Communication

The a_{leu207→arg} Mutation in F_{1}F_{0}-ATP Synthase from Escherichia coli

A MODEL FOR HUMAN MITOCHONDRIAL DISEASE*

(Received for publication, March 23, 1993)
Phillip E. Hartzog and Brian D. Cain‡

From the Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, Florida 32610

The mitochondrial ATPase 6 gene encodes a subunit of F_{1}F_{0} adenosine triphosphate (ATP) synthase. A mutation in the ATPase 6 gene has been genetically linked to two maternally inherited genetic diseases: neurologic muscle weakness, ataxia, and retinitis pigmentosa (NARP) and certain cases of subacute necrotizing encephalopathy (SNE). Although the severity of both NARP and SNE disease were correlated with the quantity of the ATPase 6_{leu156→arg} mutation in each patient, the mutation could not be shown to alter F_{1}F_{0}-ATP synthase activity. To investigate the biochemical effects of the ATPase 6_{leu156→arg} mutation on F_{1}F_{0}-ATP synthase, the a_{leu207→arg} mutation was constructed in the F_{1}F_{0}-ATP synthase from Escherichia coli to serve as a model for the disease mutation. Characterization of the model bacterial enzyme revealed that the mutation abolishes detectable ATP synthesis via oxidative phosphorylation. The a_{leu207→arg} mutation results in a structural perturbation blocking proton translocation through F_{1}F_{0}-ATP synthase. The results suggest that a structural defect in human F_{1}F_{0}-ATP synthase is the biochemical basis for NARP and SNE.

Oxidative phosphorylation is the primary aerobic pathway for adenosine triphosphate (ATP) synthase. The electron transport chain generates an electrochemical gradient of protons which is used to drive ATP production by F_{1}F_{0}-ATP synthase. Specific subunits of the electron transport chain and enzymes and of F_{1}F_{0}-ATP synthase are encoded in the mitochondrial genome (mtDNA), and abnormalities in the mtDNA result in defects in oxidative phosphorylation (1–3). Diseases of the mitochondrial genome display maternal inheritance because virtually all mitochondrial enzymes and a subunit of the electron transport chain of procaryotes (Fig. 1). Extensive site-directed mutagenesis studies of the a subunit in the E. coli F_{1}F_{0}-ATP synthase have demonstrated that the a subunit is essential for proton translocation (13–25). The location of the disease-linked ATPase 6_{leu156→arg} mutation at a site displaying strong sequence conservation suggested that the biochemical basis for NARP and SNE might be a defect in proton translocation through the human F_{1}F_{0}-ATP synthase.

Leu-207 in the a subunit of E. coli F_{1}F_{0}-ATP synthase occupies the position comparable to Leu-156 in the human ATPase 6 subunit (Fig. 1). To determine if the ATPase 6_{leu156→arg} mutation caused a defect in F_{1}F_{0}-ATP synthase accounting for the loss of oxidative phosphorylation in NARP and SNE patients, we have constructed the a_{leu207→arg} mutation in the E. coli enzyme. Biochemical characterization of the recombinant F_{1}F_{0} synthase demonstrated that the a_{leu207→arg} mutation was sufficient for a loss of oxidative phosphorylation. The present work clearly establishes the direct applicability for modeling human oxidative phosphorylation disease mutations in a bacterial system.

EXPERIMENTAL PROCEDURES

Materials—T-4 DNA ligase, T-4 polynucleotide kinase, proteinase K, and restriction endonucleases were supplied by Bethesda Research Laboratories and New England Biolabs (Beverly, MA). Radiolabeled nucleotides were purchased from Amersham Corp. Sequenase DNA sequencing materials were provided by U. S. Biochemical Corp. L-lysine, desoxyribonuclease I, pyruvate kinase, and lactate dehydrogenase were purchased from Sigma. Difco Laboratories (Detroit, MI) was the source of bacterial growth media. All other reagents and chemicals were ob-
**RESULTS**

**Effect of the Leu-207 → Arg Mutation on Oxidative Phosphorylation**—Oxidative phosphorylation is required for utilization of tricarboxylic acid cycle intermediates as carbon sources for aerobic growth, so growth of mutant strains on succinate-based medium is a sensitive test for F₁,Fₒ-ATP synthase function in vitro. The recombinant plasmid pUNCB4.70 (*a<sub>207</sub>arg*) and the control plasmids pH12 (*a*) and pH11 (*a*) were studied by complementation of the a subunit-defective *E. coli* strain RH305 (*a<sub>207</sub>arg*). Growth on succinate medium was scored as colony formation and designated as normal colonies (**+++**) or no colonies (**−**). Protein concentrations were measured using the modified Lowry procedure of Markwell et al. (32). Membrane energization (750 pg of protein/3 ml of buffer; buffer: 50 mM MOPS and 10 mM MgCl₂, pH 7.3) was assayed by fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) (33). ATP hydrolysis was measured by a coupled assay to regenerate ATP in the system (25).

