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Exchange and hydrolysis of tightly bound nucleotides in normal and photolabelled bovine heart mitochondrial F_1-ATPase

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Treatment of F_1 by threefold fast-column centrifugation or by single ammonium sulphate precipitation followed by fast-column centrifugation resulted in enzyme preparations containing 2.5–2.8 mol of bound nucleotides per mol of F_1. Short incubations of such F_1 preparations in the presence of relatively low concentrations of [^{14}C]ATP and 2-azido[α-^{32}P]ATP (100–250 μM), followed by ammonium sulphate precipitation and fast-column centrifugation, resulted in exchange of about 1 mol of the bound nucleotide per mol of F_1 not affecting the total amount of bound nucleotides. Exchange of bound nucleotides with 2-azidoATP, followed by ultraviolet irradiation, results in inhibition of the enzyme activity, full inhibition being obtained (via extrapolation) when 1 mol of 2-nitreno-adenosine 5′-tri- or diphosphate (2-N-AT(D)P) is covalently bound to the presumably catalytic site on the enzyme (Van Dongen, M.B.M., De Geus, J.P., Korver, T., Harton, A.F. and Berden, J.A. (1986) Biochim. Biophys. Acta 850, 359–368). In agreement with this, it was found that incorporated [γ-^{32}P]ATP was hydrolysed by more than 80%. Newly incorporated, not covalently bound radioactive nucleotides could be rapidly exchanged again by the addition of non-radioactive nucleotides, but a higher concentration of nucleotides was needed to fully exchange the incorporated nucleotide. Also, when F_1 was depleted of most of its bound nucleotides by repeated ammonium sulphate precipitation, part of the residual nucleotides was still rapidly exchangeable. The ability of F_1 to exchange (and hydrolyse) one of the bound nucleotides was not lost when one catalytic and one non-catalytic binding site were occupied by covalently bound 8-N-ATP. Similar results were obtained with F_1 containing 2-nitrenoATP covalently bound to one of the catalytic sites. Also, after photolabelling of up to four binding sites with 8-N([2-^{3}H]AT(D)P, part of the two remaining non-covalently bound nucleotides could still be rapidly exchanged. In this case the exchanged nucleotide was also hydrolysed. It is concluded that one of the two bound nucleotides became exchangeable when all four other sites (i.e., two catalytic and two non-catalytic) were occupied with covalently bound nucleotides. The site involved showed catalytic properties suggestive of localisation on a β-subunit.

Introduction

The F_1 part of the ATP synthase complex is known to contain the nucleotide-binding sites involved in the synthesis and hydrolysis of ATP [1]. F_1 is composed of five different subunits with the

Abbreviations: 2(8)-N-ATP, 2(8)-nitrenoadenosine 5′-triphosphate; Mops, 4-morpholinepropanesulphonic acid; NBfCl, 4-chloro-7-nitrobenzofurazan; P_i, inorganic phosphate.

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following stoichiometry: 3α, 3β, 1γ, 1δ and 1ϵ [2]. The α- and β-subunits contain the six nucleotide-binding sites [3–5]. Up to now, the precise role of all the different binding sites has not been clarified: at least two sites are known to be directly involved in catalysis [4,6], and some sites might be involved in regulation [7–9]. A clear distinction between the two types of site could be made using tritiated 8-azidoAT(D)P [4]: two interacting catalytic binding sites were found to be located on the β-subunits and it was proposed that two non-catalytic sites are located on the interface between α- and β-subunits, the latter two possibly being involved in regulation. Three cooperative catalytic sites have been proposed by Gresser et al. [10] and by Cross and Nalin [11], on the basis of kinetic data and nucleotide-binding studies, respectively. The same number was assumed by Cross et al. [12], although only two sites were found. Recent electron-microscopy data [13] show asymmetry in the F_1, which indicates that one β-subunit is distinct from the other two β-subunits. Isolated F_1 from different sources is known to contain nucleotides tightly bound to the enzyme [3,4,14,15]. These nucleotides are defined as those being present on the F_1 after several ammonium sulphate precipitations or fast-column centrifugation steps. Three tightly bound nucleotides were found in isolated F_1 after fast-column centrifugation [4]. After photolabelling of such F_1 with 4 mol of 8-azidoAT(D)P, two tightly bound nucleotides were still present which could not exchange with 8-azidoAT(D)P [3,4]. On the basis of the photolabelling data, these two remaining tightly bound nucleotides are presumably located on a β and an α/β interface site. The two remaining tightly bound nucleotides could be removed by incubation with high concentrations of LiCl and it was found that the vacant sites could be partially filled with 2-azidoATP [4]. Tightly bound nucleotides have often been identified with non-exchangeable, non-catalytic sites [11,15]. Indeed, some bound nucleotides were shown to remain bound to the F_1 even after thousands of turnovers [11]. In addition, however, it has been shown that nucleotides can be bound with high affinity to catalytic sites on the enzyme [16,17]. In this paper, it is demonstrated that, in agreement with our earlier conclusions a fraction of the tightly bound nucleotides was subject to rapid exchange when turnover took place. The exchanged tightly bound nucleotides were subject to hydrolysis, indicating the catalytic character of one tightly binding site. These results are in agreement with the demonstrated high-affinity binding to a catalytic site [6,12]. Moreover, the question has been examined whether F_1 labelled to different extents with 2- or 8-azido-AT(D)P still exhibits the same high-affinity binding to a catalytic site. After having photolabelled 0–2 catalytic sites a third site was shown to be able to bind with high affinity. Secondly, the cooperativity between catalytic binding sites has been investigated in photolabelled F_1, giving further information about the interaction between catalytic binding sites on the F_1.

