinity for a catalytic site, but the measured labelling by 2-nitreno-ADP was very low since they used 2-azido-[β-^{32}P]ADP and most radioactivity may be lost as a consequence of the formation, after covalent binding to a β-site, of 2-nitreno-AMP. This phenomenon is well established [26,45,56]. Jault and Allison found additional binding of 2-azido-ADP at two α-sites and this result fits completely with our data: in our enzyme, one α-site still contained bound ADP and one site bound 2-azido-AXP, so two α-sites were occupied. The fourth site to be occupied in our experiments is the T-site, but in the experiments of Jault and Allison, no extra label at a β-site was detected. The explanation, however, is evident: the binding is weak at pH 8.0 (even at pH 6.4 a much higher concentration of 2-azido-ATP is required than used by Jault and Allison) and in addition, if some 2-nitreno-ADP is bound at this site, most of it may be present as 2-nitreno-AMP, not detected because of loss of the radioactive β-^{32}P.

We now have to discuss how the one experiment is explained that seemingly proves rotational catalysis by F_1, the experiment of Noji et al. [4], welcomed by the research community as final evidence for the postulate that catalysis by F_1 is accompanied by rotation. However, such a conclusion cannot be drawn, for several reasons.

First, the absence of tightly bound nucleotides in TF_1 [57] indicates that the nucleotide at the first β-site dissociates off quite easily, even in the intact enzyme, and this rate can be fast enough to be compatible with the (relatively) slow rate of multi-site

catalysis observed in the experiments of Noji et al. [4].

Second, the used enzyme was not a complete enzyme. It has been reported for the *E. coli* enzyme that the slow rate of catalysis by the (single) first site is not enhanced upon addition of a high ATP concentration (inducing rapid multi-site catalysis), when the enzyme is complete [18]. A strong enhancement of the rate of catalysis at the first site upon induction of multi-site catalysis is a necessary requirement for participation of this site in multi-site catalysis. Only without δ-subunit such enhancement occurs and then the first site may participate in rapid catalysis. With the incomplete enzyme from PS3 the same phenomenon may be present.

Third, only a certain (low) percentage of the molecules shows in fact a rotational movement. The rate of ATP hydrolysis is not measured, but it seems very likely that also the much larger number of molecules that do not rotate, but show a different ATP-dependent movement (wobbling), hydrolyses ATP. If this is the case, it means that in the used preparation hydrolysis of ATP can be accompanied by rotation, but can also go on without rotation. A real conclusion is that in the used preparation of TF_1 subunits all three β-sites are intrinsically capable of participation in catalysis, and that rotation is possible, although not obligatory.

In MF_1, the tightly bound nucleotides do not dissociate at all, also not during multi-site catalysis. We have to stress again that the observed enhancement of catalysis at the site performing single site catalysis, as reported by Penefsky's laboratory [19,20], is probably not an enhancement of the catalysis at the first β-site, but of the catalysis at the second site. In the enzyme used by these investigators, isolated in the same way as our enzyme preparations, one β-site is occupied with a tightly bound non-exchanging nucleotide (ATP) and the observed single-site catalysis can only be performed by the second β-site, a high-affinity catalytic site. In the enzyme as prepared by the group of Allison, however, which contains two bound ADP molecules at two α-sites, single-site catalysis is performed by the first β-site. But with this preparation, the rate of catalysis at the single site is only slightly enhanced by induction of multi-site catalysis [16].

4.3. *Can the conclusion on the participation of only two catalytic sites in multi-site catalysis by isolated F_1 be extended to membrane-bound F_1 and to catalysis of ATP synthesis?*

The nucleotide binding properties of MF_0F_1 are very similar to those of isolated MF_1, as has been carefully analysed by Beharry and Bragg [53]. This means that also in the membrane-bound enzyme the ATP at the T-site is tightly bound, although some exchange of the nucleotide at this site was detected. F_1 in phosphorylating submitochondrial particles also contains about four bound nucleotides [50,58]. After hydrolysis with 2-azido-ATP as substrate only one is exchanged with a 2-azido-AXP that can be retained on the enzyme after two column centrifugation steps and is bound at a catalytic site [52,60]. Also, after an incubation during which 2-azido-ATP was synthesised, only one 2-azido-ADP remained tightly bound [52]. Three tightly bound nucleotides, therefore, do not exchange during ATP hydrolysis, nor during ATP synthesis. And since of the two low-affinity sites one is an α-site [59], only two sites with high affinity can be α-sites and one has to be a β-site. In the localisation of bound nucleotides and behaviour of the nucleotide binding sites, therefore, no substantial differences exist between isolated MF_1, isolated MF_0F_1 and F_1 in phosphorylating submitochondrial particles. And the same sites catalyse ATP hydrolysis and ATP synthesis. Since in preparations of phosphorylating submitochondrial particles, the enzyme contains at least 2 ATP per F_1 [50,58] it is likely that also in particles, just as in isolated F_1, the first β-site contains an ATP.

In recent papers from Boyer's laboratory [61–63], it was concluded that two sites can perform multi-site catalysis, and the authors assumed that a third site remains empty as long as the ATP concentration is not very high. The authors further postulated that all three sites are equivalent, each of them being empty one after the other, in this way conserving the postulate of a rotatory mechanism. The analysis of bi-site catalysis by Milgrom et al. [63] is very solid and fully agrees with our data, but the postulate of a third catalytic site that is filled only at very high ATP concentrations is in contradiction with many data

on binding and exchange of nucleotides. A catalytic site with a K_d for ATP above 1 mM has never been observed. In addition, the solid data presented by Weber et al. [64] convincingly show that the occupation of the third β-site parallels catalytic activity. The postulate of the existence of a catalytic site with very low affinity is based on the observation that TNP-ATP does not seem to compete with ATP for a binding site with an affinity for ATP in the order of the K_m of ATP for multi-site catalysis; but this observation can be explained, we think, on the basis of the assumption that the TNP moiety of the TNP-ATP, contributing heavily to the binding affinity, binds near the nucleotide binding site independent of the presence of AD(T)P in the real binding pocket and as soon as that pocket is empty, the ATP moiety of TNP-ATP occupies this pocket.

In conclusion, in membrane-bound MF_1, as well as in isolated MF_1, one β-site is continuously occupied with a nucleotide and this site is not involved in multi-site catalysis. In active isolated MF_1, and probably also in membrane-bound F_1, this first β-site is occupied with ATP, ADP at this site being inhibitory. A rotational mechanism of catalysis, involving three equally participating sites, can therefore be excluded for the mitochondrial ATP-synthase. This very likely also holds for the ATP-synthases from other sources, although it is possible that in the enzyme from the thermophilic bacterium PS3, which does not contain tightly bound nucleotides, also the first β-site can participate in multi-site catalysis.

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