Mutational Analysis of the Consensus Nucleotide Binding Sequences in the Rat Liver Mitochondrial ATP Synthase β-Subunit*

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The coupling step in the biosynthesis of ATP in biological systems is generally believed to involve an energy-requiring release of ATP bound to the β-subunit of the ATP synthase complex. A molecular description of the ATP binding site on the β-subunit is, therefore, critical to understanding the mechanism of coupling in the enzyme. Previously, we reported that a purified, bacterially expressed rat liver β-subunit binds adenine nucleotides tightly and specifically (Garboczi, D. N., Hullihen, J. H., and Pedersen, P. L. (1988) J. Biol. Chem. 263, 15694-15698). In order to assess the contribution of various regions of the isolated β-subunit to the ATP binding site we have systematically deleted four different regions: the N-terminal region, the Walker A consensus region, the Walker B consensus region (Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. (1982) EMBO J. 1, 945-951), and a “C” region, which, like the A and B regions, bears homology to adenylate kinase. Plasmids directing the expression of double deletions of A and B regions, and B and C regions were also constructed. In addition, 2 residues outside of these regions, His-177 and Tyr-345, which have been predicted to play a central role in nucleotide binding, were mutated. Rabbit antisera to synthetic peptides of the A and C regions verified the identity of the bacterially expressed mutant proteins.

Seven of the eight mutant proteins overexpressed in *Escherichia coli* were resistant to *E. coli* proteases in the preparative stages, as predicted for compact folded proteins. Furthermore, circular dichroism spectropolarimetry revealed no profound structural alterations in the purified mutant proteins. Relative to the overexpressed full-length β-subunit, the mutant lacking the A consensus region suffered a 30-fold loss of affinity for ATP and a loss of specificity for 2′(3′)-0-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP) over 2′(3′)-O-(2,4,6-trinitrophenyl)adenosine 5′-monophosphate. The mutant proteins lacking either the N-terminal region or the B region exhibited nucleotide binding properties similar to the full-length β-subunit, whereas the mutant protein lacking the C region suffered an order of magnitude reduction in affinity for ATP. The affinity of the A and B region double deletion was indistinguishable from the A region deletion in regard to TNP-ATP binding, while the double deletion mutant lacking the B and C regions was not stably expressed in the *E. coli* SE6004. Mutant proteins bearing the single-site changes, His-177 → Gln or Tyr-345 → Cys, exhibited nearly identical binding properties to that of the full-length β-subunit.

This is the first study in which regions of the isolated β-subunit of an ATP synthase complex have been systematically assessed by mutational analysis for their contribution to ATP binding. The results indicate that the A consensus sequence contributes over one-half of the total binding energy, with the C region making a significant contribution. The N-terminal region, the B region, Tyr-345, and His-177 appear to play little or no role in ATP binding.

Most cells’ energy needs are filled by ATP formed on the ATP synthase complex. This multisubunit enzyme couples the movement of protons down an electrochemical gradient (∆\(\mu\)H\(^{+}\)) to the production of ATP from ADP and \(\Pi\). As the equilibrium constant on the surface of the enzyme is near 1 (1), it has been proposed that the free energy present in ∆\(\mu\)H\(^{+}\) supports the release of bound ATP from the enzyme (2-5). Therefore, release of product and, thus, binding may be a critical step in coupling oxidative metabolism to ATP synthesis.

The ATP synthase complex is composed of two functional entities: the F\(_{1}\) portion, which mediates the passage of protons across the lipid membrane, and the F\(_{0}\) portion, which contains the nucleotide binding sites (see Refs. 6-8 for reviews). Both the isolated α-subunit (9) and β-subunits (10-12) of the F\(_{0}\) enzyme from a variety of sources bind adenine nucleotides. However, several nucleotide photoaffinity probes that inhibit catalysis label only the β-subunit (13-17). This fact, along with evidence that at least some isolated β-subunit preparations slowly catalyze ATP hydrolysis (18-20), indicates that this subunit contains, most if not all, the catalytic nucleotide binding site.

The β-subunit of the mitochondrial ATP synthase complex exhibits several regions of sequence homology with other ATP-utilizing enzymes. Four such regions of homology have been identified in the β-subunit: the Walker A (GX4GKT) and B (RXs$_{s}$,D, where X is any residue and H is any hydrophobic residue) consensus regions (21), found in adenylate kinase and a diverse group of ATP-dependent proteins (22), a third region also homologous to adenylate kinase (24), here called the C region, and a fourth region of sequence homologous to the P-type ATPases, here called the P region (25). Finally, residues in the N-terminal portion of the β-subunit have been implicated in nucleotide binding by photoaffinity labeling (26). The A, B, and C regions in the rat liver mitochondrial ATP synthase β-subunit are shown in Fig. 1. From homology arguments, these regions comprise the bulk of the catalytic site. Conservation of these motifs, and the fact that they each reside within an exon (Fig. 1), may indicate...
that they operate as modules during evolutionary processes.