Materials and Methods

Bovine heart mitochondrial F_1 was isolated according to the method Knowles and Penefsky [18] and stored in liquid nitrogen in a medium comprising 10 mM Tris-HCl (pH 7.5), 4 mM ATP, 250 mM sucrose and 4 mM EDTA. Prior to use, F_1 was subjected to ammonium sulphate precipitation and fast-column centrifugation [19] (unless otherwise indicated) in order to remove medium and loosely bound nucleotides. The Sephadex G-50 coarse used in the centrifugation columns was pre-equilibrated, either in the storage medium from which ATP had been omitted (medium A) or in medium in which ATP had been omitted and EDTA had been replaced by 6 mM $MgCl_2$ (medium B). ATP hydrolysis was measured spectrophotometrically as described previously [4]. The ATPase activity in the presence of 10 mM of the activating anion bicarbonate was found to be about 100 U/mg. Binding experiments with (radioactive) nucleotides were performed by incubating F_1, depleted of medium and loosely bound nucleotides, for 60 s in the presence of 250 μM of nucleotide at room temperature (unless otherwise indicated). After incubation, non- and loosely bound nucleotides were removed by ammonium sulphate precipitation and fast-column centrifugation and the amount of radioactivity was determined. [^{14}C]ATP was measured in scintillation fluid and [γ-^{32}P]ATP was measured as Cerenkov radiation in a Packard Tricarb liquid

scintillation counter. Tightly bound nucleotides present on F_1 were extracted from the enzyme with 5.2% perchloric acid followed by neutralisation with 0.6 M Mops/6.0 N KOH [20]. The amount of extracted nucleotides was measured with the luciferine/luciferase system. Irradiation of F_1 in the presence of the photoreactive nucleotide analogues 2- and 8-azidoAT(D)P was performed at room temperature at 360 nm using a CAMAG universal ultraviolet lamp. Covalently bound 8-N[2-^{3}H]ADP was measured after SDS-urea polyacrylamide gel electrophoresis of the labelled F_1 preparations; after staining and destaining the α/β region was cut into slices from which the radioactivity was extracted and counted [4]. Protein concentrations were determined as described by Lowry et al. [21], using bovine serum albumin as a standard. [8-^{14}C]ATP and [γ^{32}P]ATP were from Amersham International. 2-azido[α-^{32}P]ATP was synthesized in our laboratory mainly as described by Boulay et al. [22]. ATP, phospho*enol*-pyruvate, lactate dehydrogenase and NADH were purchased from Boehringer-Mannheim. All other chemicals used were of analytical grade.

Results

Fast-column centrifugation of F_1 resulted in the removal of loosely bound nucleotides from the enzyme (Fig. 1). After three centrifugation steps, 2.8 nucleotides (1.56 ATP and 1.26 ADP) per F_1 were measured. Repeated centrifugation of F_1 resulted in a further decrease of tightly bound ATP and ADP, to a final amount of about one ATP per F_1 still bound to the enzyme after 12 centrifugation steps (Fig. 1). The results indicate that at least part of the tightly bound nucleotides is removed by repeated fast-column centrifugation. Tightly bound nucleotides have often been identified with non-exchangeable nucleotides [11]. However, the presence of exchangeable nucleotides bound with high affinity to the enzyme has been demonstrated [12,23].

The ability of F_1 that has been depleted of loosely bound nucleotides by repeated fast-column centrifugation to bind added nucleotides with high affinity is shown in Fig. 2. After incubation, non- and loosely bound nucleotides were removed by ammonium sulphate precipitation and a fast

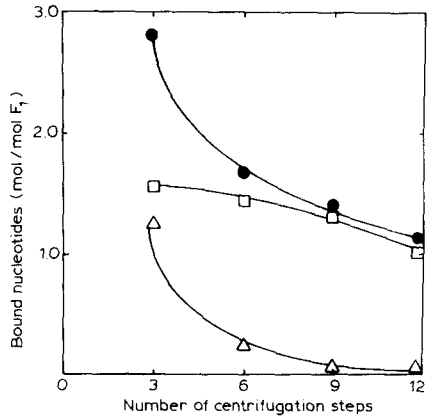

Fig. 1. F_1 (6.7 mg/ml) was applied repeatedly to a fast-centrifuge column (5×1 cm) containing Sephadex G-50 coarse pre-equilibrated in medium A. After 3, 6, 9 and 12 centrifugation steps samples were drawn; after extraction, the tightly bound nucleotides were determined by the luciferine-luciferase system. The amount of both tightly bound ATP (□) and ADP (△) and the sum of these (●) are represented. In a similar experiment the hydrolysis activity was followed, showing no loss of enzyme activity.

centrifuge column. It can be seen that, dependent on the concentration of the nucleotide used during the short incubation, up to 1.3 mol of the added radioactive nucleotide were still bound to the enzyme after ammonium sulphate precipitation and fast-column centrifugation. No difference can be seen between the extent of binding of [^{14}C]ATP and 2-azido[α-^{32}P]ATP at the different concentrations used. It can be concluded, then, that after a short incubation and subsequent removal of non-tightly bound nucleotides, at the higher nucleotide concentrations about 1 mol of nucleotide is bound to the enzyme at a tight binding site. When [γ-^{32}P]ATP was used, a maximal amount of only about 0.2 mol of label per mol of F_1 was found, showing that the newly incorporated tightly bound nucleotide was largely subject to hydrolysis and that the product [^{32}P]P_i is removed from the F_1 during the subsequent ammonium sulphate precipitation and fast-column centrifugation. This indicates that the binding site involved is catalytic. Starting with an F_1 preparation containing 0.8 mol [^{14}C]ATP/mol F_1 incorporated as described above, it was possible to exchange the incorporated nucleotide again by incubating the enzyme preparation with added non-radioactive ATP under the same circumstances as used for incorporation.

