Amino Acid Sequence of the Nucleotide Binding Region of Chloroplast Coupling Factor 1†

Arie Admon‡ and Gordon G. Hammes*
Department of Chemistry, Cornell University, Ithaca, New York 14853
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ABSTRACT: The labeling of chloroplast coupling factor 1 by 3′-*O*-(4-benzoyl)benzoyl-ATP (BzATP) was studied. When the enzyme was incubated with ~10 μM BzATP and 6 mM $MgCl_2$ at pH 7.9 for ~20 min and passed through two Sephadex G-50 centrifuge columns, three BzATP molecules were bound per coupling factor molecule. Photolysis of radioactive enzyme-bound BzATP followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography revealed that the BzATP bound primarily to the β-polypeptide. If unbound BzATP was not removed by centrifuge columns prior to photolysis, significant labeling of the α-polypeptide also occurred. After photolysis, the BzATP-labeled enzyme was treated with trypsin, and two radioactive peptides were isolated by high-performance liquid chromatography on a C_{18} column. The two peptides were sequenced and found to correspond to amino acids 360–378 and 393–397 of the β-polypeptide. For the sequence 360–378, two specific amino acids were found to be radioactive (Tyr-362 and Asp-369). This region of the polypeptide is highly conserved in several different species and probably corresponds to part of the nucleotide binding region of the catalytic site. In the case of amino acids 393–397, a very low level of radioactivity was found for all amino acids. The significance of this peptide for the binding of nucleotides to coupling factor 1 could not be established.

The ATP synthase of chloroplasts is responsible for the photophosphorylation of ADP [cf. Galmiche et al. (1985)]. It is similar to other F_1–F_0 ATP synthases from mitochondria and bacteria. The chloroplast enzyme can be dissociated into two parts: CF_0 is located in the membrane and probably contains the proton channel; chloroplast coupling factor 1 (CF_1)[1] contains the catalytic part of the enzyme and can be easily solubilized. CF_1 contains at least three nucleotide binding sites with different characteristics (Hammes, 1983). The structure of CF_1 has been extensively investigated with fluorescence resonance energy transfer [cf. Snyder and Hammes (1985) and Richter et al. (1985)].

Affinity analogues of nucleotides have been used for identifying active and regulatory sites of enzymes, including the F_1–F_0 class of enzymes [cf. Vignais and Lunardi (1985)]. The photoaffinity analogues used include 2- and 8-azidoadenyl-ATP/ADP [cf. Garin et al. (1986) and Hollemans et al. (1983)] and 3′-*O*-[3-[*N*-(4-azido-2-nitrophenyl)amino]-propionyl]-ADP/ATP (Bruist & Hammes, 1981) and 3′-*O*-(4-benzoyl)benzoyl-ATP/ADP [cf. Williams et al. (1986) and Kambouris and Hammes (1985)] with the photoreactive group located on the 3′-ribose position. The analogue 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine also has been utilized (Esch & Allison, 1978).

The photoaffinity analogue BzATP reacts by photolysis at 360 nm, with an extinction coefficient of about 160 M^{-1} cm^{-1} at this wavelength. The covalent binding of BzATP to the protein occurs when the benzophenone is excited to its triplet state and extracts a hydrogen atom from an adjacent amino acid. The advantage of this photoreactive group is its lack of specificity: this causes the probe to bind to whichever amino acid exists in its vicinity. Also, when the hydrogen is extracted from carbon, a stable carbon–carbon bond is formed between benzophenone and the protein. Another advantage is the high yield of the covalent modification by this probe: unlike azido analogues, benzoyl(benzoic) derivatives do not react with water. The major disadvantage of these derivatives is that the ester bond between benzophenone and ATP/ADP is not very stable; hydrolysis occurs under acidic or basic conditions. In addition, benzophenone is rather large, which may cause labeling at positions not directly in the nucleotide binding site. In this study, the portions of the β-polypeptide of CF_1 labeled by BzATP after binding and photolysis were determined by peptide sequencing and amino acid analysis.

