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Separate β subunits are derivatized with ^{14}C and ^{3}H when the bovine heart mitochondrial F_1-ATPase is doubly labeled with 7-chloro-4-nitro[^{14}C]benzofurazan and 5′-*p*-fluorosulfonylbenzoyl[^{3}H]inosine

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Tyrosine residues 311 and 345 of the β subunit of the bovine heart mitochondrial F_1-ATPase (MF_1) are present on the same peptide when the enzyme is fragmented with cyanogen bromide. Maximal inactivation of MF_1 with 7-chloro-4-nitro[^{14}C]benzofurazan ([^{14}C]Nbf-Cl) derivatizes tyrosine-311 in a single β subunit. Cyanogen bromide digests of MF_1 containing the [^{14}C]Nbf-*O*-derivative of tyrosine-β311 were submitted to reversed-phase HPLC, with and without prior reduction of the nitro group on the incorporated reagent with dithionite. The retention time of the radioactive cyanogen bromide peptide was shifted substantially by reduction. When a cyanogen bromide digest of MF_1 inactivated with 5′-*p*-fluorosulfonylbenzoyl[^{3}H]inosine ([^{3}H]FSBI), which proceeds with derivatizaiton of tyrosine-345 in a single β subunit, was submitted to HPLC under the same conditions, the fragment labeled with ^{3}H eluted with the same retention time as the [^{14}C]Nbf-*O*-derivative before reduction. Doubly labeled enzyme was prepared by first derivatizing Tyr-β311 with [^{14}C]Nbf-Cl and then derivatizing tyrosine-β345 with [^{3}H]FSBI with and without reducing the [^{14}C]Nbf-*O*-derivative of tyrosine-β311 with dithionite before modification with [^{3}H]FSBI. The doubly labeled enzyme preparations were digested with cyanogen bromide and submitted to HPLC. The ^{14}C and ^{3}H in the cyanogen bromide digest prepared from doubly labeled enzyme not submitted to reduction eluted together. In contrast, the ^{14}C and ^{3}H in the digest prepared from doubly labeled enzyme which had been reduced eluted separately. From these results it is concluded that different β subunits are derivatized when MF_1 is doubly labeled with [^{14}C]Nbf-Cl and [^{3}H]FSBI.

Introduction

The F_0F_1-ATP synthases are membrane-bound enzymes which couple transmembrane electrochemical potentials to the condensation of ADP with P_i. The ATP synthases can be resolved into an integral membrane protein complex, F_0, that participates in transmembrane proton conduction and a peripheral membrane protein complex, F_1, which contains the catalytic sites for ATP synthesis. Isolated F_1 catalyzes ATP hydrolysis but not multiple turnovers of ATP synthesis. The F_1-ATPases from several sources have molecular weights of about 380000 and are comprised of five different polypeptide chains, designated α–ε in order of decreasing M_r, with the stoichiometry, $\alpha_3\beta_3\gamma\delta\epsilon$ [1,2].

The F_1-ATPases contain six adenine nucleotide binding sites [3–5]. Binding of adenine nucleotides to isolated α and β subunits from bacterial F_1-ATPases [6,7] and identification of labeled subunits after reaction of intact F_1-ATPases with radioactive affinity of photoaffinity analogs of substrate have shown that the six nucleotide binding sites are on the α and β subunits [8–11]. Three of these sites, which appear to be entirely on β subunits, are potential catalytic sites which are non-selective for nucleotides [4]. The three other sites, which appear to be present at interfaces of α and β subunits [13,14], are specific for adenine nucleotides [4].

Abbreviations: FSBA, 5′-*p*-fluorosulfonylbenzoyladenosine; FSBI, 5′-*p*-fluorosulfonylbenzoylinosine; Nbf-Cl, 7-chloro-4-nitrobenzofurazan; AMEDA, P^1-(5′-adenosyl)-P^2-*N*-(2-mercaptoethyl)diphosphoramidate; IBS-, 5′-*p*-sulfonylbenzoylinosine; Abf-4-aminobenzofurazan; and FDNB, 2,4-dinitro-1-fluorobenzene.