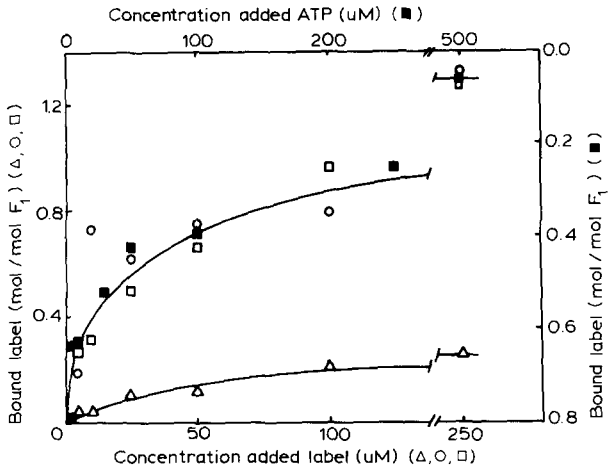

Fig. 2. F_1, pretreated by 2-fold fast-column centrifugation and dissolved in medium B (1.9 mg/ml) was incubated for 60 s at 18 °C in the presence of increasing concentrations of [^{14}C]ATP (□), 2-azido[α-^{32}P]ATP (○) or [γ-^{32}P]ATP (△). The preparations were then precipitated with 1 vol. of saturated ammonium sulphate; after 10 min at 0 °C the mixtures were centrifuged and the pellet was resuspended in medium B. This solution was applied to a fast-centrifuge column. Finally, a sample was used to measure the amount of radioactivity. After determining the protein concentration, the amount of bound label could be calculated. (■): F_1 was preloaded with 0.8 [^{14}C]ATP (mol/mol F_1) by preincubating F_1 (8.6 mg/ml) for 60 s in the presence of 250 μM of [^{14}C]ATP and subsequently treated as described above. Then, the enzyme (3.9 mg/ml) was incubated in the presence of increasing concentrations of unlabelled ATP (upper scale) under the same conditions and subsequently treated as described above in order to calculate the amount of remaining [^{14}C]ATP.

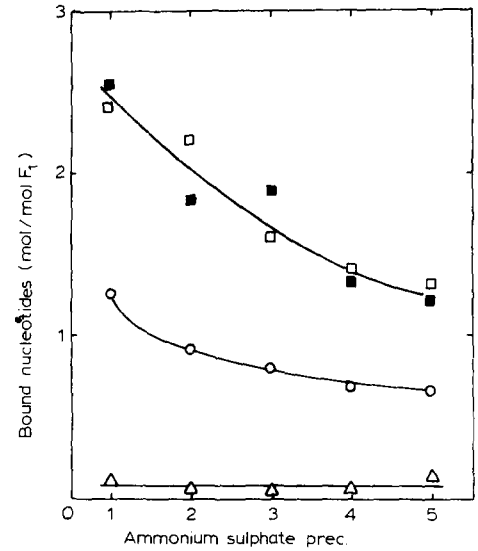

Fig. 3. F_1 dissolved in medium B was pretreated by increasing numbers of ammonium sulphate precipitation steps. After the different precipitations, samples were withdrawn and applied to a fast-centrifuge column, whereafter a sample was used to determine the amount of tightly bound nucleotides (□) (see Materials and Methods). The remaining F_1 solution (4.5–6.5 mg/ml) was incubated in the presence of 250 μM [^{14}C]ATP or [γ-^{32}P]ATP for 60 s and subsequently treated in exactly the same way as prior to incubation (increasing numbers of ammonium sulphate precipitations followed by one fast-column centrifugation). Subsequently, the total amount of tightly bound nucleotide (■) was measured as well as the amount of bound radioactivity; ○, amount of incorporated [^{14}C]ATP; △, amount of incorporated [γ-^{32}P]ATP.

However, a considerably higher concentration of ATP was needed to exchange the previously incorporated label, relative to the concentration needed for incorporation (see right-hand *Y*-axis in Fig. 2).

The consequence of the incorporation of ATP is that the exchanged nucleotide becomes part of the tightly bound nucleotides, formerly defined as being hardly exchangeable (and thus not involved in catalysis). The finding that exchanged tightly bound nucleotides are subject to hydrolysis indicates a catalytic involvement. It was interesting to investigate the exchange ability of the tightly bound nucleotides. As the amount of tightly bound nucleotides per F_1 could be lowered gradually (Fig. 1), the extent to which the tightly bound nucleotides were subject to rapid exchange was investigated at varying amounts of tightly bound nucleotides. This amount of tightly bound nucleotides was lowered from about 2.5 to 1.2 mol/mol F_1 by increasing the number of ammonium sulphate precipitations (which were followed by a fast-column centrifugation step to remove excess of salts). After a short incubation in the presence of 250 μM [^{14}C]ATP, the different samples were treated in the same way as prior to incubation. Hereafter, about the same number of tightly bound nucleotides could be measured as were present prior to incubation (Fig. 3). From this, it can be concluded that the tight nucleotide-binding sites which became vacant due to the ammonium sulphate precipitation(s) and/or fast-column centrifugation are filled during the incubation but are emptied again during subsequent ammonium sulphate precipitation(s) and fast-column centrifugation. It can be seen that a considerable number of the residual nucleotides had been exchanged

with the added [^{14}C]ATP. Even when only slightly more than one tightly bound nucleotide remained on the enzyme, half of it could be rapidly exchanged. Using [γ-^{32}P]ATP, it was found that the exchanged nucleotide was almost fully hydrolysed and P_i formed was removed. So, it can be concluded that part of the tightly bound nucleotides present on the F_1 rapidly exchanged with medium nucleotides even if slightly more than one tightly bound nucleotide is present, and that the exchanged nucleotides were subject to hydrolysis. This would suggest that nucleotides bound to a high-affinity catalytic site, as described by Grubmeyer and Penefsky [6], are as tightly bound as the non-exchangeable nucleotides. After one or two ammonium sulphate precipitations usually 2.5–3 nucleotides were still bound to F_1 (of which one is demonstrated here to be rapidly exchangeable) and it is clear that the term 'tightly bound' used for these nucleotides is only relative, and is not synonymous with non-exchangeable.

