Volume 313, number 1, 90–93 FEBS 11722 November 1992

Photolabeling of the phosphate binding site of chloroplast coupling factor 1 with [^{32}P]azidonitrophenyl phosphate

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Received 15 August 1992; revised version received 21 September 1992

Chloroplast F_1-ATPase (CF_1) was photolabeled by a radiolabeled photoactivatable derivative of P_i, 4-azido-2-nitrophenyl [^{32}P]phosphate (ANPP). The radioactivity was localized in the β subunit of CF_1. Upon cleavage of the β subunit by cyanogen bromide, the predominantly labeled peptide was recovered, which was subsequently subjected to tryptic digestion. A tryptic peptide (spanning Ile^{312}–Arg^{358}), was found to contain nearly all the covalently bound radioactivity. By Edman degradation, the labeled amino acid residues were identified as Tyr^{328}, Val^{329} and Pro^{330}. The labeled β-Tyr^{328} of CF_1 is the equivalent of β-Tyr^{311} of F_1 from beef heart mitochondria, which was previously found to be photolabeled by ANPP [J. Garin et al. (1989) Biochemistry 28, 1442–1448].

Chloroplast F_1 ATPase; Phosphate binding site; Photoaffinity; 4-Azido-2-nitrophenylphosphate

1. INTRODUCTION

Chloroplast ATP synthase (CF_0CF_1) catalyzes ATP synthesis from ADP and P_i by using the energy of the photogenerated transmembrane proton gradient. The CF_1 component is a peripheral membrane protein complex consisting of five distinct subunits, α, β, γ, δ, and ε, with a stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$ and a molecular weight of 400 kDa [1]. Although there are some data concerning P_i binding in connection with the photophosphorylation activity of membrane bound CF_1 as well as the capacity of CF_1 to synthesize bound ATP from bound ADP and exogenous P_i ([2,3] and references therein), there was a need for a probe specific to the P_i binding site to confirm the tentative structural assignment of this site next to the tight ADP binding site of CF_1 [4,5]. A previous study involving the photoactivatable P_i analogue 2-azido-4-nitrophenyl phosphate (ANPP) had led to the localization of this site on the β subunit [6]. The goal of the present work was to identify the amino acid residues of the β subunit which are labeled after photoirradiation of isolated chloroplast F_1 with [^{32}P]ANPP. The results are compared to those of the mapping studies of the P_i binding site of mitochondrial F_1-ATPase [7].

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Abbreviations: ANPP, 4-azido-2-nitrophenyl phosphate; MES, 2-(*N*-morpholino)ethane sulfonic acid; TFA, trifluoroacetic acid; CF_1, catalytic factor (soluble) of the ATP synthase complex from chloroplasts; TDAB, tetradecyltrimethylammonium bromide; EDC, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide.

2. MATERIALS AND METHODS

2.1. *Chemicals*

The sources of the chemicals were as follows: $H_3{}^{32}PO_4$ (10 mCi) New England Nuclear; Trypsin, Worthington; *S. aureus* V8 protease, Miles; Sequelon arylamine attachment kit, Millipore; EDC, Sigma. [^{32}P]ANPP was synthesized and purified as described by Lauquin et al. [8] with some modifications [7].

2.2. *[^{32}P]ANPP photolabeling and isolation of the labeled subunits of chloroplast F_1-ATPase*

