Biochemical and Genetic Studies on the Assembly and Function of the $F_1-F_0$ Adenosine Triphosphatase of *Escherichia coli*

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I am deeply appreciative of the honour of being invited to deliver the 13th Hopkins Memorial Lecture. In doing so, I am conscious that this honour reflects on the many colleagues, both academic and technical, who have worked in the laboratory in Canberra over the years. While it might seem invidious to single out individuals for special mention, and the names of many collaborators are cited in references, I must make one exception. I refer to my colleague, Dr. Graeme Cox, who has been involved in the work I will be describing since its inception, and whose contributions, both practical and conceptual, have been outstanding.

**Introduction**

It is just over a decade since the first mutant of *Escherichia coli* affected in oxidative phosphorylation was reported (Butlin *et al.*, 1971). This mutant was found to have a defective membrane-bound ATPase, providing evidence that the best characterized soluble factor concerned with coupling electron transport to oxidative phosphorylation known at that time, the magnesium-stimulated adenosine triphosphatase (ATPase), played an important role in oxidative phosphorylation *in vivo*. It was stated at that time that 'The use of bacteria with their simpler cellular organisation than eucaryotic cells, and of *Escherichia coli* in particular, with its amenability to genetic manipulations, seems a promising experimental system for a combined genetic and biochemical approach to the problem of coupling of phosphorylation to electron transport' (Butlin *et al.*, 1971).

In the first part of this Lecture I would like to outline how far this prediction has been fulfilled, and the experimental approaches that have been used. We are now entering a new phase in the study of oxidative phosphorylation and, in the second part of the Lecture, I would like to outline some recent experiments which indicate that experiments with *E. coli* are as likely to be as useful in studies on membrane assembly and the molecular mechanisms of oxidative phosphorylation as they have been in other areas of biochemistry.

At the time the work on the biochemical genetics of oxidative phosphorylation in *E. coli* started, very little was known about the ATPase in *E. coli*, other than that there existed a membrane-bound ATPase activity (Evans, 1969). The ensuing decade saw a considerable increase in our knowledge of bacterial, mitochondrial and chloroplast ATPases and the realization that there were striking structural and functional similarities between the ATPases from these three different sources. The general picture that emerges of the structure and the role of the membrane-bound ATPase complex in oxidative phosphorylation in *E. coli* can be illustrated diagrammatically as shown in Fig. 1. The ATPase complex is now conventionally divided into two portions: the first, $F_1$-ATPase, may be readily dissociated from the membrane and the soluble form is still active in hydrolysing ATP. The subunits of $F_1$-ATPase have been designated $\alpha, \beta, \gamma, \delta$ and $\varepsilon$ in decreasing order of molecular weight and the best estimate of the stoichiometry of these subunits at the moment seems to be $\alpha_3\beta_3\gamma_3\delta_6\varepsilon$ (see Dunn & Heppel, 1981).

With respect to the second portion, $F_0$ only in *E. coli* can a definitive statement be made about the number of subunits present. There are three such subunits and, although there is no generally accepted nomenclature for these subunits, I will use the designations $a$, $b$ and $c$, in order of decreasing molecular weight. In the process of oxidative phosphorylation, the $F_0$ portion of the ATPase is thought to act as a proton channel, the final synthesis of ATP taking place, by mechanisms not yet understood, at the active sites on the $F_1$-ATPase. The stoichiometry of the subunits in $F_0$ is not known, except that it is...
probable that there are multiple copies of subunit c present in the complex (see Fillingame, 1981; Sebald & Hoppe, 1981).

**Isolation and characterization of unc mutants**

Mutants of *E. coli* affected in oxidative phosphorylation (unc mutants)* have now been isolated in many laboratories (see Downie et al., 1979) and some of their properties are set out in Table 1. As *E. coli* is a facultative anaerobe, mutants affected in oxidative phosphorylation, although unable to grow on a non-fermentable substrate such as succinate, can grow on glucose by fermentation. Thus a method for isolating unc mutants is to treat cells with mutagen and screen for strains which will grow on glucose as sole carbon source, but not with succinate as sole carbon source (Suc− mutants). These strains can then be further screened by measuring aerobic growth yields on limiting glucose, and by tests on membrane preparations, to find those in which oxidative phosphorylation is impaired (Cox & Downie, 1979).

