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Catalytic and noncatalytic nucleotide binding sites of chloroplast F_1 ATPase

Photoaffinity labeling and peptide sequencing

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Exposure of chloroplast F_1 ATPase to 2-azido-ATP results in the noncovalent tight binding of 2-azido-ATP or 2-azido-ADP to noncatalytic or to catalytic sites. Subsequent photolysis results in covalent labeling of adjacent tryptic peptides of the β-subunit. Binding at noncatalytic sites results in labeling of tyrosine 385 by an ATP or an ADP moiety. Binding at catalytic sites results in labeling of tyrosine 362 by only an ADP moiety. Similar labeling patterns are observed for the heat-activated or the membrane-bound enzymes.

2-Azido-ATP; F_1 ATPase; Site labeling; Peptide sequence; (Chloroplast)

1. INTRODUCTION

The F_1 component of the chloroplast ATP synthase (CF_1) has an $\alpha_3\beta_3\gamma\delta\epsilon$ subunit composition. Nucleotide binding sites with several different characteristics have been described. A prominent view has been that only 3 nucleotide binding sites are present on CF_1, and evidence has been obtained that both catalytic and noncatalytic sites can bind ADP or ATP tightly (reviews [1–3]). Such findings were difficult to reconcile with the binding change mechanism with sequential participation of three catalytic sites. Some clarification has come from recent evidence that the CF_1, like the mitochondrial (MF_1) [4] and *E. coli* (EF_1) [5] enzymes, has six potential nucleotide binding sites, and that 2-azido-adenine nucleotides can derivatize separate catalytic and noncatalytic sites on the β-subunits [6].

2-Azido ATP and 2-azido ADP (2-azido-ANP), like ATP and ADP, can bind tightly to CF_1 and give covalent derivatization with high yield [6,7]. We report here that the azido analogs, when bound at catalytic and noncatalytic sites of CF_1, efficiently label two adjacent tryptic peptides on the β-subunit. Both peptides are derivatized on conserved tyrosine residues.

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2. MATERIALS AND METHODS

The 2-azido-ANP was prepared as described [8], and the 2-azido ADP and ATP were purified on a Bio-Rad AG MP-1 anion-exchange column. Incubations of CF_1 with azido nucleotides were in 40 mM Tricine (pH 7.8) at room temperature. After passage through Sephadex-centrifuge columns the CF_1 was exposed to ultraviolet light ('Mineralite' lamp) in a flat plastic vessel 1.5 cm from the lamp for 50 min. The long exposure to low intensity light allows the conversion of the

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bound tetrazole forms [9] to the azido form. The protein was precipitated in 0.5 M perchloric acid, washed with 80% acetone, and resuspended in 0.1 M NH_4HCO_3 (pH 8); trypsin was added (1/40 of the weight of CF_1) and the sample incubated at room temperature overnight. Ion-exchange and reverse-phase HPLC separations were performed as described in [10].

3. RESULTS

3.1. *Derivatization and separation of tryptic peptides by ion-exchange HPLC*

When latent CF_1 is incubated with 2-azido-ATP, at least 4 azido nucleotides become tightly bound [6,8]. Fig.1 shows a typical separation on the anion-exchange HPLC column of tryptic peptides obtained from latent CF_1 sample modified with 2-azido-[β,γ-^{32}P]ATP under conditions that label both catalytic and noncatalytic sites. Hydrolysis of the 2-azido-ATP allows derivatization by both an ADP and an ATP moiety. Most of the unlabeled tryptic peptides are not retained or are eluted early. The peptides modified with an ADP moiety are eluted later in the same region as free ADP (about 31 min, 28% solvent B), and those modified with an ATP moiety before free ATP (44 min, 54% solvent B). The ratios of A_{215} to ^{32}P present indicate that more underivatized peptides remain in the ADP-peptide fraction than in the ATP-peptide fraction. The recovery of modified peptides, based on the ^{32}P present, is about 85%. As reported earlier [6], no labeling of α-subunit is detected (not shown).

3.2. *Purification of peptides by reversed-phase HPLC and sequence analysis*

The fractions from the ion-exchange column were concentrated by lyophilization and further purified on the C_8 column. The ADP-peptide fraction yielded two radioactive peaks at 15% solvent B (29 min) and at 25% (39 min) with high absorption at 260 nm relative to unlabeled peptides (fig.2A). The recovery of labeled peptides for such HPLC separations is about 90% when 500 pmol or more of the peptides are loaded.