It has been reported [6,12,24] that the high-affinity conformation of one of the catalytic sites needed for tight binding disappears when a second catalytic site becomes occupied with ATP or an ATP-analogue, emphasizing the cooperative character of the reaction mechanism. Binding of 4-chloro-7-nitrobenzofurazan (Nbf), however, to one (catalytic) β-subunit does not prohibit but even induces the high-affinity conformation of the second catalytic site [25].

An important question is then what the affinity of the residual catalytic site(s) is when one or more binding sites are occupied with covalently bound nitrenoAT(D)P analogues, resulting in inhibited enzyme preparations (Fig. 4A). Photoaffinity labelling was performed with both 8-azidoATP and 2-azidoATP. F_1 was labelled with 8-azidoATP under conditions that both a catalytic (β) site and a non-catalytic (α/β interface) site are labelled [4]. Independently of the extent of inactivation of the enzyme, the same amount of [^{14}C]ATP as in the control sample was incorporated after a short-term incubation, followed by ammonium sulphate precipitation and fast-column centrifugation (Fig. 4B). Furthermore, it can be seen that the newly bound nucleotide was subject to hydrolysis, as was the case in the uninhibited control sample. From this, it can be concluded that, although the enzyme activity as measured under V_{max} conditions was inhibited by up to about 80% by covalent binding of the nucleotide analogues, the enzyme was still able to bind the same number of nucleotides with high affinity. Also, the ability of the involved high-affinity binding site to hydrolyse the bound nucleotide had not been lost. Therefore, the covalent binding of 8-nitrenoATP to a catalytic site (and to a non-catalytic site) did not

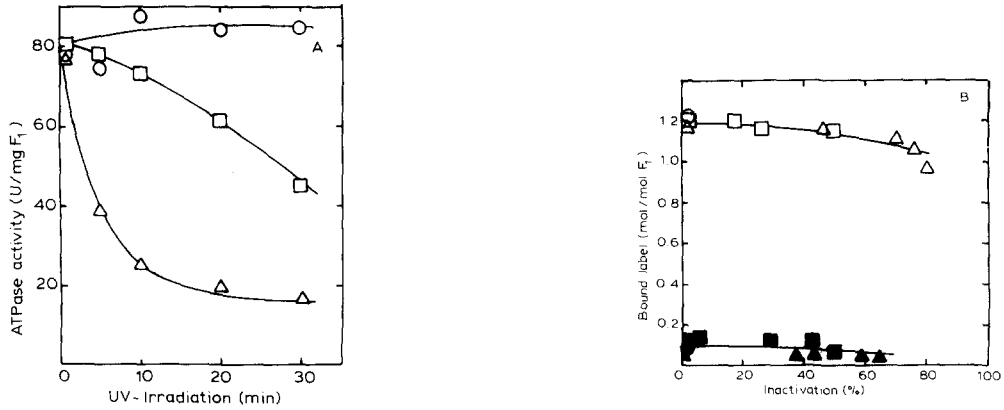

Fig. 4. (A) F_1, dissolved in medium B, was irradiated in the presence of 0.5 mM of the following nucleotides: ATP (○), 8-azidoATP (△) or 2-azidoATP (□). In the case of the 2-azido-analogue, the irradiation was preceded by an ammonium sulphate precipitation and fast-centrifuge column to remove non-bound and loosely bound photolabel. After different time intervals of irradiation, samples were withdrawn and tested for their ATPase activity. (B) Samples withdrawn as described in (A) were incubated in the presence of 250 μM of [^{14}C]ATP or [γ-^{32}P]ATP for 60 s, precipitated with ammonium sulphate and subsequently applied to a fast-centrifuge column. The amounts of bound [^{14}C]ATP (○, △, □) or [γ-^{32}P]ATP (●, ▲, ■) after irradiation were determined in the presence of ATP, 8-azidoATP and 2-azidoATP, respectively. UV, ultraviolet.

induce the low-affinity conformation of the second catalytic site. From this experiment with 8-azido-ATP, it cannot be excluded that the high-affinity binding described is only to one separate catalytic binding site on the enzyme, since it is known that 8-azidoATP cannot bind tightly [4,17] and, hence, cannot compete for the exchangeable tight site. To investigate the possibility that tight binding is to one separate binding site, 2-azidoATP was bound to the tight catalytic site. After short-term incubation with 2-azidoATP, followed by ammonium sulphate precipitation and fast-column centrifugation (conditions of tight binding), the enzyme was irradiated, and hence inactivated to different extents (Fig. 4A). Fig. 4B shows that, independently of the extent of inactivation and modification of the tight catalytic site, the same amount of [^{14}C]ATP was bound when the different samples were incubated with the radioactive label under conditions of tight binding. From this experiment, it could be concluded that high-affinity binding was not to one special binding site on the enzyme, as in that case the high-affinity binding site would have been occupied by the covalently bound 2-N-ATP. Secondly, it could also be concluded that covalent binding of 2-nitrenoATP to one site — which was previously in the high-affinity conformation — did not prevent the transfer of a second site from the low-affinity to the high-affinity conformation. Also, in this case, the newly bound nucleotides were subject to hydrolysis, again underlining the fact that high-affinity binding is to a catalytic site on the enzyme (Fig. 4B). The high-affinity binding of the 2-azido analogue has been studied before: F_1 preparations containing incorporated, tightly bound 2-azidoATP are fully inactivated when 1 mol of label is covalently bound to the enzyme upon irradiation [17].

