Journal of Bioenergetics and Biomembranes, Vol. 28, No. 6, 1996

Modification of Domains of α and β Subunits of F_1-ATPase from the Thermophylic Bacterium PS3, in Their Isolated and Associated Forms, by 3′-O-(4-Benzoyl)benzoyl Adenosine 5′-Triphosphate (BzATP)

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Received January 24, 1996; accepted May 6, 1996

Photoaffinity labeling by 3′-O-(4-benzoyl)benzoyl adenosine 5′-triphosphate (BzATP) of the adenine nucleotide binding site(s) on isolated and complexed α and β subunits of F_1-ATPase from the thermophilic bacterium PS3 (TF_1) is described. BzATP binds to both isolated α and β subunits, to complexed β subunit but not to complexed α subunit. Amino acid sequence determination of radiolabeled peptides obtained by proteolytic digestion of [γ-^{32}P]BzATP-labeled α subunit indicates that residues on both the amino-terminal (residues A41–E67) and carboxy-terminal (residues Q422–Q476) were modified by BzATP. One of the residues in the carboxy-terminal modified by BzATP is most probably αQ422. Although the binding stoichiometry of 1 mol of BzATP incorporated by either isolated or complexed β subunit was maintained, the spatial conformation of the polypeptide determines which amino acid residue(s) is more accessible to the reactive radical. CNBr derived fragments βG10–M64, βE75–M233, and βD390–M469 were labeled with the isolated β subunit. With complexed β subunit the label was found only in CNBr fragments: βE75–M233 and βG339–M389. The locations where the covalently bound BzATP was found, in the soluble and assembled subunits, indicate that different conformational states exist. In the isolated form of the α and β subunits the amino- and carboxy-termini can fold and reach the central domain of the polypeptide, the domain containing the adenine nucleotide binding site. When α combines with β to form the $\alpha_3\beta_3$ core complex the new conformation of the subunits is such that covalent labeling by BzATP of α and of the amino terminal of β subunit is excluded.

KEY WORDS: ATP synthase; F_1-ATPase; α subunit; β subunit; photoaffinity labeling; nucleotide binding site; BzATP.

INTRODUCTION

The enzyme responsible for ATP synthesis in aerobic cells and known variously as F_0F_1-ATPase or H^+-ATP synthase is a large (total M_r > 500,000) and highly organized membrane-associated protein complex composed of two discrete sectors. The F_0 sector, which lies integral to the inner membrane of mitochondria, the thylakoid membrane of chloroplasts, and the plasma membrane of aerobic bacteria, is involved in H^+-translocation across the membrane by means of a proton channel. The F_1-portion lies external to the membrane bilayer and is composed of five kinds of subunits with a stoichiometry of $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$. The minimal complexes of subunits that have ATP hydrolyzing activity are $\alpha_1\beta_1$ and $\alpha_3\beta_3$ (Kagawa *et al.*, 1989; Miwa and Yoshida, 1989; Harada *et al.*, 1991; Andralojc and Harris, 1992). The catalytic sites of F_1-ATPase are generally believed to reside on the β subunit or at

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0145-479X/96/1200-0471$09.50/0

the interfaces between the α and β subunits. This conclusion is based on the high reactivity of the β subunit in F_1-ATPases displayed with chemical modifiers, photoreactive nucleotide analogs, and from site-directed mutagenesis studies (Allison *et al.*, 1992; Pedersen and Amzel, 1993). Regarding the role of the α subunit in catalysis, much less is known. Site-directed mutagenesis of amino acid residues of EF_1-α in the region of residues 280 and 370 affected multisite but not unisite catalysis, suggesting that these regions are required for catalytic cooperativity (Maggio *et al.*, 1987; Omote *et al.*, 1994). Interactions between the α and β subunits were suggested to play a role in energy coupling. The defective energy coupling observed in a mutant of β subunit (βS174→F) was restored by introducing a second mutation in the α subunit (αR276→C) (Omote *et al.*, 1994). Isolated α subunit, as the β subunit, can bind ATP and ATP analogs but neither of them can hydrolyze ATP (Dunn, 1980; Dunn and Futai, 1980; Hisabori *et al.*, 1986; Rao *et al.*, 1988; Pagan and Senior, 1990; Bar-Zvi *et al.*, 1992; Tozawa *et al.*, 1993; Jault *et al.*, 1994). Both subunits covalently bind about 1 mol of BzATP/mol of subunit (Bar-Zvi *et al.*, 1992). While some affinity reactive nucleotide analogs have been shown to label both the α and the β subunits in a F_1 complex, nucleotide analogs containing the photoreactive benzophenone group, BzATP and BzADP, label the β subunit only (Williams and Coleman, 1982; Bar-Zvi and Shavit, 1985; Bar-Zvi *et al.*, 1985; Admon and Hammes, 1987; Aloise *et al.*, 1991; Bar-Zvi *et al.*, 1992; Allison *et al.*, 1992). The amino acid residues on the β subunit modified by several photoreactive nucleotide analogs have been identified. However, similar information for the α subunit is not yet available. The amino acid sequences of the α and β subunits show a homology extending (49.4%) through most of the polypeptide chains (Walker *et al.*, 1985). The α subunit, similar to the β subunit, possesses three domains involved in nucleotide binding: an ATP/GTP binding motif A (also referred to as P-loop (consensus $(A/G)X_4GK(S/T)$), a binding motif B (consensus RX_nh_4D), and the α and β signature motifs. On the other hand, the α subunit lacks the sequence $VXADX_3DX_8HLD$ common to the β subunit and adenylate kinase (Pedersen and Amzel, 1993).