Each ADP-peptide peak (fig.2A) consisted of a single peptide modified at a single position. The sequences obtained, and the lack of expected tyrosine residues [11] in their respective cycles

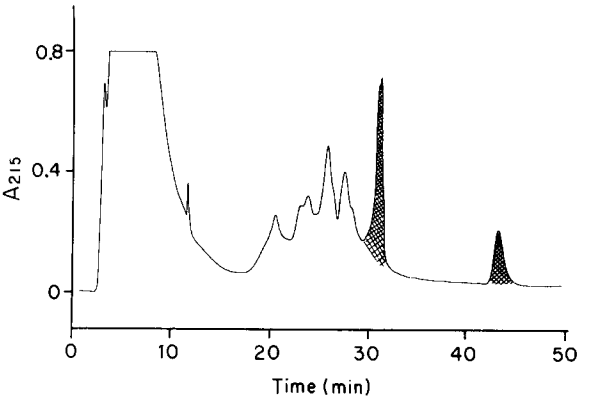

Fig.1. The separation of ADP- and ATP-labeled peptides on an ion-exchange HPLC column. Latent CF_1 (5 mg in 1 ml) was exposed to 200 μM 2-azido-[β,γ^{32}P]ATP and 1 mM Mg^{2+} for 10 min, then treated as described in section 2. Presence of ^{32}P is indicated by cross-hatching.

(table 1), showed that the first peptide was modified at Tyr-385 and the second at Tyr-362.

The ATP-peptide fraction from the ion-exchange column gave only a single peak eluting at 15% solvent B (fig.2B). Its retention time on the C_8 column was close to that of the ADP-modified peptides that eluted first, suggesting that the peptides had the same composition. This was confirmed by sequencing of the ATP-peptide.

3.3. *Correlation of labeling with catalytic or noncatalytic site occupancy*

The nature of the enzyme sites to which the nucleotide moieties are attached is readily shown by experiments using different labeling conditions. The second peak (39 min, ADP-fraction, fig.2) can be largely eliminated by a short chase with ATP before Sephadex column separation and UV irradiation [6]. The peptide is from a portion of the β-subunit that is highly conserved in the enzyme from different sources [11], that is derivatized by 2-azido-ANP in EF_1 [10] and in MF_1 [12,13] and for which the derivatization has been shown to arise from catalytic site binding in MF_1 [13] and EF_1 [10].

The first peak (29 min, ATP fraction, fig.2) is not diminished by a short ATP chase, pointing to a noncatalytic site location for the derivatizing 2-azido-ATP. Sequence data (table 1) show

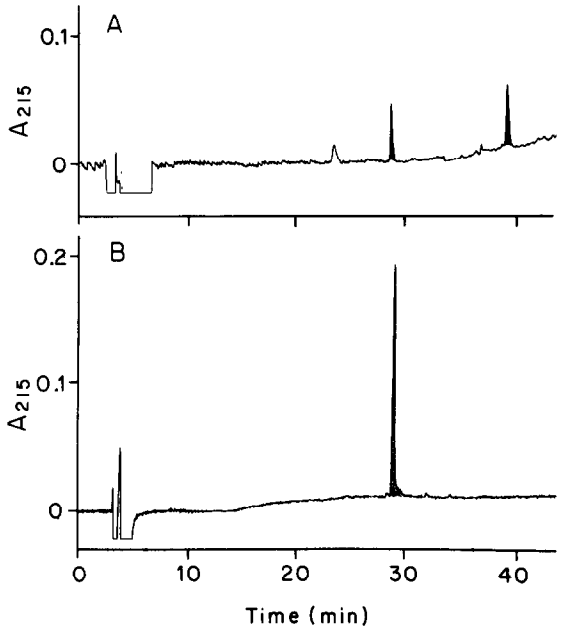

Fig.2. (A) Purification on a reversed-phase C_8 HPLC column of ADP-derivatized tryptic peptides from first-labeled peak of fig.1. Presence of ^{32}P is indicated by cross-hatching. (B) Similar data for the ATP-derivatized peptide of fig.1.

modification of Tyr-385 in a tryptic peptide adjacent to that for the catalytic site peptide. Again, the peptide is from a conserved region of the β-subunit, and corresponds to the similar peptide labeled by 2-azido-ANP at the noncatalytic site of MF_1 [13] and EF_1 [10]. This homology strengthens the identification of the ATP-labeling site on CF_1 as noncatalytic.