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The latter sites have a poorly understood functional role and thus, are called noncatalytic sites.

Chemical modification studies on F_1 have provided insights on residues that may interact with nucleotides bound to catalytic sites and noncatalytic sites [9–12,15–18]. The ATPase activity of MF_1 is maximally attenuated when Tyr-368 or His-427 are modified by FSBA in all copies of the β subunit. Inactivation of the ATPase and ITPase activities by reagents that modify Tyr-β311 or Tyr-β345 exhibit one-third-the-sites reactivity. This is consistent with the strong, positive site-to-site catalytic cooperativity that is associated with ATP and ITP hydrolysis catalyzed by the enzyme as described in detail by Boyer and his colleagues [19,20]. Therefore, it is thought that these residues are present at or near the catalytic site. Inactivation of MF_1 by FSBI [11] or 2-N_3-ATP [9,12] is accompanied by derivatization of Tyr-345 in a single β subunit. Given that FSBI probably exists in aqueous solution in a folded conformation with the benzene ring containing the reactive sulfonyl fluoride adjacent to the hypoxanthine ring [21], it has been argued that the side-chain of Tyr-β345 is near the purine ring of nucleotides bound to catalytic sites [13]. The severely attenuated hydrolytic reaction catalyzed by enzyme derivatized at Tyr-β345 with FSBI or 2-N_3-ATP appears to be the consequence of preventing one of the three catalytic sites from participating in cooperative catalysis. However, why derivatization of the side-chain of Tyr-β311 with Nbf-Cl leads to severely attenuated ATP hydrolysis catalyzed by the enzyme is unclear. It has been reported that slow catalysis by MF_1 when Tyr-311 is derivatized with Nbf-Cl in a single β subunit is caused by slow dissociation of ADP from a single catalytic site [22,23]. Cross and Nalin [24] reported that MF_1 inactivated with Nbf-Cl retained the capacity to bind 3 mol of AMP-PNP per mol. Furthermore, Wu et al. [25] have reported that AMEDA, an ADP analog with a thiol group attached to the β phosphorus through an ethyl phosphoramide linkage, can bind to the modified catalytic site and reactivate the enzyme by thiolyzing the Nbf-*O*-derivative of Tyr-β311. These observations led Bullough et al. [25] to suggest that introduction of the Nbf-group onto Tyr-311 in a single β subunit produces a sticky site that slows dissociation of product ADP from the modified catalytic site. Contrary to this view, Bragg and Hou [24] reported that different β subunits are labeled when *E. coli* F_1 is treated in succession with Nbf-Cl and 2-N_3-ATP, irrespective of the order of the chemical modifications. In similar experiments with the F_1-ATPase from spinach chloroplasts, Ceccarelli et al. [27] demonstrated that Nbf-Cl and 2-N_3-ADP, each derivatizing a single catalytic site, modify different β subunits in double labeling experiments. The Nbf-*O*-derivative of Tyr-β311 is sensitive to thiolysis, whereas the sulfonylated derivative of Tyr-β345 (IBS-*O*-Tyr-β345) formed on inactivation with FSBI is not. Therefore, it was possible to show that prior modification of the enzyme with Nbf-Cl had no effect on the rate of irreversible inactivation nor on the extent of modification of the enzyme by FSBI [11]. Given the internal folding of the 5′-*p*-fluorobenzoylsulfonylnucleoside analogs [21], it is expected that the IBS-group attached to Tyr-β345 occupies a smaller volume of the catalytic site than is occupied by ADP attached to Tyr-β345 through the 2-position of the adenine ring which occurs when the enzyme is photoinactivated by 2-N_3-ATP or -ADP [9,12]. Therefore, it was of interest to determine whether the same catalytic site is modified when MF_1 is first derivatized at Tyr-311 in a single β subunit with NBf-Cl and then treated with FSBI to modify Tyr-β345.