Spinach CF_1 was purified as described by Berger et al. [9] and stored in 2 M ammonium sulfate, 10 mM Tris-HCl, 1 mM EDTA and 0.5 mM ATP, pH 7.2, at 4°C. The CF_1 suspension in ammonium sulfate was centrifuged and the pellet was rinsed with 250 mM sucrose, 50 mM Tris-acetate, pH 7.5 (STA buffer) in the presence of 50% ammonium sulfate, and then resuspended in STA buffer. The F_1 solution was desalted using an ACA 202 column (IBF) equilibrated with STA buffer, and then passed through another ACA 202 column equilibrated in 50 mM Tris base, 50 mM MES, 1 mM $MgCl_2$, pH 7.4 (TMMg buffer), then incubated at a concentration of 10 μM with 40 μM [^{32}P]ANPP for 30 min in darkness at room temperature in a small Petri dish, and finally subjected to two sequential photoirradiations of 30 s each, using a Xenon XB100 lamp (1000 W). The enzyme solution was protected from deleterious short-wavelength radiations by a glass plate placed between the light source and the sample. Under these conditions of photoirradiation, no loss in ATPase activity occurred in the absence of probe.

The photolabeled F_1 was precipitated with 65% ammonium sulfate, centrifuged, and the pellet was resuspended using a medium consisting of 50 mM sodium succinate, 1 M sodium chloride, 0.25 M sodium

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nitrate, 0.1 mM dithiothreitol, 4 mM EDTA, pH 6.1 [10]. Dialysis against this medium was conducted overnight at 4°C.

2.3. *Fragmentation of the photolabeled β-subunit and isolation of the labeled peptide*

The isolated β-subunit was succinylated [11] and then fragmented by CNBr. The resulting peptides were separated first by gel filtration on a G75 (SF) column (100 × 2 cm) and then by reverse-phase HPLC using a Vydac™ TP C-4 column (6.3 × 250 mm). The purified peptide was subjected to tryptic and SAV8 sub-cleavage as indicated, followed by separation by gel-filtration on a Biogel P4 column (80 × 1.5 cm).

2.4. *Peptide cleavage*

CNBr cleavage was done in 80% formic acid for 6 h at room temperature in the dark with a tenfold excess of CNBr (w/w). Tryptic cleavage was realised in 100 mM NH_4HCO_3, for 6 h at 37°C, 1/50 (w/w). SAV8 cleavage was done overnight in 100 mM NH_4HCO_3, at 37°C, 1/20 (w/w).

2.5. *Sequence analysis and radioactivity elution*

The carboxyl groups of the peptide (at C-terminal and Asp or Glu residues) were reacted with EDC and were coupled to the arylamine reactive groups of Sequelon membranes. The amino acid sequence was analyzed by solid-phase Edman degradation in an automated Applied Biosystems sequenator. Radioactivity elution was optimized by extensive washing of the membrane with TFA to avoid excessive carry-over from one residue to the next. Radioactivity was determined by liquid scintillation counting.

The protein concentration was assayed by the Bradford dye binding method with bovine serum albumin as standard [12].

3. RESULTS

3.1. *Photoaffinity labeling of CF_1 by [^{32}P]ANPP and dissociation of the labeled subunits*

A solution of CF_1 (10 μM) was photoirradiated in the presence of 40 μM [^{32}P]ANPP as described in section 2. The subsequent overnight dialysis against the dissociating medium consisting of 50 mM sodium succinate, 1 M sodium chloride, 0.25 M sodium nitrate, 0.1 mM dithiothreitol, 4 mM EDTA, pH 6.1 [10] at 4°C led to partial precipitation of the protein. Following centrifugation, aliquots of supernatant and resuspended pellet were subjected to TDAB-polyacrylamide gel electrophoresis followed by autoradiography. The supernatant fraction contained only the β subunit whereas subunits α, γ, δ and ε, with little β remaining, were found in the pellet (data not shown). A similar treatment had been successfully used to separate the subunits of *E. coli* F_1 [10] and beef heart mitochondrial F_1 [13] but, in those cases, successive freezing (−70°C)/thawing steps were necessary to induce dissociation of the subunits, and all the subunits remained in solution.

