The EMBO Journal vol.4 no.9 pp.2357–2363, 1985

Proton conduction by subunit *a* of the membrane-bound ATP synthase of *Escherichia coli* revealed after induced overproduction

Kaspar von Meyenburg, Birgitte B.Jørgensen[1], Ole Michelsen, Lise Sørensen and John E.G.McCarthy[2]

Department of Microbiology, The Technical University of Denmark, 2800 Lyngby-Copenhagen, Denmark, and [2]Department of Cytogenetics, Gesellschaft für Biotechnologische Forschung, Braunschweig-Stöckheim, FRG

[1]Present address: NOVO Industry, Bagsværd, Denmark

Communicated by K.von Meyenburg

Transcriptional fusions between the phage lambda promotor *pR* and ATP synthase genes, *atp*, on plasmid pBR322 were constructed in order to study the effects upon growth and physiology of *Escherichia coli* of induced overproduction of H^+-ATPase subunits. Constitutive overproduction of the complete enzyme had earlier been found to result in decreased growth rate and cytological defects. When a 15-fold overproduction of subunit *a* alone, or together with subunit *c*, or with all other ATP synthase subunits was suddenly induced, the following effects were observed. Inhibition of growth and protein synthesis within 10 min of induction, which effect was suppressed by N,N'-dicyclohexylcarbodiimide, also when the chromosomal *atp* genes coding for the F_0 subunits *a*, *b* and *c* were deleted. Partial collapse of the membrane potential $\Delta\psi$ at 4–6 min after induction paralleled by inhibition of thiomethylgalactoside and guanosine transport. Respiration and α-methylglucoside transport was not affected. The partial collapse of $\Delta\psi$, and the specific inhibition of proton-driven transport systems is taken to show that the subunit *a* has – when suddenly overproduced and inserted into the membrane – a protonophoric activity. It is suggested that this protonophoric activity of subunit *a* is related to the function of this subunit in the F_0 sector in H^+-ATPases.

Key words: E. coli ATP synthase (F_1F_0)/proton translocation/electrochemical potential/induction of ATP synthase subunits/phage lambda promoter *pR*

Introduction

The membrane-bound ATP synthase of *Escherichia coli* has a central role in energy transduction under anaerobic and aerobic growth conditions (for reviews, see Harold, 1972; Cox and Gibson, 1974). Its eight subunits are encoded by the *atp* (*unc*) operon (for reviews, see Fillingame, 1981; Futai and Kanazawa, 1983; von Meyenburg *et al.*, 1982b). The nucleotide sequence of the entire operon (Figure 1B) has been determined (see Futai and Kanazawa, 1983). The five promoter-distal genes code for the five subunits which assemble to form the F_1-ATPase. The preceding three genes code for the three subunits *a*, *b* and *c*, which together appear to form a proton-conducting structure (F_0 sector) in the membrane which also binds the F_1 sector (Hoppe *et al.*, 1983; Friedl *et al.*, 1983; Klionsky *et al.*, 1983; Hermolin *et al.*, 1983). There is a ninth gene (*atpI*) immediately adjacent to the major promoter (Nielsen *et al.*, 1984) but this does not appear to be necessary for the assembly of a functional ATP synthase (von Meyenburg *et al.*, 1982a).

During analysis of minichromosomes carrying *atp* genes it was found that their presence could result in serious growth inhibition (von Meyenburg *et al.*, 1978, 1979; Harding *et al.*, 1982; Yamaguchi and Yamaguchi, 1983) particularly when the genes encoded the F_0 subunits. This growth inhibition could be partially overcome when N,N'-dicyclohexylcarbodiimide (DCCD) was present in the growth medium (von Meyenburg and Hansen, 1980). This reagent is known to react with the *c* subunit (Sebald *et al.*, 1979). The growth inhibitory effect was subsequently recognized to be due to the overproduction of ATP synthase subunits (Hansen *et al.*, 1981; von Meyenburg *et al.*, 1985). Constitutive overproduction of ATP synthase was also found to result in gross morphological and cytological changes in the cells as well as in an inhibition of their growth rate (von Meyenburg *et al.*, 1984).

By linking the *atp* genes to a promoter of bacteriophage lambda (*pR*) it has become possible to study the effects of overproduction of ATP synthase subunits after induction by inactivation of the heat-sensitive lambda repressor *cI857*. Here we show that the rapid induction of overproduction of specifically subunit *a* leads to a drastic growth inhibition. This effect is observed irrespective of whether overproduction of all the other seven H^+-ATPase

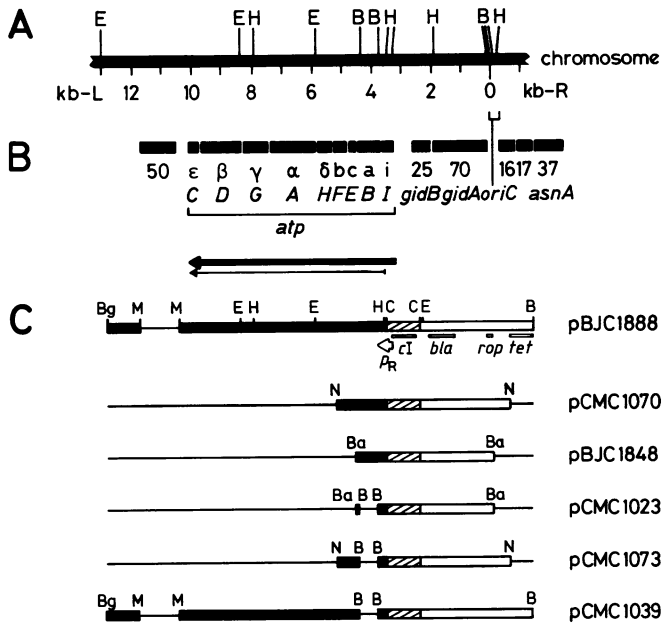

Fig. 1. **(A)** Restriction map and **(B)** genetic map of the *atp* (*unc*) operon and adjacent regions of the *E. coli* K-12 chromosome including gene and subunit designations (von Meyenburg *et al.*, 1984). Restriction sites: E = *Eco*RI, B = *Bam*HI, H = *Hind*III. Arrows indicate transcription from the major promoter *atpIp* and the minor promoter *atpB2p*, respectively (Nielsen *et al.*, 1984). **(C)** Physical and genetic maps of pBR322-derived plasmids carrying segments of the *atp* operon fused to the phage lambda promoter *pR* (hatched). Chromosomal DNA (black bars) is aligned with the maps in **(A)** and **(B)**. Open bars represent DNA of plasmid pBR322. Simple black lines indicate the extent of deletions as compared with plasmid pBJC1888. N = *Nar*I, B = *Bam*HI, Bg = *Bgl*II, Ba = *Bal*31, M = *Mlu*I.

