Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 5521–5524, August 1988
Biochemstry

cDNA sequence encoding the 16-kDa proteolipid of chromaffin granules implies gene duplication in the evolution of H^+-ATPases

(proton pump/transmembrane protein/vacuole/organelle evolution)

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Communicated by Herbert Weissbach, April 25, 1988

ABSTRACT **Vacuolar H^+-ATPases function in generating protonmotive force across the membranes of organelles connected with the vacuolar system of eukaryotic cells. This family of H^+-ATPases is distinct from the two other families of H^+-ATPases, the plasma membrane-type and the eubacterial-type. One of the subunits of the vacuolar H^+-ATPase binds *N,N'*-dicyclohexylcarbodiimide (DCCD) and has been implicated in the proton-conducting activity of these enzymes. We have cloned and sequenced the gene encoding the DCCD-binding protein (proteolipid) of the H^+-ATPase of bovine chromaffin granules. The gene encodes a highly hydrophobic protein of 15,849 Da. Hydropathy plots revealed four transmembrane segments, one of which contains a glutamic residue that is the likely candidate for the DCCD binding site. Sequence homology with the vacuolar proteolipid and with the proteolipids of eubacterial-type H^+-ATPases was detected. The proteolipids from *Escherichia coli*, spinach chloroplasts, and yeast mitochondria matched better to the NH_2-terminal part of the vacuolar protein. The proteolipids of bovine mitochondria and *Neurospora* mitochondria matched better to the COOH-terminal end of the vacuolar proteolipid. These findings suggest that the proteolipids of the vacuolar H^+-ATPases were evolved in parallel with the eubacterial proteolipid, from a common ancestral gene that underwent gene duplication.**

Proton-transporting ATPases (H^+-ATPases) play a crucial role in biological energy transduction (1). These ion pumps can be classified into three main families: plasma membrane-type, eubacterial-type, and vacuolar-type enzymes (2–5). The plasma membrane-type enzyme is present in the plasma membrane of plants, fungi, and acid-secreting gastric vesicles (6, 7). The catalysis of these enzymes involves a phosphoenzyme intermediate. The gene coding for this 100-kDa protein in yeast and *Neurospora crassa* has been cloned and sequenced (8–10).

The eubacterial-type enzyme occurs in chloroplasts, mitochondria and bacteria and operates without a phosphoenzyme intermediate (11–13). These enzymes are composed of two distinct structures, a membrane sector, which is hydrophobic, and a catalytic sector, which is hydrophilic in nature. The function of the membrane sector is to conduct protons across the membrane. This sector is composed of three or more polypeptides, one of which is an *N,N'*-dicyclohexylcarbodiimide (DCCD)-binding protein (proteolipid of about 8 kDa) that is involved in the proton conduction (14–16). The catalytic sector, which is the site of the ATPase reaction, can be readily separated from the membrane by EDTA treatment or by applying mechanical force (17).

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The vacuolar-type enzyme is present in organelles connected with the vacuolar system of eukaryotic cells and pumps protons without the involvement of a phosphoenzyme intermediate (2–5). These H^+-ATPases are composed of several polypeptides, and to our knowledge none of these have been sequenced. A 16-kDa subunit resembles the eubacterial proteolipids in that it binds DCCD and is soluble in organic solvents (18). This kind of proteolipid was isolated from clathrin-coated vesicles and was implicated in proton conductance across the organelle membranes, analogously to the eubacterial proteolipids (19, 20). Similar polypeptides were detected in several other membranes containing vacuolar H^+-ATPases, such as fungal and plant vacuoles (21–24). To ascertain the degree of correlation between the vacuolar and eubacterial proteolipid, we have cloned and sequenced the cDNA encoding this subunit of the chromaffin granule H^+-ATPase.§ Sequence information could establish whether the vacuolar and eubacterial-type proteolipids are homologous, analogous, or dissimilar (5).