The starting point for further investigation of unc mutants was the observation that two distinctly different types could be found: those which lacked membrane-bound ATPase activity (ATPase−) and those in which ATPase activity was present (ATPase+) (Butlin et al., 1971, 1973; Gutnick et al., 1972). It

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*The following designations have been proposed for the genes coding for the ATPase complex of *E. coli*: unc, Butlin et al. (1971); hcf, Rosen et al. (1978); *atp*, von Meyenburg & Hansen, (1980); *pap*, Kanazawa et al. (1981a).
was previously known that bacterial membrane-bound \( F_\text{r} \)\-ATPase could be solubilized by washing the membranes with low-ionic-strength buffer (see Downie et al., 1979). Advantage was taken of this observation to carry out experiments in which the \( F_\text{r} \)\-ATPases from \( \text{ATPase}^- \) and \( \text{ATPase}^+ \) mutants were used to see if they would reconstitute energy-linked reactions, such as electron transport-dependent phosphorylation, in the 'striped' membranes from normal and uncoupled mutants (Cox et al., 1973). In this way it was shown that in the membranes from an \( \text{ATPase}^- \) mutant the \( F_\text{r} \)\-ATPase was affected while the striped membranes (\( =F_\text{w} \)) were normal. Conversely the \( F_\text{r} \) was affected in the \( \text{ATPase}^+ \) strain, while the \( F_\text{r} \)\-ATPase was normal. It was therefore obvious that different proteins had been affected in the mutants, and the genes concerned were designated \( \text{uncA} \) and \( \text{uncB} \) respectively.

The genes affected in \( \text{unc} \) mutants were all located in the same region of the \( E. \) \( \text{coli} \) chromosome, at about minute 83.5. Therefore to distinguish \( \text{uncA} \) and \( \text{uncB} \) mutants from those affected in other genes coding for the proteins of the complex, a genetic complementation system was developed using the \( \text{uncA} \) and \( \text{uncB} \) alleles for reference. To do this a method was devised for transferring any mutant \( \text{unc} \) allele onto an \( F \)-plasmid (Gibson et al., 1977). Such \( F \)-plasmids carrying mutant \( \text{unc} \) alleles were then transferred to haploid \( \text{unc} \) strains to give partial diploids containing two mutant \( \text{unc} \) alleles. If the resulting partial diploids grew on succinate (Suc\(^+\)), it was assumed that the mutation carried on the plasmid and that on the chromosome were in different genes. Genetic complementation has, on the whole, been fairly successful in classifying mutants into different groups, although difficulties are encountered with some strains, as might be expected in dealing with a multi-protein complex containing more than one copy of some subunits. Some pairs of alleles do not complement well as tested by growth on succinate, and various biochemical tests on membranes from the partial diploids have been helpful in assessing complementation. Over the years the number of complementation groups has reached seven. i.e. \( \text{uncA}, \text{B, C, D, E, F and G} \) (see Downie et al., 1979, 1980, 1981).

As mentioned earlier, \( \text{unc} \) mutants were all found to map in the same region of the chromosome and it was therefore possible that this cluster of genes formed an operon. To test for this possibility, polarity \( \text{unc} \) mutants were obtained using the bacteriophage Mu which, when inserted in an operon, exerts a polarity effect, none of the genes past the point of insertion being transcribed. By transferring reference mutant \( \text{unc} \) alleles on plasmids into Mu-induced \( \text{unc} \) mutants it was possible to show that the genes were arranged in an operon and that the order of reading of the genes was \( \text{uncC}, \text{D, A, F, E, G} \) (Fig. 2) (see Downie et al., 1980). The polarity mutants available did not allow ordering of the \( \text{uncE} \) and \( F \) genes and these were finally ordered after cloning of the \( \text{unc} \) genes and subsequent DNA sequencing (see below).