In a previous paper we have shown that binding of BzATP to either one of the isolated α and β subunits inhibits their association into an $\alpha_3\beta_3$ complex and that the complex formed with native subunits dissociates upon covalent binding of BzATP (Bar-Zvi *et al.*, 1992). The dissociation of the complex and the concomitant loss of the ATPase activity were directly correlated to the specific binding of BzATP by the β subunit only. We report here on the properties of binding of BzATP by the isolated α subunit. We also compare the location of the incorporated BzATP by isolated and complexed β subunit. While isolated α subunit is fully capable of binding BzATP, α subunit in a complex does not bind the analog, which indicates that the full dissociation of the complex into individual subunits does not occur in solution. We suggest that labeling of the β subunit in the complex weakens the subunit–subunit interactions, resulting in the dissociation of the complex at the stage of gel filtration. Sequencing of the BzATP labeled peptides indicates that both the amino- and carboxy-termini of isolated α and β subunits are modified. However, when the subunits associate to form the $\alpha_3\beta_3$ complex, binding of BzATP to the amino terminal of β subunit and to the α subunit is prevented.

MATERIALS AND METHODS

Enzyme Preparation and Assays

TF_1 was prepared and stored as described (Yoshida *et al.*, 1977).[3] α and β subunits of TF_1 were individually expressed in *E. coli* and isolated as described (Ohta *et al.*, 1988). The $\alpha_3\beta_3$ complex was reconstituted from individual subunits by mixing equal amounts of the α and β subunits for 15 min at 30°C in a buffer containing 50 mM Tris-sulfate (pH 7.0) and 0.2 M Na_2SO_4 (Miwa and Yoshida, 1989). The $\alpha_3\beta_3$ complex and individual subunits were separated on a HPLC gel permeation column G3000SWXL at a rate flow of 0.5 ml/min at room temperature (Miwa and Yoshida, 1989). Proteins were eluted with the reconstitution buffer at a flow rate of 0.5 ml/min. Protein was determined according to Bradford (1976).

Photoaffinity Labeling

[γ-^{32}P]BzATP was synthesized as previously described (Williams and Coleman, 1982; Bar-Zvi and

[3] Abbreviations used: MF_1, CF_1, YF_1, and TF_1; the F_1-ATPase from mitochondrial, chloroplast, yeast, and plasma membranes of the thermophilic bacterium PS3; TF_1, E.C. 3.6.1.3; BzADP (BzATP), 3′-O-(4-benzoyl)benzoyl AD(T)P, respectively; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; HPLC, high-performance liquid chromatography.

Shavit, 1985). Mixes containing the protein and nucleotide analog in 25 mM Tris-Sulfate and 100 mM Na_2SO_4 buffer, pH 7.0, were irradiated at 360 nm to covalently bind the nucleotide (Bar-Zvi *et al.*, 1992). BzATP is not a substrate for TF_1 ATPase (Shavit, 1987) and is not hydrolyzed during this procedure. Covalent incorporation was quantified by acid precipitation of the labeled protein (Bar-Zvi *et al.*, 1992).

Subunit Distribution of Labeled BzATP

Irradiated and acid washed protein pellets were resuspended in SDS-PAGE sample buffer. Electrophoresis in the presence of SDS was performed according to Laemmli (1970) using a 10% polyacrylamide gel. Low-molecular-weight standards (Pharmacia Inc.) were used throughout. Following an electrophoretic run, gels were stained with Coomassie blue and exposed to x-ray film.

Proteolysis of Labeled Subunits and Peptide Sequencing

Native [γ-^{32}P]BzATP labeled α subunit was digested with trypsin, chymotrypsin, or *Staphylococcus aureous* V8 protease for 60 min at 25°C as described (Tozawa *et al.*, 1993). Samples were concentrated to 10μl by lyophilization, treated with Schagger and von Jagow's sample buffer, and loaded onto 16.5% T/6% C polyacrylamide gel with the Tricine-SDS buffer system (Schagger and von Jagow, 1987). Alternatively, labeled subunits, TF_1 holoenzyme, or $\alpha_3\beta_3$ core complex were chemically cleaved with CNBr (Pepinsky, 1983). In order to minimize the effects of free radicals, gels were precasted at least 24 h before use and 0.1 mM thioglycolate was added to the upper buffer chamber (Capasso *et al.*, 1992). Proteolytic fragments were electrotransferred onto Immobilon PVDF membrane as described (LeGendre *et al.*, 1993). Membranes were stained with Coomassie blue, and exposed to x-ray film. Radiolabeled peptides were sequenced as described (Tozawa *et al.*, 1993).

RESULTS

Nucleotide Binding by Isolated α Subunit

The nucleotide binding properties of the α subunit were further investigated by irradiation of a mix of isolated α and the photoreactive analog BzATP. The extent of covalent binding of [γ-^{32}P]BzATP is proportional to the nucleotide concentration (Fig. 1A). From the reciprocal plot it appears that under these conditions a maximum of 0.8 mol [γ-^{32}P]BzATP can be bound per mole of α subunit, and half maximal binding is obtained at 100 μM. Furthermore, Mg^{2+} had no effect on the binding affinity (Fig. 1A) or on the extent of binding (Fig. 1B). The extent of BzATP incorporation to α subunit is between 50–70% of that obtained with isolated β subunit when assayed under similar conditions (not shown). The level of nucleotide binding is affected by the intensity of the actinic light (Fig. 2) but not by prior incubation (before irradiation) of the polypeptide with the nucleotide analog (not shown).

The nucleotide specificity was studied by the effectiveness of unlabeled adenine nucleotides to displace [γ-^{32}P]BzATP in preventing covalent binding. ATP and ADP effectively prevented incorporation of [γ-^{32}P]BzATP, whereas AMP was much less effective (Fig. 3). Identical protection was obtained when the subunit was added to a mix of ATP and [γ-^{32}P]BzATP or when preincubated sequentially with one nucleotide following the other (not shown). These results suggest that without photoactivation of the benzophenone ring the binding of BzATP, as that of ATP, is fully reversible.

