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Topical Review

The Proton-ATPase of Bacteria and Mitochondria

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Introduction

Our purpose in this review is to discuss recent research on the proton-ATPase of bacterial and mitochondrial membranes. This large membrane enzyme interconverts the electrochemical potential energy of H^+ gradients and the chemical energy of the β-γ phosphoryl bond in ATP. It is the terminal enzyme in oxidative phosphorylation, and in bacteria it may hydrolyze ATP to drive nutrient uptake by symport with protons.

We shall concentrate on two major areas in which rapid advances have occurred recently. The first of these is the genetics, structure and assembly of the enzyme from *Escherichia coli*, and the second is the mechanism of proton conduction and ATP hydrolysis/synthesis. This review is not intended to be comprehensive and only selected references will be cited. Comprehensive reviews of the literature will be found in the following: Downie et al., 1979*a*; Fillingame, 1980, 1981; Cross, 1981; Dunn and Heppel, 1981; Pedersen et al. 1981; and Sebald and Hoppe, 1981. Two reviews (Senior 1973, 1979*a*) summarize earlier literature, and Maloney (1982) reviewed physiological aspects of the enzyme in this journal recently.

Structure of the *E. coli* Proton ATPase

The proton-ATPase of *E. coli* resembles generally that of mitochondria, chloroplasts and other bacteria (Senior, 1973, 1979*a*). The enzyme consists of two distinct sectors, an "F_1 sector" on which ATP hydrolysis and synthesis occur and a "membrane sector" which binds F_1 to the plasma membrane and transports protons across the membrane. (The term "F_o" is often used interchangeably with "membrane sector".) The two sectors can be separated by washing the membranes in buffer of low-ionic strength containing EDTA, which releases the F_1 in soluble form. Recombination of the two sectors is achieved by incubating purified F_1 with stripped membranes in the presence of divalent cation at normal ionic strength. Use of detergents allows purification of the whole complex intact (Foster & Fillingame, 1979; Friedl et al., 1979). Subsequent removal of F_1 from the whole complex yields purified membrane sector (F_o).

F_1 contains five different subunits, α, β, γ, δ and ε, the molecular weights of which have been calculated from the amino acid sequences and are given in the Table. The stoichiometry of these five subunits is $\alpha_3\beta_3\gamma\delta\varepsilon$ (to be discussed later, *see* p. 117) giving a total molecular weight for F_1 of 382K. F_1 contains no lipid, nor any known prosthetic groups. Tightly bound Mg and nucleotides (ATP and ADP) (Maeda et al., 1976; Bragg & Hou, 1977; Senior et al., 1980) are present in pure F_1 samples.

The membrane sector (F_o) contains three subunits (Table), which on SDS-gels run with molecular weights of 24K, 19K and 8.5K (Negrin et al., 1980; Friedl & Schairer, 1981; Schneider & Altendorf, 1982). They have been called subunits *a, b,* and *c,* or χ, ψ and ω, respectively. There is no accepted nomenclature for these subunits as yet, and since we prefer the terms "*uncB* protein", "*uncF* protein" and "*uncE* protein," for a/χ, b/ψ and c/ω, respectively, we will adopt these terms for the rest of this review. The *uncE* protein is the "proteolipid" or "DCCD-binding protein",[1] which has been characterized thoroughly (Sebald

Key Words proton transport · ATPase · *Escherichia coli* · mitochondria

[1] *Abbreviations:* DCCD, dicyclohexylcarbodiimide; FSBA, *p*-fluorosulfonylbenzoyladenosine; NBD-Cl (or Nbf), 4-chloro, 7-nitrobenzofurazan; TNP-ATP, 2′,3′-0-(2,4,6-trinitrophenyl)-ATP.

Table. Relationships between subunits and genes of the *E. coli* proton-ATPase

Subunit	Mol. Wt	No. residues	Gene
F_1			
α	55,264[a, b, c]	513	*uncA*[d]
β	50,157[e, f]	459	*uncD*[d]
γ	31,387[c, e, r]	287	*uncG*[g]
δ	19,582[b, h, i]	177	*uncH*[j]
ε	14,914[e, f, k]	138[k]	*uncC*[g]
Membrane sector			
a/χ	30,276[b, i, l, m]	271[m]	*uncB*[n]
b/ψ	17,265[b, h, i]	156	*uncF*[n]
c/ω	8,288[b, i, o, p]	79	*uncE*[n]
?	14,183[b, l, q]	130[q]	*uncI*[q]

[a] Gay and Walker, 1981*a*; [b] Gay and Walker, 1981*b*; [c] Kanazawa et al., 1981*a*; [d] Downie et al., 1979*a*; [e] Saraste et al., 1981; [f] Kanazawa et al., 1982; [g] Downie et al., 1980; [h] Mabuchi et al., 1981; [i] Nielsen et al., 1981; [j] Gunsalus et al., 1982; [k] There is disagreement between the results of Saraste et al., 1981 and Kanazawa et al., 1982. The former find ε has 132 amino acids, the latter find 138. The mol wt for the latter is given; [l] Kanazawa et al., 1981*b*; [m] Nielsen et al., 1981 find two possible initiation codons for the *uncB* protein, and discuss the possibility that the translated protein has only 201 amino acids instead of 271. The mol wt shown is for the 271 residue DNA reading frame; [n] Downie et al., 1981; [o] Kanazawa et al., 1981*c*; [p] Sebald & Hoppe, 1981; [q] The reading frame of "gene 1" of Gay and Walker, 1981*b* and that of the undesignated open reading frame described by Kanazawa et al., 1981*b* differ substantially in sequence. The reading frame of Kanazawa et al. would have 127 residues. The mol wt shown is for the 130 residue sequence of Gay and Walker 1981*b*. As described in the text, no protein corresponding to this DNA sequence has yet been seen. We have called this gene "*uncI*" to correspond to the nomenclature of genes in the *unc* operon. [r] The sequences for the γ subunit given in footnotes c and e differ at 31 positions, including two stretches of 14 and 11 residues, respectively.

& Hoppe, 1981). The amino acid sequences of it derived by either Edman or DNA sequencing are in agreement. The *uncF* protein has an N-terminal amino acid sequence corresponding to that predicted from the DNA sequence (Foster & Fillingame, 1982) so its molecular weight is therefore accurately established. The *uncB* protein would appear from the DNA sequence to have a molecular weight of 30,300, yet it runs on SDS-gels with a mobility corresponding to ~24K mol wt. Since its N-terminal sequence is unknown, the true molecular weight cannot yet be decided. A hydrophobic "leader" sequence is not apparent from the DNA sequence and the amino acid composition of the purified protein is close to that predicted from the full DNA reading frame (Steffens et al., 1982). SDS-gels may simply underestimate the molecular weight as they do for bacteriorhodopsin (*see* Henderson, 1977; and footnotes to Table for further discussion).

The stoichiometry of membrane-sector subunits is not yet known. Genetic evidence is consistent with multiple copies of the *uncE* protein being present (Friedl et al., 1980; Tamura et al., 1981). Quantitation of radioactively labeled subunits by counting of SDS-gels has given conflicting results regarding the number of copies of the *uncE* protein present in *E. coli* proton ATPase, Foster and Fillingame (1982) reporting 10 and Nielsen et al. (1981) reporting 6 to 8. Data on the homologous protein from yeast and beef-heart mitochondrial proton-ATPase (DCCD-labeling experiments) are consistent with the statement that multiple copies (possibly six) are present (Sebald & Hoppe, 1981) but are not definitive. The *uncB* protein is reported to be present to the extent of one copy and the *uncF* protein to the extent of one or two copies per complex (Nielsen et al., 1981; Foster & Fillingame, 1982). These values, based on counting of bands excised from SDS-gels might, however, be erroneous. It is not clear that 100% of radioactivity present in each subunit in the original applied samples was quantitatively recovered by either group, and the range of calculated values reported (Foster & Fillingame, 1982) is fairly broad. Independent evidence using different methods would be valuable to confirm the subunit stoichiometries of the membrane sector. At the moment the most definite we can be is to say the membrane sector contains three different subunits of aggregate molecular weight in the range of 100 to 150K, tightly integrated with lipid, of undetermined architecture, with multiple copies of at least the *uncE* protein.

Genetics of the *E. coli* Proton-ATPase

The power of genetic techniques in studying membrane enzymes is vividly demonstrated by the fact that although the first mutation affecting the *E. coli* proton-ATPase was not reported until 1971 by Butlin et al. we now know the full amino acid sequence of each of the eight different subunits of the complex. The field has indeed moved quickly, and it is appropriate here to sketch a brief history of it.

The first two mutant alleles described (*uncA401* and *uncB402*) could be shown to affect the F_1 sector and membrane sector, respectively, by the technique of stripping and rebinding F_1 from and to membranes (Cox et al., 1973). These mutants were originally selected for their inability to grow on nonfermentable substrate (suc^- phenotype) and this remains the most commonly employed proce-

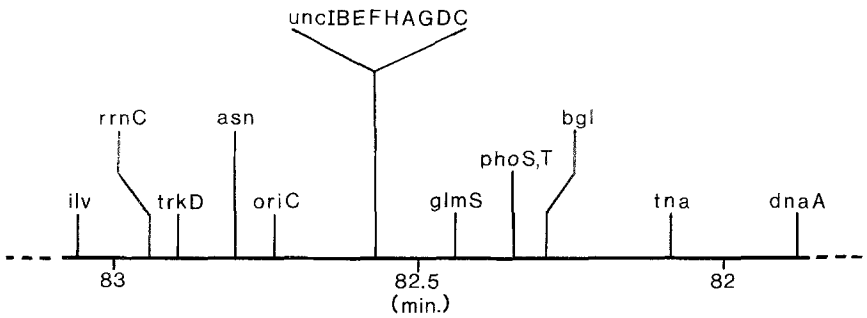

Fig. 1. Linkage map of *E. coli* around the *unc* genes. Taken from Kanazawa et al. (1981*c*), and showing the complete *unc* operon as derived from the DNA sequence (*see* text). The *uncI* gene is the first gene in the operon, called "gene one" by Gay and Walker 1981*b* (*see* footnote to Table). It is not apparently a structural gene in that no corresponding protein is found in the purified proton-ATPase. *uncBEFHAGDC* code for recognized subunits of the enzyme (*see* Table)

dure for isolating mutants affected in the proton-ATPase (Cox & Downie, 1979; Downie et al., 1979*a*). The next, and very important step forward came with the development of a complementation assay which could subdivide mutations affecting the F_1 sector and the membrane sector such that mutations in the different subunits could be distinguished from each other. This allowed the recognition of mutations affecting further structural genes for proteins of the F_1 sector (*uncC, D* and *G*) and membrane sector (*uncE, F*) (Gibson et al., 1977; Cox et al., 1978; Downie et al., 1979*b*; 1980, 1981). The use of Mu-phage to obtain polar mutations affecting the proton-ATPase then showed that the seven known genes formed an operon and the order of the genes was *B(F,E)AGDC* with the (*FE*) order unclear (Gibson et al., 1978; Downie et al., 1981). The *unc* operon maps at about minute 82.5 on the *E. coli* linkage map (Fig. 1).