**Gene–polypeptide relationships**

The relationships between the \( \text{unc} \) genes and proteins for which they code have been determined by a variety of techniques. Two-dimensional gel electrophoresis was used in the early experiments to look for electrophoretically abnormal proteins in purified \( F_\text{r} \)\-ATPase preparations or in whole membranes from mutant strains. The examination of whole membranes for subunits of \( F_\text{r} \)\-ATPase was made practicable by the finding that washing membranes in low-ionic-strength buffer containing p-aminobenzamidine removed about 70% of the membrane protein and enriched the membranes with respect to the \( \text{ATPase} \) complex (Cox et al., 1978). The relationship between the \( \text{uncD} \) gene and the \( \beta \)-subunit was the first to be determined when it was found that, in all the \( \text{uncD} \) mutants examined at that time, there was present a \( \beta \)-subunit with an altered isoelectric point which was either higher or lower than normal depending on the particular mutant allele (Fayle et al., 1978; Senior et al., 1979a). In the case of \( \text{uncA} \) mutants, some had electrophoretically altered \( \alpha \)-subunits while others were unaltered. In strains carrying some mutant alleles affecting \( F_\text{r} \)\-ATPase, no \( F_\text{r} \) aggregate could be washed off the membranes, but it was possible in some cases to use partial diploids carrying an \( \alpha \)-subunit and a mutant \( \alpha \) allele to help identify the altered protein. For example, no \( F_\text{r} \)\-ATPase aggregate could be purified from membranes of haploid strains carrying the \( \text{uncA4350} \) allele, but from the partial diploid \( \text{unc}^-/\text{uncA4350} \) an aggregate could be purified by the usual procedures and, when examined by two-dimensional electrophoresis, was found to carry both normal and electrophoretically altered \( \alpha \)-subunits.

![Fig. 2. The region of the E. coli chromosome carrying the unc genes](image)

The assumed relationship between the \( \text{uncC} \) gene and the \( \sigma \)-subunit has recently been...
confirmed by the observation that a strain carrying a mutant uncC allele has an electrophoretically altered ε-subunit (A. E. Senior & G. B. Cox, unpublished work).

In the two-dimensional gel electrophoresis system used, the α and c protein subunits of the F₆ could not be seen, therefore other methods had to be used to relate these proteins to their appropriate genes (see below).

The relationship between the uncG gene and the γ-subunit was established during the cloning of the unc operon. Thus after cloning fragments of the E. coli chromosome following digestion with the endonuclease HindIII, two plasmids were obtained (Fig. 4). One of these, plasmid pAN51, was shown by genetic complementation tests to carry genes uncB, E, F, and A, while plasmid pAN36 carried the uncD and C genes. When these two plasmids were mixed, digested with HindIII and re-ligated, it was found by genetic complementation tests that the reconstructed plasmid, pAN45, complemented an uncG⁻ mutant, which was not the case with either of the original plasmids. This experiment indicated that there was a HindIII restriction site in the uncG gene and also confirmed the gene order as uncAGD.

Protein synthesis experiments in vitro, followed by two-dimensional gel electrophoresis and autoradiography, showed that the plasmid pAN45, which carried the uncG gene, but not plasmids pAN51 or pAN36, carried the gene coding for the γ-subunit. The formation of other subunits using the DNA of these plasmids as template was consistent with the known gene-polypeptide relationships, i.e. the b- and α-subunits on plasmid pAN51; β- and ε-subunits on plasmid pAN36. It was also observed that the gene coding for the δ-subunit was carried on plasmid pAN51 (Downie et al., 1980).

The c subunit of the ATPase (the dicyclohexylcarbodi-imide-binding protein) is the best studied of the F₆-subunits from a variety of organisms. It is readily isolated and identified by extraction into chloroform/methanol and subsequent chromatography (Cattell et al., 1971; Fillingame, 1976). Two uncE mutants were found to lack this protein in their membranes and a tentative relationship between uncE and subunit c was made (Downie et al., 1981). Since that time other uncE mutants have been found to accumulate an altered protein c and we will return to these mutants later.

The relationship between the uncB gene and the α-subunit of the F₆ was established by deleting from plasmid pAN51(uncBEFA) about 100 base pairs giving a plasmid (pAN95) which did not complement with an uncB⁻ strain although it did carry functional uncE, F and A genes. Protein synthesis experiments were carried out in vitro using the DNA of the plasmid pAN95(uncB⁺E⁺F⁺A⁺) and the DNA from the parent plasmid pAN51(uncB⁻E⁺F⁺A⁺). The membranes from the crude E. coli extract in the reaction mixture were then centrifuged and washed. It was assumed that any F₆ subunits
formed would be incorporated into these membranes, which were then solubilized and examined by one-dimensional sodium dodecyl sulphate gel electrophoresis and autoradiography. When plasmid pAN51(uncB-E+F-A+) was found to code for a protein of apparent molecular weight 24,000, the plasmid carrying the deletion, in which the uncB gene had been inactivated, did not form the protein of apparent molecular weight 24,000 but formed a new protein of molecular weight 15,500, presumably a shortened uncB gene product (Downie et al., 1981).