As the extent of high-affinity binding of nucleotides could not be decreased by photolabelling a single catalytic site of F_1 with either 8-N-ATP (to the low-affinity conformation) or 2-N-ATP (to the high-affinity conformation) as the ligand, it became interesting to photolabel more binding sites on the enzyme. Such labelling has been described previously, using tritiated 8-azidoAT(D)P. After having photolabelled two binding sites with 8-azidoATP, resulting in full inhibition of enzyme

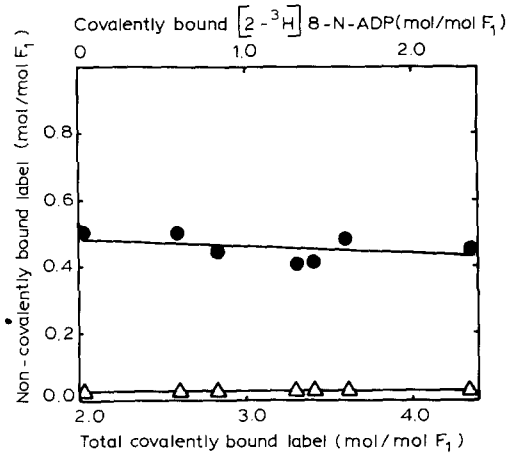

Fig. 5. F_1 (20–30 mg/ml) dissolved in medium A was irradiated in two subsequent irradiation steps of 30 min in the presence of 1.0 mM 8-azido-ATP. At different time intervals samples were drawn and tested for their hydrolytic activity; irradiation was stopped after inhibition of 95% of the enzyme activity. Subsequently, the modified F_1 (±15 mg/ml) was dissolved in medium B and irradiated in the presence of 0.5 mM 8-azido[2-^{3}H]ADP up to three irradiation steps of 25 min. At different time intervals samples were withdrawn (5–10 mg/ml) and tested for their ability to bind 2-azido[α-^{32}P]ATP (●) or [γ-^{32}P]ATP (△) with high affinity (1 min incubation in the presence of 250 μM label followed by ammonium sulphate precipitation and fast-column centrifugation). In these samples both the amount of incorporated label as well as the amount of covalently bound 8-N[2-^{3}H]ADP were determined.

activity, it is possible to photolabel another two binding sites with 8-azidoADP [4]. The latter two labelled sites have also been shown to be a catalytic and a non-catalytic binding site [4]. Such a preparation still contains two tightly bound nucleotides [3,4]. First, F_1 was fully inhibited, as measured under V_{max} conditions, with non-radioactive 8-azidoATP, so that the enzyme contained 2 mol of 8-N-ATP per mol of F_1 [4]. Subsequently, this modified enzyme was irradiated in the presence of 8-azido[2-^{3}H]ADP and labelled to various extents by varying the illumination time. The samples were tested for their amount of covalently bound 8-N[2-^{3}H]ADP and for their ability to bind 2-azido[α-^{32}P]ATP with high affinity. It can be seen in Fig. 5 that the same amount of 2-azidoATP was bound with high affinity to the enzyme, regardless of whether only two or up to four sites were photolabelled with the 8-azido analogues. Additionally, the newly bound nucleotides were still hydrolysed, as was found with [γ-^{32}P]ATP

and P_i was removed in the washing procedure. These results indicate that at least one of the two nucleotides still bound to the F_1 after the covalent binding of up to 4 mol of 8-N-AT(D)P per mol of F_1 could exchange rapidly with added nucleotides and showed properties characteristic for a catalytic site.

Discussion

In this paper it is demonstrated that catalytic binding sites in isolated F_1 can be present in a high-affinity conformation for binding nucleotides. The nucleotide, bound with high affinity to a catalytic site, is part of the tightly bound nucleotides. The high-affinity binding is in agreement with earlier results by Grubmeyer et al. [23], who showed that both the isolated and the membrane-bound enzyme are able to bind substoichiometric amounts of added nucleotide with high affinity ($K_d = 10^{-12}$ M). In this paper, high-affinity binding to catalytic sites was studied by incubating F_1 in the presence of a radioactive nucleotide, followed by ammonium sulphate precipitation and fast-column centrifugation to remove non- and loosely bound nucleotide.

The following aspects of high-affinity binding have been studied: (1) the concentration of added nucleotide during the incubation required for exchange with nucleotide bound to the high-affinity catalytic site when the concentration of ligand is higher than that of F_1; (2) which fraction of the tightly bound nucleotides participates in rapidly exchanging high-affinity binding; and (3) how many sites on the F_1 are able to participate in the high-affinity binding. In Fig. 2. it is shown that after incubation at concentrations of added nucleotide of about 250 μM and subsequent washing about 1 mol of label was incorporated. The concentration dependency shows that exchange was obtained at relatively high nucleotide concentrations, indicating that the nucleotides bind to sites in a low-affinity conformation, which is in agreement with the conclusions of Cross et al. [12] and Grubmeyer and Penefsky [6,16]. It has to be added that at the highest concentration of added nucleotide insufficient removal of added label after the incubation can result in overestimation of the amount of label incorporated. It is noticeable that the 2-azido analogue had the same binding characteristics as ATP. These results demonstrate that the azido compound and its tautomers [26] had the same binding affinity, which is in agreement with results of binding experiments previously reported by Boulay et al. [22], who showed that at low concentrations no difference in the extent of binding was observed whether or not the label was wholly in the azido form or in a mixture of tautomers. Irradiation of F_1 containing incorporated 2-azido[β-^{32}P]ATP under the conditions described in Fig. 2 results in inhibition of enzyme activity, extrapolating to complete inhibition when 1 mol of the analogue has been covalently linked to the enzyme [17]. The results indicate, again, that binding is to a catalytic site and that catalytic sites interact. The exchange of tightly bound [^{14}C]ATP (incorporated after a short-term exposure to [^{14}C]ATP and subsequent ammonium sulphate precipitation and fast-column centrifugation) with ATP (Fig. 2) was found to require higher concentrations of ATP than those required for the initial exchange of the endogenous ATP with [^{14}C]ATP, although the same circumstances were used. At a concentration of 250 μM of added ATP, some 10^4 turnovers per enzyme must have taken place during the incubation, but only two-thirds of the bound radioactivity was removed. If the incorporated [^{14}C]ATP is bound to one catalytic binding site per enzyme, and if the catalytic binding sites cooperate, the results suggest that not all binding sites containing incorporated [^{14}C]ATP are involved in turnover during the subsequent incubation [27] with ATP. ADP binding studies in isolated prokaryotic F_1 have led to comparable results: after the binding of [^{14}C]ADP, only part of the bound label could be replaced by [^{3}H]ADP under similar conditions as in the binding of the first label [28]. A possible explanation for this is that some radioactive analogue remains bound to a slowly exchanging site.