Central to the mechanism for ATP synthesis is the requirement for proton movement through F1, to act either directly at the active site on F1 (27) or indirectly, via a DeltaH+--induced conformational change within F0, or smaller F1 subunits, which is transmitted to the binding pocket (3). Two conserved residues that may be involved in a binding affinity change at the active site are Tyr-345 and His-177. Although the tyrosine residue at position 345 labeled by nucleotide photoaffinity probes (13, 14) has been predicted to play an important role in the catalytic ATP synthesis mechanism, mutagenesis studies on the E. coli F1 enzyme indicate that this residue is not required for ATP hydrolysis (28). A histidine residue at position 177, which lies close in primary sequence to the A region, has also been proposed, on the basis of homology arguments, to form part of the catalytic binding site (25).

Although many very informative mutagenesis studies have been conducted on either the complete F1 moiety or the intact membrane ATP synthase complex at the level of the beta-subunit (for example see Refs. 29-33), there has been a systematic search for isolated beta-subunit. In fact, the relative contributions of the various homologous regions to nucleotide binding, a necessary first step in describing the catalytic site, remains unknown. In experiments reported below, we have assessed the relative contributions to ATP binding of various regions throughout the beta-subunit using overexpressed, purified beta-subunit preparations rather than the beta-subunit within intact F1.

**EXPERIMENTAL PROCEDURES**

**Materials**

Restriction enzymes were obtained from New England Biolabs, Boehringer Mannheim, and Bethesda Research Laboratories. Sequenase was supplied by U. S. Biochemical Corp. T4 DNA polymerase and universal sequencing primers were from New England Biolabs. Polynucleotide kinase and dideoxynucleotide triphosphates were purchased from Pharmacia LKB Biotechnology Inc. Other oligonucleotide primers were synthesized in the Department of Biological Chemistry, Protein- Peptide facility, as were peptides used to construct the leader sequence.

**Methods**

**Plasmid Construction, Purification, and Sequencing**—The plasmid directing the overexpression of the beta-subunit, pDGH9, was constructed by ligating the EcoRI-EcoRI fragment and EcoRI-HindIII fragments of the beta-subunit cDNA (24) and a BamHI-EcoRI oligonucleotide linker with the HindIII-BamHI vector fragment of pFPG402 in which the BamHI site had been made blunt with Klenow before digestion with HindIII. After the initial ligation the second BamHI site was filled in with the Klenow fragment of DNA polymerase in the presence of dNTPs followed by precipitation of DNA ligase to close the blunt ends, thereby producing the proper reading frame. The resulting plasmid codes for an Asp-Pro that increases the efficiency of processing of the leader sequence. The plasmid pDGH9, which directs the expression of a fragment of the beta-subunit from 122 to 479, denoted as DN, previously as C4, was constructed as described previously (10). A plasmid directing the overexpression of the Delta peptide was constructed by digesting pDGH9 with Styl, gel-purifying the largest band, and ligating. Deletion of the beta-subunit cDNA between the StyI sites in the resulting pH9A4 was verified by sequencing (34). A plasmid directing overexpression of the Delta peptide was constructed in an analogous manner, but the restriction enzyme PvuII was employed and pH9AC was formed.

The overexpression of the Delta peptide was directed by the plasmid pH9A5. To form the construct, pDGH9 (3 pg) was digested with Ncol and BgII and both bands were gel-purified. The smaller band, containing base pairs 516-1320 of the beta-subunit cDNA (24), was digested with NcoI and BstXI, and the 465- and 161-base pair bands were gel-purified. These two fragments, along with the larger fragment from the Ncol-BstII digestion were ligated with a double-stranded AatI-BstXI linker to form the closed circular plasmid.

The linker contains a unique Nhel site that was used to verify the four-piece ligation. Circular plasmids, which were linearized by Nhel in the screening process, were used to transform E. coli S50004 to ampicillin resistance. Transformants were induced to express D to as described previously (34), and production of a peptide of the appropriate size was assessed by analyzing a whole cell lysis by 15% SDS-polyacrylamide gel electrophoresis. Plasmas were purified from candidate clones, and all actions from the ligation were sequenced to verify the proper construction.