Materials and Methods

Materials

MF_1 was prepared from bovine heart mitochondria by modification [28] of the method of Knowles and Penefsky [29]. The enzyme was assayed at 30 °C by coupling ATP regeneration with phospho*enol*pyruvate and pyruvate kinase to the oxidation of NADH with lactate dehydrogenase [30]. Buffer ingredients, enzymes and biochemicals used in assays, Sephadex, and cyanogen bromide were purchased from Sigma. [^{3}H]FSBI was prepared as described previously [11]. [^{14}C]Nbf-Cl was purchased from Research Products International. Sodium dithionite was from Malinckrodt and was dispensed in small vials which were stored desiccated at −20 °C. Coomassie Blue G-250 was purchased from Pierre Chemical Company.

Preparation of labeled enzyme samples

Previously described methods were used to prepare samples of 1–2 mg of MF_1 which contained about 1 mol of [^{3}H]IBS-*O*-Tyr-β345 per mol [11], about 1 mol of [^{14}C]Nbf-*O*-Tyr-β311 per mol [17], and about 0.9 mol of [^{14}C]Abf-*O*-Tyr-β311 per mol [18]. Enzyme containing both [^{14}C]Nbf-*O*-Tyr-β311 and [^{3}H]IBS-*O*-Tyr-β345 was prepared in the following manner. After removing MF_1 from storage suspension by centrifugation and decanting mother liquor, the protein was dissolved at 2 mg per ml in 50 mM Tris-H_2SO_4 (pH 7.5) containing 1 mM EDTA. Residual ammonium sulfate and ATP were removed by passing 500 μl aliquots through 5 ml centrifuge columns [31] of Sephadex G-50 which was equilibrated with the same buffer. [^{14}C]Nbf-Cl was added to the desalted protein to a final concentration of 100 μM. The resulting solution was incubated in the dark at 30 °C until about 95% of the original ATPase activity was lost as assessed by assaying 2 μl samples. One half of the inactivation mixture was passed through 5 ml centrifuge columns of Sephadex G-50 that were equilibrated with triethanolamine-H_2SO_4 (pH 7.0) con-

taining 1 mM CDTA. [^{3}H]FSBI was added to this sample to a final concentration of 100 μM and the resulting reaction mixture was incubated at 23° C until 90% activity was lost when samples were assayed in the presence of 10 mM dithiothreitol.

Excess [^{14}C]Nbf-Cl was removed from the other half of the enzyme sample derivatized with [^{14}C]Nbf-Cl by passing it through 5 ml centrifuge columns of Sephadex G-50 which were equilibrated with 100 mM Mes (pH 6.0) containing 2 mM EDTA. To the combined eluates containing enzyme derivatized with [^{14}C]Nbf-Cl was added freshly prepared 1 M sodium dithionite in 0.1 M NaOH to a final concentration of 10 mM. This solution was incubated for 10 min at 23° C, at which time it was passed through 5 ml centrifuge columns of Sephadex G-50 equilibrated with 50 mM triethanolamine-H_2SO_4 (pH 7.0) containing 1 mM CDTA. [^{3}H]FSBI was added to this solution to a final concentration of 100 μM. This reaction mixture was then incubated at 23° C for the time required to inactivate the half sample treated with [^{14}C]Nbf-Cl but not treated with dithionite by 90% when asayed in the presence of 10 mM dithiothreitol. At this time, excess [^{3}H]FSBI was removed by passing 500 μl aliquots of the reaction mixture through 5 ml centrifuge columns of Sephadex G-50 equilibrated with 50 mM triethanolamine-H_2SO_4 (pH 7.0).