Materials and Methods

Chemicals. ATP (vanadium free), dithiothreitol, and 1,1-carbonyldiimidazole were from Sigma; 4-benzoylbenzoic acid was from Aldrich; TPCK–trypsin was from Worthington. [α-^{32}P]ATP and [^{3}H]ATP were from ICN or Amersham, and 4-[^{3}H]benzoylbenzoic acid (labeled in the rings) was from Rotem Industries, Beer Sheva, Israel. Dimethyl formamide was redistilled from barium oxide under reduced pressure and stored over a dry 4-Å molecular seive. All other chemicals were high-quality commercial grades.

Enzyme. CF_1 was purified from fresh market spinach (Lien & Racker, 1971; Binder et al., 1978) and stored as a precipitate in 50% ammonium sulfate, 20 mM Tris–sulfate, 2 mM EDTA, and 0.5 mM ATP, pH 7.1 at 4 °C. Enzyme with a fluorescence ratio, 305 nm/340 nm (280-nm excitation), above 1.5 was used. For heat activation of CF_1, the enzyme was desalted by passing it through two consecutive G-50 Sephadex centrifuge columns (Penefsky, 1977) equilibrated with 50 mM Tris-HCl and 2 mM EDTA, pH 7.9, and then incubated in 50 mM Tris-HCl, 2 mM EDTA, 10 mM dithiothreitol, and 40 mM ATP, pH 7.9, at 64 °C for 5 min. The protein concentration was determined by absorbance measurements at 277 nm with an extinction coefficient of 0.483 cm^2 mg^{-1}

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‡ Weizmann Postdoctoral Fellow.

[1] Abbreviations: CF_1, chloroplast coupling factor 1; BzATP, 3′-*O*-(4-benzoyl)benzoyl-ATP; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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(Bruist & Hammes, 1981) or by the Lowry method (Lowry et al., 1951) with bovine serum albumin as the standard. The results obtained with the Lowry method were multiplied by 1.2 to give the correct CF_1 concentration (unpublished results). A molecular weight of 400000 was assumed for CF_1 (Moroney et al., 1983). The ATPase activity of the heat-activated enzyme was determined in 5 mM ATP, 5 mM $CaCl_2$, 50 mM Tris-HCl, and 2 mM EDTA, pH 7.9, by phosphate analysis (Taussky & Shorr, 1953); enzyme with an activity ≥ 12 μmol/(mg·min) at room temperature was used in this work.

Synthesis of BzATP. BzATP was synthesized with either [α-^{32}P]ATP or 4-[^{3}H]benzoylbenzoic acid (2–4 mCi/mmol) (Kambouris & Hammes, 1985). The extinction coefficients used for ATP, BzATP, and benzoylbenzoic acid were 15400, 32500, and 26000 M^{-1} cm^{-1}, respectively (Williams et al., 1986).

Nucleotide Binding and Covalent Labeling. The ammonium sulfate precipitated enzyme was centrifuged at 12000*g* for 20 min. The CF_1 pellet was suspended in a minimal volume of 50 mM Tris-HCl and 2 mM EDTA, pH 7.9, and desalted by passing it twice through a Sephadex G-50 centrifuge column (Penefsky, 1977) in the same buffer. This resulted in a small dilution of the sample (about 1.2–1.4). The ATP or BzATP was then added, followed by $MgCl_2$ to a final concentration of 6 mM. After incubation at room temperature for the specified time, the CF_1–nucleotide mixture was passed again through two consecutive centrifuge columns to remove free nucleotides. The protein concentration then was determined by direct absorbance measurement, if the amount of enzyme was sufficient, or by the Lowry method (Lowry, 1951); the amount of radioactivity coeluting with the enzyme also was measured by scintillation counting in ACS scintillation fluid. Two centrifuge columns were sufficient to remove unbound or loosely bound nucleotides.

Covalent labeling by photolysis of the BzATP was done with a 200-W Xe–Hg lamp (Hanovia); the light was passed through a Corning 360 nm wide band-pass filter. The enzyme was placed about 10 cm from the arc lamp inside a glass beaker of water to keep the sample from warming and to reduce the light intensity at wavelengths shorter than 300 nm. For labeling the tight binding sites, CF_1 was preincubated with BzATP, passed through a G-50 Sephadex centrifuge column twice, and photolyzed. For labeling the dissociable sites, CF_1 was incubated with BzATP and photolyzed; the sample was passed through the centrifuge columns after labeling.