Recently, several laboratories have been active in sequencing the unc gene operon (for references see Gay & Walker, 1981; Kanazawa et al., 1981b). This work has unequivocally established the gene order for the uncE and F genes and has established the position of the gene coding for the $\sigma$-subunit, for which the designation uncH has been suggested (Gunsalus et al., 1982), as lying between the uncF and uncA genes. The genes coding for the subunits of the ATPase complex are therefore transcribed in the order uncBEFHAGDC, the corresponding subunits being $\alpha$, $\beta$, $\delta$, $\sigma$, and $\epsilon$. A particularly interesting finding from the DNA-sequencing studies was that there is an open reading frame between the putative promoter of the operon and the first structural gene (uncB) coding for one of the ATPase subunits (Gay & Walker, 1981; Kanazawa et al., 1981b). With this new DNA sequence information, one could now attempt to determine the molecular weight about 14,000. Synthesis of this protein in vitro or in vivo has not been reported, and its possible role remains a matter for conjecture.

**Assembly of the $F_\gamma - F_\delta$ ATPase**

With the knowledge of the complete nucleotide sequences, and the corresponding complete amino acid sequences, of all the subunit proteins of the ATPase aggregate, together with the availability of mutants affected in various genes in the operon, the stage is now set for an attack on some further fundamental problems relating to oxidative phosphorylation. Such problems would include the regulation of the synthesis of the individual subunits, which occur in various proportions in the final structure, the assembly of the subunits into the functioning ATPase and the much-sought-after, but elusive, molecular mechanism of the process of oxidative phosphorylation itself. Little has been reported on the regulation of the biosynthesis of the subunits, although it seems likely that regulation is effected at the translational level (R. D. Simoni, personal communication).

For the remainder of this Lecture I wish to describe some experiments relating to the assembly of the ATPase and to the molecular mechanism of proton translocation in the complex.

Until recently the evidence available regarding the assembly of the ATPase complex has been derived from experiments in vitro. Thus, following experiments with the $F_\delta$ from the thermophilic bacterium PS3 incorporated into phospholipid vesicles, and purified $F_\gamma$-ATPase subunits, it was suggested (Yoshida et al., 1977) that the $\sigma$- and $\epsilon$-subunits bind first, but in no particular order, to the $F_\gamma$ followed by the $\gamma$-subunit and then the $\alpha$- and $\beta$-subunits. With $E. coli$ ATPase, on the other hand, it was suggested that the complete $F_\gamma$-ATPase is formed and then interacts with the $F_\delta$ (Sternweis, 1978; Sternweis & Smith, 1977; Dunn & Heppel, 1981). We have already seen that, in the unc operon, the three genes uncBEF coding for the $F_\delta$ subunits are transcribed first and then those for the $F_\gamma$-ATPase (uncHAGDC) subunits are transcribed later. This order would seem consistent with either of the sequences outlined above, but any scheme in which the $F_\delta$ is assembled first implies that, in the rapidly growing cell, there would be open proton pores in the cell membrane, a physiologically unfavourable situation.

The first hint that the assembly sequence in vivo might not involve the simple addition of pre-formed $F_\gamma$-ATPase to pre-formed $F_\delta$ came from atebrin-fluorescence quenching experiments with membranes from a particular unc mutant carrying the uncD436 allele (Cox et al., 1981). The quenching of atebrin fluorescence is now a widely used test for membrane energization and is also used as an indicator of the ‘proton leakiness’ of the membranes. The experiments depicted in Fig. 5 illustrate this test. Thus if membranes, in the presence of atebrin, are energized by electron transport following the addition of an oxidizable substrate, or by the addition of ATP, the atebrin-fluorescence is quenched (Fig. 5a). If the membranes are stripped of $F_\gamma$-ATPase neither the addition of NADH or ATP will result in fluorescence quenching (Fig. 5b). The lack of fluorescence-quenching by stripped membranes following NADH addition is thought to be due to the fact that the removal of the $F_\gamma$-ATPase has left open proton pores ($F_\delta$) and therefore an inability to maintain an electrochemical gradient of protons. If excess purified normal $F_\gamma$-ATPase is allowed to associate with the stripped membranes and the experiment repeated, the membranes now appear normal (Fig. 5c). If, however, inactive $F_\gamma$-ATPase from a mutant (e.g. uncA) is used to reconstitute the membranes (Fig. 5d), ATP is unable to energize the membranes but the addition of NADH does result in fluorescence quenching because the open $F_\delta$ in the stripped membranes has been sealed by the inactive $F_\gamma$-ATPase.

