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Catalytic and noncatalytic nucleotide binding sites of the *Escherichia coli* F_1 ATPase

Amino acid sequences of β-subunit tryptic peptides labeled with 2-azido-ATP

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Under appropriate conditions tight, noncovalent binding of 2-azido-adenine nucleotides to either catalytic or noncatalytic binding sites on the *E. coli* F_1-ATPase occurs. After removal of unbound ligands, UV-irradiation results primarily in the covalent incorporation of nucleotide moieties into the β-subunit in both catalytic and noncatalytic site labeling experiments. Minor labeling of the α-subunit was also observed. After trypsin digestion and purification of the labeled peptides, microsequencing studies identified two adjacent β-subunit tryptic peptides labeled by 2-azido-ADP or -ATP. These β-subunit peptides were labeled on tyrosine-331 (catalytic sites) and tyrosine-354 (noncatalytic sites) in homology with the labeling patterns of the mitochondrial and chloroplast enzymes.

H^+-ATPase; Oxidative phosphorylation; Photoaffinity analog; Azido-ATP; (*E. coli*)

1. INTRODUCTION

E. coli membranes contain an ATP synthase (F_1F_0 type) from which an ATPase (EF_1) is readily purified that is similar to the ATPases derived from mitochondria (MF_1) and chloroplasts (CF_1) [1–4]. The *E. coli* ATP synthase is an oligomeric enzyme composed of eight nonidentical subunits arranged in two sectors, F_1 and F_0. F_0 is comprised of three integral membrane proteins of complex stoichiometry and is known to facilitate proton transport through the membrane. *E. coli* F_1 is composed of five proteins in the stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$, is peripheral to the membrane, and contains the sites of ATP hydrolysis and synthesis.

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EF_1, like MF_1, contains six total nucleotide binding sites, three of which are potential catalytic sites, and three that are referred to as noncatalytic sites [5,6]. Noncatalytic sites are relatively specific for adenine nucleotides and do not participate directly in catalysis. Isolated EF_1 α-subunit contains a nucleotide binding site that is similar in properties to the noncatalytic binding sites of F_1 [7,8]. Catalytic sites on MF_1 and EF_1 can be induced to exchange readily bound nucleotide with medium nucleotide, while nucleotides bound at noncatalytic sites exchange with medium nucleotides very slowly with or without catalytic turnover [5,6]. Using the photoactivatable ATP analog, 2-azido-ATP, and conditions that allow the binding of nucleotide to either catalytic or noncatalytic binding sites, we have been able to modify specifically and covalently both classes of binding sites in EF_1. We report the results of the modification and sequencing of two adjacent β-subunit

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tryptic peptides corresponding to the catalytic and noncatalytic nucleotide binding sites. We briefly summarize the results of similar studies performed on MF_1 and CF_1 ATPases.

2. EXPERIMENTAL

EF_1 was prepared from *E. coli* K12 strain AN1460 [10] by the procedures described in [11,12]. Cells were grown in 150 l fermenters on either LB or supplemented minimal media. Synthesis of 2-azido-ATP was as described in [13,14]. Differential labeling of catalytic or noncatalytic binding sites was performed essentially as described in [15] except that 2-azido-ATP was substituted for imido-ATP. The incubation buffer used in each experiment contained Mg^{2+} (50 mM Tris-SO_4, 0.5 mM EDTA, 2.5 mM $MgCl_2$, pH 8.0). 3 ml Sephadex-centrifuge columns [16] equilibrated in the incubation buffer were used throughout. Irradiation, acid precipitation, and proteolysis of F_1 samples were performed essentially as described in [14]. Succinylation of F_1 when performed followed the procedures in [17]. Routine procedures were as described in [15]. Sequencing of purified peptides was performed by the automated Edman degradation method on an Applied Biosystems M470. Amino acid compositions were determined by the Waters Pico-Tag system. All of the sequences and compositions reported in this study have been reproduced.

