The predicted amino acid sequence of the α subunit of the rat liver mitochondrial ATP synthase has been obtained by sequencing a cDNA for the α subunit. Analysis of the sequence shows that it contains the A and B consensus sequences found in many nucleotide-binding proteins. Twelve amino acids of the rat liver α subunit differ from the sequence of the bovine heart α subunit; four of these involve differences in charge.

The rat liver α subunit, from arginine 15 to the C-terminal proline 510, has been overexpressed in Escherichia coli using the alkaline phosphatase promoter (phoA) and leader peptide to direct the export of the expressed protein to the bacterial periplasm. By treating the cells with lysozyme, osmotic shock, and alkaline pH washes, the α subunit can be extracted in high yield (>25 mg/liter) and in a high state of purity. The expressed α subunit remains soluble at pH 9.5 or greater and precipitates when treated with Mg2+ ions at low millimolar concentration.

The bacterially expressed α subunit interacts with 2′(3′)-O-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP), resulting in a marked fluorescence enhancement upon binding. An enhancement of fluorescence is also observed upon the interaction of the α subunit with TNP-ADP. Preincubating the α subunit with 1.5 mM ATP significantly reduces the fluorescence enhancement seen with TNP-ATP. The α subunit binds TNP-ATP with an apparent Kd in the low millimolar range (1–5 μM) and binds TNP-ADP with an affinity at least 10-fold lower.

This work shows that the rat liver α subunit can be overexpressed in E. coli to yield a large amount of functional protein. With the acquisition of the overexpressed α subunit, it is now possible to test the reconstitution of ATPase activity from a mixture of recombinant and rat liver-derived subunits and to test the formation of complexes by the overexpressed α and β subunits of the rat liver F1-ATPase.

The mitochondrial ATP synthase is responsible for the production of the majority of the cellular ATP required by eu- karyotic organisms to meet their energy needs. ATP synthesis occurs on the synthase complex and is coupled to the dissipation of the electrochemical gradient existing across the mitochondrial inner membrane. The enzyme complex is composed of two major units: F0, which is embedded within the inner membrane; and F1, which lies in the matrix region. F0 is a hydrophobic complex and is believed to be a highly selective pathway for directing protons across the inner membrane (for reviews, see Refs. 1–5). F1 is composed of five subunits (α, β, γ, δ, and ε) at a stoichiometric ratio of 3:3:1:11, respectively (6–9). Synthesis of ATP occurs on F1, and ATP is released from a tight binding site on F1, at the expense of the energy present in the electrochemical proton gradient. Although known to be necessary for the formation of an active complex, the role of the α subunit in this catalytic mechanism remains unclear. The α subunit has a magnesium-independent, adenine-specific nucleotide-binding site (10–12) which in Escherichia coli binds ATP and ADP with Kd values of 0.1 and 0.9 μM, respectively (10). Upon binding ATP, a significant conformational change in the α subunit has been detected in experiments which show that the complex of the α subunit and ATP has a decreased sensitivity to trypsin proteolysis, an increased sedimentation coefficient, an increased mobility on nondenaturing polyacrylamide gels, and changed behavior in small-angle x-ray scattering (11, 13, 14). Nevertheless, the role of this nucleotide-binding site remains unclear as studies of the E. coli α subunit suggest that it is neither a hydrolytic nor a regulatory site (2, 12, 15). In addition, it appears that reconstitution of the α, β, and γ subunits into a functional complex does not require the α subunit-binding site to be occupied (16).

In this study, the nucleotide and predicted amino acid sequences and high-yield overexpression in E. coli of a cDNA for the α subunit of the rat liver ATP synthase are reported. The rat liver α subunit sequences are compared to the other known mammalian mitochondrial α subunit sequences: the bovine heart α subunit (17) and a partial sequence of the bovine liver α subunit (18). As expected, extensive sequence homology exists between the mammalian α subunits, consistent with the identity existing between the mammalian β subunits (17, 19, 20). The rat liver α subunit overexpressed in E. coli consists of almost the entire mature α subunit, lacking only the N-terminal 14 residues. This α subunit protein is shown here to be expressed at an extremely high level, to bind nucleotides, and to exhibit properties of solubility and interaction with Mg2+ ions that closely parallel those of the α subunit protein derived from rat liver.

