Mitochondrial ATP Synthase

INTERACTION OF A SYNTHETIC 50-AMINO ACID, β-SUBUNIT Peptide WITH ATP*

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A 50-amino acid peptide predicted by chemical modification studies of F1, and by comparison with adenylate kinase to comprise part of an ATP-binding domain within the β-subunit of mitochondrial ATP synthase has been synthesized and purified. In the numbering system used for bovine heart β, the peptide consists of amino acid residues from aspartate 141 at the N-terminal end to threonine 190 at the carboxyl end.

In Tris-Cl buffer, pH 7.4, the peptide undergoes a dramatic reaction with ATP resulting in precipitate formation. Analysis of the precipitate shows it to contain both peptide and ATP. Similar to the ATPase activity of F1, and the binding of nucleotide to the enzyme, the capacity of ATP to induce precipitation of the activity is decreased markedly by lowering pH. Interaction of the peptide with the fluorescent ATP analog, TNP-ATP (2'(3')-O-(2,4,6-trinitrophenyl)-adenosine 5'-triphosphate), can be demonstrated in solution at low concentrations. A 7-fold enhancement in fluorescence is observed when 2.5 μM TNP-ATP interacts with 2.5 μM peptide. Divalent cation is neither required for ATP-induced precipitation of the peptide nor for demonstrating interaction between TNP-ATP and peptide, just as Mg2+ is not required for nucleotide binding to F1.

These results indicate that the β-subunit peptide studied here comprises at least part of a nucleotide-binding domain within the mitochondrial ATP synthase complex.

The mitochondrial ATP synthase consists of two major components, one called F0, which spans the inner membrane and the other called F1, which projects into the matrix space. F0 is thought to direct protons derived from the respiration-driven electrochemical proton gradient to F1, a water-soluble complex consisting of five different subunits in the stoichiometric ratio α3βδγε (for recent reviews, see Refs. 1-5). F1, upon binding ADP and P, synthesizes ATP on its surface with an equilibrium constant near 1, and is thought to utilize the proton gradient to effect release of bound ATP (6-10). These events are generally believed to take place on the β-subunit as this subunit binds ATP and hydrolyzes this nucleoside triphosphate at a low rate (11-13).

Recent work from several laboratories (14-18) has emphasized structural similarities between the β-subunit of F1 and certain nucleotide-binding proteins, in particular adenylate kinase, the ras p21 transforming protein, and elongation factor Tu. All of these proteins show some sequence homology in a region near a glycine-rich flexible loop. X-ray crystallographic studies of elongation factor Tu indicate that at least part of the nucleotide-binding domain may reside near the glycine-rich flexible loop (17, 18). Similar conclusions have been reached from NMR studies of MgATP binding to a 50-amino acid peptide of adenylate kinase (15, 16).

In extending these analogies to the β-subunit of F1, Duncan et al. (19) proposed a three-dimensional model for a nucleotide-binding domain, presumably the catalytic site, within the β-subunit of ATP synthase. Support for this model came from both chemical modification and genetic studies. Significantly, in this model much of the ATP-binding domain is contained within the 50-amino acid stretch depicted in Fig. 1A. In analogy to the three nucleotide-binding proteins described above, the triplyphosphate portion of ATP is placed near a glycine-rich flexible loop, in this case with the α-phosphate within binding distance of lysine 162. The adenosine portion of the molecule is embedded within the same 50-amino acid stretch between an α-helical stretch and a β-pleated sheet.

In order to test directly the above model, we have synthesized the 50-amino acid, F1-β-subunit peptide described above and examined its interaction directly with ATP, TNP-ATP, and a variety of other ligands.