The mutation in the uncD436 allele was found, by genetic complementation tests, to be a polarity mutation affecting the uncD and uncE genes but not the uncB, E, F, H, A or G genes. It was found that membrane preparations from strain AN1007 (uncD436) had neither ATPase activity nor ATP-dependent atebrin-fluorescence quenching (Table 2). Furthermore, these membrane preparations were not leaky for protons as judged by NADH-dependent atebrin-fluorescence quenching tests, even after washing in low-ionic-strength buffer (Table 2). The reason for this initially surprising proton impermeability became obvious when the protein composition of the membranes was examined by two-dimensional gel electrophoresis: no $F_\delta$ subunit $b$ was present in the membranes. It seemed therefore that a mutation resulting in lack of $\beta$- and $\epsilon$-subunits had affected the incorporation of at least one of the $F_\delta$ subunits into the membrane (the other two $F_\delta$ subunits cannot be seen in the electrophoresis system used). This conclusion was confirmed by the insertion of the plasmid pAN36 (uncD+C+) into a strain carrying the uncD436 allele. The protein composition and the atebrin-fluorescence quenching behaviour of the membranes from the plasmid-bearin strain were normal.

The results obtained using membranes from strain AN1007 (uncD436) were confirmed and extended by examining a series of Mu-induced unc mutants and the membranes from the resulting strains examined by two-dimensional gel electrophoresis. Only when the Mu phage was inserted after the uncA gene was the $F_\delta$ subunit $b$ incorporated into the membranes and the membranes made permeable to protons. This indicated that the $\alpha$-subunit, as well as the $\beta$-subunit of the $F_\gamma$-ATPase, was necessary for the formation of a proton pore. The plasmid pAN36(uncD+C+) was then used to transform the series of Mu-induced unc mutants and the membranes from the resulting strains examined by two-dimensional gel electrophoresis. Only when the Mu phage was inserted after the uncA gene was the $F_\delta$ subunit $b$ incorporated into the membranes and the membranes made permeable to protons. This indicated that the $\alpha$-subunit, as well as the $\beta$-subunit of the $F_\gamma$-ATPase, was necessary for the formation of a proton pore.
the γ-, δ- or ε-subunits and the subunit b could not be detected. In membranes from an uncG- mutant strain, on the other hand (Fig. 6b), the F₀ subunit b was present and a relatively large amount of β-subunit and a small amount of α-subunit was membrane bound.

The results of this series of experiments allowed a tentative scheme for the assembly of F₁-F₀ ATPase in E. coli (Cox et al., 1981) (Fig. 7). In this scheme it is proposed that the F₀ subunits a and c are incorporated into the membrane followed by the F₁-ATPase subunits β and α, then the F₀ subunit b, after which the F₀ is completed and further major and minor F₁-ATPase subunits added to complete the assembly. In general terms it seems that the assembly process is such that an open proton pore is not present at any stage. The α- and β-subunits block the proton pore in a number of strains with altered α- or β-subunits or with normal α- and β-subunits in the strain carrying the uncG428 allele (Cox et al., 1981). The later stages of the proposed assembly sequence are necessarily tentative at present but the scheme should form a basis for future experiments on the assembly process.

**Table 2. Effects of washing membranes from a normal strain or the uncD346 polar mutant (data from Cox et al., 1981)**

<table>
<thead>
<tr>
<th>Membranes from</th>
<th>Membranes washed?*</th>
<th>NADH-dependent atebrin-fluorescence quenching (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN248 (unc⁺)</td>
<td>No</td>
<td>69</td>
</tr>
<tr>
<td>AN1007 (uncD436)</td>
<td>Yes</td>
<td>90</td>
</tr>
</tbody>
</table>

* The membranes were washed in low-ionic-strength buffer which solubilizes F₁-ATPase from normal membranes.