**Table I**

Properties of the Leu<sup>207</sup>→Arg mutation

| Plasmid/strain<sup>2</sup> | Mutation | Growth on succinate<sup>3</sup> | Membrane-bound<sup>4</sup> | DCCD-sensitive<sup>4</sup> |
|--------------------------|----------|-------------------------------|-------------------------|-------------------------|
| pH12                     | *a*      | +++                           | 0.75 ± 0.10             | 0.32 ± 0.05             |
| pH11                     | *a*      | −                             | 0.58 ± 0.06             | 0.09 ± 0.03             |
| pUNCB4.70                | Leu<sup>207</sup>→Arg | −                             | 0.42 ± 0.05             | 0.04 ± 0.01             |
| 1100ABC                  | F₁,Fₒ<sub>Δ</sub> | −                             | 0.19 ± 0.00             | 0.01 ± 0.02             |

<sup>2</sup>Plasmids were transformed into strain RH305 (<sup>a</sup> = *a*; <sup>−</sup> = *a*<sub>207</sub>arg).<br>
<sup>3</sup>Growth on succinate was scored as colony formation and designated as normal colonies (**+++**) or no colonies (**−**).<br>
<sup>4</sup>Specific activity was measured at pH 8.0, and the values are the average of two assays obtained at two protein concentrations.<br>
<sup>5</sup>DCCD-sensitive activity is the ATPase activity lost after treatment with 50 μM DCCD for 1 h at room temperature.<br>
<sup>6</sup>The activity reported for strain 1100ABC (26) denotes membrane activity contributed by enzymes other than F₁,Fₒ-ATP synthase.

**Fig. 2.** F₁,Fₒ-ATP synthase-mediated ATP-driven vesicle acidification as determined by ACMA fluorescence quenching. Membrane vesicles and assays were performed as described previously (25). Arrows mark the addition of ATP and nigericin. Traces: *a*<sup>+</sup>, strain RH305 carrying pH12 (*a*); *a*<sup>+</sup>, strain RH305 carrying pH11 (*a*); leu<sup>207</sup>→Arg, strain RH305 carrying pUNCB4.70 (*a<sub>207</sub>arg*).
cles when Fo was properly assembled. The membranes from cells carrying plasmid pUNC4.70 (aLeu207→Arg) showed levels of membrane-associated ATPase activity equivalent to the negative control pH11 (α-), indicating that the mutation affects either assembly or stability of Fo (Table I). DCCD inhibits F1F0-ATP synthase by covalently modifying the proteolipid subunit (α subunit in E. coli) blocking proton translocation and inhibiting coupled ATP hydrolysis activity. The residual membrane-associated F1 activity from cells carrying plasmid pUNC4.70 (aLeu207→Arg) was uncoupled since DCCD-sensitive ATPase activity was far below that of the wild type control (Table I). In summary, the Leu-207 → Arg mutation apparently caused a perturbation in the structure of Fo that disrupts proton translocation and its coupling to catalytic function in F1F0-ATP synthase.

**DISCUSSION**

The results indicate that the aLeu207→Arg mutation is sufficient to abolish detectable F1F0-ATP synthase function in *E. coli*. The biochemical basis for the defect is a failure in Fo-mediated proton translocation resulting from a structural perturbation in the enzyme complex. The structural and functional similarities between *E. coli* and mitochondrial F1F0-ATP synthases suggests that both share a common molecular mechanism and that observations in the bacterial system are directly applicable to mutations occurring in the human enzyme. Therefore, the phenotype of the aLeu207→Arg mutation observed here in *E. coli* implies that the human ATPase 6Leu156→Arg mutation linked to NARP and SNE also results in a similar defect in F1F0-ATP synthase leading to a failure of oxidative phosphorylation.

Studies of the *E. coli* F1F0-ATP synthase α subunit indicated that many positions were sensitive to missense mutations (13-25). Mutations replacing the conserved amino acids in the α subunit with basic amino acids, such as occurs with the human ATPase 6Leu156→Arg mutation, frequently resulted in substantial losses of enzyme function (13, 20, 25). Similar mutations undoubtedly occur in the human mitochondrial ATPase 6 gene at positions other than Leu-156, implying that a large number of point mutations in the conserved region of ATPase 6 should be sufficient for significant losses in capacity for synthesis of ATP. Recently, mutations in mtDNA have also been linked to degenerative diseases associated with aging (2). Given that the mutation rate is 10-20-fold higher in mtDNA as compared to the nuclear genome (3), missense mutations in the ATPase 6 gene are likely to be among the most common mutations affecting human F1F0-ATP synthase.

Point mutations affecting the ATPase 6 gene are likely to remain undiagnosed in the clinical setting, making it difficult to assess the contributions of these defects to degenerative disease. NARP patients do not display the gross mitochondrial abnormalities or the lactate imbalances characteristic of mitochondrial genetic diseases associated with electron transport deficiencies (6). Other ATPase 6 gene mutations, whether inherited or arising through somatic mutation, will probably exhibit a similar pathology. Additionally, most point mutations in the ATPase 6 gene cannot be expected to change a restriction endonuclease recognition sequence, so restriction fragment length polymorphism analysis will not suffice as a definitive diagnostic approach. In view of the large number of a subunit mutations known to impair F1F0-ATP synthase function in bacteria, it seems appropriate to consider a systematic survey of tissue samples from patients with degenerative disorders for missense mutations in the ATPase 6 gene.