Gel Electrophoresis and Autoradiography. Sodium dedecyl sulfate–polyacrylamide gel electrophoresis was carried out according to Laemmli (1970) with a 13% acrylamide [0.35% bis(acrylamide)] gel. The gel was stained with 7.5% acetic acid, 50% ethanol, and 0.2% Coomassie brilliant blue for 30 min and destained overnight in 20% ethanol and 7.5% acetic acid. Acetic acid was used instead of trichloroacetic acid to reduce the possibility of hydrolyzing the BzATP.

Autoradiography was done by drying the gel and placing it on Kodak X-omat film with a Dupont Cronex intensifying screen.

Peptide Mapping. The enzyme was labeled with the BzATP as described above for the tight sites except only one centrifuge column was used before photolysis. After being labeled, the CF_1–BzATP complex was denatured by addition of solid guanidine hydrochloride and sodium sulfite to final concentrations of 5 M and 100 mM, respectively, and the cysteines were sulfonated with 2-nitro-5-thiosulfobenzoic acid (Thannhauser et al., 1984) for 10 min at room temperature. The denatured CF_1–BzATP complex was passed through a Sephadex G-50 column (1 × 25 cm) at 2 mL/min in 5 M guanidine hydrochloride. The purpose of this procedure was to remove completely BzATP that is not covalently bound. The recovery of the protein from this column was quantitative, and the same column (stored in 5 M guanidine hydrochloride) could be used for several weeks without deterioration. The denatured protein was dialyzed against 100 mM ammonium bicarbonate for a minimum of 4 h. The protein precipitated as a very fine precipitate which was treated with TPCK–trypsin (1% of CF_1, w/w) in 100 mM ammonium bicarbonate at 37 °C. The trypsin was stored at −20 °C in 0.1 mM HCl and was stable for several months without loss of activity. The proteolysis was carried out for 12–24 h with continuous mixing and was stopped by freezing at −70 °C or by direct injection into the liquid chromatograph.

The peptide mixture was resolved by high-performance liquid chromatography on a C_{18} 300-Å Vydac column (4.6 × 250 mm). The first purification was with a linear gradient of 0–30% acetonitrile in 0.1% triethylammonium phosphate in water, pH 6.5, over 60 min. The second purification was with a linear gradient of 10–40% acetonitrile in 0.1% trifluoroacetic acid in water, pH 6.5, over 30 min or with a linear gradient of 0–10% acetonitrile in 0.1% triethylammonium acetate in water, pH 5.0, over 20 min. The radioactive fractions were concentrated by lyophilization to small volumes, but not to dryness, prior to the second chromatography and for amino acid analysis and sequencing.

Peptide sequencing was performed with an Applied Biosystems Model 470A protein sequencer. The fractions of each sequencing cycle were divided: part was taken for identification of the amino acid, and part was put in scintillation fluid for measurement of the radioactivity. Amino acid analyses (Waters Pico-Tag System) and protein sequencing were carried out by the Cornell Biotechnology Program resource facility.

RESULTS

Binding of BzATP to CF_1. To characterize the binding of BzATP to CF_1, the time course and the substrate concentration dependence of the binding of BzATP and ATP to latent CF_1 was investigated. CF_1 was desalted and freed from dissociable nucleotides as described above, and incubated with [α-^{32}P]-ATP, [^{3}H]ATP, [α-^{32}P]BzATP, or [^{3}H]BzATP. After incubation for 10 min in 50 mM Tris-HCl, 6 mM $MgCl_2$, and 2 mM EDTA, pH 7.9 at room temperature, both ATP and BzATP gave a saturation curve with an apparent dissociation constant of about 2 μM. The BzATP binding saturated at about 3 BzATP/CF_1, whereas the ATP saturated at about 2 ATP/CF_1 (Figure 1). In the absence of Mg^{2+}, 0.5–1 nucleotide/CF_1 was incorporated for both BzATP and ATP after more than an hour of incubation at room temperature (data not shown). As shown in Figure 2, the BzATP binds tightly to CF_1 within a few minutes in the presence of 6 mM $MgCl_2$. If the enzyme is incubated with ATP and 6 mM $MgCl_2$ for 10 min and the free ATP is removed by passage of the solution through two centrifuge columns, incubation with BzATP in the presence of 6 mM $MgCl_2$ for another 10 min results in about one tightly bound BzATP per CF_1. In the absence of Mg^{2+}, the formation of CF_1 with tightly bound nucleotides is very slow (Figure 2).