Although isolated α subunit binds BzATP, irradiation of [γ-^{32}P]BzATP in mixes containing either $\alpha_3\beta_3$ core complex or TF_1 resulted in the specific labeling of the β subunit with no significant covalent incorporation of the analog to the α subunit. Furthermore, incorporation of the BzATP to the $\alpha_3\beta_3$ core complex resulted in the dissociation of the complex. It might be that the α subunit is inactivated by irradiation of the $\alpha_3\beta_3$ core complex with BzATP and thus loses the nucleotide binding ability. This possibility was explored by the following protocol. The $\alpha_3\beta_3$ complex, separated from the excess of individual subunits by gel permeation, was irradiated with actinic light and then dissociated into individual subunits that were separated by gel permeation HPLC with Hepes buffer as eluant. Subunits and undissociated complex were separated by gel permeation HPLC. Each fraction was irradiated with actinic light in the presence of [γ-^{32}P]BzATP. As shown in Fig. 4, the α subunit released from the $\alpha_3\beta_3$ complex is indeed capable of binding the nucleotide analog. Thus, the lack of labeling of α subunit, whether in the $\alpha_3\beta_3$ complex or in TF_1, is not due to irreversible inactivation by the actinic light or by the binding of BzATP to a neighboring β subunit.

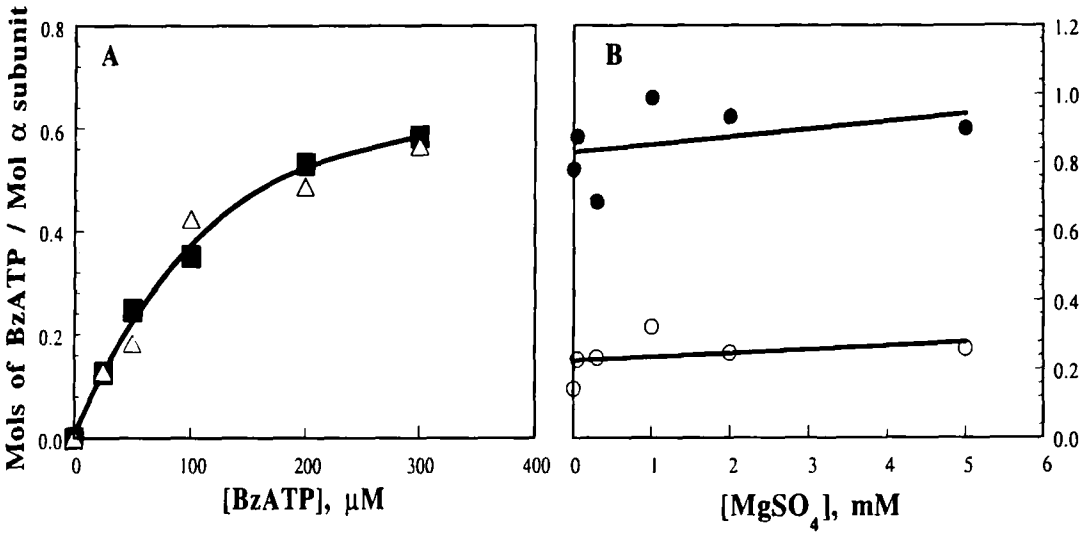

Fig. 1. Effect of nucleotide and Mg^{2+} concentrations on the covalent incorporation of [γ-^{32}P] BzATP into isolated α subunit. 8 μg of subunit was irradiated at 360 nm in the presence of the indicated concentrations of [γ-^{32}P]BzATP and Mg^{2+}. Covalently incorporated label was determined by acid precipitation. (A) △, without Mg^{2+}; ■, 2 mM Mg^{2+}. (B) ○, 30 and ●, 300 μM [γ-^{32}P]BzATP.

Mapping of [γ-^{32}P]BzATP Binding to Isolated α Subunit

The nucleotide binding site(s) were determined after proteolytic degradation of the covalently labeled [γ-^{32}P]BzATP-α subunit with trypsin, chymotrypsin, and *Staphylococcus aureous* V8 protease. Proteolytic digests were separated on Tricine-SDS-PAGE, transferred to a PVDF membrane, and exposed to X-ray film. Radiolabeled polypeptide bands of M_r ca. 3,500–25,000 were obtained (Fig. 5) and amino acid sequences of seven of the labeled proteolytic fragments were determined using the Edman degradation procedure. The sequences assigned to the radiolabeled peptides clearly show that the amino acids modified with BzATP are located at the amino and the carboxy ter-

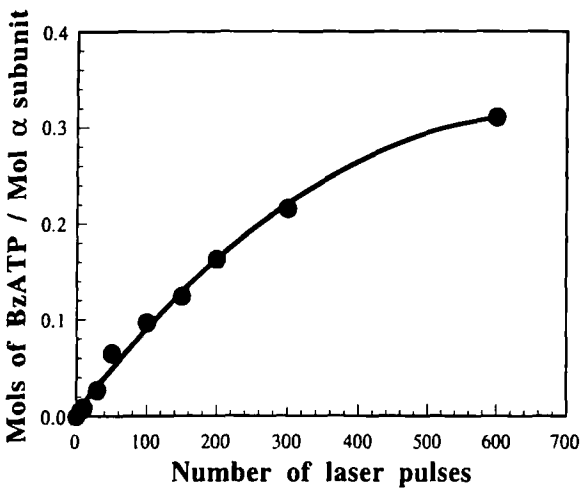

Fig. 2. Covalent incorporation of [^{3}H]BzATP to α isolated subunit. The purified subunit fractions were incubated for 30 min with 100 μM [^{3}H]BzATP, and irradiated at 308 nm with the indicated number of 16 ns pulses from a XeCl excimer laser. Covalent incorporation of label was determined as described in Materials and Methods.