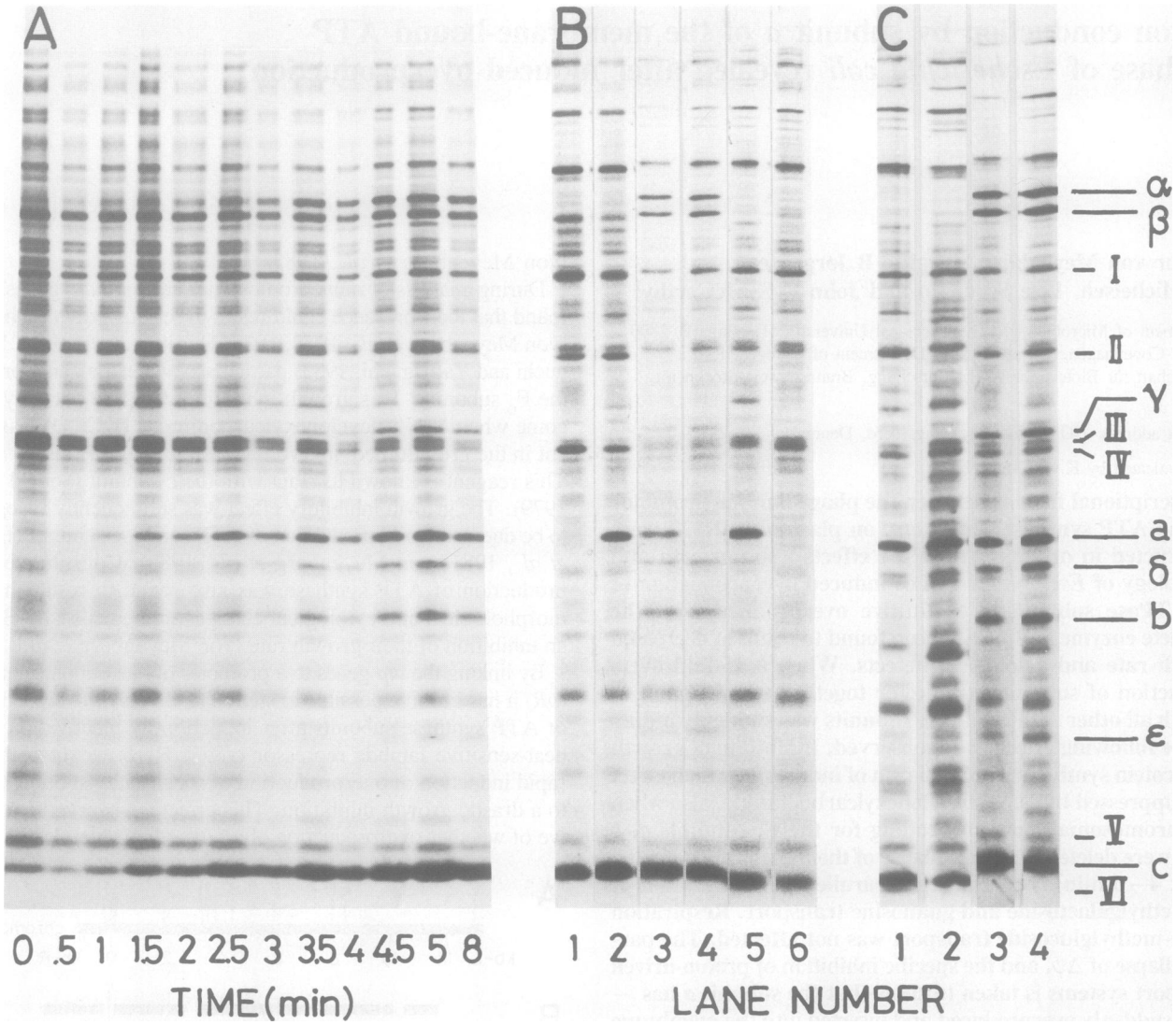

Fig. 2. Autoradiograms of [^{35}S]methionine pulse-labelled total cell proteins separated by SDS-PAGE. **(A)** Time course of induction of ATP synthase subunit synthesis: a culture of strain LM1888 pre-grown at 30°C in minimal medium with glucose was shifted to 38°C and at the times indicated aliquots were pulse-labelled with [^{35}S]methionine. **(B)** Heat-induced expression of ATP synthase subunits from different plasmids: **1:** strain CM3638 (plasmid pCMC1073), **2:** strain CM3627 (plasmid pCMC1070), **3:** strain LM1888 (plasmid pBJC1888), **4:** strain CM3638 (pCMC1039), **5:** strain LM1848 (pBJC1848), **6:** strain CM3628 (pCMC1023); pre-growth as in **(A)**, pulse-labelled after 8 min incubation at 39°C. **(C)** Effect of DCCD on ATP synthase subunit induction from pBJC1888 (strain LM1888, **lanes 3** and **4)** and pBJC1848 (strain LM1848, **lanes 1** and **2**). Cultures were pre-grown as in **(A)**; to an aliquot of each DCCD was added (50 μg/ml) **(lanes 2** and **4)** and 20 min later both the treated and untreated cultures were shifted to 39°C for 15 min and pulse-labelled. For comparison see first lane in **(A)** showing proteins synthesised in strain LM1888 before induction. Position of subunits of ATP synthase and of other proteins are indicated: (I) translation elongation factor EF-Tu, (II) major outer membrane protein OmpA, (III) lambda repressor (*cI857*), (IV) β-lactamase (*bla*), (V) lipoprotein Lpp, (VI) *rop* gene product (repressor of primer formation) of pBR322.

subunits is simultaneously induced. The growth inhibition can be overcome by DCCD. It is also accompanied by a partial collapse of the membrane potential $\Delta\psi$. Thus, the effects of subunit *a* overproduction are attributable to a proton translocating activity related to the function of this subunit in the assembled F_o sector.