MATERIALS AND METHODS

Isolation and Sequencing of Peptides Generated by Cyanogen Bromide Treatment. The H^+-ATPase was purified from chromaffin granule membranes and reconstituted as described (25, 26). The proteolipid was extracted from 2 ml of reconstituted enzyme containing about 500 μg of protein, by 2 ml of chloroform/methanol, 2:1 (vol/vol) (18). The aqueous phase was removed by pipeting and the chloroform/methanol was dried by a stream of argon gas. The dry material was suspended in 1.8 ml of 70% (vol/vol) formic acid. Then 0.2 ml of 70% formic acid containing about 10 mg of CNBr was added. The mixture was incubated overnight at room temperature in darkness and dried by a stream of argon gas. The dry material was suspended in 1 ml of 1% $NaDodSO_4$, and after centrifugation at 30,000 × *g* for 10 min the supernatant was subjected to high-performance liquid chromatography (HPLC). Since some of the peptides were highly hydrophobic, the solvent system and the method of solubilization of the peptides generated by cyanogen bromide treatment were modified. Details of this approach will be described elsewhere. The peptides were subjected to amino acid sequence analysis and the following sequences were obtained: peptide 1, Val-Phe-Ser-Ala-Leu-Gly-Ala-Ala-Tyr-Gly-Thr-Ala-Lys-Ser-Gly-Thr-Gly-Ile-Ala; peptide 2, Arg-Pro-Glu-Met-Ile. Cleavage of the proteolipid by cyanogen bromide on the Polybrene-treated glass filter of the sequenator yielded the

Abbreviation: DCCD, *N,N'*-dicyclohexylcarbodiimide.

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§The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03835).

sequence of peptide Pro-Val-Val-Met-Gly-Ile-Ile-Ala-Ile-Tyr-Leu-Val-Val-Ala-Val-Leu-Ile-Ala-Asn-Ser-Leu-Asn-Asp-Gly. The NH_2-terminal amino acid of the proteolipid is blocked and the sequence of peptide 4, Ser-Glu-Ala-Xaa-Asn-Gly-Pro-Glu-Tyr-Ala, was obtained after deblocking the HPLC-purified proteolipid or the isolated polypeptide by acid treatment on the glass filter. This hydrophilic peptide was readily isolated and was sequenced several times.

Isolation and Sequencing of the cDNA Encoding the Proteolipid. Three oligodeoxynucleotide probes were synthesized according to polypeptide 1: (*i*) d(GGCGATGCCGGTGCCG-CTTGGCGGTGCCGTAGGCGGCGCCAGGGCGCTGAA-CACCAT); (*ii*) a mixed probe in which the redundant nucleotides were replaced by all genetic code alternatives; and (*iii*) d(GCIATICCIGTICCGCTCTTIGCIGTICCITAIG-CIGCICCIAGIGCICTIAAIACCAT), in which those nucleotides were replaced by deoxyinosine. The probes were used to screen a cDNA library of bovine adrenal medulla that was obtained from U. Gubler (27). Several positive clones were obtained and the two with the largest cDNA inserts, 1.1 and 1.2 kilobases long, were sequenced. The dideoxy chain-termination technique supplemented by exonuclease III was used to determine the nucleotide sequence of the cDNA (28, 29). The amino acid sequence of the polypeptide used for generating the probes was identified in the cloned cDNA by sequencing the two opposite strands of phage M13 with the probe serving as a primer. This sequence was used to generate a second probe to serve as a primer for the second strand. Translation of this sequence revealed the amino acid sequence of the original polypeptide. This approach was time-saving, since one of the clones that was positive by hybridization displayed only partial sequence identity to the first probe and had no relation to the proteolipid gene.

RESULTS

The nucleotide and deduced amino acid sequences of the cDNA are shown in Fig. 1. The nucleotide sequence contains an open reading frame encoding a 155 amino acid polypeptide of 15,849 Da. This value agrees closely with the molecular weight estimated by $NaDodSO_4$/PAGE for the proteolipid from various vacuolar H^+-ATPases (5). This cDNA clone is likely to encode the entire proteolipid, as all of the sequenced polypeptides were identified in the translated reading frame. The blocked polypeptide corresponds to the sequence following the initiator methionine, and the amino acid composition calculated from the derived sequence correlates well with the amino acid composition determined for the purified proteolipid (Table 1). No histidine was found in the protein by amino acid analysis or by translation of the cDNA sequence. However, in regard to other amino acids this work differs from a previous report (18), probably because the previous analysis utilized a protein not purified by HPLC. As the serine residue following the initiator methionine was identified in the protein sequence, it is unlikely that incorporation of the proteolipid into the membrane involves a cleavable signal sequence. This notion is supported by the observation that the translation product of mRNA encoding the proteolipid had the same size as the mature protein (data not shown). It is possible that the charged NH_2 terminus makes contact with the membrane and that the rest of the protein, which is mostly hydrophobic, inserts directly into the membrane.