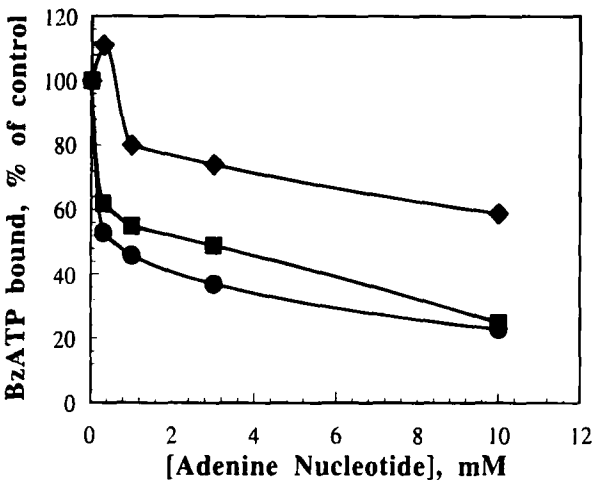

Fig. 3. Effect of adenine nucleotides on the covalent incorporation of [γ-^{32}P]BzATP into isolated α subunit. 12 μg of α subunit was irradiated at 360 nm in the presence of 100 μM [γ-^{32}P]BzATP and the indicated concentrations of unlabeled adenine nucleotides. Covalently incorporated label was determined by acid precipitation. ●, ATP; ■, ADP, and ◆, AMP.

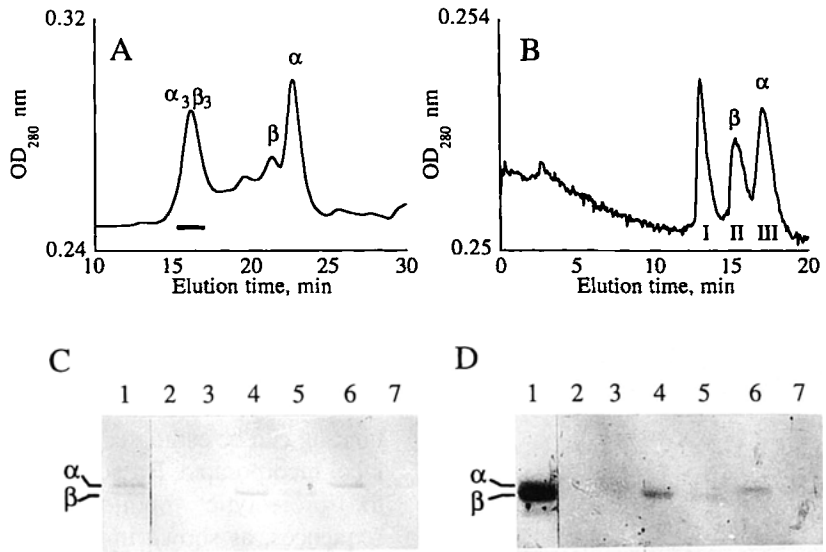

Fig. 4. Photoaffinity labeling of α and β subunits after dissociation and separation from the $\alpha_3\beta_3$ complex. (A) *Isolation of* $\alpha_3\beta_3$ *core complex:* 200 μg of α and of β subunit were incubated for 30 min at 30°C in 100 mM Na_2SO_4 and 25 mM Tris-SO_4 buffer, pH 7.0, and the $\alpha_3\beta_3$ complex formed was eluted by gel permeation HPLC on a column equilibrated and eluted with the same buffer at a flow rate of 0.5 ml/min. The fraction corresponding to the $\alpha_3\beta_3$ core complex was collected as indicated by the full bar and irradiated at 360 nm. (B) *Isolation of individual subunits:* The irradiated complex was rechromatographed on the same column equilibrated and eluted with 50 mM Hepes buffer, pH 7.3. The individual subunits eluted in Hepes buffer earlier than in the Tris-Sulfate buffer. The $\alpha_3\beta_3$ complex (from A) and fractions corresponding to the three peaks of the second column (B) were collected, irradiated in the presence of 100 μM [γ-^{32}P]BzATP, and analyzed by SDS-PAGE. Lane 1, $\alpha_3\beta_3$ core complex; lanes 2–3, peak I; lanes 4–5, peak II; lanes 6–7, peak III. (C) Coomasie blue staining. (D) Autoradiography.

mini of the α subunit (Table I). No covalently modified peptides corresponding to the "central" nucleotide binding domain of the polypeptide were detected.

Mapping of [γ-^{32}P]BzATP Binding to Isolated and Complexed β Subunit

Binding of BzATP to isolated α subunit, as well as its specific incorporation to β subunits when the TF_1 holoenzyme or the $\alpha_3\beta_3$ core complex interacted with BzATP, has been described (Bar-Zvi *et al.*, 1992). We have used peptide mapping to compare the labeling patterns of subunit β in its isolated state with that of β subunit associated in the TF_1 holoenzyme and the $\alpha_3\beta_3$ core complex. Clearly, different patterns of labeled peptides were obtained upon comparison of *Staphylococcus aureous* V8 proteolytic digests of BzATP-modified isolated β vs. β subunits in the complexed state (Fig. 6). These results are also supported by the BzATP-labeled peptide profiles obtained after CNBr cleavage of isolated β and of β in TF_1 (Fig. 7). CNBr cleavage of BzATP-modified isolated β gave several additional radioactive bands as compared to those obtained by cleavage of BzATP-modified β in TF_1. The amino termini of these proteolytic fragments were sequenced by Edman degradation (Table II). Domains containing residues β75–233 and β339–389 of the complexed β subunit in TF_1 and domains containing residues β10–64, β75–233, and β390–469 in the soluble β subunit are labeled by BzATP. The radiolabeled band F7 contained two sequences: β10–64 and β339–389. Consequently, we conclude that the residues modified are located in the peptide β339–389 since the peptide β10–74 was also obtained in the nonlabeled F6 band and that BzATP does not interact with the N-amino terminal of the complexed β subunit.

