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Adenine nucleotide binding sites on beef heart F_1 ATPase: Photoaffinity labeling of β-subunit Tyr-368 at a noncatalytic site and β Tyr-345 at a catalytic site

(oxidative phosphorylation/mitochondrial ATP synthase/nucleotide-binding domain/2-azido-ATP)

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ABSTRACT **2-Azidoadenine [^{32}P]nucleotide was bound specifically at catalytic or noncatalytic nucleotide binding sites on beef heart mitochondrial F_1 ATPase. In both cases, photolysis resulted in nearly exclusive labeling of the β subunit. The modified enzyme was digested with trypsin, and labeled peptides were purified by reversed-phase high-pressure liquid chromatography. Amino acid sequence analysis of the major ^{32}P-labeled tryptic fragments showed β-subunit Tyr-368 to be present at noncatalytic sites and β Tyr-345 to be present at catalytic sites. From the relationship between the degree of inhibition and extent of modification, it is estimated that one-third of the catalytic sites or two-thirds of the noncatalytic sites must be modified to give near-complete inhibition of catalytic activity.**

The ATP synthase F_0F_1, found in mitochondria, chloroplasts, and bacteria, couples proton translocation to ATP synthesis during oxidative phosphorylation and photophosphorylation. The F_1 component can be readily solubilized and has a $\alpha_3\beta_3\gamma\delta\varepsilon$ subunit structure. F_1 contains the catalytic sites for ATP synthesis, but when detached from the membrane it is only capable of net ATP hydrolysis (1, 2).

Of the six nucleotide binding sites present on the beef heart mitochondrial enzyme, MF_1, only three sites exchange bound nucleotide rapidly during hydrolysis of MgATP at pH 8.0 (3). Evidence has been presented that at least two (4–6) and probably all three (7–12) of the exchangeable sites participate sequentially in catalysis at high substrate concentration. The three nonexchangeable sites, referred to here as noncatalytic sites, have been suggested to play a structural or regulatory role (for example, see refs. 13 and 14).

A valuable approach to the characterization of nucleotide binding sites on F_1 has been the use of affinity and photoaffinity analogs (15). Perhaps the most promising probes are the 2-azidoadenine nucleotides (11, 16, 17). Their *anti* conformation resembles that of F_1-bound ADP and ATP; thus, they bind very tightly and serve as good substrates (18). Procedures described by Kironde and Cross (19) allow the specific loading of 2-azidoadenine nucleotides at noncatalytic or catalytic sites on MF_1. Photolysis results in the labeling of the β subunit in each case, but different labeled peptides were obtained in partial *Staphylococcus aureus* V8 protease digests (20). In this paper, we show that the labeled catalytic and noncatalytic site residues are on adjacent tryptic peptides corresponding to those labeled by 5′-[*p*-(fluorosulfonyl)benzoyl]inosine (FSBI) and 5′-[*p*-(fluorosulfonyl)benzoyl]-adenosine (FSBA), respectively (21). The catalytic-site peptide corresponds to the single tryptic peptide detected with 2-azido-ADP labeling by Garin *et al.* (22). We also report here the effects of derivatization on catalytic activity.

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MATERIALS AND METHODS

Preparation of MF_1. MF_1 was isolated from beef heart mitochondria as described (23). MF_1 containing one vacant noncatalytic site and one filled catalytic site (MF_1[2,1]) and enzyme with fully occupied noncatalytic sites and two filled catalytic sites (MF_1[3,2]) were prepared in 150 mM sucrose/10 mM Hepes/1 mM P_i/1 mM $MgSO_4$, pH 8.0 (sucrose/Hepes/P_i/Mg), as described (20). MF_1[3,0] was prepared from MF_1[3,2] by incubating at 20 μM for 2 min with 4 mM MgGTP followed by two 5-min incubations in sucrose/Hepes/P_i/Mg containing in addition 50 mM P_i. Unbound ligand was removed on a Sephadex centrifuge column (24) between each incubation, and the concentration of P_i was reduced to 1 mM during passage through the last column. Nucleotide-depleted enzyme (MF_1[0,0]) was prepared as described by Garrett and Penefsky (25).