Gene-polypeptide relationships began to be established in 1978 and 1979 when it was shown that the *uncA* gene codes for $F_1-\alpha$ subunit and the *uncD* gene codes for $F_1-\beta$ subunit (Downie et al., 1979*a*). Now all of the gene-polypeptide relationships are known, and are shown in the Table. The *uncH* gene was the last to be recognized and in fact its recognition came about not through genetic complementation experiments (no mutations in this gene have as yet been described) but from studies on restriction mapping of *unc* operon DNA, and the expression of the genes in *in vitro* protein synthesis experiments (Gunsalus et al., 1982). This work, together with the work on DNA sequencing to be described below, has shown the order of structural genes to be *uncBEFHAGDC*.

The Sequence of unc Operon DNA

Contemporaneously with the work on the genetics of the proton-ATPase, other workers were studying the origin of replication of *E. coli* chromosomal DNA (called *oriC*) and had mapped it close to the position at which the *uncA* and *uncB* genes mapped (Fig. 1). Two groups prepared specialized transducing phages (*λ asn*) which complemented the *uncA401* or *uncB402* mutations and carried *oriC* (von Meyenburg et al., 1978; Miki et al., 1978). It was likely therefore that in each case the *λ asn* contained the *unc* genes. Kanazawa et al. (1979) demonstrated this by showing that on thermo-induction, lysogenic strains carrying the *λ asn* increased synthesis of F_1 subunits and had higher membrane ATPase activity. Further confirmation was obtained by Foster et al. (1980) who showed increased synthesis of all eight subunits of the ATPase complex occurred on thermo-induction. Hansen et al. (1981) also located the eight *unc* genes on their *λ asn* isolates by genetic and functional analyses.

The *λ asn* preparations therefore allowed extensive restriction mapping of the *unc* DNA and contiguous regions, and it was possible to clone fragments in multicopy plasmids for DNA sequencing (Kanazawa et al., 1980*a*, 1981*a–c*, 1982; Gay & Walker, 1981*a, b*; Mabuchi et al., 1981; Nielsen et al., 1981; Saraste et al., 1981). Cloning of *unc* genes from hybrid-plasmid pools of restricted *E. coli* chromosomal DNA or by Mu-mediated transposition of the chromosomal *unc* genes onto a transmissible plasmid was also carried out (Downie et al., 1980, 1981) allowing mapping of the operon and *in vitro* protein synthesis experiments which demonstrated the gene-polypeptide relationships for the membrane sector subunits. More recently Cox et al.[2] sequenced cloned re-

[2] Cox, G.B., Langman, L.P., Jans, D.A., Downie, J.A., Senior, A.E., Gibson, F., Fimmel, A.L., James, L.B., Ash, G. (*submitted for publication*).

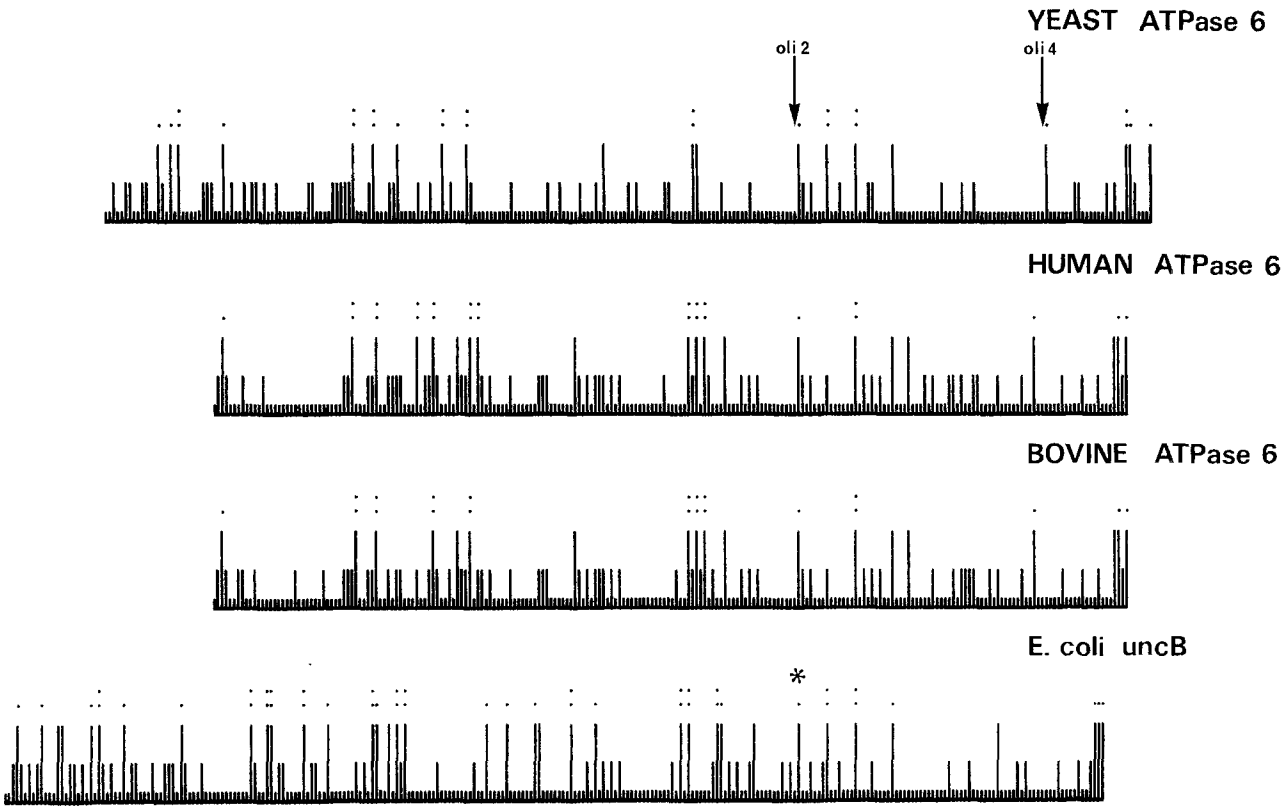

Fig. 2. "Polarity profiles" of the amino acid sequences of *E. coli uncB* protein and the homologous "ATPase 6" subunit of mitochondrial ATPase. Full bars represent His (no dot), Lys or Arg (two dots) or Glu, Asp or C-terminal (one dot). Half-bars represent Ser, Thr, Asn or Glu. *"Oli 2"* and *"oli 4"* are mutations in the yeast protein conferring resistance to the inhibitor oligomycin. The asterisk denotes the conserved glutamic acid residue (*see* Fig. 3 and text) which is residue 196 in *E. coli*. The four sequences are aligned so that this conserved Glu residue occupies the same position in each.

striction fragments to ascertain the nature of point mutations in the *uncE* gene.

The DNA sequence of the *unc* operon actually contains nine genes. The first, called *uncI* by us, is an open reading frame which if transcribed and translated would code for a protein of molecular weight of 14,183 (Gay & Walker, 1981*b*, and *see* Table footnote). No one has yet detected the actual protein. It does not occur as a subunit of the enzyme. It does not appear to be homologous in sequence to the unidentified reading frame URF A6L which occurs in front of the gene coding for "ATPase 6" (homologous to *uncB* protein) in human and bovine mitochondrial DNA (Anderson et al., 1981, 1982). Gay and Walker (1981*b*) point out that the protein would be hydrophobic and basic, and the codon usage in the gene indicates it may be expressed at only low levels. These workers suggest the protein may be a "pilot protein" to "guide assembly of the membrane sector of the enzyme complex." Prediction of its secondary and tertiary structure suggests it could be an intrinsic membrane protein containing four transmembrane helical segments (A.E., Senior, *unpublished*). Since complementation occurs in partial diploids containing mutations in different membrane sector genes carried on F-plasmid and chromosome (e.g. Downie et al., 1981), mixing of membrane sector subunits translated on different mRNA transcripts must be possible during assembly. Therefore if the *uncI* protein is involved in assembly it probably acts on the assembling subunits directly. However, one can postulate other roles for the *uncI* gene product and we feel the important point to stress here is that its role is currently unknown.

The second gene is the *uncB* gene, coding for a protein showing marked sequence homology to the "ATPase 6" subunit of yeast (Macino & Tzagoloff, 1980), human (Anderson et al., 1981) and bovine (Anderson et al., 1982) mitochondrial proton-ATPase (Fig. 2). Prediction of the secondary and tertiary structure of this protein suggests it contains seven helical transmembrane segments, similar to bacteriohodopsin[3]. The homologous yeast protein carries two oligomycin-resistance

[3] A.E. Senior (*manuscript submitted*).

Fig. 3. Amino acid sequences around conserved Glu residues in *uncB* protein of *E. coli* and "ATPase 6" subunits of mitochondrial proton-ATPase. *A* The glutamic acid residue next to the *oli* 2 locus in the yeast protein is conserved in all four species shown and lies in a hydrophobic sequence. ↓ indicates *oli 2* locus, * indicates conserved glutamic acid. *B*. The glutamic acid residue next to the *oli 4* locus in the yeast protein is conserved in yeast, human and bovine, but is deleted in the *E. coli uncB* protein. ↓ indicates oli 4 locus, * indicates conserved glutamic acid

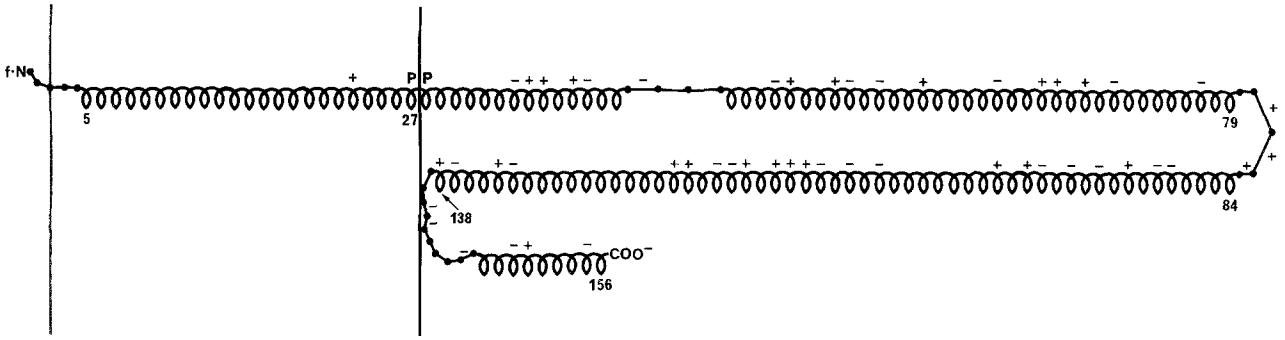

Fig. 4. Predicted secondary and tertiary structure of the *E. coli uncF* protein. The method of prediction is described elsewhere (A.E. Senior, *submitted*) and is based on application of modified Chou-Fasman rules in conjunction with a polarity profile of the *uncF* protein derived as in Fig. 2 or Fig. 6. ∿∿∿, α-helical residue; ●—●, random coil residue or β-turn residue

loci, *oli* 2 and *oli* 4 (Macino & Tzagoloff, 1980). The sequences around these two loci are conserved in human and bovine proteins (Fig. 3). In the *E. coli uncB* protein only the region around *oli* 2 is conserved, the region around *oli* 4 is deleted (Fig. 3), suggesting this potential binding site for oligomycin is absent in *E. coli*. It is established that oligomycin does not inhibit the proton-ATPase of *E. coli* whereas it is a potent inhibitor of mitochondrial proton-ATPases.

