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ADP tethered to tyrosine-β345 at the catalytic site of the bovine heart F_1-ATPase is converted to tethered AMP by Mg^{2+}-dependent hydrolysis when the enzyme is photoinactivated with 2-N_3-ADP

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Abstract

Comparison of profiles of radioactive peptides resolved by HPLC from tryptic digests of the bovine heart F_1-ATPase depleted of nucleotides (nd-MF_1) which had been photoinactivated with 2-N_3-[β-^{32}P]ADP, on the one hand, and 2-[8-^{3}H]ADP, on the other, shows that the β phosphate of ADP tethered to tyrosine-β345 is slowly hydrolyzed in the presence of Mg^{2+}. When nd-MF_1 was photoinactivated with 2-N_3-[8-^{3}H]ADP in the absence of Mg^{2+}, hydrolysis of the β phosphate from ADP tethered to tyrosine-β345 was not observed. Subsequent addition of Mg^{2+} initiated conversion of ADP tethered to tyrosine-β345 to tethered AMP suggesting that functional groups at the catalytic site participate in the hydrolytic reaction.

Key words: MF_1-ATPase; 2-N_3-[β-^{32}P]ADP; 2-N_3-[8-^{3}H]ADP; Photoinactivation; Catalytic site

1. Introduction

The F_oF_1-ATP synthases catalyze the condensation of ADP with P_i which is driven by proton electrochemical gradients generated by electron transport processes in energy transducing membranes [1]. The F_o moiety acts as a transmembrane proton channel, whereas the F_1 moiety, which is peripheral to the membrane, bears the catalytic sites. When F_1 is removed from F_o in soluble form, it functions only as an ATPase. F_1-ATPases are composed of five polypeptides in a stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$ and have molecular weights of about 380,000 [2]. They contain six binding sites for nucleotides. Three of these are potential catalytic sites [3]. Since a clear functional role for the other three sites has not been defined, they are called noncatalytic nucleotide binding sites. However, it is clear that these sites modulate F_1-ATPases under in vitro conditions [4–8].

Whereas the catalytic sites reside mostly, if not entirely, in β subunits [9,10], the noncatalytic sites clearly span α and β subunits. The major part of noncatalytic sites is in α subunits, but the adenine moiety of adenine nucleotides bound to these sites interacts with a domain near the C-termini of β subunits [11–14]. Hence, when enzyme is irradiatated after loading catalytic sites with 2-N_3-AD(T)P, tyrosine-β345 is derivatized, whereas when irradiation follows loading of noncatalytic sites, tyrosine-β368 is labeled [15,16].

After photoinactivating the CF_1-ATPase with 2-N_3-AD(T)P, Melese et al. [17] detected by two-dimensional gel electrophoresis what appeared to be AMP covalently bound to a catalytic site. They suggested that the covalently bound AMP could arise from covalently bound ADP by hydrolysis or by a transphosphorylation reaction akin to that of adenylate kinase. It was suggested earlier by Cross et al. [15] that the two nucleotide binding sites on the β subunit might be sufficiently close to allow transphosphorylation between adenine nucleotides bound to adjacent catalytic and noncatalytic sites. More recently, Vogel and Cross [18], on the basis of inhibition of MF_1 with bis-adenosine polyphosphates and homology of the β subunit with adenylate kinase, presented a model which places nucleotides bound to catalytic and noncatalytic binding sites on a single β subunit in an orientation that would allow an adenylate kinase-like transphosphorylation.

We report here that irradiation of nucleotide depleted MF_1 (nd-MF_1) with 2-N_3-[8-^{3}H]ADP in the presence of Mg^{2+} leads to detection of part of the photoaffinity label tethered at a catalytic site as [8-^{3}H]AMP. However, the [8-^{3}H]AMP derivative is detected even when noncatalytic sites are empty, therefore excluding a transphosphorylation mechanism. Instead, the [8-^{3}H]AMP detected at the catalytic site arises from Mg^{2+}-dependent hydrolysis of [8-^{3}H]ADP tethered to the catalytic site during and subsequent to irradiation.

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Abbreviations: MF_1, the bovine heart mitochondrial F_1-ATPase; nd-MF_1, the MF_1-ATPase depleted of endogenous nucleotides; CF_1, the F_1-ATPase from spinach chloroplasts; and HPLC, high performance liquid chromatography.