**EXPERIMENTAL PROCEDURES**

**Materials**

Restriction enzymes were purchased from New England Biolabs, United States Biochemical Corp., Bethesda Research Laboratories.
Pharmacia LKB Biotechnology Inc., and Boehringer Mannheim and were used according to the manufacturers' instructions. A rat liver mRNA cDNA library was obtained from Clontech Laboratories. Sequencing and Klenow fragment were from United States Biochemical Corp. T4 DNA ligase, universal sequencing 17-mer primers, plasmid pUC18, and bacterial strain JM101 were purchased from New England Biolabs. Deoxy- and dideoxynucleotide triphosphates and random primers for cDNA synthesis were from Pharmacia LKB Biotechnology Inc. Labeled dATP was from Amersham Corp., and 32P-labeled dATP was from ICN. Laboratory chemicals were from Sigma and J. T. Baker Chemical Co. The bovine ascorbic acid protein assay was from Pierce Chemical Co. Acrylamide and gel reagents were purchased from Bio-Rad, and amorphous dithionite from FMC Corp. Phosphatase, NTA (diethyldithiocarbamate), DEAE (DEAE-cellulose) membranes were obtained from Schleicher & Schuell. The DNA Synthesis Facility of the Department of Biochemistry of The Johns Hopkins School of Hygiene and Public Health prepared the five 17-mer oligonucleotide primers. TNP-nucleotides were purchased from Molecular Probes Inc., and their purities were confirmed by chromato- graphy on polyethyleneimine-cellulose plates (C-200 PEI, Brinkmann Instruments) in a solvent system containing 2 M formic acid and 0.5 M LiCl. The polynucleotide heart a subunit cDNA was a gift from Dr. J. E. Walker (Medical Research Council, Cambridge, Great Britain). Expression plasmid pFOG402 and bacterial strain SE6004 were the gifts of Dr. D. Shortle (The Johns Hopkins School of Medicine).

Methods

Library Screening—A rat liver cDNA library was screened with a [32P]dATP-labeled partial bovine heart a subunit cDNA using the method of Benton and Davis (21). The hybridization probe was prepared by labeling the cDNA by a method using random primers and the Klenow fragment of DNA polymerase I (22). During the first round of screening, 50,000 plaques were screened at a density of 300–400 plaques/cm2. Potential positive plaques were picked and carried through two more rounds of screening by plating and hybridization at lower plaque densities. Phage DNA was then isolated as described in Maniatis et al. (23).

Analysis of Clones—Restriction digests of the clones were analyzed, after separation by electrophoresis on 0.8% agarose gels, by hybridization of the bovine heart cDNA with the digests transferred into nitrocellulose filters. The filters were hybridized with 32P-labeled cDNA; washed to a stringency of 0.3 M sodium chloride, 30 mM sodium citrate, 0.1% SDS, pH 7.0, and 60 °C; and subjected to autoradiography at −70 °C with an intensifying screen. The largest inserts were excised from the phage DNA with EcoRI, isolated on an agarose gel, recovered from the gel using DEAE membranes according to the manufacturer's instructions, and ligated into the EcoRI site of pUC19. Plasmids were transformed into bacterial strain DH5α for phosphate, respectively; AMP-PNP, adenosine 5'-β,γ-iminotriphosphate; Bicine, N,N-bis(2-hydroxyethyl)glycine; MOPS, 3-(N-morpholino)propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; CAPS, 3-(cyclohexylamino)propanesulfonic acid; SDS, sodium dodecyl sulfate.

Chemical Co. The bicinchoninic acid protein assay was from Pierce Chemical Co. Acrylamide and gel reagents were purchased from Bio-Rad, and amorphous dithionite from FMC Corp. Phosphatase, NTA (diethyldithiocarbamate), DEAE (DEAE-cellulose) membranes were obtained from Schleicher & Schuell. The DNA Synthesis Facility of the Department of Biochemistry of The Johns Hopkins School of Hygiene and Public Health prepared the five 17-mer oligonucleotide primers. TNP-nucleotides were purchased from Molecular Probes Inc., and their purities were confirmed by chromato- graphy on polyethyleneimine-cellulose plates (C-200 PEI, Brinkmann Instruments) in a solvent system containing 2 M formic acid and 0.5 M LiCl. The polynucleotide heart a subunit cDNA was a gift from Dr. J. E. Walker (Medical Research Council, Cambridge, Great Britain). Expression plasmid pFOG402 and bacterial strain SE6004 were the gifts of Dr. D. Shortle (The Johns Hopkins School of Medicine).