Results

Induction of ATP synthase subunit a overproduction leads to inhibition of growth and protein synthesis

Strain LM1888 harbors the plasmid pBJC1888 carrying the promoter *pR* of phage lambda fused to the eight ATP synthase genes, *atpB* to *atpC* (Figure 1B,C). Inactivation of the heat-sensitive lambda repressor protein, which is expressed from the *cI857* gene on the same plasmid, leads to a rapid induction. It results in sequential increase of the rate of synthesis of the eight ATP synthase subunits (Figure 2A) – in accordance with the order of their genes in the operon (Figure 1B). Synthesis of subunit *a* increases first, closely followed by the increase in synthesis of subunits *c* and *b* (Figure 2A). At 5 min after induction ~3% of the total incorporated radioactivity is found in the *a* subunit band and ~20% in the *c* subunit band, reflecting a 12- to 15-fold increase in the rates of synthesis of these two subunits.

Upon induction, we noticed that growth of strain LM1888 ceased within 10 min of the temperature shift. This growth inhibition was paralleled by inhibition of protein synthesis as shown in Figure 3C. In strain LM948 (which harbors the vector plasmid pBR322) the temperature shift had no negative effect (Figure 3A).

To determine the synthesis of which of the subunits was responsible for the observed inhibitory effects, plasmids were constructed by deleting various parts of the *atp* genes from plasmid pBJC1888 (Figure 1C; Materials and methods). The effects on

growth (not shown) and protein synthesis of induction of expression of the various genes fused to *pR* were investigated. Strains harboring plasmids pCMC1070 or pBJC1848, from which subunits *a* and *c* or subunit *a* alone (together with the *rop* gene product) were expressed (Figure 2B), ceased growing (not shown) and the rate of protein synthesis was markedly inhibited upon temperature shift (Figure 3D) as in strain LM1888 (Figure 3C), also when the *atpI, B, E, F, H* and *A* genes (Figure 1B) were deleted from the chromosome (Tables I and III). The rate of protein synthesis started to decrease within 4–5 min after the temperature shift, reaching 20–30% of the initial value 20 min later (Table I). On the other hand, strains containing plasmids pCMC1023, pCMC1073 or pCMC1039 from which either none of the ATP synthase subunits, the *c* subunit alone, or all subunits except subunit *a* were expressed (Figures 1C, 2B), did not cease growing and the rate of protein synthesis did not decrease; rather, growth rate increased by 50% as a result of the temperature increase. The measured rates of protein synthesis are summarized in Table I.

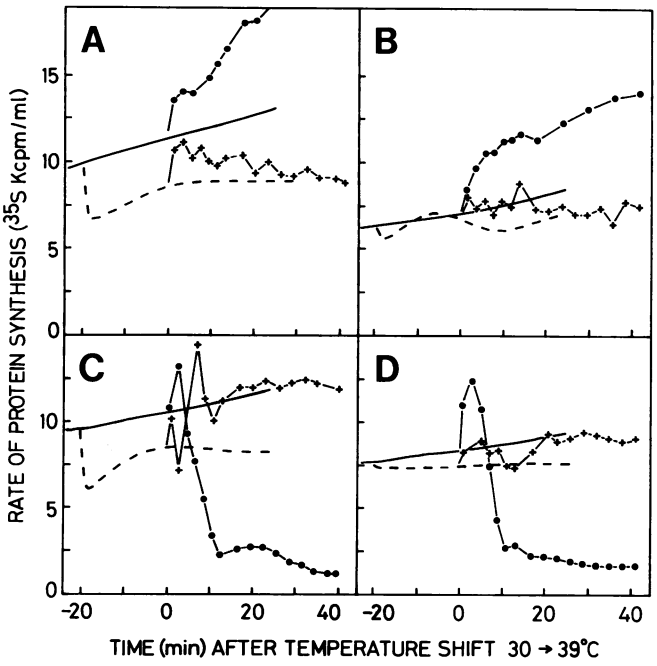

Fig. 3. Rate of protein synthesis before and after a temperature shift from 30° to 39°C in the absence and presence of DCCD (50 μg/ml) of strains carrying heat-inducible *atp* plasmids. Strains were grown in minimal medium with glucose; DCCD was added at −20 min. **(A)** Strain LM946 (= CM2443/pBR322), **(B)** CM1470, **(C)** LM1888 (= CM1470/pBJC1888), **(D)** LM1848 (= CM1470/pBJC1848). Strain CM1470 carries the *atp* deletion *atp-706* (Table III). Rate of protein synthesis was determined by pulse-labelling with [^{35}S]methionine: (●) at 39°C in the absence of DCCD, (+) at 39°C in the presence of DCCD. Rate of protein synthesis at 30°C is shown as full line (−DCCD) and dashed line (+DCCD), respectively.

The inhibition of growth and of protein synthesis can be unequivocally ascribed to the induced overproduction of subunit *a*. The *rop* gene product, which is also induced from pBJC1848 (Figure 2B) did not affect growth when induced alone from pCMC1023 (Figures 1C, 2B, Table I).

DCCD suppresses the growth inhibition after induction of a subunit overproduction

We originally suspected that growth inhibition in the case of strain LM1888, where the overproduction of all the ATP synthase subunits was directed by pBJC1888, might be due to proton leakage through additional F_0 sectors that had assembled upon induction before the synthesis of the appropriate equivalent of F_1 sectors (Figure 2A). It was indeed found that DCCD prevented the inhibition of protein synthesis in this strain (Figure 3C). However, DCCD also suppressed the inhibition of growth and protein synthesis due to the induction of subunit *a* alone in an *atp-706* deletion strain (Figure 3D) as well as in *atp*$^+$ strains (Table I). The presence of DCCD did not prevent induction of overproduction either of subunit *a* from pBJC1848 or of all subunits from pBJC1888 (Figure 2C). Moreover, all of the subunits including subunit *a* synthesised after induction appeared to enter or bind the membrane since we found them to sediment with the membrane fraction (not shown).

Induced overproduction of subunit a decreases the membrane potential, Δψ, and inhibits proton-driven transport systems

The adverse effects on growth and protein synthesis induced by overproduction of subunit *a* and its suppression by DCCD suggested that subunit *a* alone allowed for translocation of protons across the membrane leading to uncoupling.