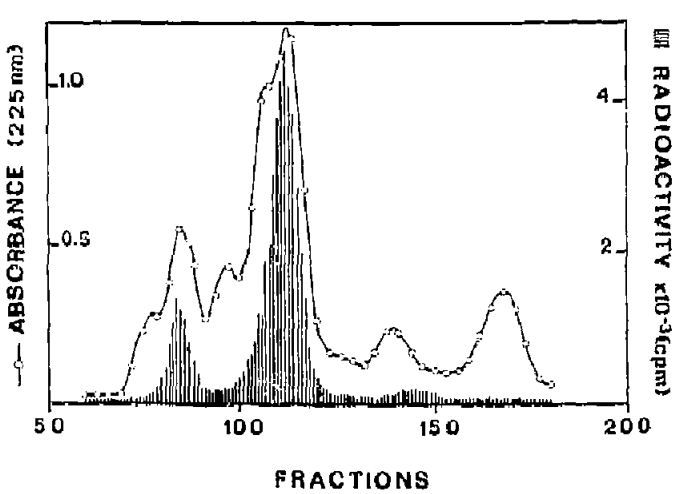

Fig. 1. Fractionation of CNBr cleavage products of photolabeled β-subunit. The resulting peptides were chromatographed on a Sephadex G-75 SF column (120 × 2 cm) in 50 mM ammonium bicarbonate. During elution, 3-ml fractions were collected and analysed for 225 nm absorption and radioactivity (10 μl aliquots were counted). Fractions 105 to 118 were pooled, and corresponded to 75% of eluted radioactivity.

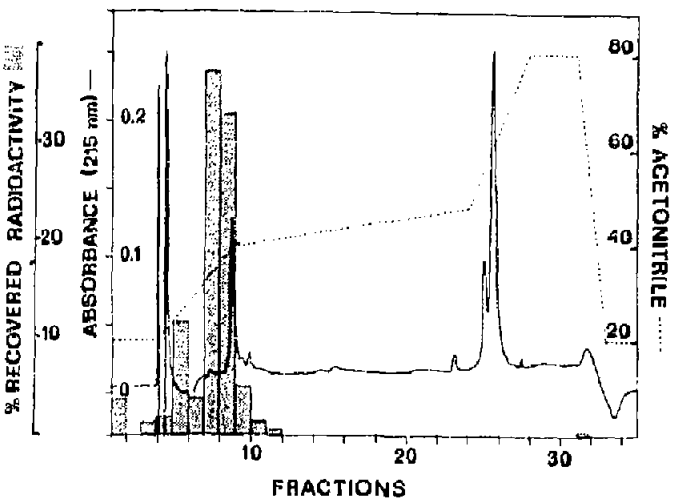

Fig. 2. Purification of the major radioactive peptide fraction obtained by CNBr cleavage. The VYDAC™ column was equilibrated in 75% buffer A (0.1% TFA) and 25% buffer B (80% acetonitrile, 0.1% TFA). The material corresponding to the pooled fraction (Fig. 1) was injected, and the column was subjected to an acetonitrile gradient at a flow rate of 1 ml · min^{-1}. Aliquot fractions were analysed for radioactivity at 1 min intervals. 80% of the radioactivity eluted in fractions 7 and 8.

The peculiar behaviour of CF_1 in the dissociating medium was a convenient basis for the direct purification of the β subunit and the demonstration that labeling occurred only on the β subunit as previously shown by SDS-PAGE of photolabeled CF_1 followed by autoradiography [6]. Guanidinium chloride was added to a final concentration of 7 M to the supernatant fraction, and succinic anhydride was added in 2-fold excess (w/w) for succinylation of the β subunit. After desalting on an ACA 202 column equilibrated in 50 mM NH_4HCO_3, stoichiometries of 0.12 and 0.18 covalently bound [^{32}P]ANPP/β subunit were determined with two different preparations of CF_1.