The *uncE* protein ("proteolipid" or "DCCD-binding protein") is encoded by the third gene. The sequence of this extremely hydrophobic protein was actually known prior to the DNA sequencing work, and much has already been learned about its structure and function (Sebald & Hoppe, 1981). It is discussed in more detail below. The *uncF* protein is encoded by the fourth gene. It is a singular protein, having a hydrophobic N-terminal segment which probably traverses the membrane (33 residues, containing but one charged residue at Lys_{23}) then a highly charged segment (123 residues, of which 51 are charged) predicted to be highly helical and probably external to the membrane bilayer (Walker et al., 1982*a*; A.E. Senior, *submitted*). A possible structure for the *uncF* protein is presented in Fig. 4. This protein may extend about 80 Å from the membrane surface and could form a stalk around which F_1 subunits are clustered.

The five F_1 subunits are coded by the last five genes (Fig. 1 and Table). The α and β subunits show weak sequence homology with each other, extending through the whole sequence (Saraste et al., 1981; Kanazawa et al., 1982). The β subunit shows an impressive 75% sequence homology with the bovine mitochondrial F_1–β subunit (Saraste et al. 1981) and 67% with the spinach chloroplast

$F_1-\beta$ subunit (Zurawski et al., 1982). The *E. coli* β subunit has segments clearly homologous to those around a unique Tyr residue which reacts with FSBA (an ATP analog) in mitochondrial $F_1-\beta$ subunit (Esch & Allison, 1978) and around two Glu residues which react with DCCD and may be sites for Mg binding in bovine mitochondrial and PS3 $F_1-\beta$ subunit (Esch et al., 1981; Saraste et al., 1981; Yoshida et al., 1981; Kanazawa et al., 1982). A nucleotide binding fold may be present in the β subunit, formed by 90 residues that occur in the middle of the sequence (residues 240 to 330), before the FSBA-reactive Tyr (residue 354) and after the DCCD-reactive Glu residues (181 and 192) (Kanazawa et al., 1982). Thus one may speculate that the regions 180 to 200 and 350 to 360 of the β subunit may come together to form the binding domain for the $\beta-\gamma$ phosphoryl groups of ATP. The δ subunit is predicted to be highly helical and contains one unusually long helical segment (~ 50 residues, Mabuchi et al., 1981). α, β, γ and ε subunits are not unusual in predicted helical content and are probably globular.

Some processing of F_1 subunits occurs after translation. The N-terminal f-Met is removed from β, γ and ε subunits and the formyl group is removed from the N-terminal Met of α and δ (Kanazawa et al., 1981*a*; Mabuchi et al., 1981; Walker et al., 1982*b*). There is only one copy of each gene in the *unc* operon, and yet some subunits are present in multiple copies in the membrane-bound enzyme, and other subunits are present in single copy only. How is expression of the genes regulated to achieve the final stoichiometry? There is a promoter region recognizable before the first gene (*uncI*) (Gay & Walker, 1981*b*) and a transcription terminator region after the *uncC* gene (Saraste et al., 1981; Kanazawa et al., 1982). The intergenic sequences are short, and there is not good evidence for any other promoter site. Therefore it is likely a single mRNA transcript forms, as the experiments with Mu-phage had earlier indicated (Gibson et al., 1978). Usage of "common" vs. "rare" codons may affect expression of the different mRNA segments (Gay & Walker, 1981*a*; Kanazawa et al., 1982) but it is difficult at this time to see how exact stoichiometries can be arrived at in this way, and further, in *in vitro* protein synthesis experiments directed by plasmids carrying *unc* DNA fragments, increasing tRNA concentrations by 10-fold increased the relative amounts of the more abundant subunits, rather than those thought to be present in single copy (Brusilow et al., 1982). These workers felt selective degradation of subunits did not occur after translation *in vitro* or *in vivo* but that some regulation at the stage of initiation of translation could be occurring. The regulation of expression of the genes is therefore a puzzle at the moment.

One problem that has arisen concerns nomenclature. Some workers have introduced new designations for the genes (*bcf, atp, pap*) instead of the conventional designation *unc* (Bachmann & Low, 1980). This will lead to confusion (e.g. Yoneda et al., 1973) and we urge all workers to conform to the established *unc* designation for *E. coli* proton-ATPase genes.

Comparison of the Proton-ATPase of *E. coli* with that of Mitochondria and the Bacterium PS3

The α, β and γ subunits of F_1, the *uncB* protein (homologous to "ATPase 6") and the *uncE* protein (homologous to "DCCD-binding protein") are seemingly common to both mitochondrial and *E. coli* enzymes. Walker et al. (1982*c*) showed that the δ subunit of *E. coli* F_1 is homologous to the subunit "OSCP" (Senior, 1971) of the mitochondrial enzyme, and that the ε subunit of *E. coli* F_1 is homologous to the δ subunit of mitochondrial F_1. At the time of this writing the ε subunit of mitochondrial F_1 and the "F_6" subunit of the mitochondrial enzyme appear to lack counterparts in *E. coli*. Mitochondrial $F_1-\varepsilon$ subunit has a mol wt of ~ 7.5 K (Senior, 1979*a*) and "F_6" has a mol wt of ~ 8 K (Kanner et al., 1976). They have not been seen in preparations of the *E. coli* enzyme and no genes corresponding to them occur in the *unc* operon.[4]

Conversely, at the time of writing no counterpart of the *E. coli uncF* protein has been seen in mitochondrial proton-ATPase. It is not encoded in yeast, human or bovine mitochondrial DNA.[5] Preparations of the whole proton-ATPase complex from mitochondria contain numerous components (Galante et al., 1979; Ryrie & Gallagher, 1979;

[4] The unpublished preliminary amino acid sequence of F_6, kindly sent to us by E. Racker and R. Bradshaw, shows resemblance to the C-terminal end of the *uncF* protein of *E. coli* in that F_6 is very polar (25 charged residues out of 69 total). No actual sequence homology between F_6 and *uncF* protein was detected by us, however. Neither could we detect any sequence homology between F_6 and the partial sequences of OSCP published by Walker et al. (1982*c*), suggesting F_6 is not a fragment of OSCP.

[5] There is, however, an unidentified reading frame (URF 6) in both human and bovine mitochondrial DNA (Anderson et al., 1981, 1982) which is homologous at the N-terminal end (~ 30 residues) with the *uncF* protein of *E. coli*, and which codes for a putative protein of mol wt ~ 19 K.

Soper et al., 1979; Ludwig et al., 1980; Todd et al., 1980; Berden & Henneke, 1981). A subunit of molecular weight of 18 to 19 K (corresponding to the mobility of the *E. coli uncF* protein on SDS-gels) has not been noted consistently by the workers in the field. However, undefined components of mol wt 20 to 24 K have been noted. When one examines the published data on mitochondrial membrane sector preparations (Glaser et al., 1980; Alonzo et al., 1981; Galante et al., 1981;) the same conclusion is apparent, and because OSCP (of mol wt 18 to 21 K on SDS-gels) is removed from the first two of these preparations, a potential ambiguity is also removed. No protein corresponding to the *E. coli uncF* protein (which stains well) has been consistently noted by workers in the field. If it is true that, as speculated above, the *uncF* protein of the *E. coli* enzyme forms a structural link between membrane sector and F_1 subunits, it would be surprising if it were not conserved in the mitochondrial enzyme. The F_6 subunit of the mitochondrial enzyme is known to be involved in binding F_1 to the membrane sector (Senior, 1979*a*) and the relationship between F_6 and the *uncF* protein is of interest therefore.

Another difference is that mitochondria contain an F_1-ATPase-inhibitor protein, thought to be regulatory in function (Pedersen et al., 1981). No such protein has been seen in *E. coli* although its existence cannot be excluded. At one time the ε subunit of *E. coli* F_1 was thought to be an inhibitory protein analogous to the mitochondrial inhibitor protein, but this is now known to be not so (Dunn & Heppel, 1981). There may also be other differences. Whereas the total number of components in the *E. coli* ATPase is clearly eight, all preparations of mitochondrial ATPase contain several more than this and the actual constitution of the mitochondrial membrane sector is undecided.

A lot of work has been published on the proton-ATPase of the thermophilic bacterium PS3. The F_1-sector from this bacterium contains five different subunits (Yoshida et al., 1975) similar to *E. coli* and there is present in PS3 membrane sector a "DCCD-binding protein" homologous to the *uncE* protein of *E. coli* (Sebald & Hoppe, 1981). A preparation of the whole proton-ATPase complex of PS3 contained the five F_1 subunits, DCCD-binding protein and two other major components of molecular weight 19 K and 13.5 K, both of which were precipitated with the other subunits by anti-F_1 antibody (Sone et al., 1975). Gel electrophoresis profiles of preparations of PS3 membrane sector ("TF_o") showed the 19 K, 13.5 K and DCCD-binding protein as major components, together with about six other minor bands (Okamoto et al., 1977; Sone et al., 1978). The stoichiometry of the membrane sector subunits derived from radioactive labeling was reported as 19 K = 1 copy, 13.5 K = 2 copies and DCCD-binding protein = 5 copies (Kagawa et al., 1976). (Later calculations by Foster and Fillingame (1982) using a correct molecular weight gave a value of 3 copies of DCCD-binding protein.) It was evident from the earlier papers on PS3 that the membrane-sector was considered to contain three components.

However, Sone et al. (1978) described a further treatment of the PS3 membrane sector consisting of treatment with 4 M urea and 0.25% Triton X-100, passage through CM-cellulose, and precipitation with ammonium sulfate in the presence of 2% cholate. The new preparation ("CM − TF_o") contained the DCCD-binding protein, the 13.5 K component and 5 to 6 other minor components, but apparently lacked the 19 K component. It was reported to have activities similar to those of the original membrane sector preparation. Based on this evidence Sone et al (1978, 1979, 1981) now state that the membrane sector of PS3 proton-ATPase is actually composed of two subunits only – the 13.5 K subunit and the DCCD-binding protein. The 13.5 K component had TF_1 binding capability when incorporated by itself into liposomes and Sone et al. (1978, 1979, 1981) assume that this F_1 binding is representative of the physiological binding of F_1 to PS3 membrane sector, although evidence for this is incomplete. As pointed out later, genetic evidence suggests that in *E. coli* all three membrane sector subunits are required for normal F_1 binding. Therefore, at the moment the composition of PS3 membrane sector seems unclear. Whether or not the 19 K or 13.5 K proteins are homologous to *E. coli uncB* or *uncF* proteins is an interesting question yet to be answered.