Preparation and fractionation of cyanogen bromide digests of the labeled enzyme samples

After excess reagent had been removed from modified samples, the derivatized proteins were precipitated by the addition of solid ammonium sulfate to 55% saturation. The precipitates were recovered by centrifugation and dissolved in 6 M guanidine-HCl. The resulting solutions were then dialyzed against 1 liter of distilled water for 1 h. The precipitates formed on dialysis were recovered by centrifugation and dissolved in 1 ml of 70% formic acid. After adding 100 μg of cyanogen bromide, the samples were incubated at 23° C for 18 h, at which time solvent was removed by vacuum centrifugation The dried samples were dissolved in a minimal volume of 6 M guanidine-HCl prior to injection onto a C-4 reversed phase column that was equilibrated with 10 mM HCl. The columns were then developed with the gradients of acetonitrile described in the figure legends.

Analytical methods

Peptide separations were performed on a Brownlee C-4 reversed phase column (0.46 × 23 cm; 5 μm particle size) in tandem with a guard column containing the same material using an Altex 322 liquid chromatograph equipped with an LKB Uvicord S detector. Protein determinations were performed with Coomassie blue G-250 using the procedure described by Bradford [32]. Liquid scintillation counting was carried out in Liquidscint from National Diagnostics using a TmAnalytic 6895 counter.

Results

Resolution of radioactive peptides in cyanogen bromide digests of the [^{14}C]Nbf-O-Tyr-β311, [^{14}C]Abf-O-Tyr-β311 and [^{3}H]IBS-O-Tyr-β345 derivatives of MF_1 by reversed-phase HPLC

Since the β subunit of MF_1 is heterogeneous when submitted to isoelectric focusing, possibly due to a frayed amino terminus [33], the two-dimensional methods introduced by Bragg and Hou [26] to examine double labeling of *E. coli* F_1 could not be used to investigate the specificity of labeling of MF_1 by radioactive Nbf-Cl and FSBI. Since Tyr-311 and Tyr-345 are present on the same cyanogen bromide fragment derived from the β subunit, it was possible to monitor radioactivity in effluents when cyanogen bromide digests of labeled enzyme were submitted to HPLC to determine if derivatization of Tyr-β311 with [^{14}C]Nbf-Cl followed by modification of Tyr-β345 with [^{3}H]FSBI proceeds with derivatization of the same or different β subunits. Preliminary experiments showed that the [^{14}C]Nbf-*O*-Tyr-β311 and [^{3}h]IBS-*O*-Tyr-β345 derivatives of MF_1 were stable in the presence of cyanogen bromide in 70% formic acid for 18 h. Fig. 1A shows the profile of radioactive peptides that were resolved when a cyanogen bromide digest of MF_1 which had been inactivated with [^{14}C]Nbf-Cl to contain 1.4 mol of covalently bound reagent per mol was submitted to reversed-phase HPLC on a C-4 column. Under these conditions, Andrews et al. [17] showed that the inactivation observed is due to modification of Tyr-311 in a single β subunit and that the additional radioactivity incorporated represents non-specific modification of lysine residues. The cyanogen bromide fragment containing the [^{14}C]Nbf-derivative of Tyr-β311 eluted from the column as a major peak of radioactivity at about 60 min as shown in Fig. 1A. When an enzyme sample derivatized with [^{14}C]Nbf-Cl was treated with dithionite prior to cleavage with cyanogen bromide and the resulting digest submitted to HPLC under the conditions of Fig. 1A, the majority of radioactivity eluted at 80 min as shown in Fig. 1B.