Plasmas directing the overexpression of DELTA and DELTAC were constructed as for pH9A5 and pH9AC plasmids described above, except that pH9A5 was used as the starting material rather than pH9AC. All plasmids were purified from alkaline/SDS lysates of the host strain (35). Bands from agarose gel separations of restriction digests were purified with DEAE paper according to the manufacturer's instructions. Sequencing templates were formed using a polyethylene glycol precipitation step and alkaline denaturation (34). Sequencing was carried out using the dideoxy chain termination method (36). All other molecular biological methods are described by Sambrook et al. (37).

**Single-site Mutagenesis**—Site-directed mutagenesis of Tyr-345 was carried out directly on the plasmid by mutant primer extension on a denatured double-stranded sequencing template; A mixed oligonucleotide primer (50 ng), 5'-GCC ATC T(G, C,T)T CCA GCT GTG-3', denatured double-stranded sequencing template. A mixed oligonucleotide primer (50 ng), 5'-GCC ATC T(G, C,T)T CCA GCT GTG-3', with an equal proportion of G, C, and T in the number 9 position was phosphorylated with T4 kinase (7.5 units) and 10 mM ATP in 50 mM Tris-Cl, 10 mM MgCl2, 5 mM dithiothreitol, pH 7.5, for 1 h at 37 °C. This reaction was terminated by addition of 15 mM DTT and subsequent heating to 70 °C for 8 min. The phosphorylated primer mix (12 ng) was hybridized 45 min at 37 °C and 2 h at 25 °C to 500 ng of uridine containing pDGH9 template prepared as previously described (34). T4 polymerase (10 units) was used to extend the A reaction mutant primer to generate antisense primers for the expression plasmid construction; AA, AN, C, H177Q, AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamine; H177Q, mutant AN P-subunit peptide in which histidine 177 was mutated to glutamin
displace the newly synthesized strand if extension should complete the circuit around the template. The reaction was initiated for 10 min at 4 °C, continued for 10 min at 25 °C, and completed for 8 h at 37 °C. The mixture was used to transform CaCl2-competent E. coli SE6004 to ampicillin resistance. Plasmids from 11 ampicillin-resistant colonies were isolated, denatured, and sequenced. One plasmid contained the mutant codon (TTT) coding for a cysteine residue at position 345.

The single-site mutation H177Q was selected from a group of mutations generated by a random misincorporation procedure previously described (34) and was verified by direct DNA sequencing.

Peptide Synthesis, Antibody Production, and Western Blotting—Peptides were synthesized on an Applied Biosystems model 430A peptide synthesizer using the solid phase method (41). Two peptides were synthesized: one corresponding to the A region and one corresponding to the C region. Both peptides were conjugated through a cysteine residue to BSA (Fraction V), emulsified in adjuvant, and injected into New Zealand White rabbits. The adjuvant for the first injection was Freund’s complete adjuvant; subsequent injections were Freund’s incomplete adjuvant.

For immunodetection of the overexpressed β-subunit peptides, SDS-polyacrylamide gels were rinsed in 10 mM CAPS, pH 11.1, 10% methanol for 15 min and then transferred to Immobilon polyvinylidenedifluoride membranes at 250 mA for 3 h in the same buffer. Membranes were blocked three times for 15 min each in 300 ml 0.1% gelatin, 0.2% Brij-58, 150 mM NaCl, 10 mM Tris-Cl, pH 7.5. Partially purified IgG fractions (42) of the rabbit antisera, preabsorbed with 1% BSA and 1% E. coli acetone powder (43), were reacted with the blots for 1 h at 25 °C. Antisera were diluted in gelatin blocking buffer with 0.1% BSA and stored frozen. Blots were detected with the enhanced chemiluminescence method as per the manufacturer’s recommendations, except the secondary antibody was used at a dilution of 1:20,000 and membranes were washed with the gelatin blocking buffer, with a brief rinse in water immediately prior to detection to remove the detergent.

Circular Dichroism Spectroscopy and Prediction of Secondary Structure—Spectra were collected on an AVIV 60DS spectropolarimeter thermostatted to 20 °C in a 0.01-cm path-length Helma Suprasil demountable cuvette. A step size of 1 nm, a bandwidth of 1.5 nm, and a dwell time of 2 s were used to collect five scans. Purified β-subunit peptides were in 2 mM Tris-Cl, pH 7.5. The β-subunit fraction obtained from rat liver F1 (39) was in 50 mM Tris-Cl, pH 7.4. CD spectra were deconvoluted into their constituent secondary structures using the basic reference curve set derived by convex constraint analysis (44) and the Lincomb program (45). Secondary structures were predicted from the β-subunit primary sequence using two separate algorithms (46, 47).