Photoaffinity Labeling. To label a noncatalytic nucleotide site, in a typical experiment 60 μl of 14.5 mM 2-azido-[β,γ-^{32}P]ATP was added with rapid mixing to 450 μl of 75 μM MF_1[2,1] and incubated for 15 min. Unbound ligand was removed on a 3-ml centrifuge column, and MgATP was added to a final concentration of 15 mM to displace 2-azido-ANP from catalytic sites. After 1 min, the enzyme was passed through a second 3-ml centrifuge column and diluted to 2 ml. Four 500-μl aliquots were placed on a shallow plastic tray 8 mm from a Minerallight lamp and photolyzed at the short wavelength setting for 30 min.

To label the catalytic site, 50 μl of 50 mM MgGTP was added with rapid mixing to 450 μl of 75 μM MF_1[3,2] and incubated for 1 min. Unbound ligand was removed on a centrifuge column. 2-Azido-[β,γ-^{32}P]ATP was then added at a concentration equal to that of MF_1. After 1 min, the sample was photolyzed for 5 min as described above. MgGTP was then added to a final concentration of 4.2 mM to displace noncovalently bound adenine nucleotide from catalytic sites, and the enzyme was passed through a 3-ml centrifuge column. A second cycle of photolabeling was performed by mixing MF_1 in the effluent with an equimolar amount of 2-azido-[β,γ-^{32}P]ATP. After 1 min, the sample was diluted to 1 ml, and two 500-μl aliquots were irradiated for 5 min as described above.

Abbreviations: MF_1, soluble ATPase portion of beef heart mitochondrial ATP synthase; MF_1[*x*,*y*], MF_1 containing *x* mol of ANP at noncatalytic sites and *y* mol of ANP at catalytic sites per mol of enzyme; ANP, adenine nucleotide; FAB, fast-atom bombardment; FSBA and FSBI, *p*-(fluorosulfonyl)benzoyl derivatives of adenosine and inosine, respectively.

Small aliquots of the photolyzed reaction mixtures were taken for determination of total ^{32}P bound, ^{32}P covalently bound, protein concentration, and analysis by tetradecyltrimethylammonium bromide/polyacrylamide gel electrophoresis (20). The rest was used for preparing samples for sequencing as described below. In a typical experiment, the amount of analog noncovalently bound prior to photolysis was 1.0 and 0.7 mol/mol of MF_1 for noncatalytic and catalytic sites, respectively. Following photolysis, 0.6 mol of noncatalytic sites and 0.4 mol of catalytic sites were covalently modified per mol of MF_1.

Succinylation and Digestion. Photolabeled MF_1 was precipitated by addition of 0.1 volume of 0.15% deoxycholate followed 10 min later by addition of 0.1 volume of 72% trichloroacetic acid. Samples were incubated on ice for 2 min and spun at 4°C for 15 min at 2800 rpm in an IEC table-top refrigerated centrifuge. Trichloroacetic acid was extracted from the pellets by Vortex mixing with 500 μl of 80% acetone. Protein was collected by centrifugation and dissolved in 2 ml of 0.2 M trimethylamine (pH 9.0). Guanidine hydrochloride was added (1.28 g) and the pH was adjusted to 9 with 4 M NaOH. Forty milligrams of succinic anhydride was added slowly with mixing over a 40-min period, pH was maintained at 9 with 4 M NaOH. Samples were diluted to 11 ml with water, and the protein was precipitated as described above. The pellet was dissolved in 1 ml of 100 mM NH_4HCO_3, and trypsin was added to give a trypsin/MF_1 (mg/mg) ratio of 1:40. The mixture was incubated at room temperature overnight. Two hours before separation of peptides by HPLC, a second equal amount of trypsin was added.

Purification of ^{32}P-Labeled Peptides. Peptides were purified by reversed-phase HPLC using a Vydac C_4 or a Brownlee C_8 column (4.6 mm × 25 cm) and a two-pump Waters system as described under *Results*.

Microsequence Analysis and Fast-Atom Bombardment (FAB) Mass Spectrometry. A gas-phase microsequencer (built at City of Hope) was used for the automated Edman chemical degradations (26). The phenylthiohydantoin amino acid derivatives were detected and identified by reversed-phase HPLC on an Ultrasphere ODS column (4.6 mm × 25 cm; Altex, Berkeley, CA) and were quantitated by an integration program on a Spectra Physics 4000 computer (27). FAB mass spectra were taken with a JEOL HX-100HF mass spectrometer utilizing a 6-kV xenon atom primary beam.