3.2. *Protein cleavage and peptide isolation*

The photolabeled and succinylated β subunit was fragmented using cyanogen bromide, and the resulting peptides were fractionated by gel filtration on Sephadex G75 SF at 4°C (Fig. 1). A major radioactive fraction

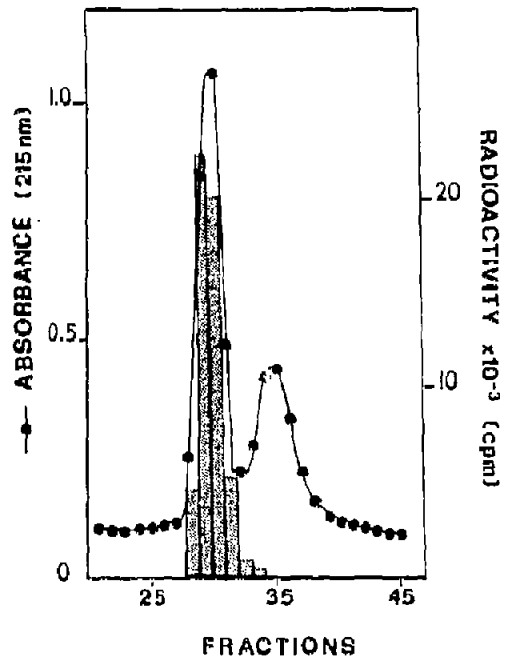

Fig. 3. Fractionation of the tryptic sub-cleavage of the purified labeled peptide (Fig. 2). The pooled fraction was submitted to tryptic cleavage as indicated in section 2. The digest was chromatographed on a Biogel P4 column (1.5 × 100 cm) equilibrated with 100 mM ammonium bicarbonate. Fractions of 1.5 ml were collected and analysed for their absorbance at 215 nm and their radioactivity content (20 μl aliquots were counted).

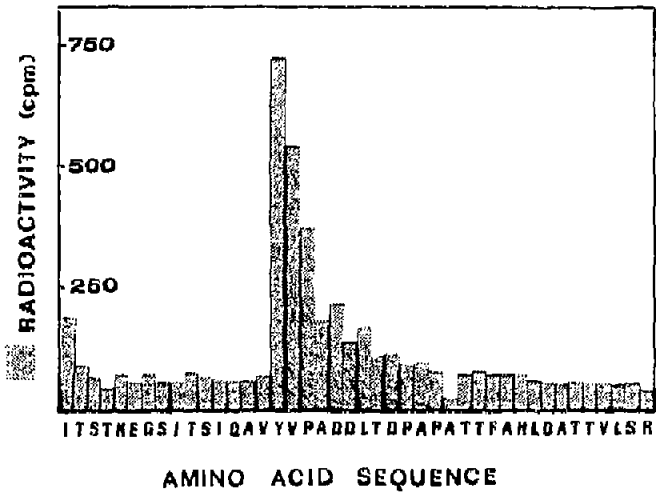

Fig. 4. Radioactivity elution from the Edman degradation of the [^{32}P]ANPP-labeled tryptic subfragment of chloroplast F_1 (10,000 cpm were linked covalently to the filter). Raw data are given, unmodified for either reaction yield or background.

containing 80% of the label was further analyzed by reverse-phase HPLC using a VYDAC column. The radioactive label was associated with a single early peptide (spanning Gly^{307}–Met^{374}) (Fig. 2) whose elution characteristics were strongly reminiscent of mitochondrial F_1 β-subunit 'CB9' peptide (nomenclature Runswick and Walker [14]) (spanning Gln^{293}–Met^{358}) that was labeled by the same phosphate analogue [^{32}P]ANPP [7]. Subsequently, this peptide was cleaved with trypsin and the resulting peptides were fractionated by gel filtration on Bio-Gel P-4. Radioactivity eluted with the higher molecular weight peptide (Fig. 3).