A hydrophobicity plot reveals four transmembrane segments (Fig. 2). Segments I and II are very hydrophobic and contain no charged amino acids. Segment III is the least hydrophobic and contains an arginine residue (Arg-107) in the middle of the segment. The transmembrane segment IV, although very hydrophobic, contains a glutamic residue (Glu-139) in the middle. The position of Glu-139 indicates a potential DCCD binding site. Glu-139 may be positioned in close proximity to Arg-107 of segment III, which could result in formation of an ion pair inside the membrane. Two other polar amino acid residues located in the transmembrane

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GCGGCTGTCGGGCCGGGTGCTTCGCAGCCTTTCGGACACAGCTTAGCTTTCGCGCAAGCCGCCGCCCGCCGCGCC   75

CGGCTTCGCACCTCGCCCCGGCCTGGTCCGTTGAACTGCCCCTTCCCAACCGCAGACATGTCCGAGGCCAAGAAC  150
                                                        M  S  E  A  K  N

GGCCCCGAGTACGCTTCCTTTTTCGCGGTCATGGGTGCCTCAGCCGCCATGGTCTTCAGCGCCCTTGGCGCCGCC  225
G  P  E  Y  A  S  F  F  A  V  M  G  A  S  A  A  M  V  F  S  A  L  G  A  A
      ^                          ^                          ^

TACGGTACAGCCAAGAGCGGCACGGGCATCGCAGCCATGTCTGTCATGCGGCCAGAGATGATCATGAAGTCCATC  300
Y  G  T  A  K  S  G  T  G  I  A  A  M  S  V  M  R  P  E  M  I  M  K  S  I
            ^                          ^

ATCCCGGTGGTCATGGCGGGGATCATCGCCATCTATGGTCTGGTGGTGGCAGTCCTCATTGCCAACTCCCTGAAT  375
I  P  V  V  M  A  G  I  I  A  I  Y  G  L  V  V  A  V  L  I  A  N  S  L  N
      ^                          ^                          ^

GACGGCATCAGTCTCTACAGGAGTTTCCTTCAGCTGGGCGCAGGCTTGAGTGTGGGCCTGAGCGGGCTGGCGGCA  450
D  G  I  S  L  Y  R  S  F  L  Q  L  G  A  G  L  S  V  G  L  S  G  L  A  A
            ^                          ^

CGTTCGCCATCGGCATTGTTGGGGACGCAAGGCCGTGCGTGCACCGCCCAGCAGCCGCGGCTCTTCGTGGGCATG  525
R  S  P  S  A  L  L  G  T  Q  G  R  A  C  T  A  Q  Q  P  R  L  F  V  G  M
      ^                          ^                          ^

ATCCTCATCCTCATCTTCGCCGAGGTGCTCGGCCTCTACGGTCTCATCGTCGCCCTTATCCTCTCCACAAAGTAG  600
I  L  I  L  I  F  A  E  V  L  G  L  Y  G  L  I  V  A  L  I  L  S  T  K
                  ^                          ^