Other Methods. ^{32}P was assayed by measuring Cerenkov radiation. MF_1 was quantitated using a modified Lowry procedure (28). ATPase activity was measured at 30°C using an ATP-regenerating system coupled to the oxidation of NADH and monitored spectrally at 340 nm.

2-Azido-AMP was prepared from 2-chloroadenosine and phosphorylated to 2-azido-[β,γ-^{32}P]ATP using 3-phosphoglycerate kinase and adenylate kinase as described (11). 2-Azido-[β-^{32}P]ATP was prepared from 2-azido-[β-^{32}P]ADP by incubation with 20 mM potassium phospho*enol*pyruvate and 1 μM pyruvate kinase at room temperature for 2 hr. 2-Azidoadenine nucleotides were purified by ion-exchange and gel-filtration chromatography using Dowex AG-MP-1 and Sephadex G-10. Concentrations were determined by measuring the absorbance of an equilibrium mixture of the azido and tetraazole isomers (pH 7) at 271 and 310 nm, using extinction coefficients of 10.3 and 7.0 $mM^{-1}\cdot cm^{-1}$, respectively (18).

RESULTS

Isolation of Peptides Derived from Modified Catalytic and Noncatalytic Sites. 2-Azidoadenine nucleotide was specifically loaded at catalytic or noncatalytic sites on MF_1, and the complexes were photolyzed and digested with trypsin as described above. The initial tryptic digests were separated by reversed-phase HPLC using a C_8 column developed with a gradient of increasing acetonitrile concentration in 0.1% trifluoroacetic acid. Fig. 1 *Upper* shows the elution of peptides derived from MF_1 having a photolabeled noncatalytic site. The major ^{32}P-labeled peaks (solid circles) are eluted when solvent B reaches 14% (T-NC-14) and 20% (T-NC-20). When MF_1 having a modified catalytic site is analyzed (Fig. 1 *Lower*), the major ^{32}P-labeled peak is eluted at 27% B (T-C-27). Similar patterns were obtained whether single or multiple sites of a given type were modified.

The profiles for ^{32}P-labeled peptides are clearly different for labeled catalytic and noncatalytic sites, as previously indicated by gel electrophoresis of partial *S. aureus* V8 protease digests (20). However, the specificity obtained for loading catalytic and noncatalytic sites appears not to be absolute. For the noncatalytic site (Fig. 1 *Upper*), a small amount of ^{32}P-labeled material is eluted at 27% B, corresponding to the major peak for the catalytic site (T-C-27). The final step in loading the noncatalytic site is to "chase" 2-azido-[^{32}P]ANP from catalytic sites by addition of MgATP. Subsequent trace labeling of a catalytic site confirms previous results indicating the presence of a small fraction of sluggish enzyme that retains nucleotide bound at the catalytic site during the few seconds it takes to hydrolyze the amount of ATP added in the nonradioactive chase (19). For the sample containing labeled catalytic site (Fig. 1 *Lower*), trace amounts of ^{32}P are eluted at 14% and 20% B, corresponding to the major noncatalytic-site peaks (T-NC-14 and T-NC-20). An additional ^{32}P-labeled peak for the photolabeled catalytic site is eluted at 35% B. We believe this may be due to the presence of incompletely digested material, since the ratio of radioactivity (cpm) eluted at 27% B to that at 35% B increases with increased time of digestion or amount of added trypsin.

Preparation of peptide fractions suitable for sequence analysis required two additional HPLC separations. Fractions containing radioactivity were pooled and subjected to further purification on an HPLC column equilibrated with 0.1% ammonium trifluoroacetate (pH 6.5). A linear gradient was developed using 0.1% ammonium trifluoroacetate in 90% acetonitrile (solvent F). A single ^{32}P-labeled peptide was eluted at 11% F for T-NC-20, and a series of closely spaced ^{32}P-labeled peptides were eluted between 13% and 15% F for T-C-27. These separated components were then subjected to a final HPLC purification using solvents A and B as described for Fig. 1.