Purification of peptides for sequencing took advantage of the anionic character of peptides modified with ADP or ATP (ANP) moieties by first employing anion-exchange HPLC. Nonsuccinylated tryptic peptides from EF_1 samples labeled with 2-azido-ATP were directly injected into a Waters/Millipore HPLC system employing a Whatman Partisil PXS25/SAX10 ion-exchange column (4.6 × 250 mm). After a 5 min isocratic elution, the labeled peptides were eluted by a 50 min gradient from 100% solvent A (29:71, acetonitrile:0.01 M NaH_2PO_4, pH 4.0) to 100% solvent B (29:71, acetonitrile:0.4 M NaH_2PO_4, pH 3.0). Absorbance was followed at 215 and 260 nm. Fractions (collected manually) were analyzed for radioactivity by Cerenkov counting. ^{32}P-containing peaks with high 260/215 nm absorbance ratios were individually lyophilized and separated by HPLC on a Vydac C_8 reversed-phase column. Gradient elution from 0.1 vol% trifluoroacetic acid to 0.1% trifluoroacetic acid, 90 vol% acetonitrile resulted in purification of the labeled peptides. Estimations of recoveries were based on the specific radioactivity of the ^{32}P-azidonucleotide used.

3. RESULTS

3.1. *Differential labeling of catalytic or noncatalytic binding sites by 2-azido-ATP*

Fig.1 shows an HPLC elution profile of tryptic peptides of EF_1 photolabeled by 2-azido-ANP bound at catalytic sites, separated only by a single C_4 reversed-phase column. Briefly, ammonium sulfate-precipitated EF_1 (5 mg) was redissolved in 0.4 ml of incubation buffer and desalted by a Sephadex-centrifuge column. Noncatalytic sites were first filled with nonradioactive nucleotide (1 mM ATP, 30 min). Removal of free ATP, ADP and P_i by a Sephadex-centrifuge column was followed by incubation in the dark with 2-azido-[β,γ-^{32}P]ATP (0.75 mM, 15 min) to allow the exchange of medium 2-azido-ATP with F_1-bound ANP present at catalytic sites. Following a final Sephadex-centrifuge column, the sample was irradiated for 30 min, acid-precipitated, succinylated and digested with trypsin. Conversion of tightly bound to covalently incorporated 2-azido-ANP moieties occurred with approx. 50% efficiency (50% acid-precipitable cpm). This high conversion efficiency is typical if protein concentrations are kept at ≤ 5 mg/ml during photolysis. The entire sample was injected into a Vydac C_4 reversed-phase column and eluted by the same gradient described in section 2 for the C_8 column. The resulting A_{215nm} and radioactivity profile are shown in fig.1. Catalytic site-labeled peptide(s) eluted at approx. 60 min or about 30% solvent B.

The bottom panel of fig.1 also shows the radioactivity profile of similar tryptic peptides of EF_1 photolabeled by 2-azido-ANP bound at noncatalytic sites. Labeling and HPLC methods were identical to that described above except that the first incubation (15 min) contained 2-azido-[β,γ-^{32}P]ATP (1.7 mM), and the second incubation (5 min) contained ATP (15 mM) and $MgCl_2$ (15 mM). Noncatalytic site-labeled peptide(s) eluted at about 40 min or about 20% solvent B. Comparison of fig.1 catalytic site labeling with

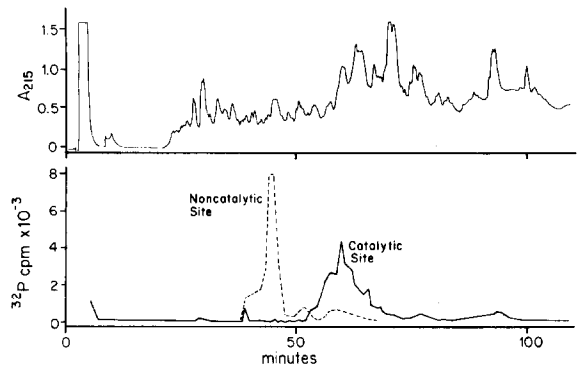

Fig.1. Reversed-phase HPLC separation of catalytic and noncatalytic site 2-azido-ANP-labeled tryptic peptides. The top panel shows the $A_{215\,nm}$ trace of the catalytic site sample. The bottom panel shows the radioactivity profiles of both catalytic and noncatalytic site-labeled samples.

noncatalytic site labeling demonstrates a clear distinction between the different labeled tryptic peptides. Both MF_1 and CF_1 tryptic peptides labeled at noncatalytic or catalytic sites by 2-azido-ANP show similar HPLC elution patterns [14,17,18].