CTCTGCGGGCCGCCAGCCACAGAATACAATTGATGTCAAGACCACCCCCTTCTCATTCCACAACGAACAGCCTGA  675

CACACGCACGGGCAGCCGCCCGCCAGTAGTCGGTCTTGTAAATGCGCAGTGTCCCAGTGCCCACCGTCTGTTGCC  750

CCAGCCTCGCCCCTGCCCGCCCCGCCCCGTGCTGTGGACATCTGGGCCCACCAGTCCCCACCCCGGCCCTGACCA  825

GTGAGGACGCCGGCCTCCCGCCCCGCCCATCTGCCCTAGAGTGCTCTGTGTATAAGGATGAATTAGAGTTGTCAT  900

TTTCTCTTCACTCGGATGTTTATTTATAAAGATTTGACCTGTTCATACGTCTGTGGAGCAGCTCTCGTCTCCAAC  975

TCTATAGTAACCTTAGGTAGACTGTTGTTGCGTTGTGCGGTTACCGTTTACCCTGAGACCCGTTGGATGGAACCA 1050

CCTCTTGCAGCCCTGGTTCGCGGGCCAGTGTGACGGGCCGCTGGCGTGGTGCCGCTCCGTGTCCAATAAAGCTCT 1125

CAGATGTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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FIG. 1. Nucleotide and encoded amino acid sequences of chromaffin granule H^+-ATPase proteolipid cDNA. The amino acid sequences of isolated polypeptides obtained by amino acid sequence analysis are underlined. Amino acids are shown in standard one-letter symbolism.

Table 1. Amino acid composition of the proteolipid of the H^+-ATPase from chromaffin granules

Amino acid	No. of residues	
	Analyzed	Predicted
Aspartic acid	5.6	4
Threonine	3.9	5
Serine	16.4	15
Glutamic acid	7.7	8
Proline	ND	5
Glycine	21.4	18
Alanine	23.8	24
Cysteine	ND	1
Valine	12.3	12
Methionine	7.5	9
Isoleucine	12.3	14
Leucine	17.5	20
Tyrosine	4.9	5
Phenylalanine	6.8	6
Histidine	0.2	0
Lysine	3.8	4
Arginine	5.0	5
Tryptophan	ND	0

Amino acid analysis was performed on HPLC-purified proteolipid. The amount of each amino acid was normalized for 149 amino acids, which is the total number of residues in the proteolipid minus 5 prolines and 1 cysteine. ND, not determined.

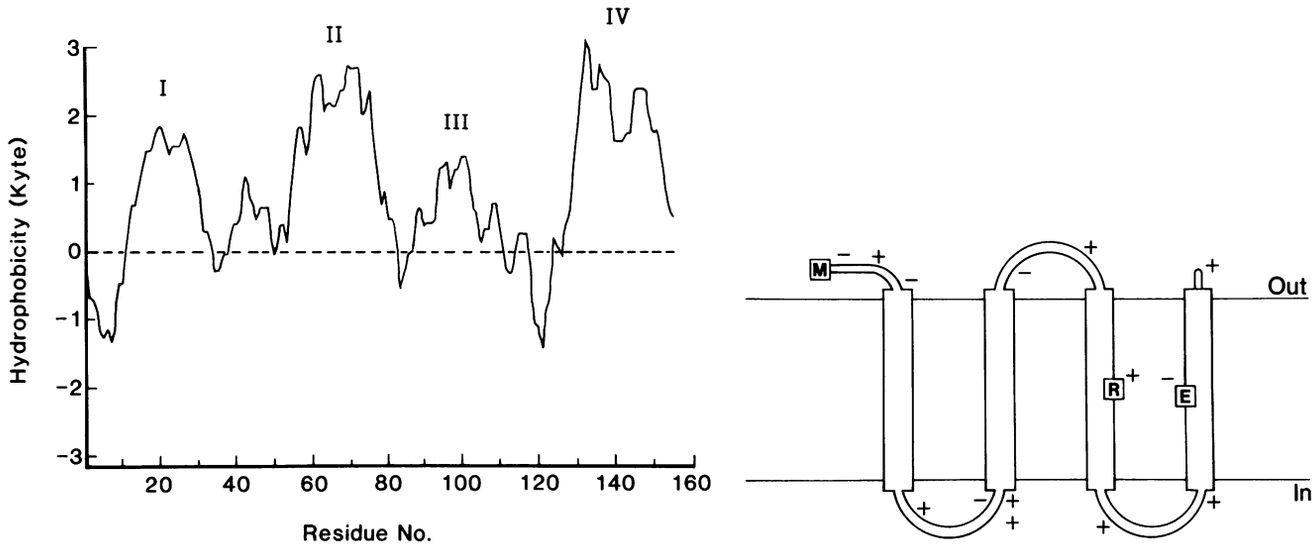

FIG. 2. Hydropathic profile of the proteolipid from chromaffin granule H^+-ATPase (*Left*) and proposed arrangement in the membrane (*Right*). Analysis was performed by the DNASTAR program according to Kyte and Doolittle (30), with a window size of 11 amino acids. M, Met-1; R, Arg-107; E, Glu-139.

segments, Tyr-68, and Tyr-144, may also play a role in the protonation–deprotonation process.