Table I. Effects of induced overproduction of ATP synthase subunits on the rate of protein synthesis in the absence and presence of DCCD

Strain[a]	Chromosomal *atp* allele	Plasmid[b]	ATP synthase subunits synthesised from plasmid[c]	Relative rate of protein synthesis 20 min after induction at 39°C[d]	
				−DCCD	+DCCD[e]
CM2443	+	−	−	1.50	1.20
CM1470	706	−	−	1.55	1.05
LM1888	706	pBJC1888	*a,c,b,δ,α,γ,β,ε*	0.22 ± 0.03	1.80 ± 0.3
CM3625	+	pCMC1070	*a,c*	0.33 ± 0.03	n.d.
CM3627	706	pCMC1070	*a,c*	0.33	1.42
LM1849	+	pBJC1848	*a*	0.27 ± 0.02	1.00 ± 0.1
LM1848	706	pBJC1848	*a*	0.33 ± 0.06	1.00 ± 0.1
CM3618	+	pCMC1023	−	1.55 ± 0.2	1.40
CM3628	706	pCMC1023	−	1.70	1.40
CM3644	+	pCMC1073	−,*c*	1.62 ± 0.05	n.d.
CM3638	706	pCMC1073	−,*c*	1.55	1.37
CM3620	+	pCMC1039	−,*c,b,δ,α,γ,β,ε*	1.50 ± 0.1	n.d.

[a]See Table III.
[b]See Figure 1C.
[c]See Figure 2B.
[d]Calculated as the ratio of the rate of protein synthesis at 20 min and at 0 min after the temperature shift from 30° to 39°C (see Figure 3).
[e]DCCD (50 μg/ml) was added 20 min before the temperature shift.

We determined $\Delta\psi$ at 30°C and 39°C in strain CM12 with the plasmids pBJC1848, pCMC1070, pCMC1073 or pCMC1023 by measuring the uptake of the lipophilic cation tetraphenylphosphonium (TPP^+) labelled with ^3H (Hirota *et al.*, 1981) (Figure 4A). At 30°C the estimated $\Delta\psi$ was approximately −151 to −156 mV. After 10 min at 39°C a 10- to 20-fold reduction of [^{3}H]TPP^+ uptake was observed for the strains in which synthesis of subunit *a* was induced (strain CM3659 and strain CM3675, Figure 4A) while it was unchanged when no ATP synthase subunits (strain CM3676), or only subunit *c* was overproduced (strain CM3657, Figure 4A). The time course of the change in [^{3}H]TPP^+ uptake is shown in Figure 4B. The reduced level of TPP^+ uptake corresponded to a $\Delta\psi$ of −84 to −90 mV.

Estimates of $\Delta\psi$ were also made from the uptake of another lipophilic cation ($BTPP^+$: butyltriphenylphosphonium) into the described K-12 strains after treatment with EDTA and measured with an ion-selective electrode (McCarthy *et al.*, 1981). The results obtained with $BTPP^+$ were generally consistent with those described above for [^{3}H]TPP^+ but were not as reproducible, probably because of the damaging effects of EDTA treatment.

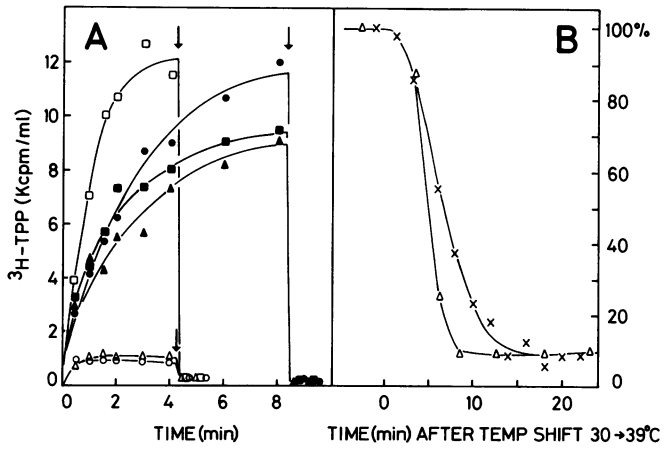

Fig. 4. (**A**) Uptake of [^{3}H]TPP^+ at 30°C (closed symbols) and 10 min after shift to 39°C (open symbols) of strains CM3657 (□), CM3659 (△) and CM3675 (○) harboring plasmids pCMC1073, pCMC1070 and pBJC1848 from which ATP synthase subunits *c*, *a* and *c*, and *a* alone were expressed, respectively (Figure 1C, Figure 2B). CCCP (50 μM) was added at 8.5 and 4.5 min at 30° and 39°C, respectively (arrows). Growth of strains was in minimal medium with glucose. (**B**) Uptake of [^{3}H]TPP^+ at 30°C (△) and intracellular [^{14}C]TMG concentration (×) after temperature shift from 30° to 39°C of a culture of CM3659. For the determination of the time course of intracellular TMG concentration, cells were pre-loaded with [^{14}C]TMG at 30°C for 10 min before the shift and radioactivity in the cells determined at intervals by collecting cells on membrane filters. Values for [^{3}H]TPP^+ uptake were obtained by adding [^{3}H]TPP^+ to aliquots of the culture at different times and measuring the intracellular concentration as in **A**.

Respiration was found not to be inhibited after induction of subunit *a* synthesis. Rather a 10−20% increase in the respiration rate was recorded 10−20 min after induction, i.e., at a time when $\Delta\psi$ was drastically decreased.

Furthermore, we probed the proton motive force Δp by measuring the rate of uptake of the lactose analog [^{14}C]thiomethylgalactoside ([^{14}C]TMG) by the lactose permease and the rate of uptake of [^{3}H]guanosine by the nucleoside permease, respectively. Active transport of these solutes depends on the Δp (West, 1970; West and Mitchell, 1972; Munch-Petersen *et al.*, 1979). $\Delta\psi$ is the main component of Δp at pH values near 7.0 in the growth medium (Shioi *et al.*, 1980). The rate of [^{14}C]TMG as well as of [^{3}H]guanosine uptake was found to be decreased severely upon induction of subunit *a* synthesis (Table II); the induction of subunit *c* alone had, by contrast, no effect on the uptake of these substrates. α-Methylglucoside, which is taken up through the phosphoenolpyruvate sugar phosphotransferase system (Dills *et al.*, 1980), was transported well in all strains at 30°C and after 20 min of induction of ATP synthase subunit synthesis at 39°C (Table II). These results represent independent evidence for a specific negative effect on the proton motive force of increased synthesis of subunit *a* without concomitant inhibition of other membrane functions.