**Assembly and function of F₁-F₀ ATPase in mutants carrying known amino acid changes in the F₀ subunit c**

As mentioned earlier the F₀-ATPase subunit c, or the dicyclohexylcarbodi-imide-binding protein, because of its unusual physical properties and low molecular weight (about 8500), is a subunit of the F₁-F₀ ATPase about which a great deal is known. The amino acid sequence of the subunit has been determined for the proteins from E. coli, the thermophilic bacterium PS3, Neurospora crassa, Saccharomyces cerevisiae, beef heart mitochondria, spinach chloroplasts, the blue-green alga Mastigocladus laminosus and Streptococcus faecalis (see Sebald & Hoppe, 1981; Ovchinnikov, 1981). F₀ subunits c from all these sources have a number of features in common. Thus the proteins have two hydrophobic regions and a connecting hydrophilic region and it has been suggested that the assembled protein has a 'hairpin' structure with the two hydrophobic segments each spanning the membrane (see Sebald & Hoppe, 1981; A. E. Senior, personal communication). There is a conserved acidic group, Asp₄₁ in E. coli, thought to be buried in the membrane and to play an important role in proton translocation. Reaction of Asp₄₁ with dicyclohexylcarbodi-imide inhibits proton translocation (see Sebald & Hoppe, 1981) and replacement of Asp₄₁ by glycine in a mutant strain of E. coli
Fig. 6. Two-dimensional gel electrophoresis of membrane preparations from strains carrying the uncF469 or uncG428 alleles

Unlabelled arrows indicate the normal positions of the γ and δ-subunits of the F₁-ATPase and the F₉ subunit b (18K) (Cox et al., 1981).

Fig. 7. Proposed sequence for the in vivo assembly of the ATPase complex in E. coli (after Cox et al., 1981)

The proposed tertiary structures of the F₀-subunits (A. E. Senior, personal communication) are indicated. Some strains carrying mutant uncE alleles incorporate the α and β- F₁-ATPase subunits and the F₉ subunit b although F₉ subunit c is not inserted. In those uncE mutant strains which insert a mutant F₉ subunit c into the membranes, a complete F₁-F₉ ATPase structure is formed.
abolishes proton translocation (Hoppe et al., 1980a; Wachter et al., 1980). In the case of mutants which are partially resistant to dicyclohexylcarbodi-imide, Ile, has been shown to be replaced by either threonine or valine but the ATPase complex is still functional with respect to oxidative phosphorylation (Hoppe et al., 1980b).

We have recently examined strains carrying the uncE410 or uncE408 mutant alleles in detail (G. B. Cox, L. P. Langman, D. A. Jans, J. A. Downie, A. E. Senior, F. Gibson, A. L. Fimmel, L. B. James & G. Ash, unpublished work) and have shown that these mutations affect function or both assembly and function. Strains carrying the uncE410 allele assemble an altered F0 subunit c into their membranes. This mutant protein blocks proton translocation as judged by fluorescence quenching (Table 3) and causes lack of ability to form ATP as a result of an artificially imposed proton gradient (Table 4). The amino acid substitution which has occurred a result of the mutation carried by the uncE410 allele has been determined. Thus the F0 subunit c is cleaved by CNBr to give a number of small fragments and one large insoluble fragment (Fig. 8). The soluble fragments are readily separated by high-performance liquid chromatography and analyses of the fractions obtained from strain AN1036 (uncE410/uncE410) and those from the corresponding protein from a normal strain are shown in Fig. 9. The results show that, in the product of the uncE410 allele, the proline normally at position 64 has been replaced by leucine.

Strains carrying the uncE408 mutant allele present quite different properties from those carrying the uncE410 allele. Haploid strains carrying the uncE408 allele assemble little, if any, mutant protein in their membranes but diploid or multicopy plasmid-bearing strains carrying multiple copies of the uncE408 allele do assemble a mutant F0 subunit c into the membrane. This protein c appears to be partially functional as judged by increased growth yield over the haploid strain, ability to grow on succinate, and atebrin-fluorescence quenching tests. Examination of the isolated mutant protein by CNBr cleavage, high-performance liquid chromatography and amino acid analysis of these fragments shows that the proline residue at position 64 is replaced by leucine.