Several proteolipids of eubacterial-type ATPases have now been sequenced (11, 12), permitting comparison to the proteolipid of the chromaffin granule H^+-ATPase (Fig. 3). It is apparent that sequence homology exists between these two classes of proteolipids. The computer program matched the proteolipids from *Escherichia coli* (not shown), spinach chloroplasts, and yeast mitochondria to the first half of the proteolipid of the chromaffin granule H^+-ATPase. On the other hand, the proteolipids of bovine mitochondria and *Neurospora* mitochondria were matched to the second half of the vacuolar H^+-ATPase. Whereas the proteolipids that match the first half are encoded by organellar or bacterial DNA, the ones that match the second half are encoded in chromosomal DNA in the nucleus and are translated on cytoplasmic ribosomes. The glutamic acid that binds DCCD in the *Neurospora* and bovine proteolipid matched perfectly with Glu-139 of the vacuolar proteolipid. Moreover, when the proteolipids of yeast mitochondria and spinach chloroplasts were forced to match with the second half of the vacuolar proteolipid, the DCCD binding site of the organellar proteolipids was positioned at Glu-139 of the vacuolar protein (data not shown). The percent identity of amino acids and the numerous conserved changes that were observed suggest a homology between the two classes of proteolipids.

DISCUSSION

The sensitivity of proton pumping into chromaffin granules and other organelles of the vacuolar system of eukaryotic cells to inhibitors suggested the existence of a new class of H^+-ATPase (32). Purification and reconstitution of the chromaffin granule enzyme substantiated the singularity of this class of proton pumps (25, 26, 33). Preparations of purified H^+-ATPase from chromaffin granules, synaptic vesicles, fungal and plant vacuoles, and clathrin-coated vesicles have been found to contain a proteolipid subunit of about 16 kDa that binds DCCD (19–24). Upon binding, DCCD inhibits ATP-dependent translocation of protons across the membrane in a fashion resembling that of mitochondria, chloroplasts, and bacteria (11–15). However, the identification of the proteolipid of the vacuolar enzymes as the proton-conducting subunit is unclear. A recent report of proton conduction by the isolated proteolipid from clathrin-coated vesicles (20) and the homology with the eubacterial-type enzymes reported in this study suggest an involvement in proton conduction in the vacuolar system.

The sequence alignment shown in Fig. 3 suggests that the proteolipid of the eubacterial H^+-ATPases and the vacuolar H^+-ATPases evolved from a common ancestral gene that existed prior to the segregation of the various organelles (34, 35). As depicted in Fig. 4, the ancestral gene might have

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                                10        20        30        40        50        60        70        80        90       100
                                |         |         |         |         |         |         |         |         |         |
Spinach chloroplast             MNPLIAAASVIAAGLAVGLASIGPGVGQGTAAGQAVEGIARQPEAEGKIRGTLLLSLAFMEALTIYGLVVALALLFANPFV
                                 .   :. : : A ::.: A : :::G.: ::::: .GIA  :    .:  . ::::.. . ::IYGLVVA: :  :
H+-ATPase (adrenal)             MSEAKNGPEYASFFAVMGASAAMVFSALGAAYGTAKSGTGIAAMSVMRPEMIMKSIIPVVMAGIIAIYGLVVAVLIANSLNDGISLYRSFLQLGAGLSVGLSGLAA
                                   .:    A.:::.  :: ::: :::G A   A   .G::  : :: .:: .:I:  .::. .::: L:V: L:  ::
Yeast mitochondria                 MQLVLAAKYIGAGISTIGLLGAGIGIAIVFAALINGVSRNPSIKDTVFPMAILGFALSEATGLFCLMVSFLLLFGV