DISCUSSION

The isolated α subunits of F_1-ATPases bind adenine nucleotides (Dunn, 1980; Dunn and Futai, 1980;

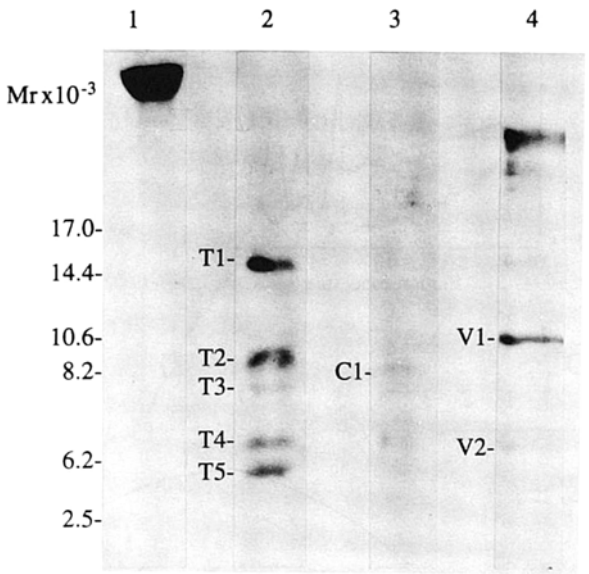

Fig. 5. Proteolytic digestion of labeled α subunit. 360 μg of α subunit was irradiated at 360 nm in the presence of 100 μM [γ-^{32}P]BzATP. Samples containing 45 μg protein were then incubated 60 min at 25°C in the absence of protease (lane 1) or in the presence of 50 ng trypsin (lane 2), 1 μg of chymotrypsin (lane 3), and 250 ng of *Staphylococus aureus* V8 protease (lanes 4). Reaction mixtures were then concentrated to 10 μl by lyophilization, separated on Tricine-SDS-PAGE, transferred to PVDF membrane, and exposed to X-ray film, as described in Materials and Methods. The numbered peptides were sequenced (see Table I).

Hisabori *et al.*, 1986; Pagan and Senior, 1990) and adenine nucleotide analogs (BzATP, 2-N_3-ATP, and pyridoxal 5′-diphospho-5′-adenosine) (Rao *et al.*, 1988; Bar-Zvi *et al.*, 1992; Jault *et al.*, 1994). In general, the affinity of binding of nucleotide analogs is much lower than that of the corresponding adenine nucleotides (Fig. 1A and Dunn, 1980; Dunn and Futai, 1980; Hisabori *et al.*, 1986; Rao *et al.*, 1988; Pagan and Senior, 1990). The binding of BzATP to the isolated α subunit and its displacement by AT(D)P but not by AMP (Fig. 3) demonstrates that the analog interacts with the nucleotide binding site(s) as expected from the specificity of binding of adenine nucleotides by F_1-ATPases. However, we cannot rule out the possibility that a small fraction of the incorporated label represents nonspecific binding. The level of nonspecific binding can be estimated as less than 20% of the total label incorporated. Based on the degree of labeling of the proteolytic fragments and their overlapping sequences, as shown in Fig. 5 and Table I, the level of potential nonspecific binding is much less than that estimated from the protection by ATP (Fig. 3). Thus, the level of nonspecific binding does not significantly affect our conclusion regarding the site(s) modified.

Isolated α and β subunits bind up to 1 mole BzATP per mole protein while CF_1, TF_1, and the $\alpha_3\beta_3$ core complex bind up to 3 moles BzATP per mole enzyme and the nucleotide analog is preferentially incorporated to the β subunit (Fig. 4 and Bar-Zvi *et al.*, 1992; Jault *et al.*, 1994). It is interesting that the α subunit is not labeled upon irradiation of the $\alpha_3\beta_3$ complex with [γ-^{32}P]BzATP since full dissociation of the complex is induced by covalent binding of BzATP and should release a soluble α subunit capable of interaction with medium BzATP. The fact that α is not labeled indicates that the $\alpha_3\beta_3$ complex is not fully dissociated upon binding of BzATP to the complexed β. It appears that labeling of complexed β subunit weakens the interactions between the different subunits and full dissociation of the complex occurs during the gel filtration step, which follows the BzATP binding step (Bar-Zvi *et al.*, 1992).

Considering the known molar stoichiometry of nucleotide binding to F_1-ATPases (Ysern *et al.*, 1988; Boyer, 1993), we propose that the lack of covalent binding of BzATP by the α subunit in the holoenzyme and in the $\alpha_3\beta_3$ complex is due to its conformation in these complexes which restricts the access of the reactive radical. The lack of labeling of α by BzADP in the soluble CF_1 and its labeling when associated in the membrane-bound CF_1 complex (Bar-Zvi *et al.*, 1983; Bar-Zvi and Shavit, 1985) support the notion of different conformations. Incorporation of BzATP into either soluble α or β structurally modifies the polypep-

Table I. Amino Acid Sequence Determination of Radiolabeled Polypeptides Obtained by Digestion of [γ-^{33}P]BzATP · α Subunit with Different Proteases

Peptide	Sequence determined	Sequence assigned[a]
T1	QQIEN	αQ14–R164
T2	QDLHQPI	αQ422–Q502
	TVEVLK	αT416–Q502
T3	?DLHQP	αQ422–R476
T5	AHGLDNV	αA41–K83
C1	KQDL	αK421–Q502
V1	SQIQVSD	αS21–E101
V2	SQIQVSDV	αS21–E67

[a] Amino acid sequences of the N-termini of ^{32}P-labeled proteolytic fragments were determined as described in Methods and Materials. Peptides are designated as described in Fig. 5. N-termini of sequenced fragments are in agreement with the amino acid sequence of α subunit and the known specificity of the proteinases employed.