After photolysis of the enzyme with tightly bound nucleotides, sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography showed that predominantly the β-polypeptide was labeled with BzATP (Figure 3). This result was obtained by using autoradiography for detection of labeled polypeptide or by cutting the gel and measuring the radio-

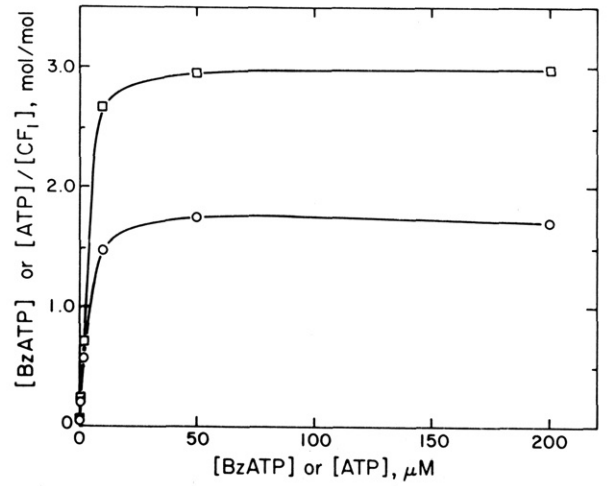

FIGURE 1: Concentration dependence of the binding of nucleotides to latent CF_1. CF_1 at about 1 mg/mL was incubated in 50 mM Tris-HCl, 6 mM $MgCl_2$, and 2 mM EDTA, pH 7.9 at room temperature, for 10 min with the specified concentration of ATP (○) or BzATP (□). The free nucleotides were removed by passage of the enzyme through two consecutive centrifuge columns of Sephadex G-50 (coarse), equilibrated with the same buffer mixture as described above, before determination of the binding stoichiometry.

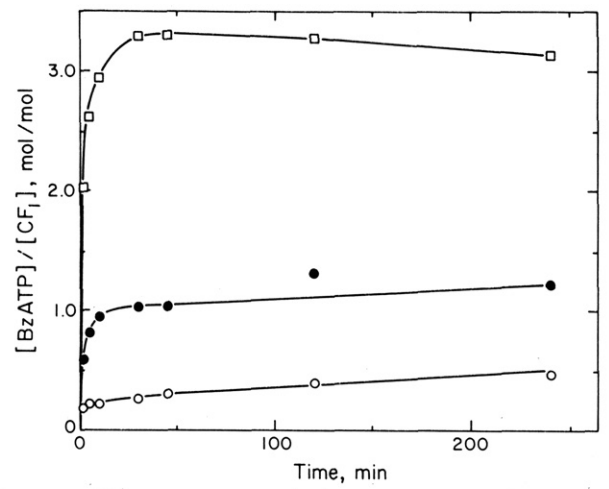

FIGURE 2: Time course of the binding of BzATP to latent CF_1. The enzyme was desalted and incubated with 10 μM [α-^{32}P]BzATP at a concentration of about 1 mg/mL in 50 mM Tris-HCl and 2 mM EDTA, pH 7.9, with (□) and without (○) 6 mM Mg^{2+}. For the data designated by (●), MgATP was added to the enzyme, and unbound nucleotide was removed prior to reaction with BzATP and Mg^{2+}.

activity of the pieces dissolved in 30% H_2O_2 (followed by sufficient catalase to remove excess H_2O_2). If photolysis is carried out without removal of the free nucleotide, some labeling of the α-polypeptide was observed (up to 30% of the radioactivity; results not shown).

About 70% of the BzATP tightly bound to the CF_1 (coeluted with the CF_1 through the G-50 centrifuge columns) was covalently bound after photolysis for 5–10 min. This is based on determination of the radioactivity of the enzyme before photolysis and after passage through the guanidine hydrochloride column.