Identification of Sites of Labeling. The purification of peptides from fraction T-NC-20 (Fig. 1) by the procedures described above yielded a single ^{32}P-labeled peptide that was analyzed by gas-phase microsequencing and FAB mass spectrometry. This noncatalytic-site peptide (Table 1) can be aligned with the reported MF_1 β-subunit sequence (29) starting at the corrresponding tryptic peptide that begins with Ile-357. In cycle 3, aspartic acid rather than asparagine was detected, in agreement with the FSBA-labeled peptide sequenced by Esch and Allison (30). No phenylthiohydantoin derivative could be detected at cycle 12 (Xaa). We surmise from the known β-subunit sequence that the 12th amino acid is a modified tyrosine. A tyrosine bearing the aminoadenylyl diphosphate moiety would have very limited solubility in the sequencer solvent 1-chlorobutane, thus precluding its detection. Indeed, although radioactivity was not recovered at cycle 12, it was present on the glass-fiber sequencer loading disk, consistent with the failure to extract the charged derivative. The FAB mass spectral analysis gave a molecular ion of 2557 atomic mass units. This result is consistent with the amino acid sequence shown if the peptide bears a photolabel, (−NH)ADP, and three succinyl groups resulting from the exhaustive succinylation of the protein prior to proteolysis. These data collectively support the identification of β Tyr-368 as the site of photo-crosslinking of 2-azido-ADP

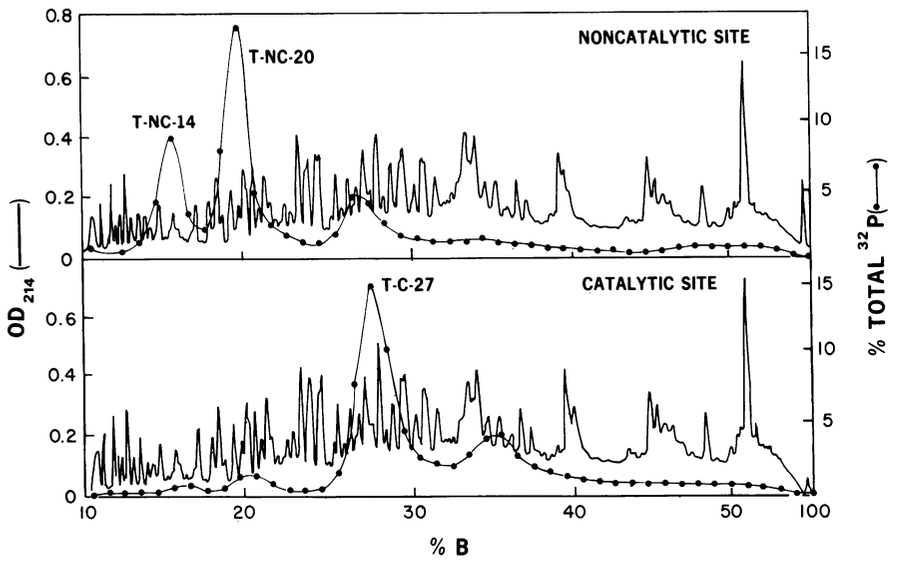

FIG. 1. Elution profile of ^{32}P-labeled peptides from initial tryptic digests. Peptides were separated by HPLC on a C_8 column using a linear gradient developed at 2% B/min from 0–10% B and at 0.5% B/min from 10–50% B. Solvent A was 0.1% trifluoroacetic acid, and solvent B was 0.1% trifluoroacetic acid in 90% acetonitrile. Of the total radioactivity applied, 84% was recovered in the experiment shown in the upper panel and 81% in the lower panel.

bound at a noncatalytic site. The second major ^{32}P-labeled fraction derived from the photolabeled noncatalytic site (T-NC-14, Fig. 1) was further purified and sequenced. The ^{32}P-labeled peptide proved to be a carboxyl-terminal fragment of the noncatalytic site peptide shown in Table 1, beginning with Ile-362 (cycle 6).

Several ^{32}P-labeled fractions that migrated close together were obtained upon further purification of the catalytic-site fraction T-C-27 (Fig. 1). The amino acid sequence of one of the major ^{32}P-labeled peptides (16% of the total radioactivity in the group of peaks) is shown in Table 1. The sequence can be aligned with the known MF_1 β-subunit sequence beginning with Ala-338. No phenylthiohydantoin derivative was detected at cycle 8 (Xaa). An adjacent ^{32}P-labeled peak (also 16% of the total radioactivity) gave an identical sequence. Both fractions contained minor comigrating impurities that may have been responsible for the small peak separation. For reasons similar to those given above, we surmise that the 8th amino acid in this sequence is a modified tyrosine. These data allow the identification of β Tyr-345 as a site of photocrosslinking to the catalytic site. The other minor ^{32}P-labeled peaks, not present in sufficient amounts for sequencing, might represent modification of other residues on the same catalytic peptide. This would be consistent with the results of a 2-azido-ADP labeling study by Vignais' laboratory (22).