3.3. *Peptide sequencing and determination of photolabeled amino acids*

The labeled tryptic peptide was covalently attached to arylamine residues on a filter with EDC and subjected to Edman degradation. Essentially all the radioactivity was eluted at the cycles corresponding to the amino acid residues Tyr^{328}, Val^{329} and Pro^{330} (Fig. 4). One cannot exclude that the labeling attributed to Val^{329} and Pro^{330} may represent carry-over of the elution of the radioactivity. A similar procedure after cleavage of this peptide with SAV8 protease which shortened the peptide by six amino acid residues on the N-terminal side confirmed Tyr^{328} as the major target for [^{32}P]ANPP labeling (data not shown).

4. DISCUSSION

The low affinity of CF_1 for P_i sets it apart from the bacterial and mitochondrial enzymes (respective K_d values of 10 μM [15] and 37 μM [8,16]). Pougeois et al. [6] found little or no binding of P_i to isolated CF_1 whereas Huchzermeyer [17] found a P_i binding site with a K_d value of 170 μM, characterized by a rapid dissociation of the enzyme–ligand complex. This raises the question of the validity of the photolabeling approach of the topography of the P_i binding site in CF_1 even though labeling is prevented by P_i binding [6]. That the low affinity P_i binding site is not an artifact resulting from thylakoid membrane solubilization is ruled out by the examination of the P_i binding parameters during the course of photophosphorylation by the thylakoid-bound enzyme. These studies gave a K_m for P_i of 580 μM and a K_i of 95 μM for thiophosphate, a competitive inhibitor of P_i binding [18]. Recently, Zhou and Boyer [2] reported a K_m for P_i of 700 μM. Several competitive inhibitors of P_i binding (noncompetitive towards ADP) have been explored by Shinohara and Sakurai [19,20]; their K_i values ranged between 1 and 4 mM, compared to a K_m for P_i of 250 μM; among them was a phenylphosphate with a structure very similar to that of the probe used in the present study.

An indication that P_i binding to isolated CF_1 also takes place next to a catalytic nucleotide binding site comes from the ability of the isolated enzyme to synthesize bound ATP from the tightly bound ADP and high concentrations of exogeneous P_i in the presence of Mg^{2+} [3].

The effects of P_i and sulfite on catalysis are also consistent with a P_i binding site located close to a nucleotide binding site [4,5,21]. Their binding results in a change

of conformation. Either P_i and sulfite induce a high energy state similar to that resulting from light-induced generation of a proton gradient [4] or they facilitate the conformational change induced by MgATP binding at another catalytic site [5].

ANPP was previously utilized in studies directed to the identification of the amino acid residues involved in the P_i binding site of F_1-ATPases from mitochondria [7,8], *E. coli* and PS3 [15] and the mitochondrial P_i carrier [22]. ANPP was also found to be a potential probe for CF_1 [6]. In the present study, the target residues at the P_i binding site of CF_1 have been investigated. β-Tyr^{328}, together with β-Val^{329} and β-Pro^{330} were identified as the photolabeled amino acid residues of CF_1. The residue of CF_1 predominantly labeled by ANPP, β-Tyr^{328}, is homologous to mitochondrial β-Tyr^{311} also found to be photolabeled by ANPP [7]. So the same region of CF_1 and MF_1 is involved in the binding of P_i even though the binding parameters of P_i to these two enzymes are different. This is in agreement with the high degree of conservation of this region in the two enzymes as shown below [1,14].

Bovine MF_1 β301–320: KGSITSVQAI YVPADDLTDP

Spinach CF_1 β318–337: EGSITSIQAV YVPADDLTDP

The functional similarity between β-Tyr^{328} in CF_1 and β-Tyr^{311} in MF_1 is emphasized by the fact that these residues in CF_1 and MF_1 are the ones that are labeled by nitrobenzofurazan [23,24].

Acknowledgements: We thank Mathilde Vinçon for skillful technical assistance at the sequencing step, Dr. Eamonn Rooney for careful reading of the manuscript, and Jeannine Bournet for excellent secretarial assistance.

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