In Vivo Assembly of the *E. coli* Proton-ATPase Complex

The *in vivo* assembly of the enzyme is an ordered process (Cox et al., 1981), and is depicted in Fig. 5. This Figure is derived from Fig. 8 of Cox et al. (1981) and is intended to convey the order of incorporation of subunits, but not necessarily the accurate morphology or correct stoichiometry of subunits.

The first event was inferred to be the incorporation of the *uncB* protein into the membrane (Cox et al., 1981). The *uncE* gene is next to be translated

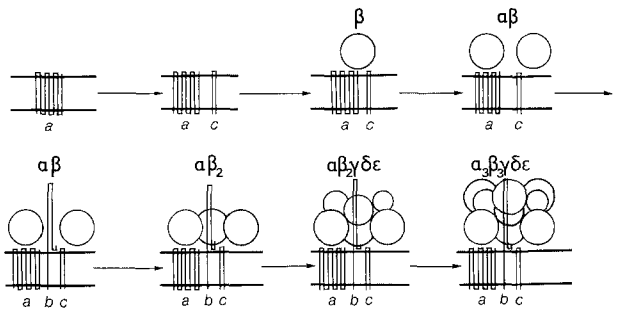

Fig. 5. Assembly of the *E. coli* proton-ATPase *in vivo*. α, β, γ, δ and ε are F_1 subunits. The membrane sector subunits are represented by *a* (*uncB* protein), *b* (uncF protein) and *c* (*uncE* protein; "DCCD-binding protein). The stoichiometry of membrane sector subunits is not established, and the morphology, with *uncF* protein (*b*) projecting from the membrane, is strictly hypothetical. The stoichiometry of α and β subunits at intermediate steps is not established with certainty. Adapted from Cox et al. (1981)

after the *uncB* gene and the *uncE* protein may be properly incorporated into the membrane immediately. In the original paper (Cox et al., 1981) we hedged on this question, since although the *uncE* protein was shown to be present in the membranes of mutant strains which contained no *uncF* protein or any F_1 subunits, it was not sure that the *uncE* protein was properly integrated with the *uncB* protein at this stage. It did appear to facilitate some binding of intact F_1 to stripped membranes (25 to 30% of normal; *see* Fig. 2 and Table 3 of Cox et al., 1981) and was reactive with DCCD. More recent work (Friedl et al., 1981) used a Mu-induced mutant strain which incoporates the *uncB* and *uncE* proteins and no other subunits into the membranes. The sub-stoichiometric DCCD reactivity of the *uncE* protein which is characteristic of normal membranes, and is believed to reflect cooperativity among individual monomers of the assembled *uncE* protein oligomer, was seen in this mutant strain, suggesting the normal *uncE* protein oligomer was already assembled. No proton conduction is seen at this stage (Cox et al., 1981; Friedl et al., 1981).

The next event is the binding of $F_1-\beta$ and α subunits (in that order) to the *uncB* protein in the membrane. Both β and α (probably one copy of each) must be bound before the *uncF* protein can be incorporated. After the incorporation of the *uncF* protein the complete membrane sector is formed but the membranes remain proton-impermeable due to the β and α subunits which are bound. Addition of a full complement of α and β subunits, and of γ, δ and ε subunits then occurs to form the complete F_1. The order of addition of these later F_1 subunits, and details of any structural rearrangements which take place as they are added, remain unexplored. The later steps in Fig. 5 are therefore tentative.

The process may be ordered purposefully to prevent open proton pores occurring in the membrane during assembly, a potentially debilitating situation. It was shown previously that mutant β or mutant α subunits could block proton conduction across the membrane sector in the absence of an F_1 aggregate (Fayle et al. 1978; Senior et al., 1979*a*, *b*), and one therefore has to be careful when studying mutant strains impaired in proton-transport to be sure that the mutation is not in fact in F_1 α or β subunits. The work of Cox et al. (1981) showed that in strains carrying the *uncG428* point mutation, or in the Mu-induced mutant strain which was $uncB^+E^+F^+H^+A^+GDC$ and was transformed by plasmid pAN 36 (D^+C^+), a functionally competent membrane sector was present which was blocked in proton translocation by normal α and β subunits.

ATPase activity is not normally found in the cytoplasmic fraction when *E. coli* cells are broken and subcellular fractions are prepared. However, considerable F_1-ATPase is found in the cytoplasm in strains carrying mutations in *uncB*, *uncF* or *uncC* genes (Downie et al., 1979*a*, *b*; 1980, 1981). The origin of this activity is not yet certain. F_1 subunits may be polymerized in solution into active F_1 aggregates *in vitro*, but it is not established that this would occur in the cytoplasm under the normal conditions of intracellular pH and aqueous environment (*in vitro* repolymerization is optimal at pH ~6 in glycerol or methanol-containing buffers). Alternatively the cytoplasmic F_1 may in some cases initially form on the membranes and then become dislocated.

Roles of Individual Subunits in the *E. coli* Proton-ATPase

Effects of Mutations

Since the assembly of the proton-ATPase complex is an ordered process, mutations can cause functional derangement by interruption of the correct assembly or by yielding a structurally unstable enzyme. One therefore has to exercise care in interpreting the effects of mutations on function. However, in many cases correct stable assembly is accomplished and important information on function has been gleaned from studies of *unc* mutants.

Both *uncB* and *uncE* proteins seem to be required for proton translocation since mutations in

either gene block proton conduction (reviewed by Fillingame, 1981). Only two *uncF* mutants are known and neither assembles the *uncF* protein into the membrane (Downie et al., 1981). Both have proton-impermeable membranes suggesting the *uncF* protein must be correctly assembled for proton conduction to occur. Data derived from the studies on assembly of the complex cited above (Cox et al., 1981; Friedl et al., 1981) also imply that *uncB* and *uncE* proteins together are not sufficient for proton conduction. It is worth reiteration that the amino acid sequence of *uncF* protein suggests it may interact with both membrane sector and F_1 subunits and if so it may play a central role in functional integration of the two sectors. Mutations in *uncB*, *uncE* or *uncF* genes cause impaired binding of F_1 to the membrane and it seems that all three proteins of the membrane sector are required for fully normal F_1 binding (Downie et al., 1979*a*, *b* 1981; Fillingame, 1981). Several mutations in the *uncA* gene ($F_1-\alpha$ subunit) are known to cause inactivation of ATP hydrolysis and ATP synthesis and these effects are discussed in detail later, in relation to the mechanism of catalysis on F_1. Several *uncD* mutants ($F_1-\beta$ subunit) with lower than normal F_1 catalytic activity are also referred to later.

Of the many reported *unc* mutant alleles (*see* Fillingame, 1981, for list) only a handful have been defined in terms of the nature and position of the amino acid substitution. Those that have been so defined all occur in the *uncE* protein and will be discussed later in relation to the molecular mechanism of proton transport.

Studies on Isolated Native Subunits

The impact of genetics has been substantial, but in actuality the *E. coli* system presents an additional experimental advantage over proton-ATPases from all other sources, which is that the F_1 may be depolymerized in chaotropic salts, the subunits isolated in pure form, and effective reconstitution ($\sim 70\%$) of the original F_1-ATPase activity may be regained by repolymerization (Dunn & Heppel, 1981). This work, started by Vogel and Steinhart (1976), has been extended by Heppel and coworkers to studies of the roles of individual subunits in F_1. Only in the PS3 proton-ATPase have similar reploymerization experiments been possible. In the intitial reported reconstitutions in PS3 the maximum regain of F_1-ATPase activity after repolymerization was $<9\%$ of the original (Yoshida et al., 1975, 1977*a*), suggesting that extensive denaturation of the PS3 F_1 subunits had occurred under the harsh conditions used to separate them. Later work used less harsh conditions of depolymerization and regain of up to 46% of the original PS3 F_1-ATPase was reported (Kagawa & Nukiwa, 1981).

Dunn and Heppel (1981) have reviewed the work on isolated *E. coli* subunits, and so here we will only describe the major discoveries, and discuss points of ambiguity. The minimum combination of subunits which gives ATPase activity on repolymerization is $\alpha\beta\gamma$.[6] Isolated α subunit from *E. coli* F_1 binds ATP ($K_d = 0.1$ μM) or ADP ($K_d = 0.9$ μM) to the extent of 1 mol/mol apparently in the absence of divalent cation (Dunn, 1980; Dunn & Futai, 1980; Paradies, 1980). ATP·α might be expected to be the form of newly synthesized α that becomes assembled into the proton-ATPase. ATP·α is more compact than α alone and the dissociation of ATP is very slow indeed (Dunn, 1980; Paradies, 1980). As Dunn and Heppel (1981) suggest, the α subunit is likely to carry the "tightly bound nucleotides" which occur in F_1. Isolated β subunit from *E. coli* F_1 also appears to carry a nucleotide binding site (Lunardi et al., 1981) which, as we discuss later, is likely to be the catalytic site.[7] Isolated γ subunit does not bind nucleotides; its role in the $\alpha\beta\gamma$ complex appears to be that of an organizer protein. Experiments with trypsin showed that in fact a complex consisting of α, β and a 10 K mol wt fragment of γ retained ATPase activity (Smith & Wilkowski, work cited in Dunn & Heppel, 1981).

Repolymerized $\alpha\beta\gamma$, $\alpha\beta\gamma\delta$ or $\alpha\beta\gamma\varepsilon$ aggregates (all of which have ATPase activity) do not rebind to stripped membranes containing intact membrane sector. Only the full $\alpha\beta\gamma\delta\varepsilon$ aggregate rebinds, and Dunn and Heppel (1981) suggest that δ and ε bind to the membrane sector and connect the $\alpha\beta\gamma$ aggregate to the membrane, primarily by binding to γ. However, despite evidence that isolated δ and ε retain their native conformations (e.g. the repolymerized $\alpha\beta\gamma\delta\varepsilon$ aggregate is active in oxidative phosphorylation) no direct binding of isolated δ or ε or δ plus ε ($\pm\gamma$) to the membrane sector has been shown. Thus the presumed connecting function of δ and ε is not proven (Dunn & Heppel, 1981). Isolated γ and ε subunits combine to form $\gamma\cdot\varepsilon$ complex with high affinity *in vitro*

[6] Kagawa and his co-workers had claimed that isolated β and γ subunits from PS3 F_1 could combine to form an active $\beta\gamma$ complex. This conclusion was later retracted, however (Kagawa & Nukiwa, 1981) and $\alpha\beta\gamma$ is now thought to be the minimal aggregate for ATPase activity with PS3 subunits.

[7] Isolated α and β subunits from PS3 have also been reported to each contain a nucleotide binding site (Ohta et al., 1980).

and are probably associated in intact F_1 (Dunn, 1982).