2. Materials and methods

MF_1 was prepared from bovine heart mitochondria with a previously described modification [19] of the method of Knowles and Penefsky [20] and was depleted of endogenous nucleotides by two successive passes through a Sephadex G-50–80 column equilibrated with 50% glycerol

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(v/v) as described by Garrett and Penefsky [21]. Protein concentrations were determined by the Bicinchoninic acid method described by Smith et al. [22].

The synthesis and purification of 2-N_3-[β-^{32}P]ADP was performed as described previously [8]. The same procedure was used to synthesize 2-N_3-[8-^{3}H]ADP except that 2-Cl-[8-^{3}H]adenosine (10 Ci/mmol) from Moravek Biochemicals was used as starting material [24]. The concentration of 2-N_3-[β-^{32}P]ADP or 2-N_3-[8-^{3}H]ADP was deterimed in 0.1 M HCl at 274 nm (ε = 15,200 $M^{-1} \cdot cm^{-1}$) [24].

Irradiation, trypsin digestion and separation of tryptic peptides by HPLC were carried out as previously described [8]. The C_4 reversed-phase column (Brownlee; 220 × 4.6 mm, 7 mm particle size) was connected to a guard column (Brownlee; 30 × 4.6 mm, 7 mm particle size) and developed with a gradient of CH_3CN into 0.1% HCl as follows: 0–10 min, 0% CH_3CN; 10–100 min, 0–24% CH_3CN; 100–115 min, 24–48% CH_3CN; and 115–120 min, 48–80% CH_3CN [8].

Biochemicals were purchased from Sigma Chemical Company. Bicinchoninic acid was from Pierce Chemical Company. Radioactivity was detected with a Packard 1600 TR liquid scintillation counter using Ecoscint from National Diagnostics.

3. Results

When a tryptic digest prepared from nd-MF_1 which had been photoinactivated with 200 μM 2-N_3-[β-^{32}P]ADP in the presence of Mg^{2+} was submitted to reversed-phase HPLC, two major peaks of radioactivity were resolved as illustrated in Fig. 1. Previous studies [8,15] have shown that the peak eluting at about 61 min is the tryptic peptide derived from the noncatalytic binding site which is derivatized at Y-β368, whereas the peak eluting at about 86 min contains the peptide derived from the catalytic site which is labeled at Y-β345. When a tryptic digest of nd-MF_1 which had been photoinactivated with 225 μM 2-N_3-[8-^{3}H]ADP was submitted to reversed-phase HPLC under the same conditions, a major peak of radioactivity eluting at about 81 and a minor one eluting at 86 min were detected as illustrated in Fig. 1B. In contrast, the peak eluting at 81 min was not detected in a tryptic digest of nd-MF_1 photoinactivated with 90 μM 2-N_3-[8-^{3}H]ADP in the absence of Mg^{2+} as shown in Fig. 2A. However, when a sample of the same inactivation mixture was incubated for 40 min with 2.5 mM Mg^{2+} prior to digestion with trypsin, the radioactivity associated with Y-β345 eluted at 81 and 86 min as illustrated in Fig. 2B. This indicates that Mg^{2+} promotes conversion of the original derivative of Y-β345 to one that has lower affinity for the reversed-phase ma-