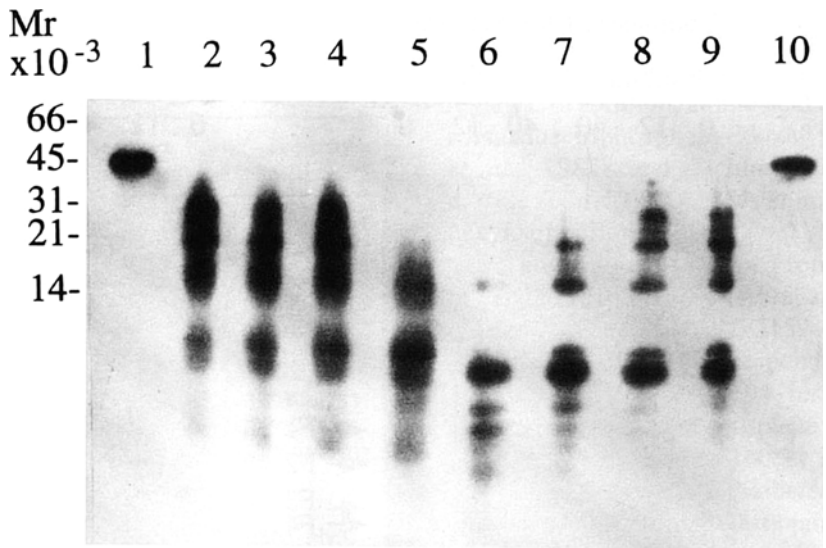

Fig. 6. Proteolytic digestion of labeled or isolated and complexed β subunit. Isolated β subunit (lanes 1–5) or TF_1 holoenzyme (lanes 6–10) were irradiated in the presence of 100 μM [γ-^{32}P]BzATP. Protein was resolved on SDS-PAGE stained with coomassie blue. Bands corresponding to β subunit were excised and loaded on a 15% acrylamide SDS-PAGE. The wells were supplemented with 0 (lanes 1, 10), 25 (lanes 2, 9), 50 (lanes 3, 8), 125 (lanes 4, 7), or 375 (lanes 5, 6) ng of *Staphylococus aureus* V8 protease. Gel running conditions were as described (Shavit 1987). Gels were dried and exposed to an X-ray film without staining.

tides, preventing their association with the complementary native subunits to form the $\alpha_3\beta_3$ complex (Bar-Zvi *et al.*, 1992). Changes in the conformation due to interaction with ligands are known. A rather large change in the conformation of isolated $EF_1 \cdot \alpha$ upon interaction with ATP (Dunn, 1980) and the dissociation of the $\alpha_3\beta_3$ complex to $\alpha_1\beta_1$ (Harada *et al.*, 1991) or to the individual subunits (Bar-Zvi *et al.*, 1992) upon interaction with nucleotides and nucleotide analogs was reported.

Modification of $TF_1 \cdot \alpha$ by BzATP only in its isolated state but not of complexed α might also be due to a lower binding affinity for BzATP by α in the complex or because α has no reactive amino acid residue accessible to the benzophenone radical. Weber *et al.* (1993) have shown that the photoaffinity labeling pattern of EF_1 with 2-azido-ATP depends on the nature of the modifiable amino acid residues. In wild type EF_1, 89% of the analog was incorporated to βY354 while βY354F-mutated EF_1 incorporated 2-azido-ATP only to α subunit. Furthermore, in αR365Y-mutated EF_1 38% of the label was found in α whereas the equivalent αR365F-mutated EF_1 was labeled on the β subunit only. These results suggest that covalent photolabeling of a reactive amino acid residue by an activated nitrene group occurs when the adenine ring of the nucleotide lies in the proximity of the reactive residue. In its absence, the activated nitrene group will interact with other residues having now the highest reactivity. Similar preferential reactivity of the photoreactive BzATP was also demonstrated (Dorman and Prestwich, 1994).

Abrahams *et al.* (1994) proposed three structural domains in the α subunit of F_1-ATPase: an N-terminal six stranded β-barrel (α19–95), a central domain common to α–β containing the nucleotide binding site (α96–379), and a C-terminal bundle of 7 helices (α380–510). In other ATPases, the central domain of $YF_1 \cdot \alpha$ (Falson *et al.*, 1993) was shown to bind adenine nucleotides and in EF_1 (Weber *et al.*, 1993) the base moiety of the adenine nucleotide lies close to αR365 at the nucleotide binding site. Sequence analysis of $BzATP \cdot \alpha$ labeled peptides derived by proteolysis of isolated α shows that BzATP modifies mainly residues located in the αA41–E67 region (Fig. 5 and Table I, overlapping regions of peptides V2 and T5) and residues located in the carboxy terminal region of αQ422–R476 (Fig. 5 and Table I, peptide T3). These results show that BzATP modified residues in both the N-terminal and C-terminal domains but not in the central domain of the nucleotide binding site. Consequently, amino acid residues located in domains potentially