3.2. *Purification of 2-azido-ANP-labeled noncatalytic and catalytic site peptides*

Fig.2 presents data from a 2-azido-ATP catalytic site-labeling experiment analogous to the experiment of fig.1 with the following modifications. EF_1 (15 mg) was incubated for 10 min with 1 mM ATP and passed through two consecutive Sephadex-centrifuge columns. 2-Azido-[β,γ-^{32}P]-ATP (0.9 mM) was added and incubation continued for 1 h. After a final Sephadex-centrifuge column, the sample was irradiated for 1 h and then acid precipitated. NH_4HCO_3 (1 ml at 0.1 M, adjusted to pH 8 with 2 M NH_4OH) and TPCK-trypsin (250 μg) were added and incubation at 37°C was continued for 16 h. The sample was centrifuged (the pellet contained <5% of total radioactivity) and the supernatant (18 nmol of labeled peptides) was separated by ion-exchange HPLC as described in section 2. Fig.2A shows the resulting A_{215} profile for the ion-exchange step. Fraction I, corresponding to the approximate elution time of free ADP (about 28% solvent B), contained 12 nmol of labeled peptide. Fraction II,

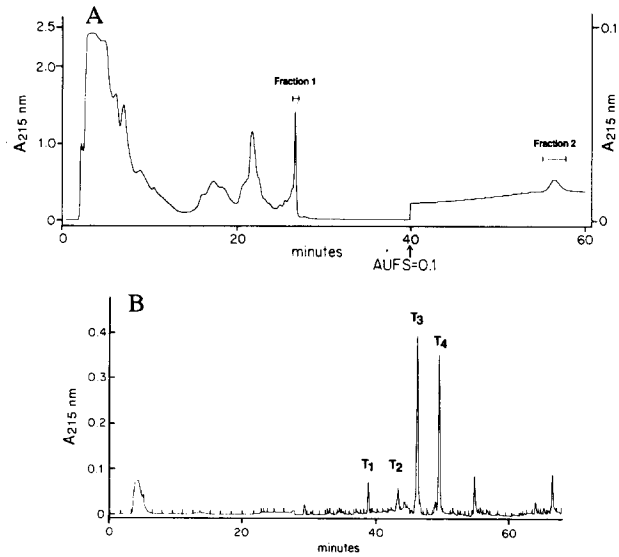

Fig.2. HPLC purification of labeled peptides. (A) HPLC anion-exchange separation of predominantly catalytic site-labeled tryptic peptides. (B) Reversed-phase HPLC separation of fraction I from panel A. Details are described in the text.

corresponding to the approximate elution time of free ATP (about 82% solvent B), contained 0.2 nmol of labeled peptide. No other fractions except the initial flow-through fraction contained significant amounts of radioactivity.

Fig.2B shows the elution profile when 10 nmol of ^{32}P-labeled peptides from fraction I above was applied to a C_8 reversed-phase column (described in section 2). Only peaks T_1, T_2, T_3 and T_4 (eluted between 25 and 29% solvent B) were radioactive and contained 0.5, 0.6, 3.5, and 2.2 nmol labeled peptide, respectively. The noncatalytic site peptides, T_1 and T_2, correspond to 7 and 9%, while the catalytic site peptides T_3 and T_4 correspond to 51 and 32% of the total radioactivity injected in this predominantly catalytic site-labeled EF_1 sample.

In separate experiments (not shown) where noncatalytic sites were predominantly photolabeled, peptides T_1 and T_2 were observed to be the major radioactive fractions while T_3 and T_4 were the minor radioactive components. Such experiments confirmed that peptides T_1 and T_2 were from noncatalytic sites and peptides T_3 and T_4 were from catalytic sites. The efficacy of the two step

purification scheme outlined in section 2 is evident from comparison of fig.1A with fig.2B. The scheme may prove useful in purifying other phosphate-moiety derivatized peptides.