What causes increased retention time of the radioactive cyanogen bromide peptide after treating enzyme containing [^{14}C]Nbf-*O*-Tyr-β311 with dithionite is unclear. Although the product or products formed on treating Nbf-*O*-tyrosine derivatives with dithionite have not been established, the following observations suggest that increased hydrophilicity should accompany treatment with dithionite. After treating Nbf-*O*-tyrosine ethyl ester with dithionite, Verburg [34] reported that absorption due to the nitroaryl group was eliminated, presumably by conversion of the nitro group to a primary

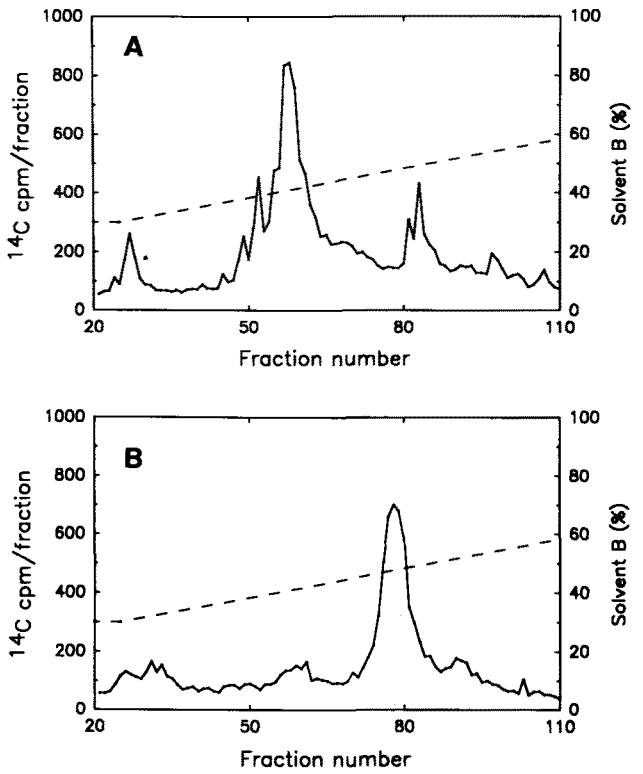

Fig. 1. The elution profiles of ^{14}C in cyanogen bromide digests of MF_1 inactivated with [^{14}C]Nbf-Cl before and after reduction with dithionite. Preparation of labeled enzyme, reduction with dithionite, cyanogen bromide digestion, and reversed phase chromatography on a C-4 column are described in detail in Materials and Methods. The 1 ml collected fractions were submitted to liquid scintillation counting. (A) HPLC of a cyanogen bromide digest of MF_1 labeled with [^{14}C]Nbf-Cl and not submitted to reduction with dithionite. (B) HPLC of a cyanogen bromide digest of MF_1 labeled with [^{14}C]Nbf-Cl which was submitted to reduction with dithionite.

amino group. Whether the furazan ring is modified by dithionite is not known. Joshi and Wang [35] have reported that ^{35}S is incorporated into MF_1 when the NBF-*O*-Tyr-β311 derivative of the enzyme was treated with [^{35}S]dithionite. Given that Riordan and Sokolovsky [36] reported that a small amount of an acidic component, tentatively identified as an amino sulfonate, was fomed during reduction of 3-nitrotyrosine with dithionite, it is possible that incorporation of ^{35}S observed by Joshi and Wang [35] may represent the formation of a sulfonic acid at the derivatized site. Whether or not treatment of peptidyl Nbf-*O*-Tyr-β311 with dithionite is accompanied by formation of a primary amine and/or a sulfonic acid, one would expect that the retention time of the reduced peptide bearing the derivative would be decreased when chromatographed on a reversed phase matrix, rather than increased as is observed. This anomolous behavior may be related to the large size of the cyanogen bromide peptide containing Tyr-β311 which has 66 residues. Evidence is accumulating which suggests that amphiphilic peptides are often structured in the hydrophobic environment of a reversed phase matrix [37]. To explain the anomolous behavior observed, it is possible that conversion of the Nbf-group attached to Tyr-β311 to a more polar substituent alters the conformation of the peptide in a manner that increases its affinity for the reversed phase matrix.