Table 3. Atebrin-fluorescence quenching in membranes from strains carrying normal or uncE mutant alleles

<table>
<thead>
<tr>
<th>Strain</th>
<th>Membranes</th>
<th>Stripped membranes</th>
<th>Membranes</th>
<th>Stripped membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN1460 (pAN45, unc+)</td>
<td>75 / 3</td>
<td>60 / 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AN1036 (uncE410/uncE410)</td>
<td>85 / 85</td>
<td>0 / 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AN1871 (pAN128, uncE408)</td>
<td>85 / 77</td>
<td>75 / 0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4. ATP synthesis driven by an artificially imposed proton gradient in strains carrying normal or uncE mutant alleles

The basal reaction mixture contained sodium acetate buffer (pH 4.5), membranes (1 mg of protein), valinomycin, ADP and p-amino benzamidine. After 4 min pre-incubation a solution containing Hepes buffer (pH 8.2), magnesium acetate, KCl, glucose, hexokinase and KH2PO4 was added. After a further 1 min the reaction was terminated by the addition of trichloroacetic acid.

<table>
<thead>
<tr>
<th>Additions to basal reaction mixture</th>
<th>AN1460 (pAN45, unc+)</th>
<th>AN1036 (uncE410/unc+)</th>
<th>AN1871 (pAN128, uncE408)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.57</td>
<td>0.14</td>
<td>0.24</td>
</tr>
<tr>
<td>Carbonyl cyanide m-chlorophenylhydrazone (CCCP)</td>
<td>0.19</td>
<td>0.16</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Fig. 8. Amino acid sequence of the F0 subunit c (the dicyclohexylcarbodi-imide-binding protein) of E. coli

The arrows indicate the fragments produced by CNBr treatment of the peptide. The designations of the fragments (B1 to B9) follows that of Wachter et al. (1980).
analysis indicated that the smaller CNBr fragments were normal. In order to obtain the amino acid sequence of the larger insoluble fragment B6 (Fig. 8) the DNA sequence of the cloned uncE408 gene was determined and compared with the sequence of the wild type gene from which the mutant was obtained. The results (Fig. 10) show that the mutation present in the uncE408 allele has resulted in Leu, being replaced by phenylalanine.

The results of atebrin-fluorescence quenching tests on membranes from strains which had incorporated the mutant uncE408 protein indicated that the stripped membranes were impermeable to protons (Table 3). This result was unexpected in view of the fact that these strains grew on succinate medium. The inability of such membranes to carry out ATP synthesis (Table 4) driven by an artificially produced proton gradient was consistent with the results of the atebrin-fluorescence quenching tests. However, an appreciable rate of phosphorylation coupled to the oxidation of NADH was obtained (Table 5), a result which is in accord with the growth yield on glucose which although somewhat lower than normal is by no means an 'uncoupled' yield (Table 5).

It would appear therefore that membranes from strain AN1871 (pAN128, uncE408) or from the diploid strain AN1332 (uncE408/uncE408) are unable to phosphorylate using a pH gradient but are able to couple phosphorylation to electron transport. Further, it would appear that the F, of such strains is impermeable to protons. These observations can be rationalized by proposing that protons involved in oxidative phosphorylation may enter the F, via a pathway within the membrane when supplied by electron transport. However, the lowered growth yield of the strains carrying multiple copies of the uncE408 allele might suggest a pathway dependent on a pH gradient may also operate in the normal E. coli cell. Strains carrying another independently isolated mutant allele uncE463 have been shown to have the same properties as those carrying

Fig. 9. High-performance liquid chromatography of the soluble fragments of CNBr-treated F, subunit c from strains AN1460 (pAN45, uncE) or AN1036 (uncE410/uncE410)

Fig. 10. Nucleotide sequence of the DNA coding for fragment B, of the F, subunit c from E. coli together with the corresponding amino acid sequence

The base change and the corresponding amino acid change due to the uncE408 allele are shown.
Table 5. NADH-dependent phosphorylation and aerobic growth yields in strains carrying normal or uncE mutant alleles

<table>
<thead>
<tr>
<th>Strain</th>
<th>ATP synthesis (nmol/min per mg of protein)</th>
<th>O₂ uptake (nmol of O₂/min per mg of protein)</th>
<th>P/O</th>
<th>Growth yield (final turbidity on 5 mm glucose; Klett units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN1460 (pAN45, unc⁺)</td>
<td>22</td>
<td>47</td>
<td>0.46</td>
<td>200</td>
</tr>
<tr>
<td>AN1036 (uncE410/uncE410)</td>
<td>&lt;1</td>
<td>53</td>
<td>&lt;0.02</td>
<td>130</td>
</tr>
<tr>
<td>AN1871 (pAN128, uncE408)</td>
<td>15</td>
<td>94</td>
<td>0.16</td>
<td>180</td>
</tr>
</tbody>
</table>

![Fig. 11. Proposed secondary and tertiary structure of the F₅ subunit c from E. coli](image)

The amino acids affected by the mutations described are indicated.

the uncE408 allele and to have an identical amino acid substitution. Caution, however, should be exercised with respect to the above interpretations until a wider variety of mutants affected in the F₅ have been examined in detail.

