An Asp–Asn substitution in the proteolipid subunit of the ATP-synthase from *Escherichia coli* leads to a non-functional proton channel

J. Hoppe, H.U. Schairer, P. Friedl and W. Sebald

Department of Stoffwechselregulation, GBF, Gesellschaft für Biotechnologische Forschung mbH., Mascheroder Weg 1, D-3300 Braunschweig-Stöckheim, FRG

Received 22 June 1982

1. INTRODUCTION

A carbodiimide binding proteolipid subunit has been identified in all ATP synthase complexes [1–8]. Due to its oligomeric form it constitutes the major part of the membrane factor F_0 which in *Escherichia coli* is composed of three different subunits *a, b, c* (proteolipid) [7,8]. Dicyclohexylcarbodiimide binds covalently to the proteolipid leading to an inhibition of the proton translocation through the membrane [1–8]. Sequence analysis of ATP synthase proteolipid subunits from various organisms (*Neurospora crassa, Saccharomyces cerevisiae*, bovine heart, chloroplasts (spinach), *mastigocladus laminosus* (blue green algae) and bacteria (*E. coli, PS-3, Bacillus acido caldarius*) revealed an invariant acidic amino acid located in a very hydrophobic stretch of amino acids which is selectively modified by the hydrophobic reagent dicyclohexylcarbodiimide [9–15].

From *E. coli* numerous ATP synthase mutants of the *uncB* phenotype have been isolated [16,17]. These mutants contain a functional F_1 moiety but the F_0-part does not translocate protons after removal of F_1. Previously, we described the characterization of the *uncB* mutant DG 7/1 which contains a glycyl residue instead of the carbodiimide reactive aspartyl residue in position 61 in the amino acid sequence of the proteolipid subunit [10]. It was shown that the mutant proteolipid effectively competes with the wild type proteolipid in assembling the oligomeric structure thus adopting a similar conformation like the wild type protein [18].

Here we describe *uncB* mutants where the DCCD reactive aspartyl residue is exchanged by an asparagine. This in sterical terms smallest alteration leads to a non-functional proton channel. Thus, an acidic amino acid side chain at this position seems to be essential.

2. MATERIALS AND METHODS

Escherichia coli K12 Y_{mel} (λ), F^- (*lacI, fadR, but12, rha, ilv, metE*) and its *uncB*-type derivatives DG 18/3 and 3/2 were used. The isolation of the mutants is described in [10,17]. Strains were analyzed for the binding of [^{14}C]DCCD to the proteolipid subunits. Those containing a non-reactive proteolipid were further analyzed. Growth of cells, isolation of the proteolipid subunit, preparation of membranes, measurement of enzymic activities, amino acid and sequence analysis were performed essentially as described [9–15,17]. Cyanogenbromide fragments were first chromatographed on P30 in 80% formic acid to separate the large fragment B6. The small fragments were pooled, lyophilized and dissolved in trifluoroacetic acid. Separation of these peptides was performed by chromatography on a reversed phase HPLC column (Lichrosorb RP 18 Merck 5 μm) 0.4 × 25 cm using a linear gradient from 0%–80% acetonitrile in 0.2% aqueous trifluoroacetic acid in 45 min.

3. RESULTS

Figure 1 shows the separation of the small CNBr-peptides during HPLC chromatography. To identify the peptides aliquots of the fractions were analyzed by thin-layer chromatography [10].The

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

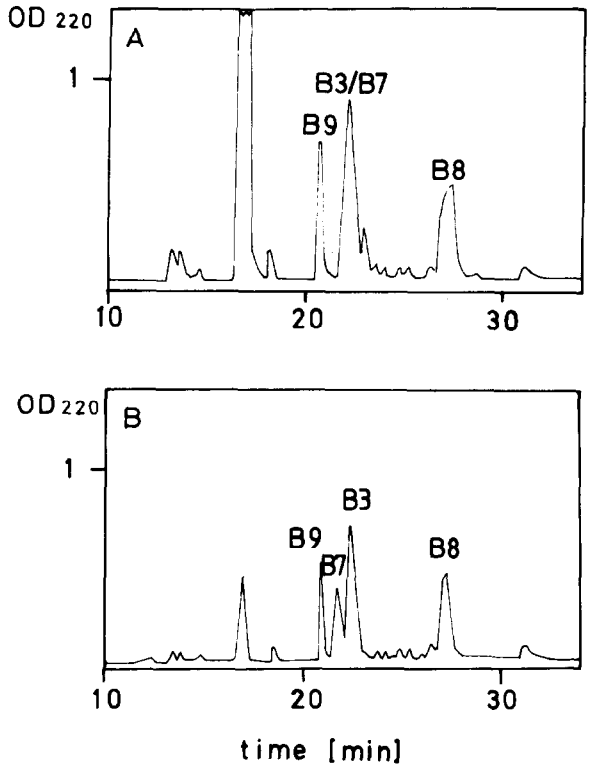

Fig.1. HPLC chromatography of the small CNBr-fragments on Lichrosorb RP-18. (A) fragments from the wild type protein. (B) fragments from the mutant proteins DG 3/2 or DG 18/3.