RESULTS

Production, Overexpression, and Purification of Mutant β-Subunit Peptides—To systematically assess the importance

![Diagram](https://example.com/diagram.png)

**Fig. 1.** Regions of sequence homology in the β-subunit. A, four regions and 2 residues suggested to be involved in nucleotide binding and mutated in the present study are illustrated schematically. B, primary, predicted secondary, and exon boundaries in the β-subunit. The primary sequence of the rat liver β-subunit is shown in single-letter amino acid code (24). The secondary structure is the consensus prediction of two methods (46, 47); α-helices are represented as coils, β-strands are represented as arrows, and predicted turns are shown as T. The exon boundaries for tobacco β-subunit (61) (open stars) and human (60) (closed stars) are shown above the primary sequence. Deletions and truncations are indicated by the boxed sequence. Consensus sequences predicted to be involved in nucleotide binding and His-177 and Tyr-345 are indicated by the solid black bar above the primary sequence.
of specific β-subunit sequences to nucleotide binding function we have employed molecular genetics to delete these regions from the overexpressed β-subunit. Four separate regions of the β-subunit were removed in this manner: the N-terminal region, the glycine-rich loop or A region, the B region, and the C region (Fig. 1). Overexpression of the β-subunit mutants in E. coli SE6004 under control of the phoA promoter was induced by low phosphate (10). β-Subunit peptides were purified from the bacterial cultures by selective release under alkaline conditions (34). Analysis of the molecular weight of the overexpressed peptides by electrophoresis on SDS-polyacrylamide (10%) gels indicates that a polypeptide of the appropriate size was made for each construct (Fig. 2A), except for the ΔAC double deletion, which cannot be detected with protein stain in the gel. The E. coli appear to efficiently remove the leader sequence directing the recombinant β-subunit into the periplasmic space, as processing is evident when whole cell lysates are loaded on the gel (Fig. 2A) and because the apparent molecular weight on the gel is not large enough to account for the presence of the leader sequence (Table I). The overexpressed β-subunit peptide, as well as the N-terminal deletion, ΔN, and ΔN lacking either the A region, ΔA, the B, ΔB, or the C, ΔC, always are greater than 93% pure (Table I).

Purified immunoglobulins produced against synthetic peptides corresponding to the A and C regions of the β-subunit verified the identity of the overexpressed peptides (Fig. 2, B and C). Antibodies directed against the A region do not interact with the overexpressed ΔA peptide and antibodies directed against the C region do not bind to the overexpressed ΔC peptide. Interestingly, the C region antibody did not interact with the E. coli β-subunit, and neither the A peptide nor the C peptide antisera had any reactivity against the rat liver α-subunit of F1, even though this subunit has some homology with the β-subunit (9). (The A peptide antibody apparently cross-reacts with the endogenous E. coli F1 β-subunit, because a band is detected at approximately 50 kDa (Fig. 2B). Although this band is also detected in the purified β-subunit peptides, indicating the presence of some contaminating E. coli β-subunit, the Coomassie-stained gel shows that the level of contamination is extremely low (Fig. 2A)).

Two single-site mutations of residues predicted to be important in nucleotide binding and or hydrolysis (13, 14, 25) were also overexpressed and purified. Mutation of tyrosine 345 to cysteine, Y345C, was performed using a synthetic oligonucleotide with a mismatch changing the codon TAT to TTT. The mutant containing an asparagine residue in place of histidine at position 177, H177Q, was isolated from a collection of random misincorporation mutants that we produced previously (34). Both single-site mutants were overproduced at high levels and were purified (Fig. 2A). Both also were immunoreactive with the synthetic peptide antibodies (Fig. 2, B and C).

Secondary Structure of Purified β-Subunit Peptides—In order to assess the effect of these mutations on the secondary structure of the β-subunit CD spectropolarimetry was applied. Fig. 3 shows the spectra of the deletion mutant peptides and