Kagawa and co-workers showed that binding of isolated PS3 δ, ε, or δ plus ε subunits to a preparation of the PS3 membrane sector incorporated in liposomes does occur (Yoshida et al. 1977*b*). This result is therefore different from what has been found in *E. coli*. However, scrutiny of the data reveals that the binding of PS3 δ or ε or δ plus ε may not have been specifically to the membrane sector proteins. Yoshida et al. (1977*b*) further reported that addition of a mixture[8] of isolated γ, δ and ε subunits to PS3 membrane sector reconstituted into liposomes reduced K^+-gradient-driven proton influx through the membrane sector. This is an interesting experiment because it represents the only direct evidence in the literature that one or more of the three minor subunits of bacterial F_1 can bind directly to proteins of the membrane sector. However, we believe the experiment should be interpreted with caution because the membrane sector preparation used appears to be ~85% denatured in respect to its F_1 binding capability (Okamota et al., 1977; Yoshida et al., 1977*b*, and compare with Sone et al., 1981) and Yoshida et al. (1977*b*) reported regain of only 4% of the original F_1-ATPase specific activity (*cf.* Yoshida et al., 1975) on repolymerization of their preparations of isolated F_1 subunits, raising the possibility that the γ subunit preparation used could be up to 96% denatured. Also, the $\Delta\bar{\mu}H^+$ in this experiment was reversed with respect to the normal situation. No such effect on proton permeabilitiy of membrane sector has been noted using isolated *E. coli* γ, δ and ε subunits (Dunn & Heppel, 1981).

We conclude that the suggested role of δ and ε in attaching γ, and thus α and β, to the membrane sector as suggested (Yoshida et al., 1977*b*; Dunn & Heppel, 1981) presently lacks unambiguous experimental support. The evidence is clear that δ and ε are required for $\alpha\beta\gamma$ to bind to the membrane but more work will be required to understand exactly how this is achieved. The reported homology between OSCP and *E. coli* $F_1-\delta$ subunit (Walker et al., 1982*c*) would certainly support a binding function for the *E. coli* $F_1-\delta$ subunit since OSCP has been found to bind to both membrane sector and F_1 sector in the mitochondrial enzyme (Senior, 1979*a*). Nevertheless, the recent description of the unusual primary structure of the *E. coli uncF* protein, and the demonstration that in *E. coli*, normal F_1 α and β subunits do bind to the membrane in the absence of γ, δ or ε subunits during the initial stages of the normal *in vivo* assembly of the complex, clearly introduce new elements into this area of investigation.

[8] It was not shown that subunit γ reduced proton conduction when added to membrane sector already containing bound δ plus ε subunits. Such an experiment was not described. It should also be noted that the molar ratio of total γ subunit to native membrane sector was 18:1 (partial block of H^+ conduction) or 36:1 (more complete block of H^+ conduction) in these experiments.

Mechanism of Proton Transport across the Membrane Sector

All three membrane sector subunits of the *E. coli* proton ATPase seem to be required for proton translocation to occur, as indicated from the evidence on mutants described above (*see also* Friedl et al., 1981 for discussion of this point). None of the amino acid substitutions in *E. coli uncB* or *uncF* mutant strains has been defined. However, present information on the *uncE* protein of *E. coli* leads to some interesting considerations.

"Polarity profiles" of six homologous DCCD-binding proteins including the *E. coli uncE* protein are shown in Fig. 6. This type of diagram readily enables one to discern hydrophobic and polar domains, and it is obvious that each of the six proteins is composed of two hydrophobic segments separated by a polar segment. A prediction of the secondary and tertiary structure of the *E. coli uncE* protein appears in Fig. 7. The model is composed of two α-helical transmembrane segments, with the polar segment connecting them. There is marked conservation of sequence in the polar connecting segment, but not at the C- and N-terminal ends of the protein (Sebald & Hoppe, 1981) suggesting the polar connecting segment is involved in binding F_1 subunits inside the cell, and that C- and N-terminal segments are on the outside of the membrane.

A salient feature of the *uncE* protein is the buried, conserved acidic group, occurring as Asp_{61} in the *E. coli* protein (Figs. 6 and 7). This is the group which reacts with the inhibitor DCCD (*see* Sigrist-Nelson & Azzi; 1979; Sebald & Hoppe, 1981). A buried DCCD-reactive acidic group also occurs in cytochrome oxidase subunit III (Prochaska et al., 1981) and actually lies within a sequence showing homology with *E. coli uncE* protein. DCCD reaction blocks proton transport in both cytochrome oxidase (Azzi, 1980) and the proton-ATPase (Sebald & Hoppe, 1981). This evidence immediately directs attention to the buried acidic residue as being involved in proton translocation. Mutant strains of *E. coli* in which Asp_{61} of the *uncE* protein is substituted by Gly or Asn

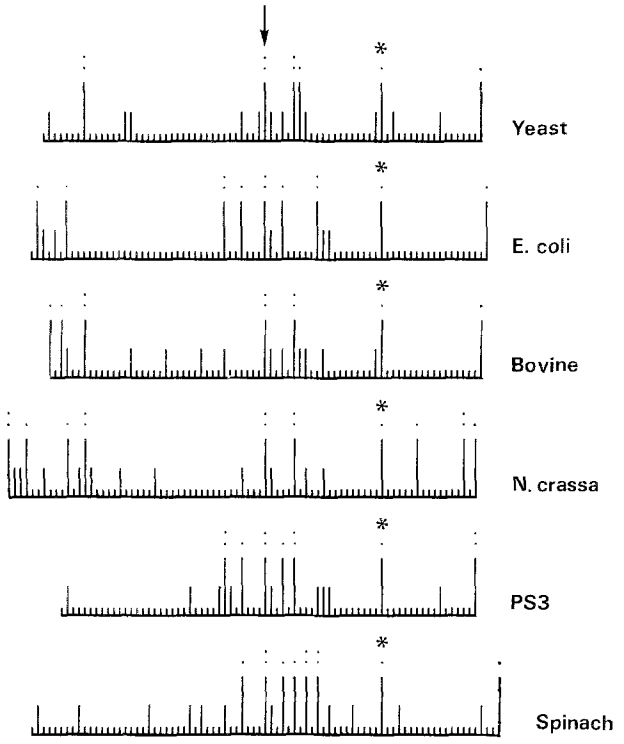

Fig. 6. Polarity profiles of the amino acid sequences of the *uncE* protein of *E. coli* and five homologous "DCCD-binding" proteins. The sequences were taken from Sebald and Hoppe (1981) and are centered around a conserved Arg (Arg_{41} in *E. coli*) shown by ↓. The asterisk denotes the conserved, buried acidic group (Asp_{61} in *E. coli*) which reacts with DCCD. Full bars are His (no dot); Lys, Arg, N-terminal (2 dots); Asp, Glu, C-terminal (one dot). Half-bars are Ser, Thr, Asn or Gln

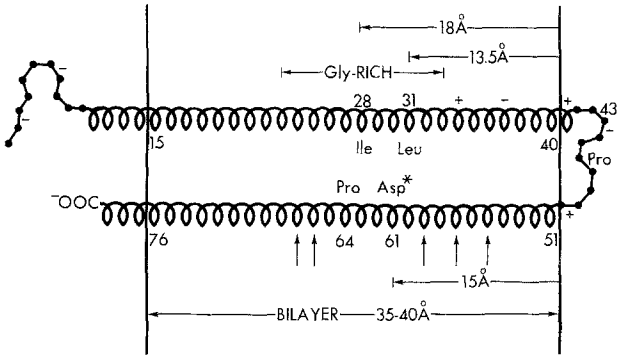

Fig. 7. Predicted secondary and tertiary structure of *E. coli uncE* protein. The method of prediction is described elsewhere (A.E. Senior, *submitted*), and is based on application of modified Chou-Fasman rules in conjunction with the polarity profile derived in Fig. 5. ∿∿∿, α-helical residue; ●–●, random coil residue or β-turn residue. Effects of mutations at positions 28, 31, 61 and 64 are discussed in the text. The homologous proteins of five other species shown in Fig. 6 were predicted to have exactly the same secondary and tertiary structure as the *E. coli* protein from position equivalent to residue 15 of *E. coli* through position equivalent to residue 76 of *E. coli*. Thus variations among the six species occurred only in N- and C-terminal extramembrane segments. The *arrows* represent positions of mutations in *N. crassa* or *S. cerevisiae* which confer resistance to oligomycin

do not show proton translocation (Hoppe et al., 1980*a*; 1982; Wachter et al., 1980). Understandably, radioactive DCCD does not label the *uncE* protein in these strains, and the bound F_1 is not DCCD-sensitive. Therefore these experiments confirm the idea that the Asp_{61} is involved intimately in proton conduction.

Six strains carrying mutations at Ile_{28} of the *E. coli uncE* protein have been described (Hoppe et al., 1980*b*; Wachter et al., 1980) and they all lead to "DCCD resistance". In four cases the substitution is $Ile_{28} \rightarrow$ Val and in two cases $Ile_{28} \rightarrow$ Thr. Proton translocation and oxidative phosphorylation are normal; the mutants differ from normal only in that the concentration of DCCD needed to inhibit is higher and DCCD labels the *uncE* protein Asp_{61} to a lesser extent. The effects can be reasonably explained by assuming that DCCD first binds noncovalently to the *uncE* protein before it reacts covalently with Asp_{61}, and that Ile_{28} provides a noncovalent binding site. The results imply therefore that Ile_{28} and Asp_{61} lie close together as in Fig. 7 (Sebald & Hoppe, 1981).

Two separate mutant *uncE* alleles isolated four years apart were found to cause the same amino acid substitution $Leu_{31} \rightarrow$ Phe (*uncE408* and *uncE463* alleles, Cox et al, (*submitted for publication*). Strains carrying these alleles did not assemble the *uncE* protein into their membranes unless multiple copies of the mutant gene were present. Strains in which the mutant protein was assembled had membranes which had enhanced reactivity to DCCD, showed oxidative phosphorylation and were not permeable to protons after F_1 was stripped off. These results are unusual, and cannot yet be fully explained. However, it seems reasonable to presume that Leu_{31} is close to Asp_{61} in the *uncE* protein as Fig. 7 predicts, and that the bulky Phe side-chain (projecting ∼2 Å further than Leu from the helix) displaces the Asp_{61} carboxyl leading to the unusual properties of membranes from strains carrying multiple copies of the *uncE408* and *uncE463* mutant alleles.

The *uncE410* mutant allele (Downie et al., 1979*b*; Cox et al., (*submitted*), directs the amino acid substitution $Pro_{64} \rightarrow$ Leu. Normal assembly of the *uncE* protein occurs in haploid strains, the membranes are proton-impermeable and do not show oxidative phosphorylation. The bound F_1 is DCCD-insensitive. It seems reasonable to assume these effects are due to the interaction of the substi-

tuted bulky Leu side chain with the Asp_{61} carboxyl. The position equivalent to Pro_{64} is occupied by a small amino acid (Pro, Gly or Thr) in the sequences of other species (Ovchinnikov, 1981; Sebald & Hoppe, 1981).

Strains carrying the *uncE429* allele, whether haploid or multiploid, do not assemble the *uncE* protein into their membranes (Ash, 1981). The amino acid substitution here is $Gly_{23} \rightarrow Asp$ (F., Gibson, G.B., Cox, A. Fimmel, D., Jans and L. Langman, *personal communication*), and an explanation for the effect of the mutation could be that assembly is prevented because burying the additional carboxyl group is too energetically expensive (Engelman & Steitz, 1981; von Heijne, 1981). Gly_{23} lies in a Gly-rich sequence (Fig. 7) which is conserved in other species (Sebald & Hoppe, 1981) but whose function is unknown. The Gly-rich region may have only weak helix-forming propensity, and may undergo a coil/helix transition during assembly.

