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Inactivation of the F_1-ATPase from the thermophilic bacterium PS3 by 5′-*p*-fluorosulfonylbenzoylinosine at 65 °C is accompanied by modification of β-tyrosine-364

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A major radioactive peptide, T_1, was resolved by high-performance liquid chromatography from a tryptic digest prepared from the F_1-ATPase from the thermophilic bacterium PS3 which had been inactivated with *p*-fluorosulfonylbenzoyl[^{3}H]inosine. Two radioactive peptides, T_1P_1 and T_1P_2, were isolated from a peptic digest of T_1 by high-performance liquid chromatography. The sequences of T_1P_1 and T_1P_2 were shown to be E-E-H-X-Q-V-A-R and E-E-H-X-Q, respectively, where X corresponds to derivatized Tyr-364 of the β subunit.

The F_0F_1-ATP synthases utilize electrochemical gradients generated across energy-transducing membranes by electron-transport processes to drive the condensation of ADP with P_i. These enzymes can be resolved into two components, F_0, an integral membrane protein complex which mediates proton translocation, and F_1, a peripheral membrane protein complex which contains the catalytic sites for ATP synthesis. When resolved from the membrane, the F_1 component catalyzes net ATP hydrolysis.

The structural characteristics of TF_1, the F_1-ATPase from the plasma membranes of the thermophilic bacterium PS3 are very similar to those of the F_1-ATPases from other energy-transducing membranes [1–3]. TF_1 has a molecular weight of about 380000 and is composed of five different polypeptide chains, designated α–ε in order of decreasing mass, which are present in the stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$ [4]. As is found in other F_1-ATPases, TF_1 contains six binding sites for adenine nucleotides [5].

Chemical modification studies have provided evidence that the catalytic sites are present on the β subunits [6–8]. From the observation that the α and β subunit isolated from TF_1 and from the F_1-ATPase of *Escherichia coli* can each bind about 1 mol of adenine nucleotide, it was thought that the catalytic sites are present exclusively on β subunits and the non-catalytic sites are present exclusively on α subunits [9,10]. Recent findings with other F_1-ATPases suggest that this is not the case. Khananshvili and Gromet-Elhanan [11,12] have shown that the β subunit isolated from chromatophores of *Rhodospirillum rubrum* has the capacity to bind two mols of adenine nucleotide

Abbreviations: FSBA, *p*-fluorosulfonylbenzoyladenosine; FSBI, *p*-fluorosulfonylbenzoylinosine, FSBG, *p*-fluorosulfonylbenzoylguanosine; FSBεA, *p*-fluorosulfonylbenzoyl-1,N^6-ethanoadenosine; Pth, phenylthiohydantoin; Mops, 4-morpholinepropanesulfonic acid; P_i, inorganic phosphate.

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per mol. Using heterobifunctional photoaffinity analogs of adenine nucleotides, Schäfer and his colleagues [13] have provided evidence for the presence of nucleotide binding sites at interfaces of α and β subunits. Consistent with these findings, it has been shown that inactivation the F_1-ATPase from bovine heart mitochondria with [^{3}H]FSBA is accompanied by labeling either Tyr-368 or His-427 in all three copies of the β subunit [14]. In contrast, inactivation of the bovine heart mitochondrial F_1-ATPase with [^{3}H]FSBI is accompanied by labeling of Tyr-345 in a single copy of the β subunit [15]. From these results, we proposed that FSBA reacts with each of the non-catalytic sites and FSBI reacts with a single catalytic site. In addition, it has been shown that after prior modification of bovine-heart mitochondrial F_1-ATPase with FSBI, each of the β subunits are labeled with FSBA at either Tyr-368 or His-427, demonstrating directly that each β subunit contains at least part of two nucleotide binding sites (Bullough, D.A. et al., unpublished results). These observations are supported by recent reports from Boyer and his colleagues [17–20]. By loading nucleotide binding sites in the F_1-ATPases from bovine heart mitochondria [17], spinach chloroplasts [18,19] and *E. coli* plasma membranes [20] with 2-azidoadenine nucleotides under different conditions and then photolyzing, they showed that β-Tyr-345 of mitochondrial F_1-ATPase, or its equivalent in the other ATPases, were labeled when catalytic sites were loaded, whereas Tyr-368 of mitochondrial F_1-ATPase, or its equivalent in the other ATPases, were labeled when non-catalytic sites were loaded.