A proposed secondary and tertiary structure for the membrane-bound F₅ subunit c based on the 'polarity profile' and on Chou–Fasman (Chou & Fasman, 1978) rules modified for membrane proteins (A. E. Senior, personal communication) is shown in Fig. 11 and a summary of the effects of mutations affecting this protein is given in Table 6. The examination of the effects of amino acid substitutions in a larger number of mutants will be necessary to make generalizations as to the effects of amino acid substitutions in different regions of the molecule on the assembly and function of the ATPase complex. Nevertheless, it is striking that the amino acid substitutions in subunit c of E. coli, which have now been characterized, although well separated on the polypeptide chain, are probably clustered in the region of the Asp₁₅ (Fig. 11) which is thought to be vital for proton translocation. Thus the effects on proton translocation of the replacement of Leu₁₅ by phenylalanine, with its bulkier side-chain, may indicate that precise positioning of the carboxyl group of Asp₁₅ is vital for the flow of protons from outside the membrane in response to a pH gradient.

I think it can be said that studies with E. coli are fulfilling their early promise. With the benefit of hindsight, a strategy for the study of oxidative phosphorylation in E. coli could be outlined as set out in Fig. 12. Although the actual investigations have not always followed tidily the steps outlined, we are now well along the path. It could be pointed out that with respect to the application of biochemical genetics to the electron transport pathway and its precise relationship to the phosphorylation system, there is still much to be done. If there is a pathway for protons operating within the membrane, then the connection between electron transport and the ATPase complex is an integral part of the overall process of oxidative phosphorylation. The three types of mutants which are currently available to assist in the study of the aerobic electron transport pathway in E. coli are those connected with the transport and metabolism of iron (ent, fep and fec mutants), those affected in ubiquinone biosynthesis (ubi mutants) (see Gibson & Cox, 1973; Downie & Cox, 1978) and those affected in haem biosynthesis (hem mutants) (see Haddock & Jones, 1977; Brookman et al., 1979). The approximate chromosomal location of at least some of the genes coding for the cytochromes is known (Shipp et al., 1972) and a mutant affected in cytochrome d has been isolated (J. A. Downie & G. B. Cox, unpublished work).

There is a tendency to stress the similarities between the ATPase complexes from widely different sources. While there is no doubt that the ATP-synthesizing system is highly conserved in all living cells, there are also some striking differences, particularly in relation to the biogenesis of the ATPase complex in mitochondria, chloroplasts or bacteria. It seems likely that there may also be differences in the details of regulation of the F₅-F₆ ATPase expected in, for example, mammalian mitochondria and E. coli. The former functions in a fairly constant environment, but the latter is subject to a wide variety of environmental conditions including widely varying oxygen tensions. However, it seems likely that information obtained on the molecular mechanisms of ATP synthesis using any organism will have implications for all F₅-F₆ ATPases and that mutants of E. coli will play a significant role in the elucidation of this long-standing problem.

1983
One wonders how Frederick Gowland Hopkins would have viewed the experimental approaches and rapid advances over the last 30 years that have been made possible by the integration of biochemistry and genetics. He was no stranger to the use of bacteria in approaching biochemical problems, having used them in the study of tryptophan metabolism as early as 1902 (see Stephenson, 1949). There is no doubt that his encouragement of the work in Cambridge on bacterial metabolism and nutrition, and on biological oxidations and phosphorylation, provided an important part of the foundations for the work I have been describing today. It is my impression from his writings that his reaction to the current state of biochemical knowledge would be that of pleasure, rather than surprise.

I am grateful to my colleagues for permission to use their unpublished results and to A. E. Senior and G. B. Cox for their helpful discussions during the preparation of the manuscript for this lecture.


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