EXPERIMENTAL PROCEDURES

Materials

All the Boc-protected amino acids, Boc-Thr(OBz1)-OCH3-PAM-resin, and the reagents and solvents for the peptide assembly were purchased from Applied Biosystems, Inc. Trifluoroacetic acid, p cresol, p-thiocresol, and dimethyl sulfoxide were supplied by Aldrich. Hydrogen fluoride was from Matheson Gas Products, Inc., and acetonitrile and methylene chloride (both HPLC grade) were obtained from American Burdick & Jackson, Inc. Amino acid standards and phenylisothiocyanate were obtained from Pierce Chemical Co. ATP and ADP were obtained from Pharmacia LKB Biotechnology Inc. ITP, GTP, AMP-PNP, MgCl2, Tris-Cl and Tris acetate were purchased from Sigma, whereas P, was obtained from J. T. Baker Chemical Co. TNP-ATP was purchased from Molecular Probes and its purity confirmed by chromatography on polyethyleneamide-lose plates (Cel-300 PEI, Brinkmann Instruments) in a solvent system containing 2 M formic acid and 0.5 M LiCl. SDS, acrylamide, and bisacrylamide were purchased from Bio-Rad. Radioactive nucleotides were obtained from ICN Radiochemicals and Budget-Solve from Research Products International.

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1 The abbreviations used are: TNP-ATP, 2'(3')-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate; AMP-PNP, adenyI-5'-ylimidodiphosphate; HPLC, high pressure liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Boc, t-butoxycarbonyl.
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RESULTS AND DISCUSSION

Purity and Characterization of the F, β-Subunit Peptide (PP-50) Used in These Studies—

Methods

Synthesis of the 50-Amino Acid Residue Peptide (PP-50)—The peptide was synthesized on an Applied Biosystems Model 430A peptide synthesizer by the solid phase method developed by Merrifield (20). Peptide assembly was carried out starting with Boc-Thr(Obz1)-2-chlorobenzyloxycarbonyl for the c-amine of lysine; 2-bromobenzyloxycarbonyl for the phenolic hydroxyl group of tyrosine. Aspartic acid was protected with phenylisothiocyanate to yield the corresponding mercapto anhydride activation procedure. Side chain protecting groups were as follows: benzyl ether for the hydroxyl group of threonine and benzyl ester for the carboxyl group of aspartic and glutamic acid; benzyl ether for the hydroxyl group of threonine 190 and includes lysine 162 and glutamate 188, known to be essential for F₁,β-ATPase activity (30-32). It is this 50-amino acid residue peptide (PP-50) that was synthesized and used in these studies. B, amino acid sequence of PP-50. Note that in addition to aspartic acid 141 at the N-terminal end of PP-50 to alanine aspartic acid 141 at the N-terminal end of PP-50 to alanine was equilibrated with buffer A at a flow rate of 2 ml/min and about was eluted with a gradient of 0-60% B over 60 min to fractionate the peptide. The fractions were analyzed and those containing only the pure peptide were pooled and freeze-dried. The yield of pure peptide was 12.5 mg.

Amino Acid Analysis—This was performed using the PICO-TAG amino acid analysis system of Waters-Millipore. The peptide (about 120 μg) was hydrolyzed with 6 N HCl containing 1% phenol by volume at 110 °C for 24 h. The hydrolysate was dried and the amino acids derivatized with phenylisothiocyanate to yield the corresponding phenylthiocarbamyl derivatives following standard procedures (21). These derivatives were analyzed on the PICO-TAG amino acid analysis system which had been previously calibrated with a standard mixture of amino acids.

Quantification of PP-50—PP-50 was quantified by the method of Lowry et al. (22) using bovine serum albumin as standard. This procedure gave values for PP-50 standards within 5% of those given by quantitative amino acid analysis. For stoichiometry calculations a molecular weight for PP-50 of 5815 was used. This was based on the known amino acid composition of PP-50 and corrected for water split out during peptide bond formation and trifluoroacetic acid salt formation with seven amino functional groups.

Interaction of PP-50 with TNP-ATP—This was monitored fluorometrically in a 4 ml quartz cuvette (Starna Suprasil) containing in a total volume of 2 ml, 10 mM Tris-Cl, pH 7.4, and concentrations of TNP-ATP and PP-50 indicated in the legend to Fig. 5. A Gilford Fluoro-4 Fluorometer set at an excitation wavelength of 410 nm and emission wavelengths indicated in the legend to Fig. 5 was used for these studies.