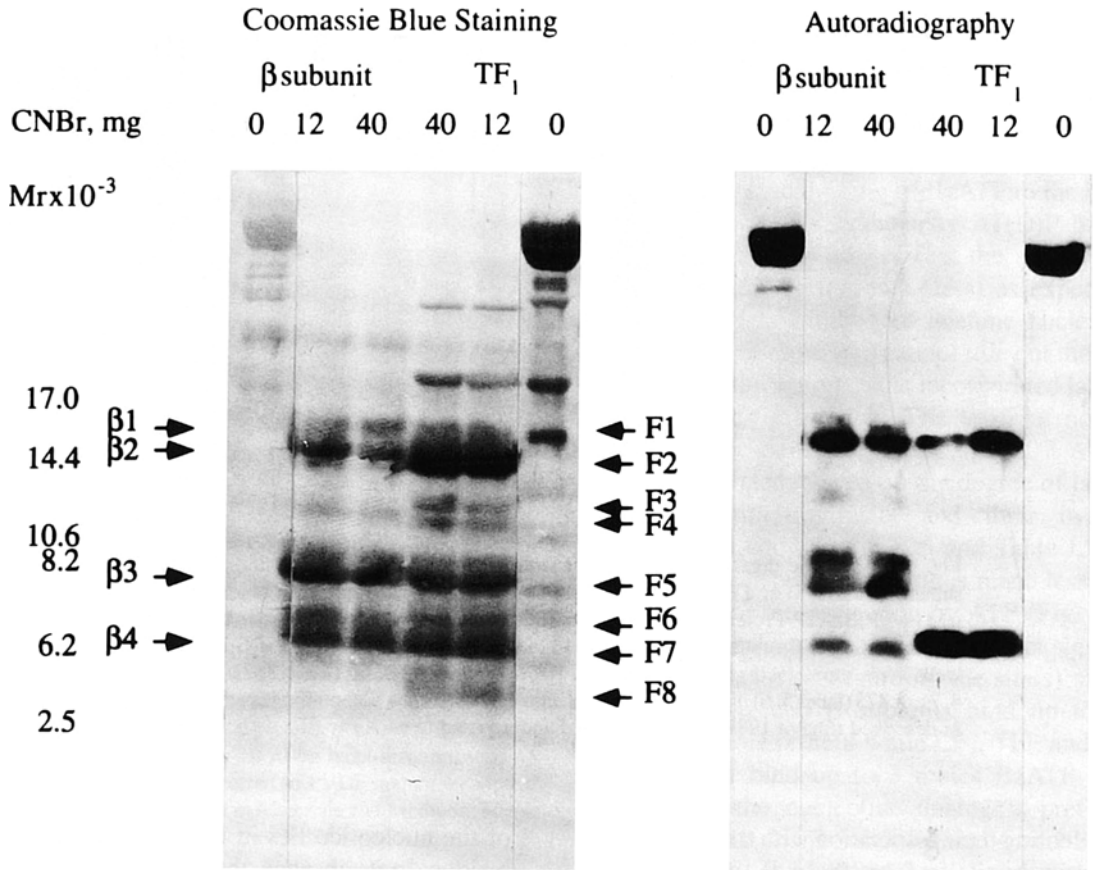

Fig. 7. Cleavage of [γ-^{32}P]BzATP-β subunit and [γ-^{32}P]BzATP-TF_1 with cyanogen bromide. Isolated β subunit (β subunit) or TF_1 holoenzyme (TF_1) were covalently labeled with [γ-^{32}P]BzATP. The labeled proteins were treated with the indicated amounts of cyanogen bromide (CNBr) in acidic solution. Mixes were lyophilized and resolved on Tricine-SDS polyacrylamide gels. After electrophoresis the proteins were electroblotted to a PVDF membrane, stained with Coomassie blue (left panel), and exposed to an X-ray film (right panel). Bands derived from isolated β subunit (β1-β4) and from TF_1 (F1-F8) were subjected to amino acid sequencing.

involved in nucleotide binding such as the ATP/GTP binding motif A (169–176), the nucleotide binding motif B (250–261), and the α and β signature motifs (355–363) were not modified by BzATP. The differences in the labeling of domains of the α and β subunits by BzATP, in the case of isolated vs. complexed forms, may reflect different conformational states of the polypeptide chains rather than specific binding to different nucleotide binding sites. These results also indicate that photoaffinity labeling of proteins with BzATP could serve to monitor protein conformational changes which occur in the vicinity of the binding site of the photoreactive analog.

The interaction between the carboxy terminal of α and the ATP binding site was suggested by Tozawa *et al.* (1993). Proteolytic digestion of the α subunit has shown that most of the proteolytic peptides remain associated after proteolysis to maintain the gross structure of the subunit. However, two proteolytic peptides from the carboxy terminal are released from association with other peptides, one of about 80 amino acid residues and another of 105 residues (C2) (Tozawa *et al.*, 1993). The release of C2 was prevented in the presence of Mg·ATP, and the ATPase activity in reconstituted $\alpha_3\beta_3$ complexes was enhanced. These results led to the suggestion that binding of Mg·ATP to α induces some structural change in the carboxy terminal domain (Tozawa *et al.*, 1993) lying close to the nucleotide binding site and that this structural change might be one of the sequential events needed to regulate the ATPase activity. The proximity of the α carboxy terminal domain to the central nucleotide binding domain is also supported by structural studies (Abrahams *et al.*, 1994). These studies demonstrate that

Table II. Amino Acid Sequence Determination of Radiolabeled Polypeptides Obtained by Chemical Cleavage of Isolated [γ-^{33}P]BzATP · β Subunit or [γ-^{32}P]BzATP · TF_1 with CNBr

Peptide	Radioactivity	Sequence determined	Sequence assigned[a]
Isolated β subunit			
β1	+	EVID	βE75–M233
β2	−	EVID	βE75–M202
β3	+	DELS	βD390–M469
β4	+	GPVV	βG10–M64
TF_1 complexed β subunit			
F1	+	EVID(E)GAP	βE75–M233
F2	−	EVIDTGA	βE75–M202
		KKVAGLT	αK376–Q502
F3	+	EVGIGE	?
		XYPXXV	?
F4	−	ALNLEEN	αS66–M95
F5	−	DELSDEDK	βD390–M469
		EVPVGE(A)L	αE96–M138
F6	−	GPVVDV	βG10–M74
		GQLQER	βG286–M338
F7	+	GPVVDVK	βG10–M64
		GIYPAVD	βG339–M389
F8	−	SIRAEEIS	αS2–M48
		ALNLEENN	αA66–M95

[a] Amino acid sequences of the N-termini of ^{32}P-labeled fragments after CNBr cleavage were determined as described. Peptides are designated according to their source. β 1–4: isolated β subunit; F1–7: TF_1-ATPase. N-termini of the sequenced fragments are preceded by a methionine residue in the subunit amino acid sequences, as expected from CNBr cleavage.

residue MF_1·αQ432 located in the carboxy terminal domain is part of the nucleotide binding site in the α subunit. Vicinal amino acids in these domains may thus be near the nucleotide binding site and could react with the analog. One of the residues that could be modified by BzATP is TF_1·αQ422, possibly the unidentified residue in the sequence of the tryptic peptide T3 (Table I). Residue TF_1·αQ422 is homologous to MF_1·αQ430, which is only two residues away from MF_1·αQ432 at the nucleotide binding site.