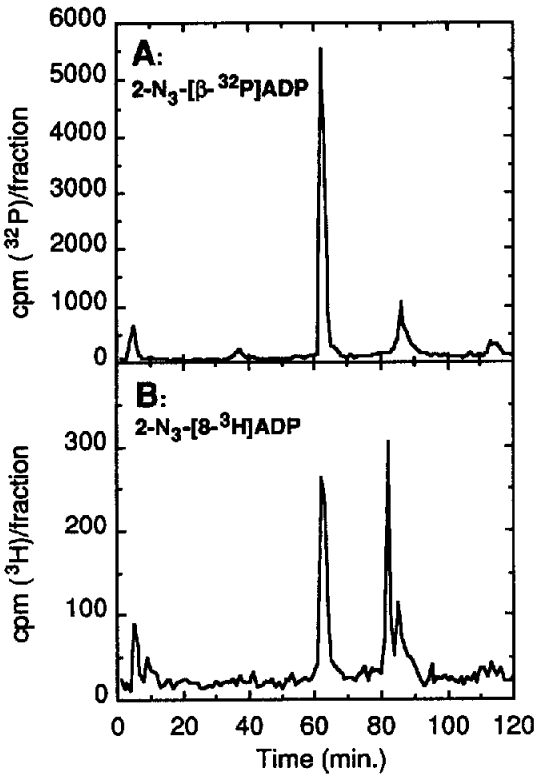

Fig. 1. Resolution by reversed-phase HPLC of radioactive peptides in a tryptic digest of nd-MF_1 photoinactivated with 2-N_3-[β-^{32}P]ADP or 2-N_3-[8-^{3}H]ADP in the presence of Mg^{2+}. (A) A 125 μl solution containing 3 μM nd-MF_1, 200 μM 2-N_3-[β-^{32}P]ADP, 2.5 mM $MgCl_2$, 0.48 mM EDTA, 12.6% glycerol (w/v) and 90 mM Tris-SO_4, pH 8.0, was incubated at 23°C at which time it was irradiated for 40 min. (B) The conditions were the same as in A except 225 μM 2-N_3-[8-^{3}H]ADP was used instead of 2-N_3-[β-^{32}P]ADP. Irradiation, preparation and fractionation of the tryptic digests were carried out as previously described [8].

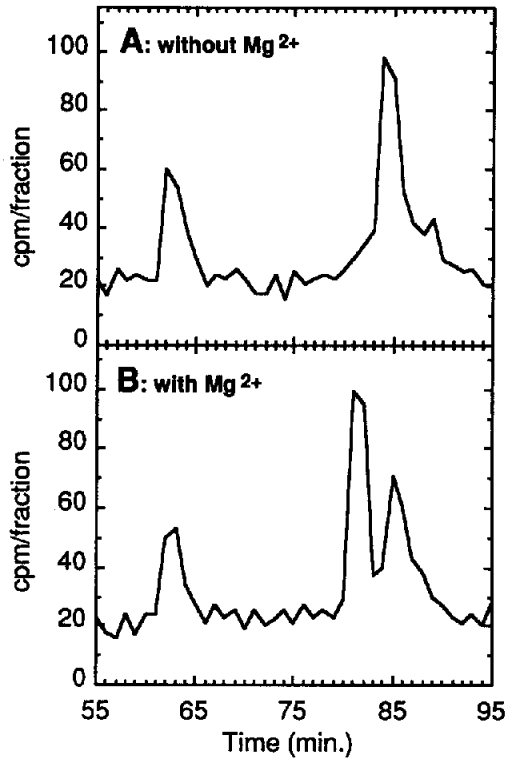

Fig. 2. Mg^{2+}-induced transformation of [8-^{3}H]ADP tethered to the catalytic site of nd-MF_1 to tethered [8-^{3}H]AMP. The experimental conditions were the same as described in Fig. 1 except that the reaction mixture contained 90 μM 2-N_3-[8-^{3}H]ADP in the absence of Mg^{2+}. After irradiation, $MgCl_2$ was added to a final concentration of 2.5 mM to one half of the reaction mixture (Profile B). For a control, the other half was diluted by the same amount with water (Profile A). The samples were then incubated for 40 min at 23°C, at which time they were prepared for tryptic digestion as previously described [8].

trix. The peak eluting at about 81 min is not detected in tryptic digests prepared from nd-MF_1 inactivated with 2-N_3-[β-^{32}P]ADP, Therefore, loss of the β phosphate is responsible for the conversion. Fig. 3 shows that substantial loss of the β phosphate from covalently bound [8-^{3}H]ADP occurs within 15 min after introducing Mg^{2+}. However, the conversion slows considerably after 15 min.

To determine whether conversion of [8-^{3}H]ADP covalently bound to Y-β345 to covalently bound [8-^{3}H]AMP involves transphosphorylation to ADP noncovalently bound to a noncatalytic site as postulated by Melese et al. [17], 3 μM nd-MF_1 was photoinactivated with 3 μM 2-N_3-[8-^{3}H]ADP in the presence of Mg^{2+}. These conditions should allow exclusive labeling of a catalytic site as previously reported [25]. That this is the case is illustrated by Fig. 4 which shows the profile of radioactivity obtained after submitting a tryptic digest of enzyme photolabeled with stoichiometric 2-N_3-[8-^{3}H]ADP to HPLC. Only slight labeling of the noncatalytic site occurs under these conditions indicating that the photolabel is bound nearly exclusively to a catalytic site. Nevertheless, most of the labeled Y-β345 is present as the [8-^{3}H]AMP derivative, indicating that a nucleotide bound to an adjacent noncatalytic site does not participate in loss of the β phosphate.