Changes in the intracellular concentration of [^{14}C]TMG and the uptake of [^{3}H]TPP^+ followed virtually identical time courses (Figure 4B). Comparison of [^{3}H]TPP^+ uptake with the rate of protein synthesis (Figure 3C,D) reveals that the latter decreases in parallel with an apparent delay of ~3 min. Thus, the partial collapse of $\Delta\psi$ appears to parallel the cessation of proton-driven solute transport and to precede the inhibition of protein synthesis.

Discussion

Transcriptional fusions between the phage lambda promoter *pR* and ATP synthase genes *atp* on plasmid pBR322 were constructed to study the effects of induced overproduction of ATP synthase subunits upon the growth and physiology of *E. coli*. Constitutive overproduction of complete ATP synthase had earlier been found to result in decreased growth rate and inhibition of cell division presumably due to the formation of intracellular membrane vesicles (von Meyenburg *et al.*, 1984).

The following effects were observed after temperature shift with strains carrying plasmids from which the 15-fold overproduction of all ATP synthase subunits (pBJC1888), subunits *a* and *c* (pCMC1070) or subunit *a* alone (pBJC1848) was directed after inactivation of the heat-labile lambda repressor *cI857* (Figures 1C, 2): (i) cessation of growth and protein synthesis within 10 min of induction, which effect was suppressed by DCCD, also when the chromosomal *atp* genes coding for the F_0 subunits *a*, *b* and *c* were deleted; (ii) partial collapse of the

Table II. Effects of induced overproduction of subunit *a* of ATP synthase on membrane transport

Substrate	Strain	ATP synthase subunit synthesised from plasmid	Relative rate of transport[a]	
			During growth at 30°C	After 20 min growth at 39°C
[^{14}C]thiomethylgalactoside	CM3657	*c*	100[b]	100
	CM3659	*a,c*	75 ± 5	2 ± 1
[^{3}H]guanosine	CM3657	*c*	100[c]	100
	CM3659	*a,c*	100	8
[^{14}C]α-methylglucoside	CM3657	*c*	100[d]	100
	CM3659	*a,c*	80	55

[a]The transport rates determined for strain CM3657 were set at 100; the actual rates for the culture growing at 30°C were 10[b], 10[c] and 2[d] nmol/min per 4×10^8 cells, respectively.

membrane potential $\Delta\psi$ between 4 and 6 min after heat induction without concomitant inhibition of respiration; rather, a 10−20% stimulation of the rate of oxygen consumption was observed; (iii) 92−98% inhibition of thiomethylgalactoside as well as of guanosine transport while α-methylglucoside transport was little affected.

These effects were not observed when inducing overproduction of all ATP synthase subunits minus subunit *a* (plasmid pCMC-1039) or of subunit *c* alone (pCMC1073) or *rop* gene product alone (pCMC1023). Rop protein was also highly expressed from plasmid pBJC1848 besides subunit *a* (Figure 2B).

We conclude that it is the induced overproduction of the F_0 sector subunit *a* which is responsible for all the effects observed. The partial collapse of $\Delta\psi$ preceded the inhibition of protein synthesis (compare Figure 3C,D and Figure 4B) and was roughly simultaneous with the decrease in proton motive force-driven transport activities (Figure 4B).

Kanazawa *et al.* (1984) reported that plasmids carrying the *atpB* gene and the *atp* operon promoters could not be transformed into *E. coli* K-12 wild-type strains (see also Yamaguchi and Yamaguchi, 1983). This phenotype was eliminated when the *atpB* gene was mutated or deleted. Similarly, we have found that *atpB* plasmids leading to an increased constitutive synthesis of the *a* subunit result in low growth rates and yield (unpublished results). These effects, as well as the growth inhibition due to the presence of minichromosomes from which the beginning of the *atp* operon were expressed (von Meyenburg *et al.*, 1978, 1979) − an inhibition that was partially relieved by DCCD (von Meyenburg and Hansen, 1980) − can now be interpreted as having resulted from a partial collapse of $\Delta\psi$.

The question arises as to whether the effects brought about by overproduction of subunit *a* are attributable to an unspecific action or to a specific activity which manifests itself upon integration of this subunit into the membrane. The simplest interpretation of the data presented here is that subunit *a* possesses an ionophoric activity which can be expressed in the absence of other subunits of the H^+-ATPase. It is inserted into the membrane irrespective of the presence of the other subunits (or of DCCD). Subunit *a* could therefore catalyze the translocation of ions across the membrane, thus reducing the magnitude of the membrane potential, and thereby also inducing the other observed effects. The dual effect of excess subunit *a* upon the membrane potential and on proton motive force-linked transport systems is similar to that obtained by uncouplers, i.e., dissipation of the proton motive force by proton translocation across the membrane. The observations that α-methylglucoside transport was unaffected and that cellular respiration was not inhibited but rather stimulated by subunit *a* overproduction are also consistent with a specific protonophoric activity.

DCCD suppresses the inhibitory effect of subunit *a* overproduction upon protein synthesis (Figure 3). It is important to note that this suppression also occurred in the absence of any subunit *c*, i.e., when subunit *a* was overproduced in the *atp* deletion strain (*atp-706*) which lacks the *atpE* gene (subunit *c*) (Table I). This effect could be due to a direct interaction of DCCD with subunit *a* which results in blocking of the protonophoric activity. Proton translocating activities of other proteins notably of cytochrome *c* oxidase, of the intact F_0 sector of the ATP synthases of many different organisms, of mitochondrial transhydrogenases and possibly of the cytochrome *b-c1* complex are blocked by DCCD (for review, see Azzi *et al.*, 1984). We also found that the hydrophilic carbodiimide ECD, which affects the activity of the F_0 sector of the ATP synthase of *E. coli* (Loetscher *et al.*, 1984), partially suppressed the inhibition of protein synthesis due to subunit *a* overproduction.