Although 1 mole of BzATP is incorporated by either isolated or complexed β subunit, the amino acid residue(s) likely to interact with BzATP will depend on the spatial conformation of the subunit. CNBr derived fragments, βG10–M64, βE75–M233, and βD390–M469, were labeled in isolated β subunit while in complexed β the label was found in CNBr fragments: βE75–M233 and βG339–M389. The βE75–M233 modified both in isolated and complexed β contains the glycine-rich ATP binding motif. This sequence (βG158–βT165) is found in many nucleotide binding proteins (see Futai *et al.*, 1992 for review). In addition, amino acid residues modified by adenosine triphosphopyridoxal (PLP-ADP) (Tagaya *et al.*, 1987) and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (Nbf-Cl) (Andrews *et al.*, 1984) and dicyclohexylcarbodiimide (DCCD) (Yoshida *et al.*, 1981, 1982) are within this region. From the crystal structure of bovine MF_1-ATPase (Abrahams *et al.*, 1994) two clusters of residues, βG159–V164 and βE188, βR189, and βE192 (corresponding in TF_1 to β161–166, β190, β191, and β194, respectively), appear to be located close to the phosphate groups of the nucleotide, while residue βV160 is located close to the ribose. The CNBr BzATP-labeled fragments, βG339–M389 and βD390–M469, obtained from complexed and isolated β subunits, respectively, contain the central domain of β subunit (Abrahams *et al.*, 1994). Labeling of the central domain of β is in agreement with the affinity binding of complexed β by 2-N_3-ATP (Cross *et al.*, 1987), FSBA (Bullough and Allison, 1986), and BzATP (Admon and Hammes, 1987; Aloise *et al.*, 1991), reagents known to modify tyrosine residues corresponding to TF_1-βY341. Furthermore, βY364 was modified by FSBA and 2-N_3-ANP. The BzATP-labeled CNBr fragment, βD390–M469, contains the site modified by FSBA and also residues βF414, βA417, βF420, and βT421 (corresponding to residues 418, 421, 424, and 425 in the β subunit of bovine MF_1), and was proposed to be in the vicinity of the adenine moiety of the bound nucleotide. In addition, mutation βS174→F in *E. coli* was suppressed by a second mutation of βL400→Q (Miki *et al.*, 1990), suggesting that these residues interact with each other. The difference in the labeling of these regions in complexed vs. soluble β could result from differences in the conformation of the same nucleotide binding site. This suggestion is also supported by the affinity labeling of MF_1 with the bifunctional analog 8-N_3-FSBA (Zhuo *et al.*, 1992). The analog was shown to bind covalently to two residues βY368 to βH427 (residues 364 and 423 in TF_1) within the same β subunit. The first residue is located in the CNBr fragment labeled in complexed β, whereas the second residue is located in the CNBr-labeled fragment in soluble β. On the other hand, identical labeling patterns of complexed β by BzATP, either when in the $\alpha_3\beta_3$ core complex or in TF_1, were observed (not shown), suggesting that the single copies of the smaller subunits present in these complexes had no effect on the interaction with the ligand.

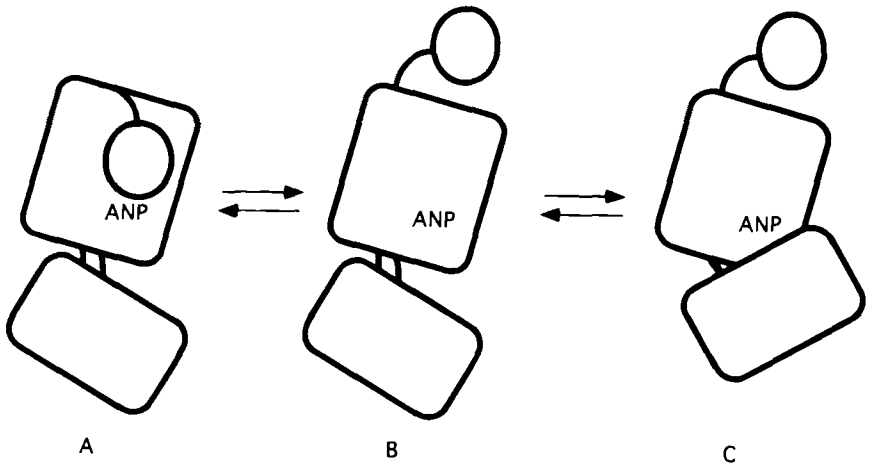

Fig. 8. Postulated conformational states of isolated and complexed α and β subunits. Subunit structures are shown schematically. The shape and size of the domains are in accordance with the structural features determined by Abrahams *et al.* (1994). B represents the conformation of the subunit in the holoenzyme. The domains (from top to bottom) are amino-terminal, central, and carboxy-terminal. Adenine nucleotide binding sites are marked by "ANP." A and C represent conformations of the isolated subunits in which the N- and C-termini are in close proximity to the nucleotide binding site.

Unlike the domains discussed above, amino acid residues lying within the βG10–M64 CNBr fragment, a domain labeled only in the case of soluble β, do not contain residues located in the nucleotide binding site(s) or in their vicinity. We propose that when α and β subunits are associated in a complex, the structure is rather rigid and the termini are not free to rotate, restricting accessibility and interaction with a ligand. We assume that the labeling of both termini of the molecule results from their spatial proximity to the reactive moiety of BzATP and it does not demonstrate different nucleotide binding sites on isolated α subunit. The N-terminal domains of α and β subunits form a rather rigid crown in the structure of MF_1 (Abrahams *et al.*, 1994), a structure which may not be formed by the isolated subunits, allowing therefore the six-stranded β-barrel N-terminal domains to move rather freely. This motion will bring the N-terminal domain in the proximity of the nucleotide bound at the central domain (Fig. 8). Binding of BzATP to the termini of α alters its conformation in such a way that association with β subunit is prevented (Bar-Zvi *et al.*, 1992).

ACKNOWLEDGMENTS

We wish to acknowledge the excellent technical assistance of Ms. Zvia Conrad-Maimon. D. Bar-Zvi is the incumbent of the Judith and Murray Schusterman Career Developing Chair in Microbiology.

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