Fig. 2 shows that a major peak of radioactivity eluted at about 62 min when a cyanogen bromide digest of MF_1, which had been inactivated with [^{3}H]FSBI and contained about 1 mol of reagent per mol of enzyme, was submitted to reversed-phase HPLC under the conditions described in the legend of Fig. 1. Therefore, the cyanogen bromide peptide containing the [^{14}C]Nbf-derivative of Tyr-β311 has nearly the same retention time as the peptide containing Tyr-β345 derivatized with [^{3}H]FSBI.

Resolution of radioactive cyanogen bromide peptides derived from MF_1 doubly labeled with [^{14}C]Nbf-Cl and [^{3}H]FSBI

In this experiment, MF_1 was first inactivated by 95% with [^{14}C]Nbf-Cl in the dark and then submitted to gel permeation chromatography through centrifuge columns of Sephadex G-50. The gel-filtered enzyme was then treated with [^{3}H]FSBI, with or without prior reduction of the [^{14}C]Nbf-group with dithionite as described under Materials and Methods. When a cyanogen bromide digest of the doubly labeled enzyme which had been prepared without prior treatment with dithionite, was submitted to HPLC using the gradient illustrated in Fig. 3, only a single, major peak of radioactivity was eluted at about 50 min which contained both ^{14}C and ^{3}H. However, when doubly labeled enzyme was prepared in which [^{14}C]Nbf-*O*-Tyr-β311 had been converted to [^{14}C]Abf-*O*-Tyr-β311 by dithionite

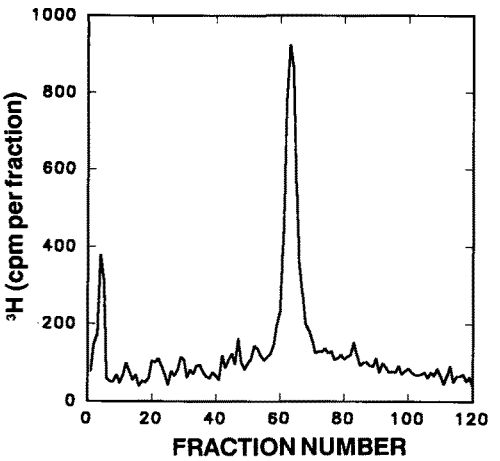

Fig. 2. The elution profile of ^{3}H in a cyanogen bromide digest of MF_1 labeled with [^{3}H]FSBI. Preparation and processing of MF_1 labeled with [^{3}H]FSBI is described in detail in Materials and Methods. The C-4 column was eluted with the same gradient illustrated in Fig. 1. The 1 ml collected fractions were submitted to liquid scintillation counting.