Identification of Labeled Peptides. After labeling and proteolysis, the peptide mixture was resolved by reverse-phase high-performance liquid chromatography. On the first chromatography with triethylammonium phosphate (see Materials and Methods), two major peaks of radioactivity were observed: the first peak could be observed only after prolonged proteolysis and eluted at about 5% acetonitrile (Figure 4A). After this peak was rechromatographed on the same column

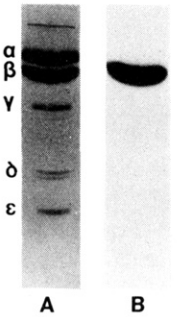

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of CF_1 labeled with tightly bound [α-^{32}P]ATP as described under Materials and Methods. (A) Coomassie blue stained gel; (B) autoradiograph of gel.

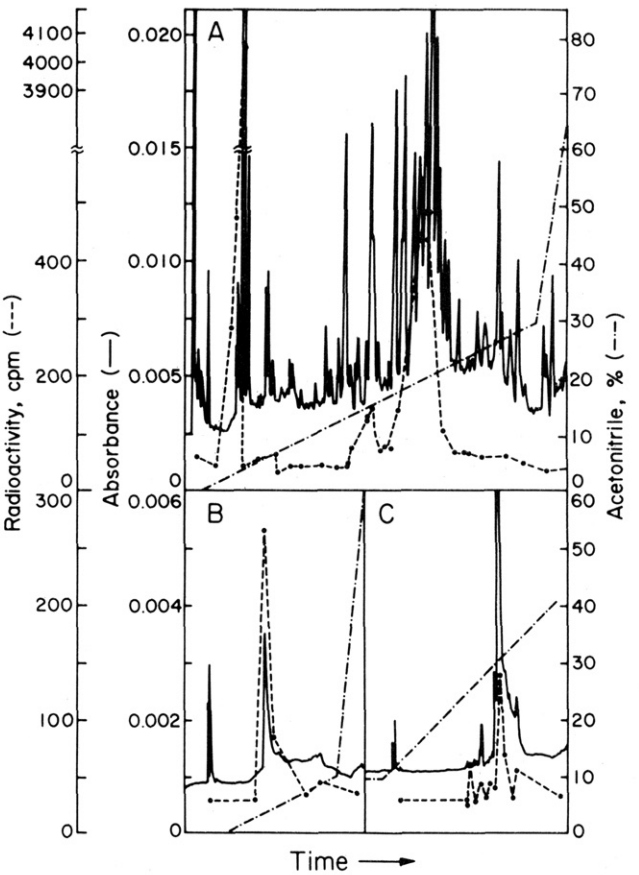

FIGURE 4: High-performance liquid chromatography of CF_1 after treatment with trypsin. The CF_1 labeled with [^{3}H]BzATP was reacted with trypsin for 24 h as described under Materials and Methods. About 2.5 mg of enzyme was loaded on a Vydac reverse-phase column. The absorbance at 214 nm (—) and the radioactivity (---) are shown. (A) The first dimension is a linear gradient of 0–30% acetonitrile (-·-) in 0.1% triethylammonium phosphate, pH 6.5. (B) The fraction containing the first radioactive peak from (A) was rechromatographed on the same column with a linear gradient of 0–10% acetonitrile (-·-) in 0.1% triethylammonium acetate, pH 5.0. (C) The second radioactive peak (from a different experiment) was rechromatographed as in (B) with a linear gradient of 10–40% acetonitrile (-·-) in 0.1% trifluoroacetic acid.

with a 0–10% acetonitrile gradient in triethylammonium acetate (Figure 4B), this peptide was identified as residues 393–397 of the β-polypeptide by amino acid analysis, peptide sequencing, and comparison with the known sequence of the β-polypeptide (Zurawski et al., 1982; Figure 5). The radioactivity associated with the individual amino acids obtained from the sequencer was about 15–20 cpm above background for all amino acids. The filter pad of the sequencer was found to be quite radioactive (several hundred cpm).

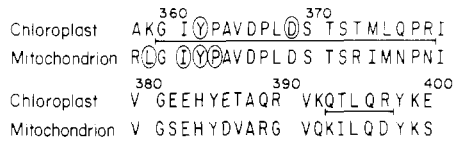

FIGURE 5: Partial amino acid sequence of the β-polypeptide from chloroplast CF_1 (Zurawski et al., 1982). The homologous mitochondrial sequence also is shown (Runswick & Walker, 1983), with the amino acid numbering as in Walker et al. (1985). The two peptides labeled by BzATP are underlined. The amino acids found to be radioactive are circled. For the mitochondrial enzyme, the circled amino acids were labeled with 2-azido-ATP (Garin et al., 1986).