Having two temperature optima, one at 23°C and the other at 65°C, TF_1 is unique among the F_1-ATPases characterized. Whereas TF_1 is slowly inactivated by FSBA at 23°C and pH 7.0, it is rapidly inactivated by the reagent at 65°C and pH 6.4. Using [^{3}H]FSBA, it was shown that the inactivation was accompanied by selective modification of β-Tyr-364 [21] which is homologous with β-Tyr-368 of mitochondrial F_1-ATPase [22]. Since inactivation of bovine heart mitochondrial F_1-ATPase by FSBI is accompanied by labeling of β-Tyr-345 and not β-Tyr-368, it was of interest to characterize the modification of TF_1 by [^{3}H]FSBI at 65°C.

TF_1, is inactivated slowly by 1 mM FSBI at 23°C and pH 7.0. Under these conditions, only about 30% of the original ATPase activity was lost in 60 min. Labeling of the enzyme with [^{3}H]FSBI under these conditions was not sufficiently specific to provide useful information on the site or sites of modification associated with the inactivation observed. This is based on the profile of radioactivity observed when a tryptic digest of TF_1, inactivated with [^{3}H]FSBI at 23°C and pH 7.0 and separated from excess reagents, was subjected to reversed-phase HPLC on a C_{18} column. Although several radioactive peaks were detected, none was present in a sufficient amount to warrant further analysis. At pH 6.4 and 65°C, the enzyme was inactivated rapidly by 1 mM FSBI, with 76% of the original activity lost in 30 min. To identify the side-chain modification leading to the inactivation, 5 mg of TF_1 at 1 mg/ml was treated with 0.5 mM [^{3}H]FSBI in 50 mM triethanolamine HCl (pH 6.4) at 65°C for 20 min, followed by a second addition of the same amount of reagent. After a total of 40 min, when 68% inactivation had occurred, excess reagent was removed by passing the reaction mixture through a column of Sephadex G-50, equilibrated with 50 mm triethanolamine-HCl (pH 7.0). After recipitating the labeled enzyme by the addition of solid ammonium sulfate to 55% saturation, the protein pellet, recovered by centrifugation, was dissolved in 6 M guanidiniumchloride adjusted to pH 7.0 with 1 M Tris and subjected to high-performance gel permeation chromatography on a column of TSK G-2000, equilibrated with the same buffer, to remove residual reagent.

The mixture of α, β, and γ subunits was dialyzed against 1 mM HCl for 4 h at 4°C, adjusted to pH 7.0 by the addition of 1 M Mops-NaOH to a final concentration of 50 mM, and digested with trypsin (1 : 20, w/w) for 8 h at 37°C. The tryptic digest was subjected to reversed-phase HPLC with a Brownlee C_{18} column equilibrated with 0.1% HCl using the gradient of increasing acetonitrile concentration illustrated in Fig. 1. The major peak of radioactivity, T_1, that eluted at 92 min, representing about 30% of the total label eluted from the column, was collected and subjected to centrifugation under vacuum to remove acetonitrile. The pH was adjusted to 2.5, by addition of 1 M

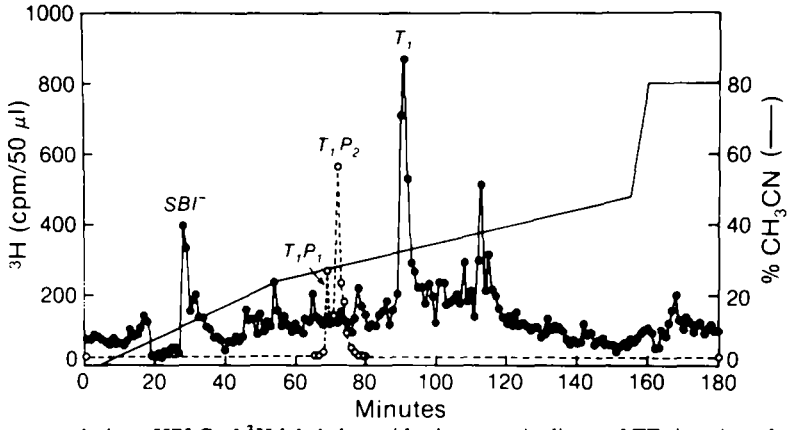

Fig. 1. Fractionation by reversed-phase HPLC of ^{3}H-labeled peptides in a tryptic digest of TF_1 inactivated with [^{3}H]FSBI and in a peptic digest of ^{3}H-labeled tryptic peptide, T_1. The tryptic digest was prepared from TF_1 inactivated with [^{3}H]FSBI at 65 °C and subjected to reversed-phase HPLC on a C_{18} column as described in the text. Samples, 50 μl each, of the 1 ml collected fractions of the effluent of the tryptic digest (●——●) were subjected to liquid scintillation counting. The same procedure was used to monitor the radioactivity of the effluent of the peptic digest (○——○).