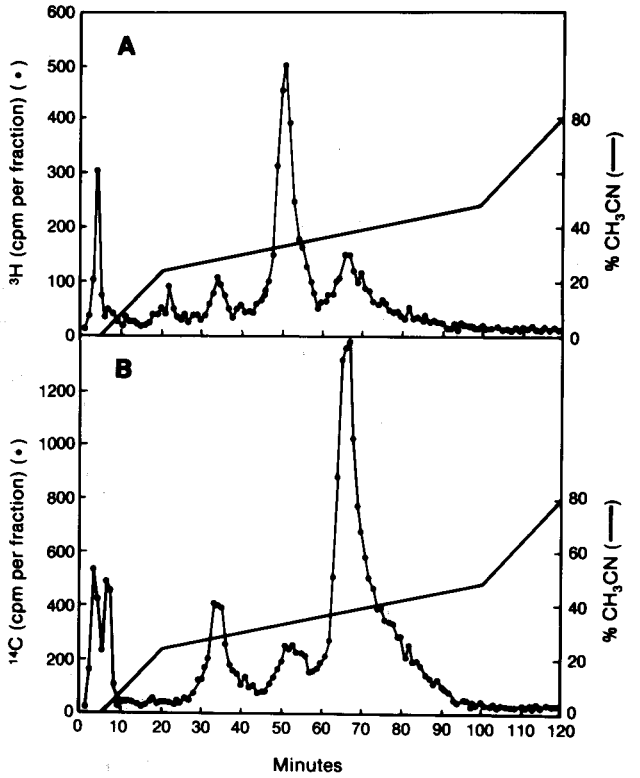

Fig. 3. The elution profiles of ^{14}C and ^{3}H of cyanogen bromide digests of MF_1 doubly labeled with [^{14}C]Nbf-Cl and [^{3}H]FSBI before and after reduction with dithionite. Preparation of doubly labeled enzyme, reduction with dithionite, cyanogen bromide digestion, and reversed phase HPLC on a C-4 column are described in detail in Material and Methods. The 1 ml collected fractions were submitted to liquid scintillation counting for: (A) ^{3}H; and (B) ^{14}C.

reduction prior to labeling Tyr-β345 with [^{3}H[FSBI, the patterns of ^{3}H-labeled and ^{14}C-labeled peptides shown in Fig. 3A and 3B, respectively, were revealed by reversed-phase HPLC of a cyanogen bromide digest of the doubly labeled enzyme. With the gradient used in Fig. 3, which differed from that used in Figs. 1 and 2, the majority of the ^{3}H eluted at about 50 min, whereas the majority of the ^{14}C eluted at about 70 min. When a cyanogen bromide digest of labeled enzyme, prepared by first inactivating MF_1 with non-radioactive Nbf-Cl followed by reduction by dithionite and subsequent labeling with [^{3}H]FSBI, was submitted to reversed-phase HPLC under the conditions illustrated in Fig. 3, no radioactivity was eluted from the column with a retention time greater than 60 min. Therefore, the peak of radioactivity in Fig. 3A eluting at about 70 min appears to represent detection of ^{14}C by the ^{3}H channel of the scintillation counter, and not double labeling of the same β subunit. When MF_1 which had been derivatized with [^{14}C]Nbf-Cl was irreversibly inactivated with [^{3}H]FSBI and then treated with dithionite before digestion with cyanogen bromide, essentially the same results were obtained as illustrated in Fig. 3 on submitting the digest to reversed-phase HPLC. From the combined results we conclude that catalytic sites in different β subunits are labeled by the two reagents.

Discussion

The results presented clearly show that prior modification of Tyr-β311 of MF_1 with Nbf-Cl effectively blocks labeling of Tyr-345 in the same β subunit with FSBI. These results are consistent with those of Bragg and Hou [26] and Ceccarelli et al. [27], who reported qualitative evidence suggesting that double labeling of the F_1-ATPases from *E. coli* plasma membranes and spinach thylakoids with Nbf-Cl and 2-N_3-ADP was mutually exclusive with respect to β subunits. Results qualitatively similar to those reported here were also obtained when MF_1 was submitted to double labeling with 2-N_3-[α-^{32}P]ADP and [^{14}C]Nbf-Cl using the reagents in either order (Zhou, S. and Allison, W.S., unpublished experiments, 1988). However, labeling of both Tyr-β345 and Tyr-β368 with 2-N_3-[α-^{32}P]ADP was observed in these experiments, irrespective of the loading conditions. Lability of the β-phosphate of the covalently bound [α-^{32}P]ADP derivative led to further heterogeneity. Therefore, complicated profiles of radioactivity were observed when cyanogen bromide digests of enzyme labeled with the radioactive reagents were submitted to reversed-phase HPLC that were difficult to interpret quantitatively.

That neither the 2-azido-derivatives of ATP or ADP nor FSBI label Tyr-345 in the same β subunit previously derivatized with Nbf-Cl is inconsistent with the conclusion of Wu et al. [25] that AMEDA reactivates the Nbf-*O*-Tyr-β311 derivative of MF_1 by binding reversibly to the derivatized catalytic site before thiolysis occurs. The kinetics of reactivation of the Nbf-*O*-Tyr-β311 derivative of MF_1 by AMEDA reported by Wu et al. [25] were complex. Two pathways for reactivation were exhibited. One was characterized with apparent binding of AMEDA with a K_d of 15 μM, which was sensitive to micromolar concentrations of ADP and ATP. The other was a bimolecular pathway that was unaffected by 20 mM ATP. Considering the results presented here and those of Bragg and Hou [26] and Ceccarelli et al. [27], it is possible that the complicated kinetics of reactivation of the Nbf-*O*-derivative of MF_1 by AMEDA [25] are caused by positive modulation of bimolecular reactivation by AMEDA binding to underivatized catalytic sites or to an open noncatalytic site [38].