Gel Electrophoresis of PP-50 in SDS—SDS-PAGE was carried out by a modification of the Weber and Osborn procedure (23) in 10% polyacrylamide gels as described previously (24).

Methods Relevant to F₁,ATPase—F₁ was purified to homogeneity from rat liver mitochondria by the procedure of Catterall and Pedersen (25). Binding of α-labeled [βγδ]ATP to this enzyme was assessed by the column centrifugation procedure of Garrett and Penefsky (26) exactly as described previously (27). Initial ATPase rates were monitored by quantifying the release of inorganic phosphate by the procedure of Omori (28).

Measurement of Radioactivity—Radioactivity was measured in a Beckman LS 100C series spectrometer. PP-50 precipitated in the presence of α-labeled [βγδ]ATP was dissolved in 150 μl of NaOH; 50 μl was added to 10 ml of Budget-Solve and assessed for radioactivity and 100 μl was used to determine protein as described above. When binding of α-labeled [βγδ]ATP to F₁ was carried out, 100 μl of the eluate from the centrifugation column was added to 10 ml of Budget-Solve and assessed for radioactivity.

RESULTS AND DISCUSSION

Purity and Characterization of the F, β-Subunit Peptide (PP-50) Used in These Studies—

Methods employed in the synthesis and purification of PP-50 are described in detail under “Methods.” Two batches of PP-50 have been synthesized de novo and exhibit essentially identical purity and behavior patterns. As shown in Fig. 2, three methods were used to examine the purity of PP-50 prior to investigating its capacity to interact with ATP. Fig. 2A shows that, upon HPLC chromatography on a μBondapak C₁₈ column, PP-50 elutes as a single peak, nearly gaussian in shape. The inset in Fig. 2A shows that PP-50 migrates as a single band upon SDS-PAGE in a 10% polyacrylamide gel system. As expected from its predicted molecular weight of 5815, PP-50 migrates noticeably faster than cytochrome c (Mₐ, 11,700). Significantly, there is no suggestion from either the HPLC chromatogram or from SDS-PAGE of larger molecular weight species that might have resulted during chemical synthesis. As shown in Fig. 2B the amino acid composition of PP-50 compares favorably (within experimental error) with the predicted amino acid composition of the peptide. Finally, in data not presented here we have confirmed the sequence from aspartic acid 141 at the N-terminal end of PP-50 to alanine 176.

Interaction of PP-50 with ATP—Initial experiments showed that when ATP is added to PP-50 in 50 mM Tris-Cl buffer, pH 7.4, a rather dramatic precipitation of PP-50 occurs which can be observed visually. Precipitation is visually ob-
Fig. 2. Evidence for purity of PP-50. A, HPLC chromatogram of PP-50. Chromatography was carried out on a 3.78 x 30-cm µBondapak C8 column. PP-50 (10 µg) was injected in a volume of 10 µl. Elution was effected with a gradient from 30 to 60% buffer B which consisted of acetonitrile + 0.03% trifluoroacetic acid. PP-50 in the eluate was detected at 220 nm using an ultraviolet detector. Inset, SDS-PAGE of PP-50 relative to that of cytochrome C, which had an electrophoretic mobility of 11,700. In each case, 2.5 µg of protein was electrophoresed. B, amino acid composition of PP-50. The predicted amino acid composition of PP-50 is compared with that obtained experimentally by carrying out amino acid analysis by the PICO-TAG procedure (see "Methods").

The ligands indicated below were added at a final concentration of 1 mM to 27 µg of PP-50 in 10 mM Tris-Cl, pH 7.4, (final volume, 0.1 ml). The mixture was allowed to incubate at room temperature for 2.5 min in a Brinkman microfuge. The amount of peptide in the sediment was then quantified exactly as described under "Methods." Values are averages of duplicate determinations.