In Fig. 3 it is clearly shown that the newly incorporated nucleotides contribute to the tightly bound nucleotides. As the newly incorporated nucleotide was hydrolysed, it can be concluded that this newly bound nucleotide was bound at a catalytic site. Hydrolysis of this tightly bound ATP cannot be ascribed to single-site catalysis as described by Cross et al. [12], and this occurs at a very slow rate ($V_{max} = 10^{-4}$ s^{-1}). It is likely then,

that hydrolysis occurs during the exchange incubation, which take place at ATP concentrations high enough to induce multi-site catalysis [12]. The experiment in Fig. 3 further demonstrates that even if only slightly more than one tightly bound nucleotide is present on the enzyme (after five ammonium sulphate precipitations and one fast-column centrifugation), half of the number of nucleotides can still be rapidly exchanged with radioactive ATP. These results can be explained if one assumes that the one remaining tightly bound nucleotide (after five ammonium sulphate precipitations and one fast-column centrifugation) is bound to a catalytic site in part of the enzyme preparation and to a non-catalytic site in the other part of the preparation. The definition of tightly bound nucleotides (nucleotides bound to the F_1 after repeated ammonium sulphate precipitation or fast-centrifuge columns) need not be changed by these results. However, the rapid exchangeability demonstrated in this paper indicates that tightly bound nucleotides cannot be regarded as being hardly exchangeable or not exchangeable at all. The results can be explained very well if exchange of a fraction of the tightly bound nucleotide occurs at the rate of multi-site turn-over of the enzyme. It is likely that in the experiment of Fig. 3 part of the non-exchangeable tightly bound nucleotides has been removed by (the repeated) ammonium sulphate precipitations and fast-column centrifugations.

The catalytic character of the high-affinity binding site is clearly demonstrated by the finding that tightly bound nucleotides are being hydrolysed after binding. The finding that mainly ADP is found as tightly bound to a catalytic site is not in disagreement with the enzyme-bound ATP synthesis from tightly bound ADP and added P_i in the presence of DMSO [33,34]. Under our conditions no added P_i is present, shifting the equilibrium towards ADP. Both exchange of one of the tightly bound nucleotides and hydrolysis were also found in photolabelled F_1 preparations (Figs. 4 and 5). From the experiment with F_1 photolabelled with 8-azido-ATP, it can be concluded that the hydrolysis of the newly bound nucleotide was not eliminated when up to one catalytic and one non-catalytic binding site were occupied with covalently bound analogues, and when the multi-site catalysis (values measured under V_{max} conditions) has been reduced extensively. Single-site catalysis according to the method of Cross et al. [12] is too slow to account for the hydrolysis. However, the hydrolysis can be explained by assuming that we are dealing with the single-site catalysis described by Gresser et al. [10] and Roveri and Calcaterra [29]. We have argued previously that this catalysis ($K_m = 4\ \mu M$, $V_{max} = 2$ μmol/min per mg) is single-site catalysis when a regulatory site is occupied [30]. With regard to the K_m value for binding of ATP to this regulatory site, it can be concluded that this site must be occupied during the 1 min incubation with 250 μM ATP. These results can also be interpreted by assuming the presence of one separate rapidly exchanging, high-affinity binding site. However, the finding that tight binding (and hydrolysis of the newly bound nucleotide) was also found after filling this separate site by 2-azido-ATP and subsequent irradiation to covalently bind the analogue, demonstrates that more than one binding site is able to perform rapid exchange at a high-affinity binding site. In contrast to when ATP is bound [12], these results indicate that the second catalytic site behaves as if the first site is empty. From earlier work in our laboratory, it is known that it is possible to photolabel F_1 with up to 4 mol of 8-azidoAT(D)P per mol F_1 [4]. On the basis of the distribution of radioactivity, it could be concluded that in that case two catalytic β and two non-catalytic α/β interface binding sites were labelled. One of the two exchangeable non-catalytic sites can probably be identified with the slowly exchanging non-catalytic site on F_1 (which exchanges in the presence of EDTA) [27], which was previously considered to be non-exchangeable [11]. The two remaining binding sites after labelling F_1 with 4,8-azidoAT(D)P, could not be labelled with the (*syn*) 8-azido-nucleotides, even if the two residual tightly bound nucleotides were removed with high concentrations of LiCl [4]. It has been demonstrated that after this LiCl treatment, the remaining binding sites could be partly labelled with 2-azidoATP [4]. These experiments, however, do not inform us about the possible exchangeability of the two remaining sites. When labelling with 2 mol 8-N-ATP per mol F_1 was followed by covalent binding of up to 2 extra mol of 8-N[2-^{3}H]ADP