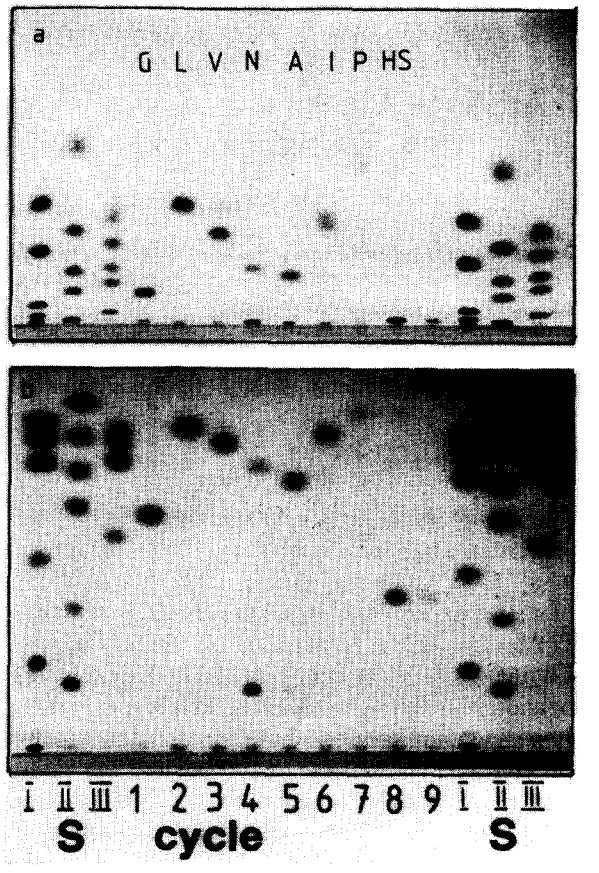

Fig.2. Amino acid sequence of the CNBr-fragment B7 of the proteolipid from mutant DG 3/2. The amino acid derivatives released during the cycles 1–8 of automated solid-phase Edman degradation were identified by thin-layer chromatography using: (a) chloroform/ethanol 98/2; (b) chloroform/methanol 90/12 to identify the polar amino acid derivatives. The calibration standards (S) of the phenylthiohydantoin amino acids contained (I) leucine (L), methionine, lysine, threonine, glutamine, arginine, (II) proline (P), valine (V), alanine (A), glycine (G), serine, asparagine (N) and (III) isoleucine (I), phenylalanine, aspartic acid methylester, glutamic acid methylester, tyrosine, glutamic acid, aspartic acid. HS designates the derivative of homoserine.

UV peaks eluting between 10 and 20 min during HPLC chromatography did not contain peptides. Peptides B2 and B4 are eluted after 5 min (not shown). Peptides B3, B7, B8 and B9 appeared between 20 and 30 min in the gradient elution. For the wild type peptides B3 and B7 were not resolved and eluted after 22 min. For the mutants an additional peak appeared after 21 min between peptide B9 and B3. Thin-layer chromatography showed a peptide with a higher mobility than the wild type peptide B7. The amino acid analysis gave the composition of peptide B7. Sequence analysis of this peptide (fig.2) showed an asparagine residue at position 4 of the peptide corresponding to residue number 61 in the whole sequence. (It should be noted that a complete separation of all the wild type peptides is accomplished with a buffer system using 5% formic acid and ethanol.)

To prove that no other amino acid exchange occurred in the proteolipid subunit, all the seven CNBr peptides (B2,3,4,6,7,8,9) were subjected to amino acid analysis. No deviation from the amino acid compositions of the wild type peptides was found. Furthermore, it was demonstrated by sequencing the whole proteins up to residue 60 that all the other acidic groups are unaffected. Func-

Table 1

Strain	ATPase activity on the membrane (μmol/min mg)	DCCD sensitivity	ATP-driven H^+-translocation (U_{F_1}/mg)	ATP–P_i exchange (nmol/min mg)
A 1	0.6	80%	130	11.6
7/1	0.6	<1%	<1	<0.2
3/2	0.6	<1%	<1	<0.2
18/3	0.8	<1%	<1	<0.2

tional properties are summarized in table 1. Normal amounts of ATPase activities were found on the mutant membranes. In contrast to the wild type no DCCD sensitivity was found. Neither ATP-dependent H^+-translocation nor ATP–P_i exchange activities were detected in the mutant strain membranes. F_1 isolated from the mutant strains reconstituted normally with F_1 depleted wild type membranes (data not shown). Thus, in the mutants 18/3 and 3/2 the defect is located in the membrane sector F_0.