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**TABLE I**

*Characterization of β-subunit peptides*

The β-subunit peptides were constructed, expressed and purified as described under "Methods." The number of residues is for only those arising from the β-subunit and does not include the 5 amino acids resulting from the expression construct.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Residues</th>
<th>Mutation</th>
<th>pI*</th>
<th>M, (calc.)*</th>
<th>M, (SDS)*</th>
<th>Stability†</th>
<th>Purity‡</th>
</tr>
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<tbody>
<tr>
<td>β</td>
<td>469</td>
<td>None</td>
<td>4.74</td>
<td>51</td>
<td>53.4</td>
<td>Yes</td>
<td>93</td>
</tr>
<tr>
<td>ΔN</td>
<td>358</td>
<td>Deleted to 122</td>
<td>4.72</td>
<td>39.4</td>
<td>41.6</td>
<td>Yes</td>
<td>95</td>
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<tr>
<td>H177Q</td>
<td>358</td>
<td>His → Gln</td>
<td>4.66</td>
<td>39.4</td>
<td>42</td>
<td>Yes</td>
<td>98</td>
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<tr>
<td>Y345C</td>
<td>358</td>
<td>Tyr → Cys</td>
<td>4.72</td>
<td>39.4</td>
<td>42.2</td>
<td>Yes</td>
<td>85</td>
</tr>
<tr>
<td>ΔA</td>
<td>292</td>
<td>ΔN, deleted 148–213</td>
<td>4.5</td>
<td>32.6</td>
<td>33.3</td>
<td>Yes</td>
<td>98</td>
</tr>
<tr>
<td>ΔB</td>
<td>325</td>
<td>ΔN, deleted 232–285</td>
<td>4.87</td>
<td>33.8</td>
<td>34</td>
<td>Yes</td>
<td>97</td>
</tr>
<tr>
<td>ΔC</td>
<td>306</td>
<td>ΔN, deleted 316–347</td>
<td>4.91</td>
<td>36</td>
<td>38.5</td>
<td>Yes</td>
<td>98</td>
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<tr>
<td>ΔBΔC</td>
<td>273</td>
<td>ΔN, ΔB, ΔC</td>
<td>5.17</td>
<td>30.5</td>
<td>31.3</td>
<td>No</td>
<td></td>
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<tr>
<td>ΔBΔB</td>
<td>259</td>
<td>ΔN, ΔA, ΔB</td>
<td>4.65</td>
<td>29.2</td>
<td>31.3</td>
<td>Yes</td>
<td>82</td>
</tr>
</tbody>
</table>

* Calculated from the primary structure.
† Linear interpolation of molecular weight using the standards in Fig. 2A.
‡ Defined as the ability to be expressed in E. coli in amounts detectable by Coomassie staining.
§ Determined by scanning densitometry of Coomassie-stained SDS-polyacrylamide gels.
of the rat liver \( \beta \)-subunit fraction purified from rat liver mitochondria using traditional biochemical methods (see "Methods"). Significantly, the \( \Delta N \) peptide and the Y345C mutant are nearly superimposable and show the same general shape as the \( \beta \)-subunit preparation (Fig. 3). All three exhibit double minima at 208 and 222 nm and a maximum at 192 nm characteristic of proteins containing significant \( \alpha \)-helical secondary structure (48). Deconvolution of the spectra (44, 45) into the respective secondary structural components (Table II) indicates that the \( \Delta N \) peptide consists of 30% \( \alpha \)-helical structure, 40% \( \beta \)-strand and turn structures, with the remaining structure being random coil. It should be noted that the term random coil does not indicate a lack of structure, but rather a nonrepetitive structure. Although these results do not indicate whether the mutant peptides are folded into a completely native structure, they do indicate that they are folded into a compact, secondary structure containing mass.

**Nucleotide Binding to Full-length \( \beta \)-Subunit**—As previously reported for an overexpressed and purified fragment of the rat liver \( \beta \)-subunit (10), the 474 amino acid \( \beta \)-subunit peptide expressed in *E. coli* and purified also binds adenine nucleotides. Fig. 4A demonstrates the saturable interaction of the \( \beta \)-subunit peptide with the fluorescent adenine nucleotide probe TNP-ATP. The fluorescent enhancement observed when the probe binds to the peptide is directly proportional to the amount of the ligand peptide complex formed and thus provides a convenient method for determining nucleotide binding (49). Significantly, ATP competes for the same binding site (Fig. 4B). Neither 10 mM AMP, 150 mM potassium phosphate, nor 600 mM KCl reduced TNP-ATP fluorescence, and, although Tris-C1 concentrations below 50 mM reduce the fluorescence maximum, the affinity for TNP-ATP was unchanged (data not shown). Furthermore, no binding was observed when 4 M urea was included in the binding buffer, indicating that the nucleotide binding function is dependent upon the higher order structure of the overexpressed \( \beta \)-subunit. Analysis of the binding data reveals a single binding site with an affinity for TNP-ATP in the low micromolar range and in the low millimolar range for ATP (Table III).

**Effect of Deletion Mutations on Nucleotide Binding Function**—The 363-amino acid \( \Delta N \) \( \beta \)-subunit peptide, which lacks 122 N-terminal amino acids, binds TNP-ATP and ATP with affinity similar to the 474-amino acid \( \beta \)-subunit peptide (Table III, Fig. 4, C and D). Moreover, \( \Delta N \) binds TNP-ADP with an apparent \( K_d \) of 7 \( \mu \)M and a stoichiometry of 0.85 (Fig. 4C, inset). TNP-AMP fluorescence was not significantly enhanced when incubated with the \( \Delta N \) peptide. Finally, deletion of the N-terminal 122 amino acids had no apparent effect upon the stoichiometry of TNP-ATP binding, indicating that this portion of the sequence is not involved in nucleotide binding to the overexpressed \( \beta \)-subunit.