It is conceivable that the blocking by DCCD of H^+-conduction through the complete F_0 sector (*a*1,*b*2,*c*10-15: Foster and Fillingame, 1982; von Meyenburg *et al.*, 1982b) is also due to its interaction with subunit *a*. Such an interaction may have previously gone unnoticed either because it is unstable or because it was difficult to detect in relation to the DCCD binding to subunit *c* which is present in ~12 copies per subunit *a*. From the earlier studies it was concluded that the blocking of H^+-conduction through the H^+-ATPase by DCCD was due to the interaction of DCCD with subunit *c* (Fillingame, 1975) at aspartyl residue 61 (Sebald *et al.*, 1979; Hoppe *et al.*, 1980). The isolation of DCCD-resistant mutants with a mutational change in the subunit *c* amino acid sequence (Hoppe *et al.*, 1980) was interpreted as supporting this notion. Possibly, structural changes in subunit *c* indirectly affect the activity of subunit *a* as well as its interaction with DCCD. Chemical modification studies by Steffens *et al.* (1984) have indicated that carboxyl groups other than *Asp*-61 in subunit *c* are involved in H^+-conduction; they also found that histidine and other basic residues might participate in the H^+-conduction. Such residues are found in several hydrophobic domains of subunit *a* (Nielsen *et al.*, 1981; Sebald *et al.*, 1982).

The protonophoric activity of subunit *a* revealed in this study is in contradiction to the conclusion reached from genetic data (Friedl *et al.*, 1983; Klionsky *et al.*, 1983) and biochemical analyses (Schneider and Altendorf, 1984, 1985) that all three F_0 subunits *a, b* and *c* are required for proton translocation. However, in the present experiments the subunit *a* synthesis rate was almost instantaneously increased 10-fold due to derepression of the lambda promoter *pR* preceding the *atpB* gene. This may lead to insertion of subunit *a* into the membrane in an abnormal configuration able to translocate protons by itself. We suggest that it inserts in a multimeric form, first because the *a* subunit when synthesized alone at a normal rate has no effect on the membrane potential (Friedl *et al.*, 1983) and growth, second because during the high rate of synthesis the cytoplasmic pool of *a* subunits would increase thus enabling them to interact with each other possibly *via* the hydrophobic domains (Nielsen *et al.*, 1981; Foster *et al.*, 1983) before inserting into the membrane. The multimeric state may be essential for the attainment of the proton conducting activity. In the complete F_0 sector this presumably is obtained as a result of interactions between subunit *a* and the many *c* subunits and the N-terminal part of the *b* subunit (Friedl *et al.*, 1983; Hoppe *et al.*, 1983; Hermolin *et al.*, 1983; Jans *et al.*, 1984; Aris and Simoni, 1983).

We propose that the *a* subunit is the major element in the F_0 sector-mediated proton conduction which activity under particular conditions − notably high rates of overproduction − becomes apparent without the need for interaction with other F_0 sector subunits (neither of the two other F_0 subunits affected $\Delta\psi$ when overproduced). By inference we expect this also to be valid for the ATPase subunit 6 of human mitochondria (Anderson *et al.*, 1981) and of yeast (*oli*-2; Macino and Tzagaloff, 1980) which share homology with subunit *a* of the *E. coli* H^+-ATPase and with each other (Nielsen *et al.*, 1981).

The temperature-sensitive phenotype associated with the partial collapse of $\Delta\psi$ after induction of *a* subunit overproduction renders the described effects amenable to further genetic analysis. It will be possible to define segments in the *a* protein which are essential for inducing the effects either by direct selection of temperature-resistant mutants or by *in vitro* modification of the

Table III. *E. coli* strains

Strain designation	Type	Genotype and plasmids[a]	Source, reference or construction[b]
CM12	B	*leu*, several mutations giving increased permeability	A *leu* derivative of strain AS19 (Sekiguchi and Iida, 1967)
CM1470	K-12	F$^+$, *atp-706* (*ΔIBEFHA*), *asnB32*, *thi-1*, *relA1*, *spoT1*	von Meyenburg *et al.* (1982a)[d]
CM2443	K-12	F$^+$, *asnB32*, *thi-1*, *relA1*, *spoT1*, Tn*10*-112	von Meyenburg *et al.* (1982a)
MC1000	K-12	*araD139*, Δ(*ara leu*)*7697*, Δ*lacX74*, *galU*, *galK*, *rpsL*	Casadaban and Cohen (1980)
LM946	K-12	(ii) pBR322	
LM1042	K-12	(ii) pOMC11-1(*atpB*+)	Friedl *et al.* (1983)
LM917	K-12	*asnB32*, *thi-1*, *relA1*, *spoT1*, *fuc*, *recA1*, *atpG221*/pBJC917(*atpBEFHAGDC*)	von Meyenburg *et al.* (1982a)
PL135	K-12	(iii) pPLJ135 (*cI857*, *pR*)	From Per L.Jørgensen; see Materials and methods
LM1815	K-12	(ii) *recA1*/pBJC1815	This paper
LM1888	K-12	(ii) pBJC1888(*cI857*,*pR-atpBEFHAGDC*)	This paper[c]
LM1848	K-12	(ii) pBJC1848(*cI857*, *pR-atpB*)	This paper[c]
LM1849	K-12	(i) pBJC1848	Transf. from LM1848 into CM2443
CM3625	K-12	(i) pCMC1070(*cI857*, *pR-atpBE*)[c]	*Nar*I deletion of pBJC1888
CM3627	K-12	(ii) pCMC1070[c]	As CM3625 but transf. into CM1470
CM3618	K-12	(i) pCMC1023(*cI857*, *pR-rop*)[c]	*Bam*HI deletion of pBJC1848
CM3628	K-12	(ii) pCMC1023[c]	As CM3618 but transf. into CM1470
CM3620	K-12	(i) pCMC1039(*cI857*, *pR-atpEFHAGDC*)[c]	*Bam*HI deletion of pBJC1888
CM3638	K-12	(ii) pCMC1073(*cI857*, *pR-atpE*)[c]	*Bam*HI deletion from pCMC1070 (from CM3625)
CM3644	K-12	(i) pCMC1073[c]	As CM3638 but transf. into CM2443
CM3657	B	(iv) pCMC1073[c]	From CM3638 transf. into CM12
CM3659	B	(iv) pCMC1070[c]	From CM3625 transf. into CM12
CM3675	B	(iv) pBJC1848[c]	From LM1848 transf. into CM12
CM3676	B	(iv) pCMC1023[c]	From CM3618 transf. into CM12

(i) Genotype of strain CM2443. (ii) Genotype of strain CM1470. (iii) Genotype of MC1000. (iv) Genotype of strain CM12.
[a]For genetic symbols, see Bachmann (1983). [b]See also Materials and methods. [c]See Figure 1C. [d]The extent of the *atp-706* deletion has been determined by restriction analysis (Hansen *et al.*, 1981); absence of the *atp* genes from strain CM1470 was determined by genetic complementation as well as demonstration of absence of subunits *a*, *b* and *c* (Friedl *et al.*, 1983; von Meyenburg *et al.*, 1984).

atpB gene. Earlier selection for suppression of growth inhibition by minichromosomes had yielded deletions most of which affected the *atpB* gene *per se* (Hansen *et al.*, 1981). A more refined genetic approach as outlined above should also shed some light on the structure of the *a* protein in the membrane. So far only a few alternative models exist (Sebald *et al.*, 1982; Hermolin *et al.*, 1983) based on the primary sequence (Nielsen *et al.*, 1981; Gay and Walker, 1981) with six or seven hydrophobic α-helices winding back and forth through the membrane.