3.3. *Analysis of catalytic and noncatalytic site 2-azido-ANP-labeled peptides*

Table 1 presents automated Edman gas-phase microsequencing data obtained from the noncatalytic site peptide, T_1. The sequence obtained can be aligned with the amino acid sequence deduced from the DNA sequence [19] starting with residue Gln-343 of the EF_1 β-subunit. No PTH derivative was detected at cycle 12, while all other cycles identified residues in complete agreement with the amino acid sequence in [19]. The deduced amino acid sequence would have a tyrosyl residue at the corresponding cycle 12. As noted in [17], a PTH-derivatized tyrosine bearing an ADP moiety would have very limited solubility in the microsequencer solvent system, precluding its detection. We conclude that the site of labeling on the EF_1 β-subunit by 2-azido-ANP when bound at noncatalytic sites is Tyr-354.

Table 1 also presents the microsequencing data for the catalytic site peptide, T_3. This sequence can be aligned with the deduced amino acid sequence in [19] starting with residue Gln-329 of the EF_1 β-subunit. No PTH derivative was detected in cycle 8. By analogy to the reasoning given above, we conclude that cycle 8 corresponds to the modified β-subunit residue, Tyr-331.

Peptides T_2 and T_4 were resistant to Edman degradation as no detectable PTH derivatives were observed in any of the automated cycles (replicate samples of up to 0.5 nmol each of the radiolabeled peptides). Comparison of the amino acid compositions (acid hydrolysis) of the four labeled peptides suggests that T_2 is identical to the noncatalytic site peptide T_1, and that T_4 is identical to the catalytic site peptide T_3 (table 2). The differences in HPLC elution time and susceptibility to Edman degradation may reflect cyclization of the N-terminal glutamine residues of peptides T_2 and T_4.

Fraction II of fig.2A has been purified on reversed-phase HPLC and sequenced and been found to be identical to the noncatalytic site peptide, T_1 (not shown). We surmise that the cycle 12, Tyr-354 of this sample has been modified with a 2-azido-ATP moiety rather than a 2-azido-ADP moiety due to its greater anionic character. In other experiments, EF_1 was labeled with 2-azido-ANP and the β-subunits were dissociated and isolated. After the isolated β-subunits were subjected to proteolysis, purification and sequencing of the labeled peptides resulted in identification of catalytic and noncatalytic site peptides identical to those of fig.2 and tables 1 and 2 (not shown). As with CF_1 [14], only ADP-derivatized peptides were found with catalytic-site labeling, and both ADP- and ATP-derivatized peptides were found with noncatalytic-site labeling.

It is important to mention that between 5 and 10% of the 2-azido-ANP covalently incorporated into EF_1 modifies the α-subunit as determined by SDS-polyacrylamide gel electrophoresis and autoradiography (not shown). We are currently working to identify the labeled α-peptide(s).

Table 1

Microsequence analyses of 2-azido-ANP modified peptides

Cycle	PTH-amino acid residue (pmol)	
	Noncatalytic peptide, T_1	Catalytic peptide, T_3
1	Gln (39)	Gln (65)
2	Leu (26)	Ile (38)
3	Asp (33)	Ala (48)
4	Pro (28)	Ser (15)
5	Leu (17)	Leu (30)
6	Val (17)	Gly (29)
7	Val (25)	Ile (24)
8	Gly (11)	–
9	Gln (10)	Pro (31)
10	Glu (7)	Ala (22)
11	His (5)	Val (14)
12	–	Asp (22)
13	Asp (4)	Pro (16)
14	Thr (3)	Leu (9)
15	Ala (5)	Asp (10)
16	Arg (1)	Ser (2)
17		Thr (2)
18		Ser (1)
19		Arg (1)

160 pmol of peptide T_1 and 260 pmol of peptide T_3 were analyzed in these experiments. The dashes indicate that no quantifiable PTH derivative was present