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TNP-nucleotide concentration greater than 10 μM and without protein present. The line defined by the fluorescence intensities of TNP-nucleotides at least 0 μM was used as the standard. The calculated factors were then used to correct the fluorescence intensity data at TNP-nucleotide concentrations greater than 10 μM in the presence of protein.

The stoichiometries were estimated by extrapolation from the initial linear portion of the titration curves, where it is assumed that all substrates are bound, to the maximal fluorescence change. The apparent Kd values were estimated by the method of Stinson and Holbrook (30).

RESULTS AND DISCUSSION

Isolation of α Subunit cDNAs—A bovine heart α subunit cDNA fragment of ~1,400 base pairs in length was used as a probe. During the first round of screening, over 60 potential clones were identified from 750,000 plaques. After two more rounds of screening, a total of eight clones were isolated and ligated into the EcoRI site of plasmid pUC19. A blot analysis of these two subclones hybridized with the bovine heart α subunit cDNA confirmed their identity (data not shown).

Nucleotide and Amino Acid Sequence—Initially, DNA sequence information was obtained from two clones using the universal 17-mer forward and reverse primers on pUC19. To determine which portions of the α subunit mRNA were included in each plasmid subclone, the 5’ and 3’ ends of the inserts were sequenced and translated into amino acid sequence. Comparison with the bovine heart α subunit sequence (17) revealed that the larger clone did not contain all of the coding region, but consisted of α subunit sequence from glutamate 14 to a poly(A)-containing region following ~500 bases of 3’-untranslated sequence. The smaller 1.7-kilobase clone, however, coded for all of the mature α subunit in addition to some of the 5’- and 3’-flanking sequences and was chosen for complete DNA sequence analysis. Both strands of the 1.7-kilobase clone were sequenced in their entirety using the strategy outlined in Fig. 1. The nucleotide sequence of the cDNA and the predicted amino acid sequence are presented in Fig. 2. The cDNA consists of 1,629 nucleotides of open reading frame followed by a 3’-untranslated segment 89 nucleotides long. The cDNA codes for a basic 55,256-Da protein (computed isoelectric point of 8.8) containing 510 amino acids of the mature protein and 33 amino acids of a likely mitochondrial import peptide. The sequence of the import peptide is presumed to be incomplete because an in-frame initiation codon was not found and because a longer import peptide is presumed to be incomplete because an in-frame initiation codon was not found and because a longer import peptide sequence is expected by comparison to the import peptide sequence of the bovine heart α subunit (44) and the bovine liver α subunit (18), which are 43 amino acids in length.

Comparison of the rat liver amino acid sequence to the sequence of the N-terminal 316 amino acids available from the bovine liver α subunit (18) reveals no sequence differences. However, there are 12 residues that differ between the complete rat liver sequence and the complete bovine heart α subunit sequence (17), with four of these differences involving changes in the charge of the residue. The rat liver α subunit is 510 amino acids long and contains a proline residue at the C terminus. The bovine heart α subunit cDNA predicts an alanine residue predicted from the bovine heart α subunit cDNA (44). Putative nucleotide-binding domains A and B (17, 32-37) are underlined, The A domain extends from glycine 169 to asparagine 190, and the B domain extends from glycine 261 to aspartate 269. The termination codon is marked by three asterisks.
from arginine 128 to glutamate 465 (339 amino acids) contains only a single amino acid difference. These changes may explain the observed differences in the binding of nucleotides to the rat and bovine F1 ATPases, both in stoichiometry and affinity. The heart enzyme binds AMP-PNP with a Kd value as low as 0.1 mM, whereas the rat liver enzyme binds AMP-PNP with a Kd value of ~1 mM (31, 32). Also, only four nucleotide-binding sites are readily detectable in the rat liver enzyme, whereas six are observed in the bovine heart enzyme (31, 32). However, it must be emphasized that the bovine heart and rat liver α subunits share almost 97% identity at the amino acid level, indicating their close evolutionary relationship. Moreover, both proteins contain certain conserved regions which are found in many nucleotide-binding enzymes. The glycine-rich A domain and the B domain regions, which have been proposed as parts of the nucleotide-binding domain from studies of elongation factor Tu and adenylate kinase (17, 33–37), are both found in the rat liver α subunit. The conservation of these sequences among many species may indicate their involvement in the common structural and functional properties of nucleotide-binding proteins.