glycine-HCl to a final concentration of 50 mM, and the peptide mixture was digested with pepsin (50 μg) for 1 h at 30 °C. The peptic digest was submitted to reversed-phase HPLC on the C_{18} column using the same acetonitrile gradient to fractionate the tryptic digest. The chromatogram of the radioactive peptides franctionated from the peptic digest by HPLC is also shown in Fig. 1. The fractions containing radioactive peptides T_1P_1 and T_1P_2 were pooled separately and concentrated by centrifugation under vacuum. T_1P_1 contained 20% and T_1P_2 contained 80% of the radioactivity eluted from the column. The concentrated samples were subjected to automatic Edman degradation on a gas-phase sequencer [23], the results of which are summarized in Table I.

From the Pth-amino acids released during the automatic Edman degradations, the sequence of T_1P_2 is: E-E-H-X-Q-V-A-R and that of T_1P_1 is: E-E-H-X-Q, which is obviously a fragment of T_1P_2. In each of these sequences, X designates that a Pth-amino acid derivative was not identified in cycle 4. From the primary structure of the β subunit of TF_1 determined by Kagawa et al. [22] this position is occupied by Tyr-364, which corresponds to β-Tyr-368 of bovine heart mitochondrial F_1-ATPase. Therefore, unlike the mitochondrial F_1-ATPase, in which FSBI and FSBA react with different tyrosine residues in the β subunit, the inactivation of TF_1 by both FSBA and FSBI at 65 °C proceeds with modification of β-Tyr-364.

From the results presented and earlier studies which showed that the site of reaction of DCCD in the β subunit of TF_1 [24] is different from the one common to the F_1-ATPases of bovine heart mitochondria [25] and plasma membranes of *E. coli* [26], it would appear different sites of modification observed during the inactivation of TF_1 at 65 °C and mitochondrial F_1-ATPase at 23 °C might be dictated primarily by differences in enzyme structure. However, the work of Jacobson and Colman [16] suggests that the differences in the

TABLE I

AUTOMATIC EDMAN DEGRADATIONS OF THE ^{3}H-LABELED PEPTIC PEPTIDES, T_1P_1 AND T_1P_2

Cycle number	T_1P_2		T_1P_1	
	Pth-amino acid	pmol	Pth-amino acid	pmol
1	Glu	573	Glu	135
2	Glu	527	Glu	123
3	His	82	His	9
4	n.d.	n.d.	n.d.	n.d.
5	Gln	359	Gln	37
6	Val	353	–	–
7	Ala	444	–	–
8	Arg	42	–	–

folded conformations of the two affinity labels might also play a significant role in dictating the specificity of the site modified by the fluorosulfonylbenzoylnucleoside analogs. From NMR analysis of FSBA, FSBG, and NMR and fluorescence analysis of FSBεA, they showed that the benzene and purine rings in each affinity label in aqueous solution at room temperature are stacked. Thus, the affinity labels might bind to enzymes in folded conformations, the absolute structures of which differ, depending on the substituents on the purine rings. To explain the different specificities shown when FSBA and FSBI inactive mitochondrial F_1-ATPase at 23° C, it could be argued that FSBA and FSBI are folded in different conformations at this temperature, which are accomodated by non-catalytic and catalytic sites, respectively. Based on the NMR analysis of Jacobson and Colman [16] the unfolded conformations of FSBA and FSBG predominate at 65° C. Therefore, the identical specificity exhibited during inactivation of TF_1 with FSBA and FSBI at 65° C could reflect that the non-catalytic sites of TF_1 accomodate the unfolded conformations of the two affinity labels in a similar manner at this temperature or that the conformations of the catalytic and non-catalytic sites of TF_1 at 65° C differ from those of bovine heart mitochondrial F_1-ATPase at 23° C.

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