                                50        60        70        80        90       100       110       120       130       140       150
                                |         |         |         |         |         |         |         |         |         |         |
Neurospora mitochondria                                  YSSEIAQAMVEVSKNLGMGSAAIGLTGAGIGIGLVFAALLNGVARN-PALRGQLFSYAILGFAFVEAIGLFDLMVALMAKFT
                                                           . : .     .  :  :A   .G :   :   :ALL.. :R. .A : :LF   IL : F.E.:GL::L:VAL: . .
H+-ATPase (adrenal)             EMIMKSIIPVVMAGIIAIYGLVVAVLIANSLNDGISLYRSFLQLGAGLSVGLSGLAARSPSALLGTQGRACTAQQPRLFVGMILILIFAEVLGLYGLIVALILSTK
                                                                 D  :  : : . :A.::V: SG :  :  : L .     .: :.:LF   IL : ::E.:GL: L:VA:::
Bovine mitochondria                                              DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM
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FIG. 3. Aligned sequences of chromaffin granule proteolipid with bovine, yeast, and *Neurospora* mitochondrial and spinach chloroplast proteolipids. Alignment of the amino acid sequences was performed by the DNASTAR program according to the method of Lipman and Pearson (31). Gap penalty of 4 and deletion penalty of 12 were used. Conservative amino acid replacements are indicated by colons.

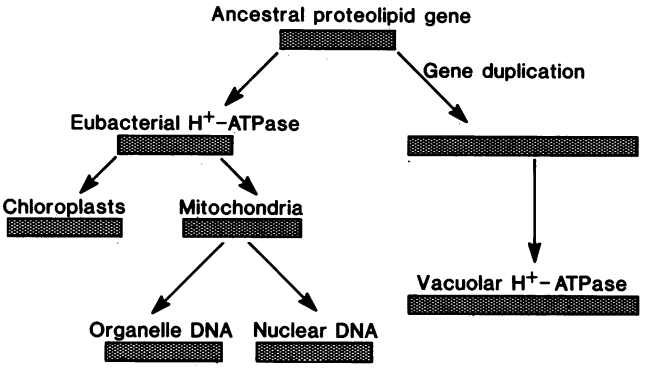

FIG. 4. Evolution of the proteolipids of eubacterial and vacuolar H^+-ATPases.

encoded a proteolipid of ≈8 kDa that was maintained in the H^+-ATPases of bacteria, mitochondria, and chloroplasts. The vacuolar H^+-ATPase gene may have undergone gene duplication. While the first half of this gene evolved in a parallel fashion to the bacterial and organellar genes, the second half evolved more closely to the proteolipid genes encoded in the nucleus. The first half lost its DCCD binding site through a conservative change of glutamic acid to serine. The second half developed motifs resembling the bacterial ATPase α subunit, as manifested by the presence of phenylalanine at positions 128 and 137 and by tyrosine at position 144 (36). These data add credence to the endosymbiotic hypothesis for the evolution of semi-autonomous organelles.

Recently it was proposed that the vacuolar-type H^+-ATPases served as ATP synthases in primitive organisms such as progenotes and some archaebacteria (5, 37, 38). The function of the H^+-ATPase in the vacuolar system of eukaryotic cells is to maintain limited acidification of the organelles connected with the system (5, 38). This may be achieved through controlling the proton-pumping activity of the enzyme by loosening the coupling between the ATPase activity and proton translocation (39). While this control mechanism was developing, the vacuolar H^+-ATPase ceased functioning in ATP synthesis. At the same time, the eubacterial-type H^+-ATPases perfected the coupling between proton translocation and ATP synthesis by further evolution of their catalytic and membrane sectors. Sequencing data from our laboratory and others support this proposed evolutionary pathway of the vacuolar and eubacterial H^+-ATPases.

We wish to thank Mr. Larry Brink for performing the amino acid analysis, Dr. Tom Curran and Dr. Patricia Reilly for critical reading of the manuscript, and Ms. Samantha Mehta for excellent assistance.