Removal of the Walker A region (21) from the \( \Delta N \) peptide decreases the affinity for TNP-ATP and for ATP by more than an order of magnitude (Fig. 5, A and B, Table III). The remaining low affinity binding is no longer specific for TNP-ATP and TNP-ADP over TNP-AMP (data not shown). Deletion of the B region has little effect upon the ability of the peptide to bind adenine nucleotides. TNP-ATP and ATP affinities for the \( \Delta B \) peptide were both within 2-fold of the wild type peptide and the stoichiometry remained close to 1 (Table III, Fig. 5). When the C homology region is removed from the \( \Delta N \) peptide, the effect upon nucleotide binding is greater than that for the \( \Delta B \) mutant peptide, but less pronounced than the loss observed for the \( \Delta A \) peptide. The affinity of the \( \Delta C \) peptide for TNP-ATP is reduced by 3-5 fold, whereas that for ATP is reduced by an order of magnitude (Table III). In order to assess if these effects are additive, double deletion mutants were constructed. Unfortunately, the \( \Delta B\Delta C \) mutant is not stably expressed in *E. coli* SE6004 (Table I). However, the \( \Delta \Delta B \) peptide exhibited TNP-ATP binding indistinguishable from that of the \( \Delta A \) mutant peptide (Fig. 5), confirming that the B region has little role in nucleotide binding.

**Effect of Single-site Mutations on Nucleotide Binding Function**—Nucleotide binding to the two single-site mutants of residues, which have previously been implicated as participants at the catalytic binding site on the basis of photoaffinity studies (13, 14) and homology arguments (25), is shown in

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**TABLE II**

Secondary structure of \( \beta \)-subunit mutants

The percentage of each secondary structure (\( \alpha \)-helix, \( \beta \)-strand, turn, and random coil) was determined for each peptide by the primary sequence based predictions of Chou and Fasman (46) (CF) and Garnier *et al.* (47) (GOR), and by deconvolution of the CD spectra (Fig. 3) into their component parts (44, 45).

<table>
<thead>
<tr>
<th>Secondary structure</th>
<th>( \Delta N )</th>
<th>( \Delta A )</th>
<th>( \Delta B )</th>
<th>( \Delta C )</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Predicted</td>
<td>CD</td>
<td>Predicted</td>
<td>CD</td>
</tr>
<tr>
<td></td>
<td>GOR CF</td>
<td>%</td>
<td>GOR CF</td>
<td>%</td>
</tr>
<tr>
<td>( \alpha )-Helix</td>
<td>37 36 30</td>
<td>40 36 25</td>
<td>36 42 26</td>
<td>34 34 26</td>
</tr>
<tr>
<td>( \beta )-Strand</td>
<td>31 27 40*</td>
<td>32.5 27 23*</td>
<td>31 26 33*</td>
<td>34 28 33*</td>
</tr>
<tr>
<td>Turn</td>
<td>13 12 30</td>
<td>10 11 52</td>
<td>13 12 41</td>
<td>19 26 41</td>
</tr>
<tr>
<td>Random coil</td>
<td>19 25 30</td>
<td>17.5 26 52</td>
<td>19 21 41</td>
<td></td>
</tr>
</tbody>
</table>

* Deconvolution of the CD spectra give a combined percentage for \( \beta \)-strand and turn structures.
Fig. 4. Nucleotide binding by the $\beta$-subunit and $\Delta N$ peptides. Nucleotide fluorescence was measured as described under "Methods." A, TNP-ATP fluorescence upon interaction with 5 $\mu M$ $\beta$-subunit in 50 mM Tris-Cl, pH 7.4, in the presence (open circles) and absence (closed circles) of 4 M urea. B, ATP competition with 50 $\mu M$ TNP-ATP for the binding site on 5 $\mu M$ $\beta$-subunit. C, nucleotide fluorescence enhancement in the presence of 5 $\mu M$ of the $\Delta N$ peptide, TNP-ATP (closed circles), TNP-ADP (open circles), and TNP-AMP (+) were incubated with the peptide in 50 mM Tris-Cl, pH 7.4. D, ATP competition with 50 $\mu M$ TNP-ATP for the binding site on $\Delta N$.