per mol of F_1, the same amount of 2-azido[α-^{32}P]ATP was bound with high affinity to the F_1, independently of the amount of covalently bound 8-N[2-^{3}H]ADP (Fig. 5). From this, it can be concluded that the LiCl treatment is not needed to bind the 2-azido analogue, as at least one of the two so-called non-exchangeable nucleotides remaining at the enzyme after 4 mol of 8-azidoAXP had been covalently bound to the enzyme could be rapidly exchanged by the 2-azido analogue. A comparable experiment shows that 1 mol of [^{14}C]ATP could be bound with high affinity to F_1 preparations containing 0–4 mol of covalently bound 8-N-AT(D)P (results not shown). The finding that under comparable circumstances only 0.5 mol of 2-azido[α-^{32}P]ATP was bound (Fig. 5) can possibly be explained if one assumes that in the case of F_1 containing 2–4 mol of 8-N-AT(D)P both the azido and the tetrazolic forms are able to bind, but that part of the three tautomeric forms is removed during the ammonium sulphate precipitation and fast-column centrifugation. These results also suggest a higher substrate specificity of (one of) the two remaining supposedly non-exchanging nucleotide-binding sites. The finding that 2-azidoATP but not 8-azidoATP binds to this third β site and exchanges, agrees with the conclusion above that the exchanging high-affinity site has a high affinity only for nucleotides in the *anti* configuration and that 8-azidoATP (*syn* configuration) does not bind well to such a high-affinity site. The results not only indicate that at least one of the two so-called non-exchangeable nucleotide-binding sites is able to exchange rapidly without losing the character of a high-affinity site, but in Fig. 5 it is also shown that nucleotides thus bound were still subject to hydrolysis. These results indicate that one of the two supposedly non-exchangeable binding sites has catalytic properties. This would suggest that that in total three cata-

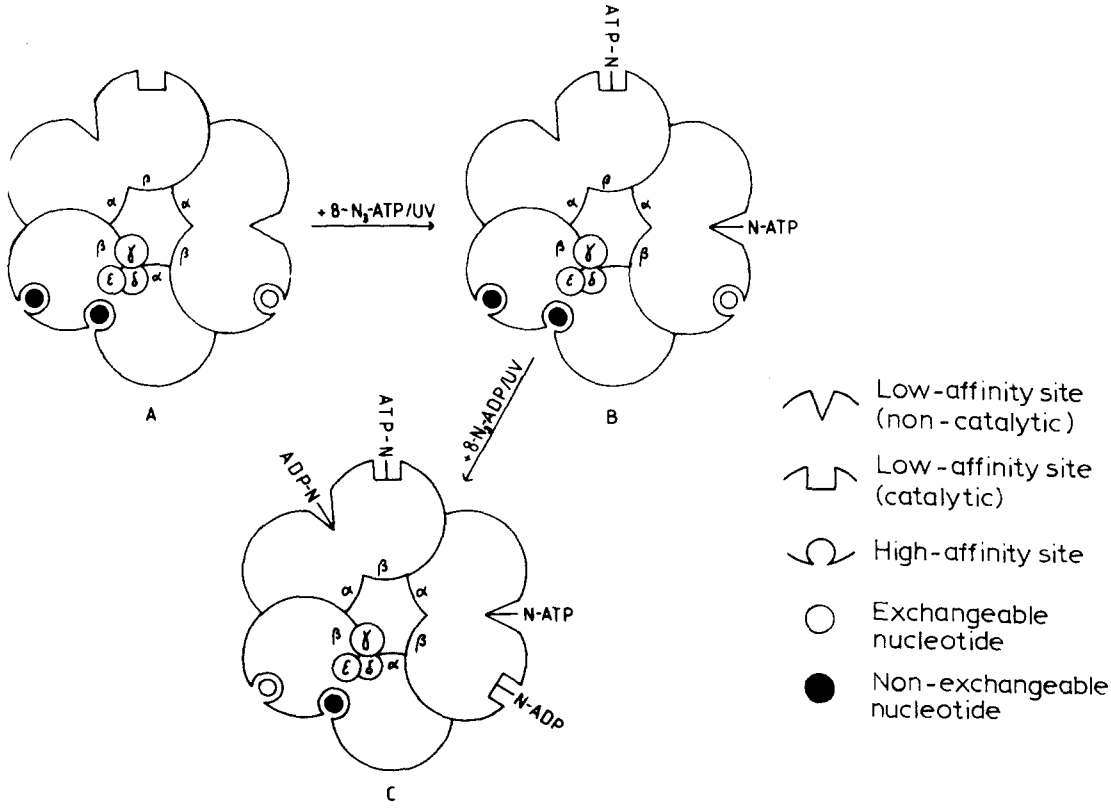

Fig. 6. Schematic representation of the nucleotide-binding sites of F_1. (A) F_1 as isolated; (B) F_1 photolabelled with 2 mol 8-N-ATP/mol F_1; (C) F_1 photolabelled with 2 mol 8-N-ATP/mol F_1 and subsequently with 2 mol 8-N-ADP/mol F_1.

lytic sites are present on F_1. A triple-site mechanism [10], however, cannot explain the experimental findings, since after tens of thousands of turnovers in non-inhibited F_1, the third 'catalytic' site (the site binding 2-azidoATP with high affinity after 2–4 sites have been labelled with 8-N-AT(D)P) still contains the original nucleotide. It has been proposed [31] that in membrane-bound F_1 at every moment two catalytic sites are involved in ATP synthesis or hydrolysis, but that a third latent catalytic site becomes catalytic as well on a larger time-scale (one of the other two becoming non-exchangeable) and that phosphorylation speeds up this rate of interchange between catalytic and non-catalytic sites. In isolated F_1, however, we have to conclude that such an interchange does not occur at a measurable rate, as the covalent binding of 8- or 2-azido-ATP to a catalytic site results in the inhibition of enzyme activity as measured under V_{max} conditions [4,17]. Reactivation of thus modified F_1 based on this proposal is currently being investigated in ourlaboratory. The asymmetry in the F_1 as visualized in X-ray studies [32] and electron microscopic studies [13] can be interpreted by assuming the presence of one α/β couple in interaction with one or more of the smaller subunits. This may be the β subunit that is not involved in multi-site catalysis but under certain conditions can be converted into a catalytic site.