In Fig. 7, several sites of mutations which confer "oligomycin-resistance" in the *N. crassa* and *S. cerevisiae* DCCD-binding proteins are shown by arrows. They lie close to the conserved, buried, DCCD-reactive acidic residue, and we feel a reasonable hypothesis is that oligomycin normally binds to these sites and shields the buried acidic residue, perturbating its interaction with protons. Each of the two "oligomycin-resistance" loci located in the "ATPase 6" protein of yeast (Fig. 2 and Fig. 3) are next to Glu residues which we predict will be buried in the membrane bilayer. The data on the loci conferring oligomycin-resistance imply that *uncB* and *uncE* proteins lie close together in the membrane. Further evidence that the two proteins interact was provided by Stephenson et al. (1981) who showed that in yeast a mutation affecting "ATPase 6" (homologous to *uncB* protein) prevented normal assembly of the DCCD-binding protein although the mutant "ATPase 6" protein was itself assembled in normal amount.

The interactions of the *uncB, uncE* (and *uncF*) proteins obviously must be better understood for us to clarify the proton-conduction pathway. If one assumes a (minimal) stoichiometry of one *uncB*, one *uncF* and six *uncE* protein monomers in the *E. coli* membrane sector, this might provide around twenty transmembrane α-helices altogether (*uncB* providing seven, *uncF* one and *uncE* twelve). From such a multi-helical assembly it may well be possible to construct a transmembrane chain of H-bonds such as is required in schemes of H^+-translocation described by Dunker and Marvin (1978) or Nagle and Morowitz (1978), although such a chain could probably not be constructed from *E. coli uncE* and *uncF* proteins without *uncB* protein. We tend to feel the evidence is against these speculative mechanisms. It is surely no coincidence that all the mutation sites in the *uncE* protein which have been found so far to interfere with function appear to be at, or clustered around, the position of the buried DCCD-reactive acidic group, Asp_{61}. Further, DCCD blocks proton translocation in cytochrome oxidase subunit III by reacting with a buried carboxyl group conserved in a sequence homologous to that around Asp_{61} of the *uncE* protein (Prochaska et al., 1981), and oligomycin appears to bind at sites very close to buried acidic residues which also show considerable conservation of sequence. To our mind present evidence suggests that research on the mechanism of interaction of specific carboxyl side-chains with protons in lipid bilayers would be most pertinent to the mechanism of proton-translocation.

Sone et al. (1979) showed that tetranitromethane and iodine inhibited proton-conduction of a preparation of the membrane sector ("TF_o") of the PS3 proton-ATPase which had been incorporated in liposomes. In the PS3 DCCD-binding protein there is only one Tyr residue (at a position equivalent to residue 74 of *E. coli uncE* protein) and no Lys, His, Cys or Trp. The purified DCCD-binding protein from the tetranitromethane-reacted membrane sector was shown to contain nitrotyrosine in the amount of one-third of tyrosyl residues present in nonreacted protein. From this it was inferred that the single tyrosyl residue in the DCCD-binding protein is involved in H^+ translocation. The experiment is not conclusive because, as pointed out above, the preparation of membrane sector used contains several components, and more than one may be required for H^+ translocation. Nitration of other proteins within the membrane sector may have occurred and the data presented do not throw any light on this point. It is noteworthy that the tyrosyl residue present in the DCCD-binding protein of PS3 is not conserved in any of six other species (Sebald & Hoppe, 1981). Tyrosine occurs at one position removed in *E. coli* but in the other five species no tyrosine would be close to this position (even considering the possible hairpin structure as in Fig. 7). We conclude therefore that one or more tyrosyl residues in the membrane sector may well be involved in proton conduction, but the subunit location of the tyrosine(s) cannot yet be specified. Succinic anhydride appeared to increase proton conduction through PS3 membrane sector preparations by fourfold (Sone et al., 1978). The DCCD-binding protein of PS3 contains no

lysine, so the site of action of succinic anhydride may be another membrane sector subunit.

Mechanism of ATP Synthesis and Hydrolysis on F_1

The stoichiometry of the F_1 subunits remained undecided at the time one of us last reviewed this area (Senior, 1979*a*) but we believe that evidence reported since then shows the stoichiometry of subunits in F_1 to be $\alpha_3\beta_3\gamma\delta\varepsilon$. Two persuasive lines of evidence show that α and β are present in three copies each. They are the sulfhydryl labeling experiments by Yoshida et al. (1979) and the affinity labeling experiments and reported actual recoveries of purified subunits by Esch and Allison (1979). Several new reports have also appeared in which subunit stoichiometry of F_1 was studied by procedures such as ultracentrifugation, covalent labeling or incorporation of radioactivity into subunits, e.g. by growth of bacteria or yeast on ^{35}S-sulfate or ^{14}C-glucose. While these reports are essentially repetitious of earlier experiments, and are open to the same criticisms and reservations, a consensus has developed because almost all the later papers report data indicative of $\alpha_3\beta_3\gamma\delta\varepsilon$ stoichiometry. Dunn and Heppel (1981) and Dunn (1982) summarize binding and reconstitution data using isolated subunits which suggest γ, δ and ε are present to the extent of one copy each per F_1. We do not believe, however, that the reconstitution experiments reported in the literature (*see* e.g. Dunn & Heppel, 1981) give dependable indication of α or β subunit stoichiometry since the actual data are variable and it is not certain that all of the added subunits become reconstituted into active F_1 aggregates. Because the DNA sequences of β subunit of beef heart, *E. coli* and chloroplast F_1[9] show such extensive amino acid sequence homology, we believe chloroplast, mitochondrial and bacterial F_1 all have $\alpha_3\beta_3\gamma\delta\varepsilon$ stoichiometry, and quite similar molecular weights.

The arrangement of the six α and β subunits of F_1 was discussed by Senior (1979*a*) and further work on this problem has also been reported. Most of these reports have used the subunit cross-linking approach, which seems to give imprecise data (the gels shown seem often to lack distinct spots). An alternating arrangement of α and β subunits seems logical because any other arrangement requires that each of the three individual α or β subunits makes unique interfacial contacts with other α or β subunits. In a hexagon (or distorted hexagon) composed of alternating α and β subunits each subunit will make two distinct interfacial contacts with adjacent α or β but these two interfaces are the same for all three α's and all three β's. Nevertheless an $\alpha_3\beta_3\gamma$ complex will have inherent asymmetry in its $\alpha-\gamma$ or $\beta-\gamma$ interfaces. Amzel et al. (1982) have recently determined the three-dimensional structure of mitochondrial F_1 in the presence of ATP to 9 Å resolution by X-ray diffraction techniques. For an $\alpha_3\beta_3\gamma\delta\varepsilon$ stoichiometry, they conclude an inherent asymmetry of α and β subunits must be present and have discussed possible structural and functional implications of the data.

The affinity labeling experiments of Esch and Allison (1978, 1979) used the ATP analog, FSBA, which inhibits soluble F_1-ATPase irreversibly. A single tyrosine residue in β subunits was shown to be labeled concomitant with inhibition and is therefore likely to lie in proximity to the $\beta-\gamma$ phosphoryl groups of ATP bound at catalytic sites. Yoshida et al. (1981) and Esch et al. (1981) documented that the covalent inhibitor DCCD labels either of two glutamyl residues contained in a short contiguous sequence of the β subunit and have plausibly suggested, largely on the basis of protection from labeling by Mg^{++} ions, that those two carboxyl groups may lie in proximity to the $\beta-\gamma$ phosphoryl groups of ATP bound at catalytic sites. As noted before, the essential tyrosyl and glutamyl residues are conserved in homologous regions of sequence in beef heart $F_1\beta$ subunit and *E. coli* $F_1\beta$ subunit, and they are also conserved in chloroplast $F_1\beta$ subunit (Zurawski et al., 1982). Two other ATP analogs with reactive substituents in position approximating that of the $\beta-\gamma$ phosphoryl groups of ATP have been shown to inhibit soluble F_1 activity and to covalently label the β subunit (Budker et al., 1977; Drutsa et al., 1979). The inhibitory, reactive analog of Pi(4-azido,2-nitrophenylphosphate) has also been shown to label the β subunit (Lauquin et al., 1980). It is established therefore that the β subunits provide the parts of the catalytic site which bind the $\beta-\gamma$ phosphoryl moieties of ATP and Pi.

It should be noted that the covalent inhibitor NBD-Cl (also called Nbf) does not apparently bind at a catalytic site (Cross & Nalin, 1982). It reacts with a tyrosyl group on the β subunit which is not the same as the catalytic site tyrosyl with which FSBA reacts (Esch & Allison, 1979). The noncovalent inhibitor aurovertin also binds to the β subunit (*see* Wise et al., 1981), but not apparently to a catalytic site (Cross & Nalin, 1982). Both NBD-Cl and

[9] β subunit of spinach chloroplast F_1 contained only one Cys (DNA sequencing; Zurawski et al., 1982) in contrast to earlier higher estimates on which subunit stoichiometry of 2β per F_1 was based.

aurovertin may prevent subunit-subunit conformational interaction required for catalysis (*described below*). The noncovalent inhibitor efrapeptin apparently binds to catalytic sites (*see* Cross & Nalin, 1982). There is evidence that arginine and lysine (and an additional carboxyl group) may also be essential residues at the catalytic sites. This evidence, based on labeling, inhibition and nucleotide binding experiments, using phenylglyoxal, pyridoxal phosphate, or similar nondirected chemical modification reagents, is less convincing as yet than that provided by Allison and coworkers above, because in no case has it been shown that reaction of a unique residue correlates with inhibition. Future work may provide such evidence and identify the sequence positions of catalytic site arginine, lysine and additional carboxyl residues.

An important contribution on the chemical mechanism of phosphate bond cleavage and synthesis at F_1 catalytic sites is that of Webb et al. (1980). This paper showed that an $F_1 \sim P$ (covalent phosphorylated intermediate) is very unlikely to occur during ATP hydrolysis and this distinguishes F_1-ATPase from Na/K-ATPase, sarcoplasmic reticulum and mammalian plasma membrane Ca-ATPase, gastric H^+/K^+ ATPase and the proton-ATPase of the plasma membrane of lower eukaryotes, e.g. yeast, *Neurospora*. On the other hand it suggests a possible similarity between F_1-ATPase and myosin-ATPase mechanism, a suggestion supported by other experiments (Matsuoka et al., 1981; Grubmeyer et al., 1982). The data of Webb et al. (1980) also strongly suggest ADP (not AMP) is the primary phosphate acceptor in ATP synthesis, congruent with most workers' views.