The results presented suggest that nucleotides cannot bind to the catalytic site of MF_1 which contains the Nbf-*O*-derivative of Tyr-β311. If this is indeed the case, one could conclude that derivatization of one catalytic site with Nbf-Cl increases the affinity of an underivatized catalytic site for product ADP formed during attenuated hydrolysis of ATP by the modified enzyme

[22,23]. Therefore, it would appear that derivatization of Tyr-β311 with Nbf-Cl in a single catalytic site affects the conformation of one, if not both, of the unmodified catalytic sites. It has also been shown that the second order rate constant for binding substoichiometric ATP to the Nbf-*O*-derivative of MF_1 is 25-fold lower than that exhibited by the unmodified enzyme, again suggesting that the conformations of the underivatized catalytic sites have been altered. It is interesting ASU particles reconstituted with the Nbf-*O*-derivative of MF_1 catalyze ATP synthesis at rates up to 60% of the rate catalyzed by particles reconstituted with underivatized MF_1 [39,40]. The catalytic properties of the modified enzyme suggest that derivatization of Tyr-311 in a single β subunit converts the conformation of the underivatized catalytic sites from a conformation geared for ATP hydrolysis to one geared for ATP synthesis.

Recently, Garin et al. [41] demonstrated that photoinactivation of MF_1 with 4-azido-2-nitrophenyl[^{32}P]-phosphate proceeded with derivatization of Ile-β304, Gln-β308 and Tyr-β311, with most of the label attached to Tyr-β311. Since inorganic phosphate protects against photoinactivation and the Nbf-group migrates to Lys-β162 [18], Garin et al. [41] suggested that the phosphate group of the reagent interacts with the ϵ-ammonium ion of Lys-β162, thus positioning the azido-group of the reagent near the side-chain of Tyr-β311. Ting and Wang [42] and Perez et al. [43] have reported that phosphate bound to a catalytic site protects MF_1 against inactivation by Nbf-Cl. It is possible that the 4-nitro-group of Nbf-Cl interacts with the ϵ-ammonium ion of protonated Lys-β162, thus aligning the 7-chloro-group with the hydroxyl of Tyr-β311. This would explain why derivatization of Lys-β162 by Nbf-Cl does not occur directly, but does occur by migration of the Nbf-group from the Nbf-*O*-derivative of Tyr-β311. It is interesting that FDNB inactivates MF_1 slowly [44,45] and that the inactivated enzyme can be substantially reactivated by thiols [45]. Furthermore, prior inactivation of the enzyme with FDNB blocks derivatization of the enzyme with [^{14}C]Nbf-Cl [45]. These observations suggest that the 4-nitro-group of FDNB might also interact with the ϵ-ammonium ion of Lys-β162, thus positioning the reactive fluoride in the vicinity of the hydroxyl group of Tyr-β311. Given these observations, it is curious that Joshi and Wang [46] have reported that Lys-β162 is substantially labeled when MF_1 is inactivated with [^{14}C]FDNB. Under the conditions of inactivation used by Joshi and Wang [46], Andrews and Allison [44] found that 70% of the original activity was regained when the derivatized enzyme was treated with dithiothreitol after removing excess FDNB. Thiols do not remove dinitrobenzene groups from ϵ-amino groups of lysine residues [47]. Given these discrepancies, the site or sites modified when MF_1 is inactivated with [^{14}C]FDNB warrants reinvestigation.

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