Table 1. Amino acid sequence analysis of ^{32}P-labeled tryptic peptides

	Noncatalytic site*		β	Catalytic site*		β
Cycle	aa	pmol	357–372†	aa	pmol	338–356†
1	Ile	163	Ile	Ala	75	Ala
2	Met	128	Met	Ile	62	Ile
3	Asp	128	Asn	Ala	30	Ala
4	Pro	85	Pro	Glu	20	Glu
5	Asn	103	Asn	Leu	33	Leu
6	Ile	80	Ile	Gly	23	Gly
7	Val	90	Val	Ile	85	Ile
8	Gly	30	Gly	Xaa		Tyr
9	Ser	10	Ser	Pro	20	Pro
10	Glu	28	Glu	Ala	48	Ala
11	His	8	His	Val	33	Val
12	Xaa		Tyr	Asp	23	Asp
13	Asp	28	Asp	Pro	18	Pro
14	Val	23	Val	Leu	25	Leu
15	Ala	23	Ala	Asp	13	Asp
16	(Arg)		Arg	Ser	NQ	Ser
17				Thr	NQ	Thr
18				(Ser)		Ser
19				(Arg)		Arg

Noncatalytic- and catalytic-site peptides were isolated from fractions T-NC-20 and T-C-27 (Fig. 1), respectively, by two additional reversed-phase HPLC steps.

*Values indicate pmol yields of the phenylthiohydantoin amino acid (aa) residues starting with approximately 400 and 140 pmol of ^{32}P-labeled peptide derived from modified noncatalytic and catalytic sites, respectively. NQ denotes residue assignments that were made but not quantitated due to insufficient chromatographic resolution for integration. Residue assignments enclosed in parentheses indicate ambiguity at those cycles.

†β-Subunit sequence from ref. 29.

The possibility that the labeling of Tyr-345 or Tyr-368 is due to the modification of a single binding site in two different conformational states is unlikely, since both catalytic and noncatalytic sites can be simultaneously occupied (3). That catalytic and noncatalytic sites are indeed distinct was confirmed by two-dimensional gel electrophoresis (11) of enzyme prepared with both types of sites labeled. Isoelectric focusing showed that some β subunits were doubly derivatized and that this was eliminated by an ATP chase to remove 2-azido-ANP from catalytic sites prior to photolysis (data not shown).

Effect of Photolabeling Catalytic or Noncatalytic Sites on Enzyme Activity. Fig. 2 shows the relationship between the extent of derivatization and the inhibition of catalysis for photolabeling catalytic sites (solid squares) and noncatalytic sites (open squares). The same percentage inhibition was obtained when the samples were assayed under conditions for either bi-site (2 μM MgATP) or tri-site (2 mM MgATP) catalysis. Extrapolation indicates that modification of one-third of the catalytic sites or two-thirds of the noncatalytic sites would suffice for stopping nearly all catalytic activity. The possibility that MF_1, completely modified at one catalytic site, might retain a low level of activity was not assessed in these experiments. Such retention has been observed with F_1 from chloroplasts (T. Melese, Z.X., and P.D.B., unpublished data).

DISCUSSION

In this study we have shown β-subunit Tyr-345 to be part of the catalytic site and β Tyr-368 to be part of the noncatalytic site of MF_1. These residues are conserved in all known sequences for mitochondrial, chloroplast, and bacterial F_1 (29). Further, they are found within highly conserved se-