The noncatalytic site location is also corroborated by tests based on the tight MgATP binding site on CF_1 demonstrated in Hammes' laboratory [3,14] and regarded as a noncatalytic site. When this site is filled by Mg-2-azido-ATP in heat-activated CF_1, using the conditions described [14], only an ATP-peptide, migrating at the same retention time as the first peak (fig.2) is obtained. In this and other experiments, the same peptide labeling patterns for catalytic and noncatalytic site binding were obtained when the azido nucleotides were bound to heat-activated CF_1 or to membrane-bound CF_1 and the same sequences demonstrated for the heat-activated CF_1 (not shown).

Our results show that exposure to 2-azido-ATP leads to catalytic site labeling only by an ADP moiety. This is true even if pyruvate kinase and phospho*enol*pyruvate are present to remove 2-azido-ADP and with the latent enzyme that has very weak hydrolytic capacity. Probably any bound 2-azido-ATP is hydrolyzed to bound 2-azido-ADP before or perhaps even after covalent insertion. Whenever a covalently bound ATP moiety has been found in our studies, it has been associated with the noncatalytic site. The noncatalytic site can also be occupied by ADP as shown in section 3.2.

Comment may be appropriate that the identification of the nucleotide in the fractions designated as ADP- or ATP-peptides is based on: (i) the expected insertion of the bound nucleotide without decomposition; (ii) the migration in the ion-exchange separation as shown in fig.1, which shows the same sequence for the peptides labeled

Table 1

Microsequence analysis of 2-N_3-ANP modified peptides

Cycle	PTH-amino acid residue (pmol)	
	Catalytic peptide	Noncatalytic peptide
1	Gly (40)	Ile (24)
2	Ile (30)	Val (72)
3	–	Gly (16)
4	Pro (19)	Glu (28)
5	Ala (36)	Glu (42)
6	Val (35)	His (5)
7	Asp (25)	–
8	Pro (10)	Glu (18)
9	Leu (22)	Ile (50)
10	Asp (18)	Ala (12)
11	Ser (5)	Gln (24)
12	Thr (7)	Arg (3)
13	Ser (5)	
14	Thr (7)	
15	Met (6)	
16	Leu (8)	
17	Gln (2)	
18	Pro (2)	
19	Arg (2)	

HPLC purified peptides were subjected to automated Edman degradations on a City of Hope-built gas-phase microsequencer as described elsewhere [13]. A dash (–) indicates that no quantifiable PTH-derivative was present

at noncatalytic sites with an ADP or an ATP moiety and thus that the migration differences did not result from differences in peptide composition; (iii) the separation of β-subunits in isoelectric focusing SDS gels which shows the expected added charge for derivatization with either an ADP or an ATP moiety [6]; (iv) the relative ratio of ^{32}P to protein after separation in 2-dimensional gels with labeling by 2-azido-[β,γ-^{32}P]ATP or 2-azido-[β-^{32}P]ADP [6]; and (v) the ratios of ^{32}P in azido-nucleotides noncovalently but tightly bound prior to photolysis to the amount of [^{3}H]ADP or [^{3}H]ATP bound under similar conditions.

4. DISCUSSION

Our results allow identification of separate catalytic and noncatalytic binding sites on CF_1. Although only labeling of the β-subunit is observed, one or both sites could be at α–β-subunit interfaces with orientation of the nitrene formed from the 2-azido group towards the β-subunit.

Previously Abbott et al. [7] showed that tightly bound 2-azido-ADP and 2-azido-ATP on chloroplast thylakoid membranes labeled the β-subunit of CF_1. Whether both catalytic and noncatalytic sites were labeled under the experimental conditions used by Abbott et al. [7] remains to be evaluated. The derivatization of Tyr-362 by 2-azido-ADP is the same as that very recently reported by Adman and Hammes [15] for labeling with 2′,3′-*O*-(4-benzoyl)benzoyl-ADP [15]. Based on results reported here, their labeling probably occurred at a catalytic site. They did not identify a separate noncatalytic site derivatization.

The observations that the same labeled peptides are obtained from the latent CF_1, the membrane-bound CF_1, or the heat-activated CF_1 have important implications. Separation of the enzyme from the membrane or heat activation apparently does not change the binding orientations at the catalytic site. Because the latent enzyme with 2-azido-ANP bound at three noncatalytic sites yields only one uniquely labeled noncatalytic peptide the conformations of all three noncatalytic binding sites are likely similar. This is of interest because only one tight MgATP noncatalytic site is readily observed with the CF_1 as usually prepared [14].

We are currently applying the procedures described in this communication to help clarify questions that have been raised about the location and function of nucleotide binding sites of the CF_1.

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