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

Semiannual Progress Report, September 1993 - February 1994

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

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Summary

N-ethylmaleimide (NEM) inhibits the ATPase of *H. saccharovorum* in a nucleotide protectable manner. The bulk of ^{14}C-NEM is incorporated into subunit I. Cyanogen bromide cleavage of labeled subunit I indicated that NEM bound to a peptide of a Mr of about 8 900. Thus, Cys 262 (*H. salinarium* numbering) may be the NEM binding site. Cyanogen bromide fragments have been submitted for sequencing. To prove the presence of three Cys residues in subunit I, alkaline cleavage following treatment with NTCB[2)] was carried out.

Thiol reagents such as p-chloromercuriphenylsulfonate also inhibited the ATPase. However, this inhibition was not nucleotide-protectable, suggesting a different location and role for the PCMS-sensitive Cys.

The proteolipid which was extracted with chloroform/methanol from the membranes of *H. saccharovorum* cross-reacted with an antiserum against subunit c (the DCCD[2)]-binding protein) of*Escherichia coli* . Following labeling of membranes from *H. saccharovorum* with ^{14}C-DCCD under conditions, which inhibited ATP synthesis (Hochstein 1992), the isotope was incorporated into one protein of M_r of about 6 500. Thus, the proteolipid of *H. saccharovorum* and the DCCD-labeled peptide may be identical. If so, these results suggest that the proteolipid is a component of the membrane sector of an archaeal F-type ATP synthase.

[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; NEM, N-ethylmaleimide; PCMS, p-chloromercuriphenylsulfonate; NTCB, 2-nitro-5-thiocyanobenzoic acid

Progress report

1. The ATPase from *Halobacterium saccharovorum* is inhibited in a nucleotide protectable manner by NEM[2)]and the bulk of the inhibitor is incorporated into subunit I (Ref.2). One goal of this Cooperative Agreement is the determination of the sequence around the NEM binding site. This information will allow a detailed comparison between the halobacterial ATPase and V-type ATPases.
The enzyme was labeled with ^{14}C-NEM and subunit I was prepared by electroelution. Following cleavage with cyanogen bromide and separation on Tris-Tricine gels, approximately 12 peptides could be identified. Autoradiography showed that the bulk of radioactivity was associated with a peptide of M_r of 8 900. A second peptide (M_r about 10 800) showed less, but still substantial incorporation of the isotope. If the sequence from subunit A of *H. salinarium* is taken as a guide (Ref.3), the first peptide would correspond to amino acids 191 - 268 and the second to amino acids 191 - 289. Assuming an incomplete cleavage at Met 268, the molecular masses as seen on gels are as expected from the amino acid sequences. The N-termini of these peptides will be sequenced to confirm the location.
(Note: It may be mentioned that the peptides were already prepared and sent for sequencing to the University of Santa Cruz in November 1993, but so far the sequencing was not performed by this institution).
Three cysteinyl residues are present in subunit A of *H. salinarium* as deduced from the DNA sequence (Ref.3). From the amino acid analysis of carboxymethylated subunit I of *H. saccharovorum* , we reported earlier 4.6 Cys (Ref.4), taking a M_r of 87 000 for the calculation. If the true M_r is 64 104 (DNA sequence), this number would be reduced to 3.3 Cys, which is in close agreement with the DNA data from *H. salinarium* .
Still, it would be desirable to show unequivocally the presence of 3 Cys residues in subunit I of *H. saccharovorum* . Treatment with 2-nitro-5-thiocyanobenzoic acid and subsequent exposure to alkaline pH results in cysteinyl-specific cleavage (Ref.5). Electroeluted subunit I was cleaved with NTCB and several fragments were obtained. The insolubility of peptides following cleavage proved to be a serious problem, as was seen before with peptides from subunit II. Acetonitrile, urea or various detergents were not sufficient to solubilize halobacterial peptides. The

best strategy appeared to keep the peptides at rather low concentrations (< 0.1 mg/ml) and have the detergent Tween 20 (final concentration 0.01 %) present in all buffers.
So far, peptides with molecular masses of about 52, 40, 32, 22 and 6 K were found to originate from subunit I following NTCB treatment. These peptides might correspond to fragments composed of amino acids 262-585; 1-308; 1-261; 309-513 and 262-308, respectively. This would account for three cysteinyl cleavage sites (including some incompletely cleaved peptides); however, the peptide corresponding to amino acids 514-585 has not been detected yet on gels. It is expected that application of longer incubation times (for cleavage) and more sensitive staining methods will resolve the fourth peptide. The results are currently being written up in a manuscript with the tentative title: Inhibition of the ATPase from *H. saccharovorum* by NEM occurs by asymmetric insertion into CYS 262 of subunit I.

2. Several thiol reagents other than NEM also inhibited the ATPase from *H. saccharovorum* . One particularly interesting reagent was p-chloromercuriphenylsulfonate because of its unusually rapid action (half inactivation possibly after about 1 min). However, this inhibition was not nucleotide-protectable, as is the inhibition caused by NEM. This suggested a different location of the PCMS-sensitive Cys and by extension, a different role, perhaps in maintaining structure. These results will be presented at the ASM 94 meeting (see below).

3. The membrane sector of the halobacterial ATPase has not been characterized. A commonly used procedure for the extraction of the membrane-spanning proteolipid (subunit c in *E. coli*) is treatment with chloroform- methanol (2:1). When applied to membranes from *H. saccharovorum* , this method yielded a preparation which was enriched in a protein of M_r of about 6 500. In Western blots, a strong cross-reaction was observed with an antiserum against subunit c. Membranes from *H. saccharovorum* were treated with ^{14}C-DCCD at a final concentration of 10 µM for 2 h. Subsequent gel electrophoresis, using the Tris-Tricine-system, and autoradiography showed a radioactive band at approximately M_r 6 500. Slicing of the gel, solubilizing the slices and liquid scintillation counting confirmed the existence of only one major product which had incorporated

the label. The estimated minimal amount of the DCCD-labeled protein in the washed membranes was 0.04 %. These results will be presented at the annual ASM meeting (see below).

Other Activities

October 1993 - January 1994: Teaching appointment at the University of Vienna, Austria, while remaining at reduced time (10%) with the SETI Institute.

References:

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5. Degani, Y. and Patchornik, A. (1974) Biochemistry 13, 1-11.

Abstracts submitted

Radax, C., Stan-Lotter, H., and Hochstein, L.I. A Proteolipid from *Halobacterium saccharovorum* with Similarity to Subunit c of *Escherichia coli.* ASM Meeting, Las Vegas, May 1994

Hochstein, L.I., Stan-Lotter, H., and Emrich, E. Inhibition of the Vacuolar-like ATPase from *Halobacterium saccharovorum* by Thiol Reagents: Evidence for different functional thiols. ASM Meeting, Las Vegas, May 1994