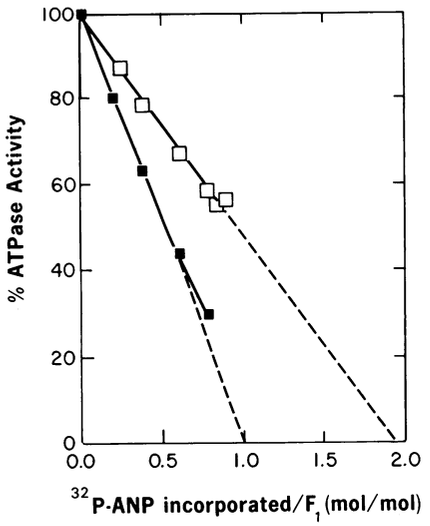

FIG. 2. Stoichiometry for inhibition of ATPase activity due to photolabeling of catalytic (■) and noncatalytic (□) sites. Noncatalytic sites were loaded by incubating MF_1[0,0] at 11 μM with 1 mM 2-azido-[β-^{32}P]ATP for 45 min in sucrose/Hepes/P_i/Mg buffer. Unbound ligand was removed on a Sephadex centrifuge column. The azido analog was displaced from catalytic sites by incubation with 4 mM MgATP for 2 min. Passage through a second centrifuge column yielded enzyme that retained 2.2 mol of 2-azido-[β-^{32}P]ANP at noncatalytic sites per mol of MF_1. Enzyme was diluted to 5 μM, and 50 μM ATP was added as a "trap" just prior to photolysis. Catalytic sites were loaded by preincubating MF_1[3,0] at 5 μM for 10 min with 50 μM 2-azido-[β-^{32}P]ATP in sucrose/Hepes/P_i/Mg buffer. Photolysis was initiated and aliquots were removed at various times from 20 sec to 16 min and assayed for covalently bound ^{32}P and ATP hydrolysis activity. Controls were treated identically except that ATP was added in place of 2-azido-ATP.

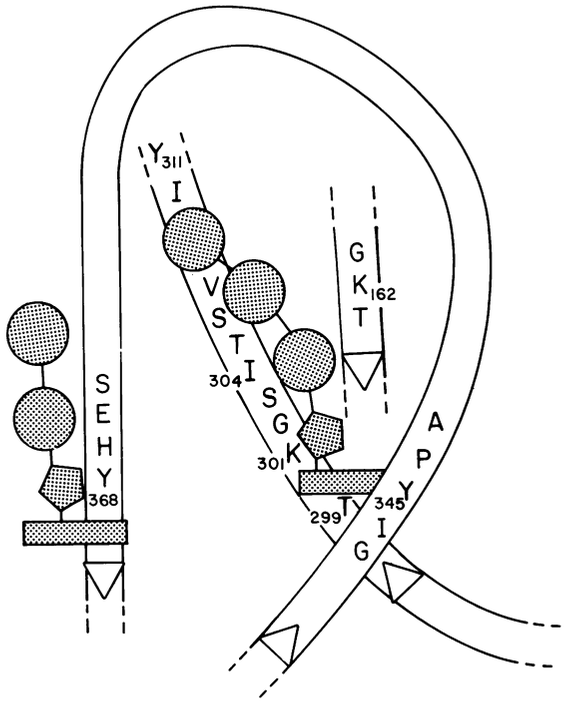

FIG. 3. A proposed partial structure for the adenine nucleotide binding sites on the β subunit of MF_1. The noncatalytic site shown to the left is occupied by ADP and is composed in part by Tyr-368. The catalytic site is occupied by ATP and is composed in part by Tyr-345. Amino acid residues are represented by standard one-letter symbols.

quences, consistent with an essential role in catalysis and perhaps regulation.

A proposed partial structure for the nucleotide-binding domains on the β subunit is given in Fig. 3. The catalytic site, to the right, is shown to be occupied by ATP with Tyr-345 positioned near the adenine ring. The noncatalytic site, composed in part by Tyr-368, is shown to contain bound ADP. The close proximity of the two sites and the positioning of β-subunit residues Thr-299 to Tyr-311 and Gly-161 to Thr-163 are based on the predicted homology between the tertiary structures of the β subunit and adenylate kinase (31, 32). The likelihood that Thr-299 to Tyr-311 contribute to the catalytic site is strengthened by the sequence homology of residues Thr-298 through Thr-305 to that found at the phosphorylation site of E_1E_2-type ATPases (33). In addition, Lys-301, Ile-304, and Tyr-311 of the β subunit of MF_1 are labeled by 8-azido-ATP (34). Tyr-311 is also modified by the adenine analog Nbf-Cl, with subsequent transfer of the 4-nitrobenzofurazan group to Lys-162 (35). Since isolated α subunit from *Escherichia coli* BF_1 has a binding site for adenine nucleotide (13), α probably contributes significantly to the noncatalytic nucleotide binding domain shown to the left. If the two nucleotide sites on β are as close as those on adenylate kinase, it would raise an intriguing question regarding a possible function. Although the catalytic mechanism does not appear to involve a transphosphorylation step (7), a slow adenylate kinase-type reaction might have a regulatory role.