Table I provides a quantitative picture of the specificity of the precipitation phenomenon should be noted. Thus, AMP, P, and Mg2+ have little capacity to induce precipitation of PP-50. Under these conditions, the ATP/PP-50 stoichiometric ratio, determined radioactively as described under "Methods," was found in five different experiments to be 0.91 ± 0.08.

Fig. 3 demonstrates that ATP-induced precipitation of PP-50 is sharply dependent on pH. Lowering the pH to 4 markedly suppresses the capacity of ATP to induce precipitation of PP-50 (Fig. 3A). Significantly, the capacity of F1-ATPase to bind AMP-PNP (Fig. 3B) and to hydrolyze ATP (Fig. 3C) is also markedly diminished as pH is decreased. It will be noted also in Fig. 3, A and B, that added Mg2+ is not required to facilitate either ATP-induced precipitation of PP-50 or binding of nucleotide to F1.

Fig. 4 shows that the reaction described above can be readily demonstrated in solution provided ATP is replaced with the fluorescent ATP analog TNP-ATP. Significantly, at 2.5 µM PP-50 and 2.5 µM TNP-ATP, a 7-fold enhancement in fluorescence of the latter species is observed (Fig. 4A). This finding is of particular interest as Grubmeyer and Penefsky (34) reported previously that TNP-ATP is a strong competitive inhibitor of F1, undergoing a 7-fold enhancement in fluorescence when added in equimolar amounts to F1. Fig. 4B demonstrates the dependence of the fluorescence enhancement response on TNP-ATP concentration. A half-maximal response is observed at less than 0.5 µM TNP-ATP demonstrating a very high apparent affinity of PP-50 for this nucleotide analog. It is shown also in Fig. 4B that excess ATP added prior to TNP-ATP to PP-50 markedly reduces the fluorescence enhancement response, consistent with interaction of both nucleotides with the same binding domain on PP-50.

Taken together, results presented here are consistent with the view that PP-50 contains at least one part of a nucleotide-binding domain. The finding that precipitation of PP-50 is induced only by ligands with a pyrophosphate or triply phosphate moiety indicates that, contained within this peptide, is a region which interacts strongly with the phosphate ester region of nucleotides.
these low concentrations of peptide, PP-50 remains in solution in the presence of ATP.) The relative fluorescence enhancement plotted in three-dimensional structure of F1.

A functional change is induced by binding substrate. Nonpolar tested promote precipitation of PP-50 suggests that a conformational change in solution throughout the catalytic/functional cycle. This is emphasized by the recent results of Cross et al. (35) who have shown that 2-azido-ATP labels tyrosine 345, suggesting that this residue may also lie near or within the catalytic domain. Although tyrosine 345 lies outside the adenylate kinase homology region, Cross et al. (35) suggest that it may then become exposed to the aqueous medium. Along these lines it is of interest to note that ATP has been shown recently to induce a similar precipitation response when added to a 19-amino acid "active site" peptide of actin (36). It is tempting to suggest, therefore, that certain ATP-binding proteins may contain one or more regions, peripheral to nucleotide-binding domains, which are critical for maintaining such domains in solution throughout the catalytic/functional cycle.

Future experiments will focus on the effect of site-directed changes on the interaction of PP-50 with ATP and TNP-ATP as well as the capacity of other synthetic β-subunit peptides to facilitate ATP hydrolysis when combined with PP-50.