4. Discussion

It is clear from the results presented that the [8-^{3}H]AMP-derivative of Y-β345 detected on photoinactivation of MF_1 with 2-N_3-[8-^{3}H]ADP arises from Mg^{2+}- dependent hydrolysis of the β phosphate of tethered [8- ^{3}H]ADP. Since AMP has very low affinity for F_1 [26], hydrolysis probably occurs after derivatization. This is supported by the observation that addition of Mg^{2+} to enzyme derivatized with 2-N_3-[8-^{3}H]ADP in the absence of the divalent metal ion initiates loss of the β phosphate. It is interesting that an [8-^{3}H]AMP derivative is not detected on Y-β368 which is part of the noncatalytic site. Melese et al. [17] reported a similar observation with CF_1. This suggests that hydrolysis of the β phosphate from ADP tethered to Y-β345 at a catalytic site might involve participation of amino acid side chains in the immediate vicinity of the covalently bound ligand. Supporting this contention is the observation that conversion of tethered ADP to tethered AMP is nearly complete when Mg^{2+} is included during the 40 min irradiation used to derivatize the enzyme, whereas the rate of conversion drops off rapidly with time when Mg^{2+} is added after irradiation. The decrease in rate of conversion observed might reflect unfolding of the derivatized enzyme to a form that can no longer utilize amino acid side chains at the catalytic site to hydrolyze the β phosphate tethered to Y-β345. This raises the intriguing possibility that ADP tethered to Y-β345 at a catalytic site might be distorted in a position that would allow the β phosphate to occupy the site normally occupied by the γ phosphate when ATP is bound to the unmodified catalytic site. In any event, no matter what mechanism is responsible for conversion of ADP tethered to Y-β345 to tethered AMP, transphosphorylation to nucleotide bound to a noncatalytic site is not involved. Therefore, the detection of AMP covalently bound to the catalytic site of MF_1 as described here or to the catalytic site of CF_1 as described by Melese et al. [17] cannot be taken as support for the regulatory transphosphorylation mechanism postulated by Cross et al. [15] and Vogel and Cross [18]. Furthermore, it is clear from the results presented that 2-N_3-[8-^{3}H]AD(T)P or 2-N_3-[α-^{32}P]AD(T)P

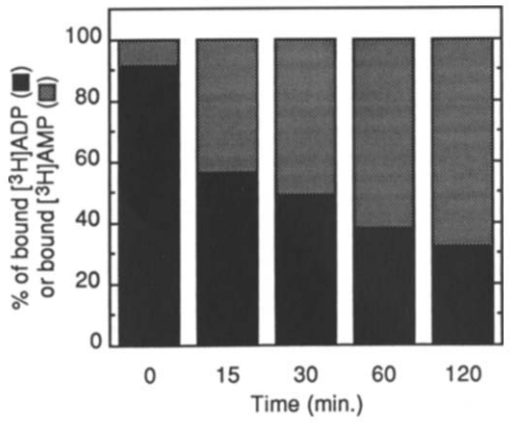

Fig. 3. Conversion of [8-^{3}H]ADP tethered to Y-β345 to tethered [8-^{3}H]AMP after adding Mg^{2+} to nd-MF_1 which had been photoinactivated with 2-N_3-[8-^{3}H]ADP. The experimental conditions were the same as in Fig. 2 except that 112 μM 2-N_3-[8-^{3}H]ADP was used. After irradiation, a sample was removed for a control. To the remaining solution, $MgCl_2$ was added to a final concentration of 2.5 mM. At the times indicated, samples were removed from the reaction mixture and processed for trypsin digestion as described previously [8].

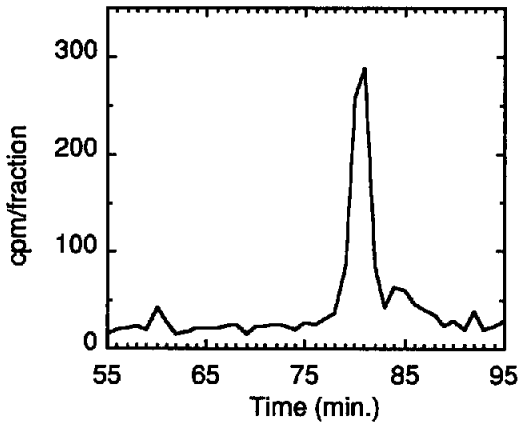

Fig. 4. The β phosphate of [8-^{3}H]ADP tethered to Y-β345 is hydrolyzed when noncatalytic sites are empty. The experimental conditions were the same as those described in Fig. 1 except that the reaction mixtures contained 3 μM 2-N_3-[8-^{3}H]ADP.

[27] are the preferred reagents for correlating the number of catalytic sites modified with the extent of inactivation when F_1-ATPases are photoinactivated with 2-N_3-AD(T)P.

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