FSBI also modifies β-subunit Tyr-345 (21), and FSBA modifies β Tyr-368 (30). Since noncatalytic sites show a much greater specificity for adenine nucleotide than catalytic sites (36), Bullough and Allison (21) have suggested that their results could be interpreted as evidence for the presence of Tyr-345 at the catalytic site and Tyr-368 at the noncatalytic site. Here we have demonstrated this directly, using an affinity probe that acts as a substrate in the dark and that is bound to specific sites under well-defined conditions. Unlike the studies with FSBI and FSBA, our studies rule out the possibility that two different conformational states of the same nucleotide site are labeled. The catalytic site was modified following the loading of one of the three exchangeable sites on MF_1 under conditions for uni-site catalysis (37), whereas the noncatalytic site was modified following the loading of a single vacant nonexchangeable site present on desalted MF_1 (19). As noted in *Results*, catalytic and noncatalytic sites can be occupied simultaneously (3), and doubly labeled β subunits were detected following photolysis of enzyme with 2-azido-ANP bound at both types of sites.

Vignais and coworkers (22) reported the labeling of β Tyr-345 and several other residues on the same tryptic peptide by 2-azido-ADP. Since desalted MF_1 normally contains a vacant noncatalytic site and since they added 2-azido-ADP in 4-fold molar excess to MF_1, it might have been expected that both β Tyr-345 and β Tyr-368 would have been detected. The specificity they obtained may have resulted from the procedure used to collect and dissolve the large quantities of MF_1 used in their studies. Ammonium sulfate storage medium contains ATP, and if not carefully removed before enzyme is dissolved in a buffer containing Mg^{2+}, a brief episode of catalysis will occur. Inadvertently, this would have resulted in the filling of the vacant noncatalytic site by medium ADP. This explanation is supported by their report (22) that the enzyme used to prepare photolabeled samples for sequencing contained an unusually large amount of endogenous nucleotide (3.7 mol/mol of MF_1).

The binding change mechanism, proposed to explain ATP synthesis by oxidative and photophosphorylation (38), has found fairly widespread use as a working model. One of the main features of this model is strong positive catalytic cooperativity between two (4) or three (7, 8) catalytic subunits. Under conditions where a single site per F_1 turns

over (uni-site catalysis), the rate of hydrolysis is too slow to detect by a normal coupled enzyme assay (37). However, rapid turnover occurs under conditions where substrate binding at one catalytic site promotes product release from a second catalytic site (bi-site catalysis). The availability of an affinity probe that binds at a catalytic site and that, following covalent incorporation, completely inactivates that catalytic site could prove useful in further refining certain aspects of the binding change mechanism. For instance, such a probe could be used to determine the minimum number of functional catalytic sites required for bi-site catalysis.

Vignais' laboratory has recently reported that reaction with 2 mol of 2-azido-ATP per MF_1 was required for near-complete inhibition (39). However, in this study they used desalted MF_1 that contained the normal amount of endogenous nucleotide. It is likely that their enzyme was in the form $MF_1[2,1]$, having two occupied noncatalytic sites and one occupied catalytic site. As Kironde and Cross suggested earlier (19), 1 of the 2 mol of reagent incorporated in their studies may have reacted at the vacant noncatalytic site. We have reexamined the stoichiometry for inhibition, using MF_1 with fully occupied noncatalytic sites (Fig. 2) and found that, as for FSBI (21), reaction with only 1 mol of reagent per mol of MF_1 suffices to block rapid catalysis. Further, there is no preferential inhibition of tri-site catalysis compared to bi-site catalysis. These results are consistent with those of van Dongen *et al.* (40) and suggest a strictly ordered participation of the three catalytic sites. It is also of interest that the modification of only two noncatalytic sites per MF_1 by 2-azido-ADP may suffice for nearly complete inhibition (Fig. 2). This would be in contrast to FSBA, which, although it reacts with the same tyrosine residue, requires reaction of close to 3 mol per mol of enzyme for inhibition (41).

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