IN TERM
IN-51-CR

SETI Institute, 2035 Landings Drive, Mountain View, CA 94043.

The Evolution of Energy-Transducing Systems. Studies with Archaebacteria.

Semiannual Progress Report, March1996 - August1996

NASA Cooperative Agreement number: NCC 2-578[1)]

Principal Investigator: Helga Stan-Lotter

Source: University of Salzburg, Institut für Ge...

Summary

The DCCD[2)]- binding site of the membrane ATPase from *Halobacterium saccharovorum* was investigated during earlier periods of this Cooperative Agreement and was localized to a cyanogen bromide fragment of subunit II from amino acids 379 (Glu) to 442 (Met). Although the exact position of the reactive amino acid (probably a glutamic acid) has not yet been determined, the data, together with recently obtained immuno reactions and sequences of CNBr fragments from *E.coli* F-ATPase, suggested subunit interactions in the halobacterial ATPase which had not been recognized before. They also provided evidence for the presence of a gamma subunit in the halobacterial ATPase, and for a stretch of a amino acids similar to the "catch" between beta and gamma in bovine F-ATPase (Abrahams et al.1994). The evolutionary implications of these findings are twofold: first, halobacterial (or archaebacterial) ATPases appear as complex as those from higher organisms - no simpler versions of these membrane enzymes are known to date; second, a monophyletic origin of the energy-transducing ATPases is becoming more apparent, and - together with other data - the split into V- and F-ATPases may have occurred much later than had been previously thought (i.e., after the split into Archaea and Bacteria).
Other work included the characterization of an extremely halophilic isolate (*Halococcus salifodinae*) from Permian salt sediments. This organism appeared to be an autotrophic halobacterium; its incorporation of CO_2 was investigated.

[1)]The NASA Technical Officer for this grant is Dr. L.I.Hochstein, NASA Ames Research Center, Moffett Field, CA 94035

[2)]Abbreviations: DCCD, dicyclohexylcarbodiimide; CNBr, cyanogen bromide

Progress report

1. The ATPase from *H. saccharovorum* is inhibited completely by approx. 50 µm DCCD[2)] when incubation takes place at acidic pH (Kristjansson and Hochstein 1985). This is similar to what is known from F-ATPases. The bulk of DCCD is incorporated into subunit II of the halobacterial ATPase, whereas it is found in the beta subunit of F-ATPases. However, nucleotides did not protect against the inhibition by DCCD, nor did divalent cations, as is the case with F-ATPases. When subunit II from DCCD-labeled ATPase was cleaved with CNBr[2)], incorporation of the inhibitor was detected in two peptides of M_r 14 000 and 6000 (14K and 6K). These peptides were N-terminally sequenced; 11 amino acids of the 6K peptide were identical with residues starting from Ala 380 of the sequence from *H. halobium* (Ihara and Mukohata 1991). The 14K fragment, which also incorporated the label, is probably due to incomplete cleavage.
The entire DNA sequences of the *H. saccharovorum* ATPase subunits have not yet been determined. However, we have now several sequences from protein work which showed that the identity between the ATPases of *H. halobium* and *H. saccharovorum* is rather high - probably more than 95 %.

The halobacterial ATPase showed immunological crossreaction with both major subunits of the V-ATPase from eukaryotes, as was seen in a Western blot with membranes from *Neurospora crassa* (prepared by Karen Tenney, University of Santa Cruz). We also screened several extremely halophilic and haloalkaliphilic archaebacteria for the presence of ATPase, using antiserum against subunit I. All cell extracts (whole cell proteins from *Natronococcus occultus, Natronobacterium pharaonis, Haloferax mediterranei, Haloarcula vallismortis, H. halobium, Halococcus salifodinae* and halophile strain 54R) contained an immunoreactive band of molecular mass of approx. 85 000, suggesting the presence of the V-ATPase.

When the F-ATPase from *E. coli* was probed in Western blots with the same antiserum, no crossreaction with any of the *E coli* subunits was found. However, antiserum against subunit II did react with both the alpha and beta subunits of *E. coli.*
Crossreaction of an antiserum against subunit B (II) of the ATPase from

Sulfolobus acidocaldarius with the beta subunits of various F-type ATPases had been reported by Lübben et al. (1987); also crossreactions with methanogen ATPase subunits were found. These data had initially supported the classification of archaebacterial ATPases as F-type ATPases. The suggestion was made that this result was not so surprising, since it is to be expected that all nucleotide-binding proteins must share similar amino acid sequences or epitopes, which would give rise to similar antigenic reactivity.
To explore this suggestion further and to determine, where exactly the crossreactions originated from, CNBr fragments of the alpha and beta subunits from *E. coli* F-ATPase were prepared and examined in Western blots. There was only a weak immuno reaction left with the alpha fragments, but there were two fragments from the beta subunit, which showed a strong reaction in the immunoblot. The molecular masses of these fragments were about10K and 17K. N-terminal sequencing confirmed that they corresponded to the CNBr peptides starting at Asp 380 and Gly 276, respectively. These fragments started with or contained the sequence DELSEED, respectively; this is a very conserved sequence in all F-type ATPases from various organisms. It has been suggested to be a binding site of the natural inhibitor protein of the enzyme. From the paper of Abrahams et al.(1994), where the 2.8 A resolution of crystals of the bovine F-ATPase was reported, it became clear, that the DELSEED stretch is located on an edge of the beta subunits, well away from the nucleotide binding sites, and that it makes contact with the gamma subunit. Walker´s group calls this the "catch" between the beta and gamma subunits. With this information, the inhibition of the ATPase by only one molecule of natural inhibitor or by a similar synthetic peptide is now envisaged as a disruption of the cooperativity between the subunits, since the inhibitor binds at the critical region and acts like a wedge between the subunits..