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REFERENCES

1. Hatefi, Y. (1985) Annu. Rev. Biochem. 54, 1015–1069
2. Amzel, L. M., and Pedersen, P. L. (1983) Annu. Rev. Biochem. 52, 801–824
3. Senior, A. E., and Wise, J. G. (1983) J. Membr. Biol. 73, 105–124
4. Wang, J. H. (1983) Annu. Rev. Biophys. Bioeng. 12, 21–24
5. Cross, R. L. (1981) Annu. Rev. Biochem. 50, 681–714
6. Sakamoto, J., and Tonomura, Y. (1983) J. Biochem. (Tokyo) 93, 1601–1614
7. Kandpal, R. P., Stempel, K. E., and Boyer, P. D. (1987) Biochemistry 26, 1512–1517
8. Grubmeyer, C., Cross, R. L., and Penefsky, H. S. (1982) J. Biol. Chem. 257, 12092–12100
9. Boyer, P. D., Cross, R. L., and Monsen, W. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 2837–2839
10. Penefsky, H. S. (1985) J. Biol. Chem. 260, 13735–13741
11. Khananshvili, D., and Groetz-Ehlanan, Z. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 1886–1890
12. Harris, D. A., Boork, J., and Baltzschetisky, M. (1985) Biochemistry 24, 8287–8288
13. Roux-Frome, M., Neumann, J.-M., Andre, F., Berger, G., Girault, G., Galimiche, J.-M., and Remy, R. (1987) Biochem. Biophys. Res. Commun. 144, 718–725
14. Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) EMBO J. 1, 945–951
15. Fry, D. C., Kubo, S. A., and Mildvan, A. S. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 907–911
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16. Fry, D. C., Kuby, S. A., and Mildvan, A. S. (1985) \textit{Biochemistry} \textbf{24}, 4680–4694
17. Jurnak, F. (1985) \textit{Science} \textbf{230}, 32–36
18. McCormick, F., Clark, B. F. C., LaCour, T. F. M., Kjeldgaard, M., Norskov-Lauritsen, L., and Nyborg, J. (1985) \textit{Science} \textbf{230}, 78–82
19. Duncan, I. M., Parsonage, D., and Senior, A. E. (1986) \textit{FEBS Lett.} \textbf{208}, 1–6
20. Merrifield, R. B. (1968) \textit{J. Am. Chem. Soc.} \textbf{85}, 2149–2154
21. Bidlingmeyer, B. A., Cohen, S. A., and Tarvin, T. L. (1984) \textit{J. Chromatogr.} \textbf{336}, 93–104
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) \textit{J. Biol. Chem.} \textbf{193}, 265–275
23. Weber, K., and Osborn, M. (1969) \textit{J. Biol. Chem.} \textbf{244}, 4406–4412
24. Pedersen, P. L., and Hullihen, J. (1978) \textit{J. Biol. Chem.} \textbf{253}, 2176–2183
25. Catterall, W. A., and Pedersen, P. L. (1971) \textit{J. Biol. Chem.} \textbf{246}, 4987–4994
26. Garrett, N. E., and Penefsky, H. S. (1975) \textit{J. Biol. Chem.} \textbf{250}, 6640–6647
27. Williams, N., Hullihen, J., and Pedersen, P. L. (1987) \textit{Biochemistry} \textbf{26}, 162–169
28. Gomori, G. J. (1962) \textit{J. Lab. Clin. Med.} \textbf{27}, 955–960
29. Parsonage, D., Wilke-Mounts, S., and Senior, A. E. (1987) \textit{J. Biol. Chem.} \textbf{262}, 8022–8026
30. Andrews, W. W., Hill, F. C., and Allison, W. S. (1984) \textit{J. Biol. Chem.} \textbf{259}, 14378–14382
31. Andrews, W. W., Hill, F. C., and Allison, W. S. (1984) \textit{J. Biol. Chem.} \textbf{259}, 8219–8225
32. Ohtsubo, M., Yoshida, M., Ohta, S., Kagawa, Y., Yohda, M., and Date, T. (1987) \textit{Biochem. Biophys. Res. Commun.} \textbf{146}, 706–710
33. Pedersen, P. L. (1976) \textit{J. Biol. Chem.} \textbf{251}, 934–940
34. Grubmeyer, C., and Penefsky, H. S. (1981) \textit{J. Biol. Chem.} \textbf{256}, 3718–3727
35. Cross, R. L., Cunningham, D., Miller, C. G., Xue, Z., Zhou, J-M., and Boyer, P. D. (1987) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{84}, 5715–5719
36. Barden, J. A., and Kemp, B. E. (1987) \textit{Biochemistry} \textbf{26}, 1471–1478