**Table III**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$K_d$ TNP-ATP ($\mu M$)</th>
<th>$n$ TNP-ATP</th>
<th>$K_d$ ATP (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$</td>
<td>5</td>
<td>0.95</td>
<td>1.1</td>
</tr>
<tr>
<td>$\Delta N$</td>
<td>8</td>
<td>0.96</td>
<td>1.2</td>
</tr>
<tr>
<td>$\Delta A$</td>
<td>&gt;70</td>
<td>0.98</td>
<td>40</td>
</tr>
<tr>
<td>$\Delta B$</td>
<td>11</td>
<td>0.98</td>
<td>2.5</td>
</tr>
<tr>
<td>$\Delta C$</td>
<td>25</td>
<td>0.96</td>
<td>13</td>
</tr>
<tr>
<td>$\Delta A\Delta B$</td>
<td>&gt;70</td>
<td>0.98</td>
<td>ND</td>
</tr>
<tr>
<td>His-177</td>
<td>7</td>
<td>1.0</td>
<td>ND</td>
</tr>
<tr>
<td>Y345C</td>
<td>4</td>
<td>1.0</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, not determined.

Fig. 6. Once again, both mutants bind TNP-ATP with a stoichiometry of 1 (Table III). Moreover, the affinity of the H177Q and Y345C mutants for TNP-ATP is similar to that determined for the $\Delta N$ peptide, ruling out a significant role in ATP binding for either of these residues in the isolated subunit under the conditions employed.

**DISCUSSION**

The release of the product ATP by the ATP synthase complex is believed to be the major energy-requiring step in ATP synthesis (see Refs. 3, 6, and 7 for reviews). Therefore, defining the molecular details of ATP binding is fundamental to understanding how the enzyme is coupled to the electrochemical gradient of protons which provides the driving force for ATP synthesis. Central to this problem is a requirement for cataloging the individual residues involved in nucleotide binding. A first step toward this goal is to define specific regions of the $\beta$-subunit that are required for adenine nucleotide binding. In the present study we have overexpressed and purified a variety of rat liver $\beta$-subunit mutants in *E. coli* and tested their ability to interact with adenine nucleotides. The results of these analyses indicate that the A consensus sequence contributes fully one-half of the total binding energy, whereas the C region contributes approximately one-third of the total. Furthermore, the results indicate that neither the N-terminal region, the B region, Tyr-345, nor His-177 contribute significantly to adenine nucleotide binding to the isolated $\beta$-subunit.

The $\Delta A$ peptide lacks 65 amino acid residues that include the Walker A region and corresponds approximately to a single human $\beta$-subunit exon (Fig. 1). The deletion removes two predicted $\alpha$-helices and two predicted $\beta$-strands from the peptide. The dramatic reduction of ATP binding affinity observed in the purified recombinant $\beta$-subunit is in agreement with site-directed mutagenesis studies of the $\beta$-subunit within intact $F_1$, and membrane preparations from *E. coli* (29, 32, 33), thermophilic bacterium PS3 (30), and yeast (31). The latter studies indicate that both lysine and glycine residues are required for maximal catalytic activity and that the threonine may be involved in chelating the Mg$^{2+}$ at the active site.

The $\Delta B$ peptide retained a wild type-like nucleotide binding
to the binding site and might be predicted to play a role in affinity explaining the specificity function (Table 111). This result was somewhat surprising, changes occurring in the coupling process.

Finally, the results presented here do not support involvement of AMP and the lack of this specificity in the AA peptide. The loop, interacts with the pyrophosphate moiety of the nucleotide synthase \( \alpha \)-subunit. The model of the catalytic nucleotide binding site on the \( \alpha \)-subunit folds in a manner analogous to adenylate kinase (50) or Rec A (25). However, it should be stressed that the \( \beta \)-strand that is predicted within the B region occupies a different position in the folding pattern of different nucleotide-binding proteins (23). Furthermore, in the \( \Delta B \) peptide similar secondary structural elements may be in position (see Fig. 1) to replace the deleted elements, consistent with the fact that the B plus C deletion is not stably expressed \( E. coli \) (Fig. 2A).

Finally, it has been suggested that the aspartate residue at the end of the \( \beta \)-strand in the B region may be involved in chelating the \( \text{Mg}^{2+} \) required for catalysis (51), and, thus, although the \( \text{Mg}^{2+} \) independent nucleotide binding is unaffected, the region may be important for catalysis as indicated by mutagenesis studies of thermophilic \( \beta \)-subunits (30).

The C region is conserved in \( \text{F}_1 \), ATP synthase \( \beta \)-subunits from all species sequenced thus far, from \( E. coli \) to human (24, 62) and lies between the two tyrosine residues at positions 311 and 345 that are labeled by photoaffinity probes (13, 14, 16). NMR studies of the ATP binding site of adenylate kinase indicate that this region is in contact with the nucleotide (51); however, crystallographic studies with artificial substrates contradict this finding (50). Significantly, deletion of this region from the overexpressed \( \Delta N \) \( \beta \)-subunit peptide caused a diminution of nucleotide binding affinity (Fig. 5), supporting the involvement of this region in the catalytic site. Future studies of the individual conserved amino acid residues within this region are needed to confirm this functional role.