The number and nature of nucleotide binding sites on F_1, which was for a long time obscure, has become much clearer in the last three years, so that we believe there is now a straightforward correlation between number and nature of nucleotide binding sites and α and β subunit stoichiometry. Briefly reviewing information derived from work on isolated subunits and catalytic site labeling described above, both isolated α and β subunits (but no other subunit) carry a nucleotide binding site. The β site is likely to be the catalytic site. The α site has characteristics which qualify it as a nonexchangeable nucleotide (ATP or ADP) binding site since it binds nucleotide very tightly in the absence of Mg and the nucleotide dissociates very slowly. These are all properties of the "tightly bound" nucleotides in F_1 (*see* Senior, 1979*b* for evidence that Mg is not bound with "tightly bound" nucleotide in F_1). Therefore one would predict that F_1 would contain six total sites, three of them catalytic and three nonexchangeable.

Recent evidence shows that this is indeed the case. Cross and Nalin (1982) were able to show that soluble beef heart F_1, which was fully depleted of nucleotide, bound 6 mol of total nucleotide per mol F_1 under appropriate conditions. There were apparently three nonexchangeable sites, and three exchangeable sites which were identified as catalytic sites from their behavior. F_1 which was not previously depleted of nucleotide (and which already contained 3 mol of nonexchangeable nucleotide per mol F_1) bound 3 mol of AMPPNP per mol F_1, showing strong negative cooperativity between the sites. The first site had extremely high affinity ($K_d = 18$ nM), similar to the K_i(14 nM) found for competitive inhibition of ATPase activity by AMPPNP, and the other two sites had considerably lower affinity ($K_d \sim 1$ μM). We have performed similar experiments on normal *E. coli* F_1 (Wise et al., 1982; J.G. Wise, *unpublished*) and have obtained similar results, Grubmeyer and Penefsky (1981*a*) reported that TNP-ATP, a hydrolyzable analog of ATP, bound to three sites on soluble beef heart F_1 preparations which contained 3 mol of nonexchangeable nucleotide per mol F_1. Strong negative cooperativity was seen between these three sites with the first site binding the TNP-ATP very tightly ($K_d \leqq 5$ nM, but could not be measured accurately). At least two of these sites were directly shown to be catalytic sites. The work of Esch and Allison (1978) already referred to, had indicated 3 catalytic sites were present, one on each β subunit, and in their 1979 paper these workers supplied evidence that more than two additional sites for ADP were still present on enzyme in which 90% of the hydrolytic sites had been blocked by reaction with FSBA. These additional sites had properties of the nonexchangeable, noncatalytic sites.

The role of the three nonexchangeable sites on α subunit is currently not understood. Their participation in catalysis has been conclusively denied (Gresser et al., 1979; Rosen et al., 1979). Regulatory roles have been postulated for them, but in a search of the literature we have found no clear "regulatory" effect likely to be expressed at physiological pH or concentrations of ATP or ADP in bacteria or mitochondria. $\alpha \cdot$ATP is the likely form of α subunit incorporated into newly synthesized F_1, and where less than 3 mol of nonexchangeable nucleotide have been found per mol F_1, this was probably due to losses caused by preparative manipulations. Our tentative conclusion at this time is that the nonexchangeable, tightly bound nucleotides have an essential structural role, analogous to some intrinsic metal in enzymes. However, it is not yet clear whether only ATP or whether both

ATP and ADP occur *in vivo* in these sites. Analyses of purified F_1 or membrane-bound proton-ATPase commonly show both ATP and ADP present. Understanding this point may help to elucidate the role of nonexchangeable sites.

In retrospect we can see that two features of intact F_1 complicated the determination of number and nature of nucleotide binding sites present. One is the strong negative cooperativity between catalytic sites, such that all three sites can be readily filled only by use of extremely tight-binding analogs of ATP, e.g., AMPPNP, TNP-ATP or FSBA. MgADP binds only to two exchangeable sites (Skerrett et al., 1981; Wise et al., 1981) unless high levels of Mg are added (Lunardi et al., 1981). The second is that these analogs, and ATP and ADP (Grubmeyer et al., 1982, discussed below), bind to the *first* catalytic site with very high affinity, much higher than the apparent K_m ATP or K_i ADP. This misled workers into thinking nonexchangeable or tight, exchangeable, noncatalytic, "regulatory" sites were being occupied in experiments where binding was to a catalytic site. It now seems improbable that a nucleotide binding site(s) is formed *de novo* during formation of F_1 from its subunits since six is the apparent total number of sites. This tends to eliminate the possibility that F_1 contains an exchangeable, regulatory, noncatalytic nucleotide binding site. Here again, although many authors have suggested the presence of such a site(s) we would take the position that no clear evidence has yet been provided for a physiologically operative regulation of catalysis by nucleotide in bacteria or mitochondria.

When just one catalytic site of soluble beef heart F_1 (containing 3 mol/mol of nonexchangeable nucleotide) binds substrate, only a very slow rate of hydrolysis ensues and dissociation of products was shown to be rate-limiting (Grubmeyer & Penesfky, 1981*a*, *b*; Grubmeyer et al., 1982). An enormous (10^6-fold) acceleration of hydrolysis at the first site occurs when a second catalytic site is filled. ATP, and analogs both hydrolyzable and nonhydrolyzable, could bring about "promotion" hydrolysis, with substrates seeming more efficacious (Grubmeyer & Penefsky, 1981 b; Cross et al., 1982). Cross et al. (1982) suggest that occupation of both second and third catalytic sites by ATP may cause a further small acceleration of hydrolysis at the first site. The data clearly show therefore that normal rates of catalysis require site-site interaction between at least two catalytic sites. Where apparent K_m values for substrates have been deduced in the previous literature, the numbers calculated are reflections of the ability of substrates studied to trigger the correct subunit-subunit interactions responsible for the "promotion," and do not reflect interaction at the actual site of hydrolysis. In fact Grubmeyer et al. (1982) calculate a K_a for ATP binding at the first catalytic site of 10^{12} M^{-1}, and a K_d for dissociation of ADP in unpromoted hydrolysis of 0.28 μM[10]. Both of these values are considerably lower than previously reported values of K_m ATP or K_i ADP derived from experiments in which "multi-site" catalysis was occurring. Cross et al. (1982) report tentative K_m ATP values for promotion by occupancy of second and third sites, respectively, of 30 and 150 μM.

The enhancement of rate of ATP hydrolysis at the first site caused by substrate binding at the second site apparently involves both acceleration of bond cleavage rate (up to 30-fold increase) and enhancement of product release (Grubmeyer et al., 1981*b*; Cross et al., 1982). The apparent binding affinity for the products at the first site was shown to decrease by a factor of 10^5 to 10^6 when the second site was occupied by substrate, and the increased rate of product release was the principal factor in the hydrolysis rate promotion. In the pathway proposed by this group, Pi dissociates after cleavage at a slightly faster rate than ADP during "uni-site" catalysis.

Using soluble F_1 in which three nonexchangeable nucleotide sites were occupied, Nalin and Cross (1982) provided a further demonstration in which site-site cooperativity changed the apparent affinity of the catalytic site for nucleotide. AMPPNP, bound at only a single catalytic site on F_1, dissociated at a negligible rate. ADP (but not ATP or AMPPNP) induced the release of AMPPNP. The effect of ADP was efrapeptin-sensitive, and enhanced by Pi, suggesting ADP and Pi bind at a second catalytic site to decrease the binding affinity of AMPPNP at the first site. This experiment may therefore mimic (in soluble F_1) a single turnover in ATP synthesis. Chernyak and Kozlov (1979) and Penefsky (1979) showed a similar requirement for ADP to release AMPPNP from membrane-bound F_1. In the membrane-bound enzyme formation of $\Delta\bar{\mu}H^+$ seemed to sharply increase AMPPNP release. In fact, during oxidative phosphorylation, levels of AMPPNP up to 1.1 mM do not appreciably inhibit (Penefsky, 1974; Peder-

[10] The exact agreement between this value and the value deduced earlier by Hilborn and Hammes (1973) for equilibrium binding of ADP to soluble F_1 should be noted. Because the value of 0.28 μM was far below the measured apparent K_i ADP, Hilborn and Hammes suggested that ADP was not binding at the catalytic site, but possibly to a regulatory site. The new data shows they were actually measuring MgADP binding at the first catalytic site.

sen, 1975) suggesting the binding affinity for AMPPNP at the catalytic site has changed by possibly 10^5-fold. Data in Grubmeyer and Penefsky (1981 *a*) also suggest the K_d TNP-ATP is considerably raised by the onset of oxidative phosphorylation. Thus site-site cooperativity, and large changes in binding affinity at catalytic sites appear to be integral features of catalysis in both ATP synthesis and hydrolysis. Grubmeyer et al. (1982) surmise that a decrease in ATP-binding affinity of up to 10^{10}-fold (from K_d 10^{-12} M to 10^{-2} M) may occur at the catalytic site during oxidative phosphorylation to release ATP. However, one should note that the K_a for binding of ATP to membrane-bound F_1 is not yet known, and that Gresser et al. (1982) suggest a lower value of K_d ATP (~ 1 μM) binding at the first catalytic site on soluble F_1, even though these workers also favor an interacting three-site model for F_1 catalyses.

Several different experimental approaches suggest that the reversible interconversion of ATP and ADP plus Pi can occur without appreciable free energy change on soluble F_1. Grubmeyer et al. (1982) deduced an equilibrium constant of 0.5 for the reaction ATP → ADP + Pi during unpromoted catalysis, whereas Feldman and Sigman (1982) (who actually measured ATP synthesis) found an equilibrium constant of 2. Bossard et al. (1980) have shown formation of the stable Pi·Cr(III)·ADP complex on soluble F_1 from either Cr(III)·ATP or Pi plus Cr(III)·ADP. Measurements of Pi⇌H_2O ^{18}O exchange reactions on soluble F_1 (Choate et al., 1979) also showed that significant interconversion of ATP⇌ADP + Pi occurred. The apparent reversible nature of the reaction in the soluble enzyme appears to derive from the extremely tight binding of ATP, and is reminiscent of myosin ATPase.