Materials and methods

Strains and plasmids

The strains used in this study are listed in Table III. Plasmid pBJC1888, a pBR322 derivative, carries the eight *atp* genes *B*, *E*, *F*, *H*, *A*, *G*, *D* and *C* of *E. coli* downstream of the lambda promoter *pR* (Figure 1C). It was constructed by ligating a 1212 bp long *Cla*I fragment from pPLJ135 into plasmid pBJC917 Δ*Mlu*I (= pBJC1815) partially cleaved by *Cla*I, and transformation into strain CM1470, selecting for growth on succinate at 30°C and screening for temperature-sensitivity. That *Cla*I fragment contains DNA from phage lambda from base pair 36 966 (*Cla*I) to 38 103 (*Bgl*II) (Sanger *et al.*, 1982; Daniels *et al.*, 1983) – containing the intact *cI857* gene and *pR* – linked to *E. coli* chromosomal DNA from the *oriC* region, coordinates 111 (*Bcl*I) to 184 (*Cla*I) (Meijer *et al.*, 1979). The desired plasmid with the 1212-bp *Cla*I fragment inserted into the *Cla*I site of pBR322 DNA in pBJC1815 with transcription from *pR* into the *atp* operon was expected to give temperature-sensitivity since ATP synthase overproduction had been found to give growth inhibition (von Meyenburg *et al.*, 1984). Plasmid pBJC1848 was constructed in an analogous way by inserting the 1212-bp *Cla*I fragment from pPLJ135 into the *Cla*I site of pOMC11-1 in front of the *atpB* gene (Friedl *et al.*, 1983; Figure 1C).

By deleting different segments from plasmids pBJC1888 and pBJC1848, respectively, plasmids expressing only some or none of the ATP synthase subunits were obtained (Figure 1C; Table II). In all cases expression was mainly from *pR* when inactivating the *cI857* repressor at temperatures >36°C. At 30°C transcription from the minor promoter *atpB2p* in front of the *atpB* gene (Nielsen *et al.*, 1984) resulted in a level of expression from these plasmids corresponding to approximately twice the level in a wild-type *atp*$^+$ strain (von Meyenburg *et al.*, 1982a).

Preparation of DNA, plasmids, cleavage by restriction enzymes, ligation and transformation were according to standard methods (Maniatis *et al.*, 1982).

Growth of bacterial cultures and analysis of protein synthesis

Growth media and measurement of growth were as described earlier (von Meyenburg *et al.*, 1982a, 1984). When appropriate DCCD was added to the cultures at a nominal concentration of 50 mg/l by diluting a concentrated solution of DCCD in ethanol at 10 mg/l. Synthesis of ATP synthase subunits was determined by radioactive labelling with [^{35}S] methionine (10 μCi/ml; 1000 Ci/mmol) of 1 ml samples of the cultures, in AB minimal medium with glucose, for 1 min at 30° or 39°C followed by a 1 min chase with 100 mg/ml methionine; the cells were harvested by centrifugation and washed and the proteins separated by SDS-PAGE and autoradiographed (von Meyenburg *et al.*, 1982a). For quantitation the autoradiograms were optically scanned on LKB UltraScan Laser densitometer 2202.

The rate of total protein synthesis was determined by pulse-labelling with [^{35}S]-methionine (0.2 μg/ml; 65 mCi/mmol) for 30 s at 34°C of 0.5 ml aliquots of the culture (growing at 30° or 39°C). The TCA-precipitated cells were collected on glassfibre filters (GF/C Whatman) and the incorporated radioactivity was determined by scintillation counting.

Uptake of [^{14}*C*]*methyl-β-D-thiogalactoside (TMG),* [^{14}*C*]*methyl-α-glucoside (α-MG) and* [^{3}*H*]*guanosine*

This was determined as described (von Meyenburg, 1971; Munch-Petersen *et al.*, 1979). Cells were grown in minimal medium with glycerol and IPTG added (5×10^{-4} M) at 30°C to induce the *lac* operon. To 4 ml aliquots of the cultures [^{14}C]TMG (4×10^{-4} M; 0.4 mCi/mmol), [^{14}C]α-MG (4×10^{-4} M; 0.4 mCi/mmol) or [^{3}H]guanosine (10^{-5} M; 100 mCi/mmol) was added at 34°C; at 20 s intervals cells from 0.3 ml samples were collected on membrane filters (SS 85, Schleicher and Schüll), washed twice with AB minimal medium, dried, and the radioactivity determined.

Determination of the membrane potential $\Delta\psi$ *by measuring* [^{3}*H*]*TPP*$^+$ *uptake*

This was essentially as described by Hirota *et al.* (1981). Strain CM12, a derivative of the antibiotic-permeable *E. coli* B strain AS19 (Sekiguchi and Iida, 1967; Pato and von Meyenburg, 1970) was found to be highly permeable to [^{3}H]TPP$^+$ similar to the *acrA* strains (Hirota *et al.*, 1981). Derivatives of this strain carrying various plasmids (Table III) were grown in minimal medium with glucose to an OD_{450} at 0.6. To 4 ml portions [^{3}H]TPP$^+$ was added (5 μM; 25 mCi/mmol) and at intervals the cells from 0.5 ml samples were collected on glassfibre filters (GF/C), washed with AB medium three times, dried and the radioactivity

in the cells determined. 85% of the cells were retained. Addition of the uncoupler CCCP at 50 μM (Hirota *et al.*, 1981) resulted in all cases in rapid and complete release of the $[^3H]TPP^+$ from the cells (Figure 4A). $\Delta\psi$ was estimated from the ratio of external to intracellular TPP^+ concentration using the Nernst equation. The intracellular volume of water was taken as 2.5 μl/mg dry weight (Hirota *et al.*, 1981). OD_{450} = 1.000 corresponds to 190 μg dry weight per ml in this strain (Andersen and von Meyenburg, 1980).