Comparison of the calculated isoelectric points of the rat liver α and β subunits suggests a possible means of association between the members of an αβ pair within the F1 complex. The isoelectric point of the α subunit is 8.84, in contrast to that of the β subunit of 4.7. At the physiological pH of the mitochondrial matrix (pH 7.5), the α subunit will be positively charged, and the β subunit will be negatively charged. Thus, charge-charge interactions may be involved in forming the pairing of the α and β subunits within F1.

Expression and Purification of Rat Liver α Subunit—Using the method developed in this laboratory by Garboczi et al. (27), the α subunit was overexpressed in E. coli. The BamHI-digested α subunit cDNA, coding for Arg-15 as the first amino acid, was ligated into expression plasmid pFOG402 directly after the alkaline phosphatase promoter and in-frame with the alkaline phosphatase leader peptide. The advantages of this vector are the strength of the promoter and the transport of the expressed protein to the periplasmic space where the leader peptide is removed (38, 39). Extracting the overexpressed protein from the periplasmic space can simplify the purification protocol. Cells containing the expression vector were induced by growth in low phosphate media (see “Methods”). The α subunit protein was obtained at almost 40% of total protein as based on the SDS-polyacrylamide gel of the culture (Fig. 3A, lane 2). Unprocessed α subunit protein is present because, most likely, the rate of expression overtakes the rate of leader peptide processing, leading to the accumulation of unprocessed protein. An immunoblot analysis of total bacterial protein with rat liver α subunit (lower arrowhead) and the processed α subunit (upper arrowhead) to differ in mobility, consistent with the size of the leader peptide; lane 3, 10 µg of the purified soluble α subunit preparation after high pH washes and dialysis (see “Methods”) now showing only a small quantity of unprocessed α subunit. B, immunoblot analysis of overexpressed α subunit. Lane 1, 50 µl of bacterial culture containing the expression plasmid. Antibody reactivity is seen at three bands: the unprocessed α subunit (upper arrowhead), the processed α subunit (middle arrowhead), and a proteolytic fragment of the α subunit (lower arrowhead) which is not visible on the SDS-polyacrylamide gel. Lane 2, 50 µl of bacterial culture without the expression plasmid. The light reactivity seen here may be due to the E. coli α and β subunits.

NaH2PO4, pH 12.5, released the α subunit protein from the membranes in high yield. During the three alkaline washes, 5 mM ATP was included in the buffer to stabilize the α subunit protein.

Dialyzing the protein in a dialysis membrane with a molecular weight cutoff of 50,000 eliminated most of the smaller contaminating proteins, resulting in a purity of ~90%. Fig. 3A (lane 3) shows the purity of the α subunit obtained through this method. A small amount (<10%) of unprocessed α subunit protein remains in the preparation. The yield of the purification procedure is ~25–30 mg/liter of culture.

Solubility of a Subunit as Function of pH—The pH-dependent precipitation of the α subunit is shown in Fig. 4A. The α subunit was soluble at alkaline pH values (pH ~9.5). At pH values below 9.5, precipitation increased dramatically, with the α subunit protein becoming completely insoluble at approximately pH 6.0.

The precipitation of the α subunit at pH 7.5 indicates that the α subunit, by itself, may not be soluble in the matrix of the mitochondrion before or during assembly of the F1 complex. Interestingly, when an equal molar concentration of β-C4 (27), a 358-residue rat liver β peptide from glutamate 122 to the C-terminal serine 479, was added to the α subunit, precipitation of the α subunit was sharply reduced (data not shown). Thus, the β-C4/α subunit mixture is more soluble than the α subunit alone at pH values found in the mitochon-
Subunit of Rat Liver Mitochondrial ATP Synthase

FIG. 4. A, solubility of α subunit as function of pH. The concentration of the α subunit was 4 μM, and the pH was varied as indicated. The percent solubility was determined after 3 h of incubation at 25 °C. The percent solubility is the amount of protein soluble divided by total protein (180 μg). The purity of the α subunit is ~90%. B, interaction of α subunit with Mg2+. The concentration of the α subunit was 4 μM in 40 mM MBG buffer, pH 9.5. The percent solubility was determined as described for A.