1. Mitchell, P. (1968) *Chemiosmotic Coupling and Energy Transduction* (Bodmin, Cornwall, England).
2. Mellman, I., Fuchs, R. & Helenius, A. (1986) *Annu. Rev. Biochem.* **55**, 663–700.
3. Rudnick, G. A. (1986) *Annu. Rev. Physiol.* **48**, 403–413.
4. Bowman, B. J. & Bowman, E. J. (1986) *J. Membr. Biol.* **94**, 83–97.
5. Nelson, N. (1987) *BioEssays* **7**, 251–254.
6. Goffeau, A. & Slayman, C. W. (1981) *Biochim. Biophys. Acta* **639**, 197–223.
7. Faller, L. D., Rabon, E. & Sachs, G. (1983) *Biochemistry* **22**, 4676–4685.
8. Serrano, R., Kielland-Brandt, M. C. & Fink, G. R. (1986) *Nature (London)* **319**, 689–693.
9. Addison, R. (1986) *J. Biol. Chem.* **261**, 14896–14901.
10. Hager, K. M., Mandala, S. M., Davenport, J. W., Speicher, D. W., Benz, E. J. & Slayman, C. W. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 7693–7697.
11. Hoppe, J. & Sebald, W. (1984) *Biochim. Biophys. Acta* **768**, 1–27.
12. Walker, J. E., Saraste, M. & Nicholas, J. F. (1984) *Biochim. Biophys. Acta* **768**, 164–200.
13. McCarty, R. E. & Nalin, C. M. (1986) *Encycl. Plant Physiol. New Ser.* **19**, 576–583.
14. Cattell, K. J., Lindop, C. R., Knight, I. G. & Beechey, R. B. (1971) *Biochem. J.* **125**, 169–177.
15. Futai, M. & Kanazawa, H. (1983) *Microbiol. Rev.* **47**, 285–312.
16. Schneider, E. & Altendorf, K. (1987) *Microbiol. Rev.* **51**, 477–497.
17. Nelson, N. (1981) *Curr. Top. Bioenerg.* **11**, 1–33.
18. Sutton, R. & Apps, D. K. (1981) *FEBS Lett.* **130**, 103–106.
19. Arai, H., Berne, M. & Forgac, M. (1987) *J. Biol. Chem.* **262**, 11006–11011.
20. Sun, S.-Z., Xie, X.-S. & Stone, D. K. (1987) *J. Biol. Chem.* **262**, 14790–14794.
21. Bowman, E. J. (1983) *J. Biol. Chem.* **258**, 15238–15244.
22. Uchida, E., Ohsumi, Y. & Anraku, Y. (1985) *J. Biol. Chem.* **260**, 1090–1095.
23. Manolson, M. F., Rea, P. A. & Poole, R. J. (1985) *J. Biol. Chem.* **260**, 12273–12279.
24. Randall, S. K. & Sze, H. (1986) *J. Biol. Chem.* **261**, 1364–1371.
25. Moriyama, Y. & Nelson, N. (1987) *J. Biol. Chem.* **262**, 9175–9180.
26. Moriyama, Y. & Nelson, N. (1987) *J. Biol. Chem.* **262**, 14723–14729.
27. Gubler, U. & Hoffman, B. J. (1983) *Gene* **25**, 263–269.
28. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
29. Henikoff, S. (1984) *Gene* **28**, 351–359.
30. Kyte, J. & Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132.
31. Lipman, D. L. & Pearson, W. (1985) *Science* **227**, 1435–1441.
32. Cidon, S. & Nelson, N. (1983) *J. Biol. Chem.* **258**, 2892–2898.
33. Moriyama, Y. & Nelson, N. (1987) *Biochem. Biophys. Res. Commun.* **149**, 140–144.
34. Margulis, L. (1974) *Evol. Biol.* **7**, 45–78.
35. Raven, P. H. (1970) *Science* **169**, 641–646.
36. Cozens, A. L., Walker, J. E., Phillips, A. L., Huttly, A. K. & Gray, J. C. (1986) *EMBO J.* **5**, 217–222.
37. Mukohata, Y. & Yoshida, M. (1987) *J. Biochem.* **102**, 797–802.
38. Nelson, N. (1988) *Plant Physiol.* **86**, 1–3.
39. Moriyama, Y. & Nelson, N. (1988) in *The Ion Pumps—Structure, Function and Regulation*, ed. Stein, W. (Liss, New York), pp. 387–394.