Table 2

Amino acid compositions of 2-azido-ANP-modified peptides

Noncatalytic site peptides			Catalytic site peptides		
Residue	T_1 (mol/mol)	T_2 (mol/mol)	Residue	T_3 (mol/mol)	T_4 (mol/mol)
D (2)	1.7	1.6	D (2)	1.4	1.5
E (3)	2.0	2.3	E (1)	1.1	1.2
S (0)	0.7	0.5	S (3)	2.1	1.9
G (1)	1.7	1.2	G (1)	1.1	1.2
H (1)	0.8	0.7	H (0)	<0.1	0.2
R (1)	1.0	1.0	R (1)	1.0	1.0
T (1)	0.8	0.9	T (1)	0.9	0.9
A (1)	0.8	1.0	A (2)	1.5	1.4
P (1)	1.0	1.1	P (2)	1.7	1.8
Y (1)	0.2	0.3	Y (1)	0.3	<0.1
V (2)	1.2	1.7	V (1)	1.5	1.1
M (0)	<0.1	0.3	M (0)	<0.1	0.1
I (0)	0.3	0.2	I (2)	1.2	1.3
L (2)	1.2	1.4	L (2)	1.4	1.5
F (0)	0.2	0.1	F (0)	<0.1	0.2
K (0)	0.3	0.3	K (0)	<0.1	0.3

D and E represent D + N and E + Q, respectively. W and C were not determined. Numbers in parentheses represent the values from the sequence determinations of peptides T_1 and T_3, respectively. Between 0.25 and 0.50 nmol of each peptide was subjected to analysis

4. DISCUSSION

It has been recognized that the major subunits of F_1-ATPases from mitochondria, chloroplasts and bacteria are similar. Indeed, in view of recent reports of the amino acid sequences of the α- and β-subunits of MF_1, CF_1 and EF_1 (see [19]), it is apparent that these proteins from very disparate species are highly conserved. One might predict that each of the three enzymes may be quite similar in the basic structures of the F_1 sector. Until recently, CF_1 was widely regarded as having only three nucleotide binding sites, leaving a discrepancy in observed structure when contrasted against the six sites observed in MF_1 and EF_1 [5,6]. Xue et al. [18], in confirmation and extension of suggestions of others, report strong evidence that CF_1, like MF_1 and EF_1, probably has a total of six nucleotide binding sites, comprised of catalytic and noncatalytic sites.

Fig.3 summarizes 2-azido-ANP labeling data from experiments on MF_1 [17,20], CF_1 [14], and EF_1 (this report). It is apparent from the figure that 2-azido-ANP modifies highly conserved β-subunit peptides at homologous tyrosyl residues when either catalytic or noncatalytic sites are filled with 2-azido-ANP. The direct demonstration of similar catalytic and noncatalytic peptides modified by 2-azido-ANP in MF_1, CF_1 and EF_1 strongly supports the structural and functional analogies between the three enzymes. The ability to directly distinguish between catalytic and noncatalytic nucleotide binding, and between incorporated ADP- and ATP-moieties, should serve to advance efforts at understanding the effects of bound nucleotides on the function of the ATP synthase.

When nucleotide sites on EF_1 are modified by 2-azido-ANP moieties, small amounts of the covalently incorporated analog are found on the α-subunit. The majority of analog incorporated into EF_1 reacts with the β-subunit presumably because

2-AZIDO-ANP-LABELED CATALYTIC β-SUBUNIT PEPTIDES

F_1 Source	Modified Residue*	Labeled Peptide
Bovine Mitochondria	Tyr-345[a,b]	A I A E L G I Y* P A V D P L D S T S R
Spinach Chloroplast	Tyr-362[c]	G I Y* P A V D P L D S T S T M L Q P R
Escherichia coli	Tyr-331[d]	Q I A S L G I Y* P A V D P L D S T S R

2-AZIDO-ANP-LABELED NONCATALYTIC β-SUBUNIT PEPTIDES

F_1 Source	Modified Residue*	Labeled Peptide
Bovine Mitochondria	Tyr-368[b]	I M N P N I V G S E H Y* D V A R
Spinach Chloroplast	Tyr-385[c]	I V G E E H Y* E I A Q R
Escherichia coli	Tyr-354[d]	Q L D P L V V G Q E H Y* D T A R

Fig.3. Catalytic and noncatalytic site tryptic peptides of MF_1, CF_1 and EF_1 labeled with 2-azido-ANP moieties. (*) Residue number refers to the site of 2-azido-ANP modification counted from the N-terminus of the respective β-subunit. Boxes indicate identically conserved residues of the relevant regions of the β-subunits from ten different species [19]. [a] From [20]; [b] from [17]; [c] from [14]; [d] from this work.

the 2-position of the enzyme bound 2-azido-ANP exists in an orientation closest to the β-subunit. The possibility that catalytic and/or noncatalytic binding sites may be formed at the interfaces of the α- and β-subunits should not be discounted.

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