4. DISCUSSION

The present studies established an amino acid exchange in the amino acid sequence of the proteolipid (subunit *c*) from the *E. coli* ATP synthase in two mutant strains (DG 3/2 and DG 18/3). Like in the previously described mutant DG 7/1 the carbodiimide reactive residue is affected. But unlike in the DG 7/1 mutant protein here the aspartyl residue is substituted by a polar residue. These two mutant strains show a high reversion rate of 10^{-5}. After a few passages a considerable amount of revertant strains is found in the cultures as indicated by the revived binding of [^{14}C]DCCD to the proteolipid. This indicates a single point mutation in the operon coding for the ATP-synthase subunits. The mutant protein effectively competes with the wild type protein in the assembly. As shown by genetic analysis using partially diploid strains [18], a strong negative complementation was found (H.U. Schairer, O. Michelsen, unpublished), thus, the mutant protein adopts a similar conformation like the wild type protein.

But most interestingly in the mutants DG 7/1, 18/3 and 3/2 ATPase activities comparable to the wild type are found on the membrane [17]. Apparently, this activity is not inhibited by the exchange of the aspartyl residue by a glycine or an asparagine. This is in contrast to the reaction of DCCD which upon binding to this acid amino acid residue inhibits the ATPase activity. Obviously, in the mutants ATP hydrolysis and proton translocation are uncoupled. This indicates an impaired assembly of ATP synthase subunits caused by the mutation. Possibly, this acidic group is also necessary to maintain a certain conformation which might be important for the contact with other subunits.

In conclusion an acidic amino acid residue which might be charged is essential for the proton conducting activity of the F_0 part. Just a polar group is not sufficient. Sterical reasons can largely be excluded since a carboxamide group has a similar size like a free carboxy group.

REFERENCES

[1] Cattel, K.J., Lindop, C.R., Knight, I.G. and Beechey, R.B. (1971) Biochem. J. 125, 169–177.
[2] Fillingame, R.H. (1976) J. Biol. Chem. 251, 6630–6637.
[3] Sone, N., Yoshida, M., Hirata, H. and Kagawa, Y. (1978) Proc. Natl. Acad. Sci. USA 75, 4219–4223.
[4] Kagawa, Y. (1978) Biochim. Biophys. Acta 505, 45–93.
[5] Sebald, W., Graf, Th. and Lukins, H.B. (1979) Eur. J. Biochem. 93, 587–599.
[6] Pick, U. and Racker, E. (1979) J. Biol. Chem. 254, 2793–2799.
[7] Friedl, P., Friedl, C. and Schairer, H.U. (1979) Eur. J. Biochem. 100, 175–180.
[8] Foster, D.L. and Fillingame, R.H. (1979) J. Biol. Chem. 254, 8230–8236.

[9] Sebald, W., Machleidt, W. and Wachter, E. (1980) Proc. Natl. Acad. Sci. USA 77, 785–789.
[10] Hoppe, J., Schairer, H.U. and Sebald, W. (1980) FEBS Lett. 109, 107–111.
[11] Hoppe, J. and Sebald, W. (1980) Eur. J. Biochem. 107, 57–65.
[12] Sebald, W. and Wachter, E. (1980) FEBS Lett. 122, 307–311.
[13] Hoppe, J., Schairer, H.U. and Sebald, W. (1980) Eur. J. Biochem. 112, 17–24.
[14] Hoppe, J. and Sebald, W. (1980) in: Chemiosmotic Proton Circuits in Biological Membranes (Skulachev, V.P. and Hinkle, P.C. eds) pp. 449–458, Addison-Wesley, Mass. USA.
[15] Sebald, W. and Hoppe, J. (1981) in: Current Topics in Bioenergetics (Sanadi, D.R. ed) pp. 2–59, Academic Press, New York, London.
[16] Downie, J.A., Gibson, F. and Cox, G.B. (1979) Ann. Rev. Biochem. 48, 257–268.
[17] Schairer, H.U., Friedl, P., Schmid, B.I. and Vogel, G. (1976) Eur. J. Biochem. 66, 257–268.
[18] Friedl, P., Friedl, C. and Schairer, H.U. (1980) FEBS Lett. 119, 254–256.