In order to find out if archaebacterial ATPases possess a DELSEED site, we used a multiple optimal alignment program and compared the published sequences of subunit II from *H. halobium*, *Methanosarcina barkeri* and *Sulfolobus acidocaldarius*. Some stretches were recognized in the halobacterial sequence which are similar to the DELSEED loop; they start at residue Asp 376, and include also some amino acids further down in the sequence. Thus, there are several potential epitopes which

could have given rise to the immunological crossreaction of the halobacterial subunit II with the *E.coli* beta subunit. A confirmation of the importance of this region was provided by the DCCD inhibition data, which suggested a disruption of cooperativity between the halobacterial subunits upon reaction with the bulky reagent DCCD.

In summary, the results suggested that there is an equivalent of a gamma subunit present in halobacterial ATPases; such a subunit has not yet been identified. Furthermore, the complex subunit interactions, as deduced from the above data, indicated a sophistication of halo- and generally archaebacterial ATPases which appears similar to that of higher eukaryotes. Together with increasing reports of the presence of V-type ATPases in eubacteria, it is conceivable that a complex, hexagon-type of membrane ATPase was already present in the last common ancestor of all extant life; that this ATPase was more similar to the extant V-type ATPases, and that the F-type ATPases were derived from this enzyme, retaining the overall subunit composition and the critical subunit contact sites.

These results were presented in a talk at the First Extremophile Congress in Estoril, Portugal (Stan-Lotter et al. 1996) and are currently being prepared for publication.

2. *Halococcus salifodinae* is a novel extremely halophilic archaebacterium which was isolated from Austrian rock salt believed to be deposited about 250 million years ago (Denner et al. 1994). Growth of this aerobic strain in a minimal medium in the absence of an added carbon source suggested its capability for autotrophy. A few halophilic archaebacteria are known to possess the enzyme ribulose biphosphate carboxylase; however, all of those appear to be heterotrophs. The incorporation of ^{14}C-labeled sodium bicarbonate into whole cells and cell free extracts of *Hc. salifodinae* was examined. The heterotrophic control organism *Haloferax mediterranei* incorporated about 1.8 x 10^6 dpm per g of cells (wet weight) within 3 h; propionic acid and NH_4Cl stimulated incorporation about 1.4 fold. Similar behaviour was found with *Hc. salifodinae*, although incubation times had to be extended to 5-

10 h. Cell free extracts of *Hf. mediterranei* , but not of *Hc. salifodinae* , incorporated $NaH^{14}CO_3$ in the presence of ribulose 1,5 diphosphate. The data suggested that *Hc. salifodinae* does not possess the enzyme ribulose biphosphate carboxylase, but employs a different, perhaps more primitive, mode of carbon fixation.

These results were presented in a talk at the 8th ISSOL meeting in Orleans, France (Stan-Lotter and Glaser 1996).

References:

Abrahams, J.P., Leslie, A.G.W., Lutter R. and Walker, J.E. (1994) Structure at 2.8 A resolution of F_1-ATPase from bovine heart mitochondria. Nature 370, 621-628.

Denner, E.B.M., McGenity, T.J., Busse, H.-J., Wanner, G., Grant, W.D. and Stan-Lotter, H. (1994) Halococcus salifodinae sp.nov., an archaeal isolate from an Austrian salt mine. Internat. J.System. Bacteriol. 44, 774-780.

Ihara,K. and Mukohata,Y. (1991) The ATP synthase of Halobacterium salinarium (halobium) is an archaebacterial type as revealed from the amino acid sequences of its two major subunits. Arch. Biochem.Biophys. 286, 111-116.

Kristjansson,H., and Hochstein,L.I. (1985) Dicyclohexylcarbodiimide-sensitive ATPase in Halobacterium saccharovorum. Arch.Biochem.Biophys. 241,590-595.

Lübben,M., Lünsdorf,H. and Schäfer,G. (1987) The plasma membrane ATPase of the thermoacidophilic archaebacterium Sulfolobus acidocaldarius. Purification and immunological relationships to F1-ATPases. Eur.J.Biochem. 167, 211-219.

Stan-Lotter,H., Hochstein, L.I. and Gruber, C. (1996) Immunological approaches to the evolution of halobacterial and eubacterial membrane

ATPases. Abstr.p.86, First Int. Congr. Extremophiles, Estoril, Portugal

Stan-Lotter, H. and Glaser, K. (1996) Carbon assimilation by Halococcus salifodinae, an archaebacterial isolate from Permian salt deposits. Abstr. c4.4, p. 54, 8th ISSOL Meeting, Orleans, France

Other Activities

March 1995 - August 1996: Teaching appointments at the University of Vienna and University of Salzburg, Austria, while remaining at reduced time (10%) with the SETI Institute.
May 8, 1996: Talk at the International Soroptimist Club of Ravensburg/Stuttgart with the title: Microorganisms and the search for extraterrestrial life.