The second large peak of radioactivity eluted at about 18–20% acetonitrile, with either trifluoroacetic acid or triethylammonium phosphate (Figure 4A). This peak is broader than the first, presumably because it contains several peptides. The major peptide purified from the peak radioactivity in this region by a second chromatography with trifluoroacetic acid–acetonitrile coelutes with the radioactivity (Figure 4C). It was identified as residues 360–378 of the β-polypeptide by amino acid analysis and peptide sequencing (Figure 5). In this peptide, Tyr-362 and Asp-369 were found to be radioactive at about one-third the level expected for 100% recovery of the amino acid. The center portion of the broad peak observed after the first chromatography was arbitrarily divided into three fractions, according to the horizontal position on the chromatogram. The amino acid analyses were identical for all fractions, suggesting that the broad peak may contain primarily a single peptide with labeling at different amino acids.

Discussion

The binding of ATP and ADP to CF_1 has been extensively characterized [cf. Carlier et al. (1979) and Bruist and Hammes (1981)]. Three ATP/ADP binding sites per CF_1 molecule with different characteristics have been identified. In the presence of Mg^{2+}, two tight binding sites were found; i.e., the nucleotides remain with the enzyme after passage of the enzyme through centrifuge columns and after dialysis. A third site binds ATP and ADP with a dissociation constant in the micromolar range. In the presence of Mg^{2+}, 3 mol of BzATP/mol of CF_1 is found after a 20-min incubation with the BzATP concentration greater than ~ 10 μM and passage through centrifuge columns (Figures 1 and 2). This tight binding requires Mg^{2+} (Figure 1), and the binding of MgATP to the enzyme prior to incubation with BzATP significantly reduces BzATP binding (Figure 1). If ATP is present in the incubation mixture, the binding of BzATP also is reduced (data not shown). These results indicate that BzATP and ATP are competing for the same binding sites on the enzyme. In the case of ATP, two of the three sites are catalytic (Leckband & Hammes, 1987); BzATP is a strong inhibitor of CF_1 ATPase activity (Bar-Zvi & Shavit, 1984). Therefore, BzATP is probably covalently bound to a catalytic site after photolysis.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography indicate that predominantly the β-polypeptide of CF_1 is labeled, in agreement with previous results for the binding of 2 BzATP/CF_1 (mol/mol) (Kambouris & Hammes, 1985). If unbound BzATP and loosely bound BzATP are not removed prior to photolysis, significant labeling of the α-polypeptide occurs. However, since the specificity of this labeling could not be ascertained, a detailed analysis of the α-polypeptide labeling was not pursued. Labeling of the α-polypeptide was found previously when the enzyme and BzATP concentrations were comparable during photolysis (Kambouris & Hammes, 1985), but the extent of labeling is insufficient for a complete characterization. BzATP has been reported to label both the α- and β-polypeptides of membrane-bound CF_1 (Bar-Zvi et al., 1983) but only the β-polypeptide of soluble CF_1 (Bar-Zvi & Shavit, 1984). When 2-azido-ADP was used as a photoaffinity label with membrane-bound CF_1, only the β-polypeptide was labeled (Czarnecki et al., (1982). With 3′-*O*-[3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl]ADP, both α- and β-polypeptides were labeled (Bruist & Hammes, 1981). For ATP synthases from other sources, labeling of both α- and β-polypeptides with nucleotide photoaffinity labels has been reported (Bar-Zvi et al., 1985; Wagenvoord et al., 1981; Schäfer et al., 1980, 1983; Hollemans et al., 1983; Garin et al., 1986). The general conclusion appears to be that specific labeling of the α-polypeptide is not nearly as extensive as labeling of the β-polypeptide. The nucleotide binding sites may be predominantly on the β-polypeptide but near the interfaces of the α- and β-polypeptides. Alternatively, the α-polypeptide might contain low-affinity nucleotide binding sites.