The deletion of the C region of the \( \beta \)-subunit also resulted in the removal of Tyr-345 at the C terminus of the consensus sequence. In order to assess whether loss of Tyr-345, rather than that of the C region, could account for the diminished binding, the tyrosine was mutated to a cysteine residue (Y345C). The Y345C mutation had no discernible effect on nucleotide binding indicating that other residues are responsible for the observed effect. However, the \( \Delta C \) peptide results do indicate that Tyr-345 may be in close proximity to the nucleotide binding site, in agreement with the results of Weber et al. (52) with the nucleotide analog \( l^- \text{in-benzo-ADP} \) on intact \( E. coli \) \( \text{F}_1 \). While the recent results indicate that Tyr-345 is not directly involved in nucleotide binding by the isolated \( \beta \)-subunit, they can not rule out a role for this residue at the binding site in \( \text{F}_1 \), or under other conditions. In this regard, it is important to note that several groups have proposed that the nucleotide binding sites on \( \text{F}_1 \) lie at \( \alpha/\beta \) interfaces (53, 54) an idea that is supported by the pattern of heavy atom labeling in the recent 3.5-\( \text{Å} \) map of the rat liver mitochondrial \( \text{F}_1 \) (55).

Another residue that has been predicted to comprise part of the binding site is the conserved His-177 (25). When His-177 was mutated to a glutamine residue, again, nucleotide binding is unaltered arguing against a role for this residue in binding, although ionization of this group cannot be ruled out as part of the binding change mechanism until more extensive investigation of this mutant and other missense mutants of His-177 is undertaken.

In the present study we have combined a single-site mutagenesis approach with a modular approach based upon the idea that certain structural motifs are heritable by some unknown means, as a unit during evolution thereby accelerating and making more efficient the process of evolution (56–59). In this second approach, deletions of the Walker A and B consensus sequences and of the C region were designed to avoid strongly predicted regions of secondary structure (as do the exon boundaries), and to correspond as closely as possible to exon boundaries (60, 61) (Fig. 1) or a gap in the alignment of known \( \beta \)-subunit sequences (62). Deletional analysis previously has been used to study the function of many other proteins (63–66). It should be noted that both the modular deletions and the single-site mutations, in some cases, may cause nonspecific structural changes that are unrelated to the actual involvement of the affected residues in nucleotide binding. Significantly, in this study we have demonstrated that the mutant peptides retain significant secondary structure (Fig. 3) and are stably expressed in \( E. coli \). In further support of the results reported here, the \( K_d \) values for the recombinant peptides studied here are in good agreement with those previously reported for \( \beta \)-subunits from \( E. coli \) (11) and chloroplasts (12). However, the low millimolar \( K_d \) observed for ATP to the isolated, overexpressed \( \beta \)-subunits is low compared to the micromolar \( K_d \) for the first binding site on rat liver \( \text{F}_1 \) (67), a result that may be due to the previously mentioned interaction with the \( \alpha \)-subunit or other modification of the catalytic site within intact \( \text{F}_1 \). Such subunit interactions may decrease the dielectric constant in the binding site thereby strengthening required electrostatic bonds. Alteration of these subunit interactions and, thus, the environment of the binding site may play an important role in affinity changes during the catalytic cycle. Although evidence has been presented indicating that the recombinant, overexpressed \( \beta \)-subunit exhibits secondary structural properties corresponding to a compact folded protein, the conformation of the \( \beta \)-subunit may be altered by its interaction with the \( \alpha \)-subunit, thus favoring tighter ATP binding.

The results presented here systematically quantify the relative contributions of four regions and two specific residues to nucleotide binding by the isolated \( \beta \)-subunit of the mitochondrial ATP synthase, a step fundamental to understanding the catalytic mechanism of the enzyme (Fig. 7). Although the results demonstrate that the A and previously unimplicated C region are involved in nucleotide binding, the specific residues within the C and A region that are directly responsible for nucleotide binding remain to be described. The evidence presented does not support a role for the B region...
in nucleotide binding, and, thus, the function of this highly conserved sequence may be limited to another step in the catalytic mechanism. Finally, we have developed specific antibodies that react with two well defined regions of the \( \beta \)-subunit. Future studies will utilize these immunoprobes to determine the surface accessibility and potential movement of the A and C sequences during the catalytic cycle.

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REFERENCES