Effects of Mg2+ on Solubility of α Subunit—Previously, it was shown by Pedersen et al. (42) that the rat liver F1 complex dissociates into two fractions in the presence of 5 mM Mg2+. They found that all of the α subunit and some of the γ subunit precipitate, whereas the β, δ, ε, and the remaining γ subunits remain soluble. Significantly, the overexpressed α subunit is also found to precipitate in the presence of high concentrations of Mg2+ (Fig. 4B). This result suggests that Mg2+ interacts with the α subunit, inducing a change to an insoluble conformation. From Fig. 4B it can be seen that 5 mM MgCl2 caused about 60% of the α subunit protein to precipitate, closely corresponding to the level of cation required to completely inhibit the ATPase activity of the native F1 complex. This loss of activity has been shown to be accompanied by the dissociation of the F1 complex and the precipitation of the α subunit (42). In this study, the addition of an equal concentration of ATP to the Mg2+ ions prevented the precipitation of the α subunit; yet when a subsequent excess of Mg2+ ions was added, the precipitation of the α subunit was again induced. Addition of AMP failed to protect the α subunit from precipitation by Mg2+, whereas the protective effect of ADP was very small (data not shown). These results show that Mg2+ ions induce precipitation of the α subunit protein, whereas Mg2+ complexed to ATP and, to a lesser extent, to ADP does not. This suggests that a low affinity binding site for Mg2+ exists on the α subunit. These results also indicate that the Mg2+-induced α subunit precipitation phenomenon observed by Pedersen et al. (42) may be due to the binding of Mg2+ ions to the α subunit of the F1 ATPase.

Interaction of α Subunit with TNP-nucleotides—To establish whether the overexpressed α subunit protein functions as a nucleotide-binding protein, its interaction with TNP-nucleotides was examined. TNP-nucleotide analogs have been shown to undergo significant fluorescence enhancement upon interaction with the beef heart mitochondrial F1-ATPase (43). The binding curves shown in Fig. 5A were generated by adding 0–20 μM TNP-nucleotides to 4 μM α subunit protein in a 4-ml quartz cuvette (see “Methods”). TNP-ATP exhibited a large fluorescence enhancement upon interaction with the α subunit. TNP-ADP also showed a fluorescence enhancement, although much smaller than that of TNP-ATP. There was no enhancement with TNP-AMP. The stoichiometries for the interactions of TNP-ATP and TNP-ADP with the α subunit were both ~1.0. The Kd value for the binding of TNP-ATP was in the low micromolar range (1–5 μM); and for TNP-ADP, the Kd value was about 10 fold higher (10 60 μM). A similar difference in binding affinities of ATP and ADP to
the E. coli α subunit has been reported (10).

The addition of 1.5 mM ATP to the α subunit prior to titration with TNP-ATP significantly reduced the subsequent fluorescence enhancement of TNP-ATP (Fig. 5B). Furthermore, the addition of TNP-ATP failed to increase further the maximal fluorescence enhancement observed when 15 μM TNP-ADP interacted with the α subunit. Also, TNP-ADP failed to increase the maximal fluorescence enhancement observed when 15 μM TNP-ATP interacted with the α subunit (data not shown). Together, these results indicate that the α subunit contains a single nucleotide-binding site which can interact with adenine nucleotides.

In summary, this work reports the cDNA cloning, the amino acid sequence, the overexpression, and some properties of the rat liver α subunit of the mitochondrial ATP synthase. The α subunit can be expressed at a very high level in E. coli and retains the capacity to interact with adenine nucleotides. The availability of large amounts of pure α subunit should facilitate further investigation of its role in the structure and function of the rat liver ATP synthase.

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Mitochondrial ATP synthase. cDNA cloning, amino acid sequence, overexpression, and properties of the rat liver alpha subunit.
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