Two radioactive peptides have been characterized. However, in the case of the peptide with amino acids 393–397, specific radioactive amino acids were not found. The possibility exists that a derivative of BzATP coelutes with the peptide sequence 393–397. If this is the case, resolution of the mixture was not achieved with several different solvent systems and gradients. Alternatively, all of the amino acids could be labeled to some extent. The increase in radioactivity with increased time of proteolysis also suggests something unusual may be occurring. The fact that the radioactive amino acids are quite hydrophobic may cause them to be washed through the sequencer by the organic solvents used. In view of these problems, the assignment of this sequence as part of (or near) a nucleotide binding site cannot be made with certainty at this time.

On the other hand, the situation with regard to the peptide containing amino acids 360–387 seems clear. Two of the amino acids are radioactive, suggesting that the peptide sequenced consists of the peptide with a mixture of two different amino acids labeled. Moreover, the amino acid analyses of different parts of the broader peak of radioactivity suggest that other amino acids in this sequence also may be labeled. The multiple labeling of neighboring amino acids is analogous to the behavior observed for labeling of the β-polypeptide of beef heart mitochondrial ATPase by 2-azido-ATP (Garin et al., 1986). The homologous radioactive amino acids in the mitochondrial enzyme are in an identical region of the β-polypeptide as those labeled in CF_1 [Figure 5; the numbering of amino acids and corresponding homologies are as in Walker et al. (1985)]. In both cases, Tyr-362 is labeled. This tyrosine is also modified by the inhibitor 5′-[*p*-(fluorosulfonyl)-benzoyl]inosine in the beef heart mitochondrial enzyme (Bullough & Allison, 1986). Amino acids 360–370 are conserved in the β-polypeptide of enzymes from tobacco, spinach, barley, wheat, and maize chloroplasts, *Escherichia coli*, *Rhodopseudomonas blastica*, *Rhodospirillum rubrium*, and yeast and bovine mitochondria (Walker et al., 1985). On the basis of the above results, the conserved amino acids are very likely part of the nucleotide binding portion of the catalytic site.

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Escherichia coli Tryptophan Synthase: Synthesis of Catalytically Competent α Subunit in a Cell-Free System Containing Preacylated tRNAs†

Robert C. Payne,‡ Brian P. Nichols,§ and Sidney M. Hecht*,‡
Departments of Chemistry and Biology, University of Virginia, Charlottesville, Virginia 22901, and Department of Biological Sciences, University of Illinois at Chicago, Chicago, Illinois 60680
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ABSTRACT: A cell-free protein biosynthesizing system prepared from *Escherichia coli* CF300 was found to synthesize *E. coli* tryptophan synthase α subunit in a time-dependent manner when programmed with pBN69 plasmid DNA. This plasmid contains the *trp* promoter from *Serratia marcescens* adjacent to the coding region of *E. coli* tryptophan synthase α protein [Nichols, B. P., & Yanofsky, C. (1983) *Methods Enzymol. 101*, 155–164]. The synthesized tryptophan synthase α subunit was found to be indistinguishable from authentic α subunit protein when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and to have the same specific activity for catalyzing the conversion of indole → L-tryptophan by tryptophan synthase β_2 subunit, as well as the conversion of indole + glyceraldehyde 3-phosphate to indole-3-glycerol phosphate. In the absence of exogenously added phenylalanine, admixture of *E. coli* phenylalanyl-tRNA^{Phe} to the protein biosynthesizing system stimulated the production of functional α protein; the analogous result was obtained when valine was replaced by *E. coli* valyl-tRNA^{Val}. The ability of a misacylated tRNA to participate in α protein synthesis in this system was established by the use of *E. coli* phenylalanyl-tRNA^{Val} in the absence of added valine. Protein biosynthesis proceeded normally and gave a product having the approximate molecular weight of tryptophan synthase α subunit; as expected, this polypeptide lacked catalytic activity.

The study of gene expression has been greatly facilitated by the development of cell-free protein biosynthesizing systems in which transcription and translation are coupled (Zubay, 1980; Yang et al., 1980). These coupled systems have varied in complexity from relatively simple S-30 homogenates (Nirenberg & Matthaei, 1961) to more complex systems reconstructed from purified, isolated components (Herrlich & Schweiger, 1974; Yang et al., 1980). Such in vitro protein biosynthesizing systems have been employed to examine the

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‡ University of Virginia.
§ University of Illinois at Chicago.

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