The results reported in this paper have been schematised in Fig. 6, in which a model is presented based on the photolabelling experiments [4,17]. In Fig. 6A, isolated (and washed) F_1 is represented, containing two non-exchangeable tightly bound nucleotides (filled circles) and one exchangeable tightly bound nucleotide (open circle). The latter is bound with high affinity to a catalytic site and can be rapidly exchanged upon the addition of ATP, resulting in tight binding of added ATP. After photolabelling F_1 with 8-N-ATP, (on a catalytic β site and a non-catalytic α/β interface site [4]), the F_1 is still able to exchange bound ATP to a high-affinity catalytic site (represented by the open circle in Fig. 6B). This same site is likely to be labelled after tight binding of 2-azidoATP and irradiation [17]. Subsequent photolabelling of F_1 containing 2 mol 8-N-ATP/mol F_1 with 2 mol of 8-N-ADP results in F_1 still with two tightly bound nucleotides (Fig. 6C). Under these conditions, however, one of the two can be rapidly exchanged and hydrolysed, suggestive of localisation on the third β subunit likely to be on a latent catalytic site.

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References

1 Pullman, M.E., Penefsky, H.S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322–3329
2 Vignais, P.V. and Satre, M. (1984) Mol. Cell. Biochem. 60, 33–70
3 Wagenvoord, R.J., Kemp, A. and Slater, E.C. (1980) Biochim. Biophys. Acta 593, 204–211
4 Van Dongen, M.B.M. and Berden, J.A. (1986) Biochim. Biophys. Acta 850, 121–130
5 Weber, J., Lücken, U. and Schäfer, G. (1985) Eur. J. Biochem. 148, 41–47
6 Grubmeyer, C. and Penefsky, H.S. (1981) J. Biol. chem. 256, 3728–3734
7 Recktenwald, D. and Hess, B. (1977) FEBS Letts. 76, 25–28
8 Ebel, R.E. and Lardy, H.A. (1975) J. Biol. chem. 250, 191–196
9 Schuster, S.M., Ebel, R.E. and Lardy, H.A. (1975) J. Biol. Chem. 250, 7848–7853
10 Gresser, M.J., Myers, J.A. and Boyer, P.D. (1982) J. Biol. Chem. 257, 12030–12038
11 Cross, R.L. and Nalin, C.M. (1982) J. Biol. Chem. 257, 2874–2881
12 Cross, R.L., Grubmeyer, C. and Penefsky, H.S. (1982) J. Biol. Chem. 257, 12101–12105
13 Boekema, E.J., Berden, J.A. and Van Heel, M.G. (1986) Biochim. Biophys. Acta 851, 353–360
14 Harris, F.A., Rosing, J., Van de Stadt, R.J. and Slater, E.C. (1973) Biochim. Biophys. Acta 314, 149–153
15 Rosing, J., Harris, D.A., Slater, E.C. and Kemp, A. (1975) J. Supramol. Struct. 3, 284–296
16 Grubmeyer, C. and Penefsky, H.S. (1981) J. Biol. Chem. 256, 3718–3727
17 Van Dongen, M.B.M., De Geus, J.P., Korver, T., Hartog, A.F. and Berden, J.A. (1986) Biochim. Biophys. Acta 850, 359–368
18 Knowles, A.F. and Penefsky, H.S. (1972) J. Biol. Chem. 247, 6617–6623

19 Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891–2899
20 Rosing, J. and Slater, E.C. (1972) Biochim. Biophys. Acta 267, 275–290
21 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
22 Boulay, F., Dalbon, P. and Vignais, P.V. (1985) Biochemistry 24, 7372–7379
23 Grubmeyer, C., Cross, R.L. and Penefsky, H.S. (1982) J. Biol. Chem. 257, 12093–12100
24 Penefsky, H.S. (1985) J. Biol. Chem. 260, 13728–13734
25 Kandpal, R.P., Melese, T., Stroop, S.D. and Boyer, P.D. (1985) J. Biol. Chem. 260, 5542–5547
26 Czarnecki, J.J. (1984) Biochim. Biophys. Acta 800, 41–51
27 Cross, R.L., Kironde, F.A.S. and Cunningham, D. (1986) Abstracts of the Fourth European Bioenergetics Conference, Prague, p. 411
28 Issartel, J.P., Lundardi, J. and Vignais, P.V. (1986) J. Biol. Chem. 261, 895–901
29 Roveri, O.A. and Calcaterra, N.B. (1985) FEBS Lett. 192, 125–127
30 Slater, E.C., Berden, J.A. and Van Dongen, M.B.M. (1986) J. Prot. Chem. 5, 177–191
31 Soong, K.S. and Wang, J.H. (1984) Biochemistry 23, 136–141
32 Amzel, L.M., McKinney, M., Narayanou, P. and Pedersen, P.L. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 5852–5856
33 Sakamoto, J. (1984) J. Biochem. 96, 483–487
34 Yohda, M., Kagawa, Y. and Yoshida, M. (1986) Biochim. Biophys. Acta 850, 429–435.
35 Gresser, M.J., Myers, J.A. and Boyer, P.D. (1982) J. Biol. Chem. 257, 12030–12038