**Acknowledgments**—We thank Dr. Philip Laipis and Dr. Michael Kilberg for comments on the preparation of this manuscript.

**REFERENCES**

1. Shoffner, J. M., and Wallace, D. C. (1990) Adv. Hum. Genet. 19, 267-320
2. Wallace, D. C. (1992) Science 256, 628-632
3. Wallace, D. C. (1990) Annu. Rev. Biochem. 61, 1175-1212
4. Gylseesten, U., Wharton, D., Jossefson, A., and Wilson, A. C. (1991) Nature 352, 255-257
5. Ashley, M. V., Laipis, P. J., and Hauswirth, W. W. (1989) Nucleic Acids Res. 17, 7325-7331
6. Holt, J. I., Harding, A. E., Perry, R. K. H., and Morgan-Hughes, J. A. (1990) Am. J. Hum. Genet. 46, 428-433
7. Tatch, Y., Christodoulou, J., Feigenschbaum, A., Clarke, J. R. T., Wherret, J., Smith, C., Rudd, N., Petrova-Benedict, R., and Robbinsen, B. H. (1990) Am. J. Hum. Genet. 50, 552-568
8. Shoffner, J. M., Fynholt, P. M., Krawiecki, N. S., Coplan, D. B., Holt, P. J., Koontz, D. A., Takan, Y., Newman, N. J., Ortiz, R. G., Polsk, M., Ballinger, S. W., Lott, M. T., and Wallace, D. C. (1992) Nature 426, 2168-2174
9. Futai, M., Noumi, T., and Maeda, M. (1990) Annu. Rev. Biochem. 59, 111-136
10. Senior, A. E. (1990) Annu. Rev. Biochem. 60, 7-41
11. Fillingame, R. H. (1990) Bacterial Energetics: The Bacterics (Kuwrich, T. A., ed) Vol. XII, pp. 345-391, Academic Press, New York
12. Walker, J. E., Saraste, M., and Gay, N. J. (1994) Biochim. Biophys. Acta 768, 164-200
13. Cain, B. D., and Simoni, R. D. (1988) J. Biol. Chem. 263, 6606-6612
14. Cain, B. D., and Simoni, R. D. (1988) J. Biol. Chem. 263, 10043-10050
15. Lightowlers, R. N., Howitt, S. M., Hatch, L., Gibson, F., and Cox, G. B. (1987) Biochim. Biophys. Acta 884, 399-406
16. Lightowlers, R. N., Howitt, S. M., Hatch, L., Gibson, F., and Cox, G. B. (1988) Biochim. Biophys. Acta 933, 74-80
17. Vik, S. B., Cain, B. D., Chun, K. T., and Simoni, R. D. (1998) J. Biol. Chem. 263, 6599-6605
18. Eya, S., Noumi, T., Maeda, M., and Futai, M. (1988) J. Biol. Chem. 263, 10956-10962
19. Eya, S., Noumi, T., Maeda, M., and Futai, M. (1988) J. Biol. Chem. 264, 2292-3300
20. Cain, B. D., and Simoni, R. D. (1988) J. Biol. Chem. 264, 270-284
21. Lightowlers, R. N., Howitt, S. M., Hatch, L., Gibson, F., and Cox, G. B. (1987) Biochim. Biophys. Acta 933, 241-248
22. Howitt, S. M., Gibson, F., and Cox, G. B. (1988) Biochim. Biophys. Acta 936, 74-80
23. Vik, S. B., Lee, D., and Marshall, F. A. (1991) J. Biol. Chem. 266, 628-632
24. Lightowlers, R. N., Howitt, S. M., Hatch, L., Gibson, F., and Cox, G. B. (1988) Biochim. Biophys. Acta 933, 241-248
25. Schaefer, E. M., Hartz, D., Cold, L., and Simoni, R. D. (1988) J. Bacteriol. 171, 3901-3905
26. Miller, J. H. (1972) Experiments in Molecular Genetics, pp. 131-135, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. Promega (1997) KRT Sequencing Systems Technical Manual, Madison, WI
28. United States Biological Corp. (1987) Step-by-Step Protocols for DNA Sequencing with Sequenase, 3rd Ed., Cleveland, OH
29. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring, NY
30. Klonsky, D. J., Brasile, W. S. A., and Simoni, R. D. (1985) J. Biol. Chem. 258, 10136-10143
31. Markwell, M. A. K., Ries, S. M., Bieber, L. L., and Tolbert, N. E. (1989) Biochim. Biophys. Acta 11207-11215
32. Geis, E., Brown, T. A., Waring, R. B., Scanzuchio, C., and Davies, R. W. (1992) Nucleic Acids Res. 10, 3531-3539
33. Morelli, G., and Macino, G. (1984) J. Mol. Biol. 178, 491-507
34. Holmes, J., and Herrmann, R. G. (1986) Mol. Gen. Genet. 205, 117-128
35. Protein Identification Resource (1992) release 31, National Biomedical Research Foundation, Washington, DC