The accumulated evidence now strongly suggests that ATP synthesis during oxidative phosphorylation occurs at the same sites as ATP hydrolysis on F_1, and one naturally questions whether these elegant recent studies on soluble F_1 help us to understand the mechanism of ATP synthesis in membrane-bound F_1. The answer appears to be a confident yes, indeed they give broad support to the "binding change mechanism" of oxidative phosphorylation proposed by Boyer and his colleagues. The basic tenets of and evidence for this mechanism are listed in Rosen et al. (1979) and have been discussed by Cross (1981). Features of this mechanism which we feel are now strongly supported are as follows. First, reversible cleavage of the $\beta-\gamma$ phosphoryl bond of ATP can proceed in both membrane-bound and soluble F_1 without significant energy input. Secondly, energy input from the proton gradient or ATP hydrolysis can effect changes in affinity of F_1 binding sites for nucleotides and Pi, such that substrate binding and product release are facilitated. Some of these changes in affinity appear large and may account for most if not all of the energy required for ATP synthesis in oxidative phosphorylation. Thirdly, nucleotide sites on F_1 show a marked degree of site-site interaction. This has been measured in soluble F_1 as negative cooperativity of AMPPNP or TNP-ATP binding, obligate dependence for release of AMPPNP from one catalytic site on ADP binding at a second catalytic site, large increases in actual hydrolytic rate at one catalytic site caused by binding of nucleotide at a second catalytic site, and large decrease in affinity of binding of products of hydrolysis at one site cause by substrate binding at a second site. The characteristics of ^{18}O exchange reactions on soluble F_1 (Choate et al., 1979; Hutton & Boyer, 1979) also imply that interaction between nucleotide sites modulates isotope exchange reactions at the catalytic site. Boyer and his co-workers have shown in extensive studies that in membrane-bound F_1 catalyzing either ATP synthesis or hydrolysis, ^{18}O isotope exchange reactions at the catalytic site are strongly influenced by binding of substrate at another catalytic site (summarized by Rosen et al., 1979). Further suggestion that site-site interaction on F_1 is a necessary feature of either ATP synthesis or hydrolysis comes from the many literature reports showing that covalent or noncovalent inhibitors (e.g. DCCD, NBD-Cl, efrapeptin) can inhibit completely when bound in amounts substoichiometric with $F_1\beta$ subunits. The most likely reason for the obligate interaction of sites seems to be to facilitate energy input in a single conformational "switching event" as product is released, substrate becomes tightly bound, and all three sites simultaneously change affinities for substrates and products.

During ATP synthesis energy input for the changes in binding affinity required at the catalytic sites to release ATP and bind ADP and Pi tightly is presumably derived from the movement of protons down the proton gradient across the membrane sector. The actual mechanism of the coupling of site-site interactions and binding affinity changes on F_1 to proton movement remains unknown. The most likely mode of mediation of this coupling is through conformational changes in the proteins. The "active protons mechanism" of Mitchell (1976) involving transport of protons directly to a buried catalytic site is not supported by the more recent data. The direct demonstration that

reversal of ATP hydrolysis can occur with $K_{eq} \sim 1$ on soluble F_1 can be held as evidence against it, and it is clear that if the release of ATP does require substantial energy input, channelling the protons into the catalytic site to alter the K_{eq} for the reaction ADP + Pi → ATP will not in itself be sufficient to achieve normal rates of free ATP formation.

The molecular mechanism of the interaction of catalytic sites on F_1 appears therefore to be an important aspect of ATP synthesis and hydrolysis. The study of *E. coli* mutants may be useful in understanding such interactions. Wise et al. (1981, 1982) and J.G. Wise (*unpublished*) showed that three *uncA* (α subunit) point mutants of *E. coli* which had no ATPase or ATP synthesis activities, but which form F_1 aggregates of normal size and stability, contained the normal number of nucleotide binding sites on F_1, i.e. 3 nonexchangeable and 3 exchangeable binding sites. Negative cooperativity between the exchangeable binding sites was retained although the affinities of the sites for AMPPNP were 2 to 5 times lower than in normal *E. coli* F_1. The rate and degree of labeling of essential Glu or Tyr residues in the $F_1-\beta$ subunit by DCCD or NBD-Cl, respectively, appeared normal, and aurovertin bound with normal K_d. In normal (*unc*$^+$) *E. coli* F_1, addition of ADP causes enhancement of bound aurovertin fluorescence (K_m ADP ~ 1 μM), but this effect was absent in the three inactive *uncA* F_1 preparations. Depolymerization of normal F_1 abolished the ADP-induced effect (but not the binding of aurovertin), and the ADP-induced effect was regained on repolymerization of normal F_1. Furthermore, binding of ATP, AMPPNP or ADP was tightened (K_d lowered by ~50%) by aurovertin in normal *E. coli* F_1, but no such effect of aurovertin was seen in the three inactive *uncA* F_1 preparations. Therefore mutation in the α subunit appeared to have blocked an intersubunit interaction between the aurovertin site and a tight exchangeable nucleotide site.

A suggested explanation of the effects is proposed in Fig. 8. The α and β subunits are arranged alternately in the model, with aurovertin sites on β, nonexchangeable nucleotide binding sites on α, and exchangeable, catalytic (active) sites located a α/β interfaces. Explanation is required for this (tentative) localization of catalytic sites and is included in the legend to Fig. 8. The arrow linking aurovertin site and exchangeable nucleotide site represents the effects measured in normal F_1, described above, and the suggested effect of the inactivating *uncA* mutations is to disrupt $\alpha-\beta$ subunit interaction, most probably at an intersubunit contact region. Such a hypothesis can also explain why these mutants have catalytically inactive F_1. If one designates the catalytic sites as A, B and C for very tight (first), intermediate affinity (second) and loosest (third), then binding of ATP at only site A in normal F_1 would yield the very slow rate of unpromoted, "uni-site", ATP hydrolysis described by Grubmeyer et al. (1982) (which would

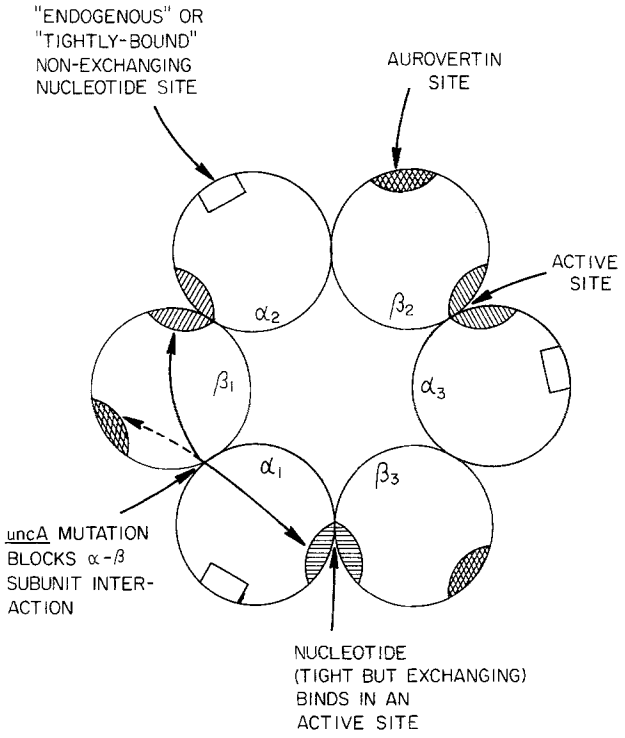

Fig. 8. Site-site interactions in F_1. Site-site interactions designated by the arrows are discussed in the text. The reason for putting the exchangeable (catalytic, active) sites at α/β interfaces is as follows. It is established that β subunit provides the portion of the catalytic site which binds the $\beta-\gamma$ phosphoryl moieties of ATP. However, there are numerous reports in the literature in which photoaffinity- or affinity-labeling analogs of ATP, containing substituents in 3′, 2′, C^6 or C^8 positions, have been used to label intact F_1 from mitochondria and bacteria under conditions where exchangeable sites should be predominantly labeled. The confusing aspect of these literature reports has been that both α and β subunits appear to become labeled. This has led some workers to postulate that "regulatory" and "catalytic" sites, on α and β, respectively, are labeled. However, as we discussed earlier, we feel it is unlikely that α carries an exchangeable regulatory site, and that a better way out of the dilemma is to propose that the adenosine binding portion of catalytic sites is formed by both α and β subunits. If we suppose that C^6 faces the β subunit, that C^8-faces the β subunit in *syn* conformation and the α subunit in *anti* conformation, and that 2′ and 3′ positions face regions of both α and β subunit, then much, although not all of the labeling data in the literature can be rationalized. Cosson and Guillory (1979), Schafer et al. (1980) and Williams and Coleman (1982) have previously discussed the possibility that α and β subunits combine to form the active sites

have gone undetected in previous assays of *uncA* mutant F_1). Binding of ATP at catalytic site B in normal F_1 would now cause acceleration of hydrolysis of ATP bound at site A by the promotion effect, and this site-site cooperativity is shown by the arrow linking active sites in Fig. 8. The promotion effect would be blocked by the *uncA* mutations, explaining the inhibition of catalytic activity. In the normal enzyme, when hydrolysis occurred, site B (containing ATP) would now become site A (tight), site C would become site B (intermediate affinity) and site A, having released its ADP and Pi products, would become site C (loosest). The catalytic cycle would proceed as "promotor" ATP bound to new site B (formerly site C). In the inactive *uncA* mutant F_1, the catalytic cycle would not proceed.

If our model does have validity (and we stress it is only a suggestion at present) then examination of F_1 preparations containing α subunits or β subunits which allow partial activity may be useful in understanding the cooperativity between catalytic sites and we are currently examining further mutants and partial revertants of inactive mutants to find examples of such α or β subunits. F_1 preparations from a strain of *E. coli* carrying the *uncA498* allele have around 50% of normal ATPase activity and do show normal ADP-induced enhancement of aurovertin fluorescence and aurovertin-induced decreases in ADP, ATP and AMPPNP binding affinity (J.G., Wise, and A.E., Senior, *unpublished*). Six *uncD* mutants of *E. coli* which form F_1 aggregates of normal molecular size (containing altered β subunits) are now available for study, but in no case has characterization yet proceeded far enough for us to understand the defect in detail. The ATP hydrolytic rate is lowered to 10% of normal (Senior et al., 1979*b*; Kanazawa et al., 1980*b*) or 0.2% to 15% of normal (Senior et al., 1983) in purified F_1 from these mutants.

It should be noted that the model in Fig. 8 is easily reversed to describe ATP synthesis in oxidative phosphorylation, as has already been proposed by Cross (1981). The central problem of oxidative phosphorylation may therefore be rephrased at this time as, what is the relationship in molecular terms between the conformational changes involved in site-site cooperativity on F_1 and the energy input from the proton gradient during ATP synthesis? It has been known for some time (reviewed by Senior, 1979*a*; Penefsky, 1979) that induction of proton gradients across the membrane causes gross conformational changes on F_1. The study of different mutations in α and β subunits may allow us to understand how the energy is channelled into the nucleotide binding sites.

Conclusions

We apologize for the fact that this review is longer than we intended; we excuse ourselves with the statement that since almost all the information we have presented was discovered since one of us last reviewed the field in 1979, we have not strayed from the topical. Remarkable advances in understanding of the mechanism of F_1-catalyzed ATP hydrolysis and synthesis, of the structure of the proton-ATPase, of the genetics of the enzyme and its assembly *in vivo* have been made in the last four years. Understanding the mechanism of proton translocation across the membrane seems now within our reach, but comprehension of the integration of F_1 catalysis and proton translocation remains somewhat elusive. We feel that understanding the roles of *uncF* protein, OSCP and F_6 is important in this regard. We expect that the rapid chemical characterization of mutation and reversion sites by DNA sequencing will become possible within the near future, and that site-specific mutagenesis may soon allow us to introduce amino acid substitutions in specific domains of specific subunits. Such capabilities would greatly expand our horizons, allowing detailed molecular probing of all the components of the proton-ATPase.

We are pleased to have had the opportunity to review the field at an opportune time. Being both Senior and Wise we shall not be surprised or distressed if our views and interpretations fail to elicit universal concurrence; but we do hope that all readers find them valuable and stimulating.

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