Rate of oxygen consumption

This was determined using a Clark type electrode (Kier *et al.*, 1976) by measuring the decrease in dissolved oxygen concentration in aliquots of the cultures at the respective temperatures.

Acknowledgements

We thank Vibeke Tjell for expert technical assistance, Flemming G.Hansen, Jørgen Nielsen, Malcolm G.P.Page, Ole Laguni, Walter Sebald, Juergen Hoppe, Peter Friedl and Hans Ulrich Schairer for discussions and suggestions and Franz Floto and Jørgen Olsen for lending oxygen electrodes. This work was supported by grants from the Danish Natural Science Research Council, the NOVO Foundation, Bagsværd, Denmark, and an EMBO stipend to J.E.G.M.

References

Andersen,K.B. and von Meyenburg,K. (1980) *J. Bacteriol.*, **144**, 114-123.
Anderson,S., Bankier,A.T., Barrell,B.G., de Bruijn,M.H.L., Coulson,A.R., Drouin,J., Eperon,I.C., Nierlich,D.P., Roe,B.A., Sanger,F., Schreier,P.H., Smith,A.J.H., Staden,R. and Young,I.G. (1981) *Nature*, **290**, 457-465.
Aris,J.P. and Simoni,R.D. (1983) *J. Biol. Chem.*, **258**, 14599-14609.
Azzi,A., Casey,R.P. and Nalecz,M.J. (1984) *Biochim. Biophys. Acta*, **768**, 209-226.
Bachmann,B.J. (1983) *Microbiol. Rev.*, **47**, 180-230.
Casadaban,M.J. and Cohen,S.N. (1980) *J. Mol. Biol.*, **138**, 179-207.
Cox,G.B. and Gibson,F. (1974) *Biochim. Biophys. Acta*, **346**, 1-25.
Daniels,D.L., Schroeder,J.L., Szybalsky,W., Sanger,F., Coulson,A.R., Hong, G.F., Hill,D.F., Petersen,G.B. and Blattner,F.C. (1983) in Hendrix,R.W., Roberts,J.W., Stahl,F.W. and Weisberg,R.A. (eds.), *Lambda II*, Cold Spring Harbor Laboratory Press, NY, pp. 519-676.
Dills,S.S., Apperson,A., Schmidt,M.R. and Saier,M.H., Jr. (1980) *Microbiol. Rev.*, **44**, 385-418.
Fillingame,R.H. (1975) *J. Bacteriol.*, **124**, 870-883.
Fillingame,R.H. (1981) *Curr. Top. Bioenerg.*, **11**, 35-106.
Foster,D.L. and Fillingame,R.H. (1982) *J. Biol. Chem.*, **257**, 209-215.
Foster,D.L., Boublik,M. and Kaback,H.R. (1983) *J. Biol. Chem.*, **258**, 31-34.
Friedl,P., Hoppe,J., Gunsalus,R.P., Michelsen,O., von Meyenburg,K. and Schairer,H.U. (1983) *EMBO J.*, **2**, 99-103.
Futai,M. and Kanazawa,H. (1983) *Microbiol. Rev.*, **47**, 285-312.
Gay,N.J. and Walker,J.E. (1981) *Nucleic Acids Res.*, **9**, 2187-2194.
Hansen,F.G., Nielsen,J., Riise,E. and von Meyenburg,K. (1981) *Mol. Gen. Genet.*, **183**, 463-472.
Harding,N.E., Cleary,J.M., Smith,D.W., Michon,J.J., Brusilow,W.S.A. and Zyskind,J.W. (1982) *J. Bacteriol.*, **152**, 983-993.
Harold,F.M. (1972) *Bacteriol. Rev.*, **36**, 172-230.
Hermolin,J., Gallant,J. and Fillingame,R.H. (1983) *J. Biol. Chem.*, **258**, 14550-14555.
Hirota,N., Matsuura,S., Mochizuki,N., Mutoh,N. and Imae,Y. (1981) *J. Bacteriol.*, **148**, 399-405.
Hoppe,J., Schairer,H.U. and Sebald,W. (1980) *FEBS Lett.*, **109**, 107-111.
Hoppe,J., Friedl,P., Schairer,H.U., Sebald,W., von Meyenburg,K. and Jørgensen,B.B. (1983) *EMBO J.*, **2**, 105-110.
Jans,D.A., Fimmel,A.L., Hatch,L., Gibson,F. and Cox,G.B. (1984) *Biochem. J.*, **221**, 43-51.
Kanazawa,H., Kiyasu,T., Noumi,T. and Futai,M. (1984) *J. Bacteriol.*, **158**, 300-306.
Kier,I., Allermann,K., Floto,F., Olsen,J. and Sortkjaer,O. (1976) *Physiol. Plant.*, **38**, 6-12.
Klionsky,D.J., Brusilow,W.S.A. and Simoni,R.D. (1983) *J. Biol. Chem.*, **258**, 10136-10143.
Loetscher,H.-R., de Jong,C. and Capaldi,R.A. (1984) *Biochemistry (Wash.)*, **23**, 4128-4140.
Macino,G. and Tzagaloff,A. (1980) *Cell*, **20**, 507-517.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*, published by Cold Spring Harbor Laboratory Press, NY.
McCarthy,J.E.G., Ferguson,S.J. and Kell,D.B. (1981) *Biochem. J.*, **196**, 311-321.
Meijer,M., Beck,E., Hansen,F.G., Bergmans,H.E.N., Messer,W., von Meyenburg,K. and Schaller,H. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 580-584.
Munch-Petersen,A., Mygind,B., Nicolaisen,A. and Pihl,N.J. (1979) *J. Biol. Chem.*, **254**, 3703-3737.
Nielsen,J., Hansen,F.G., Hoppe,J., Friedl,P. and von Meyenburg,K. (1981) *Mol. Gen. Genet.*, **184**, 33-39.
Nielsen,J., Jørgensen,B.B., von Meyenburg,K. and Hansen,F.G. (1984) *Mol. Gen. Genet.*, **193**, 64-71.
Pato,M. and von Meyenburg,K. (1970) *Cold Spring Harbor Symp. Quant. Biol.*, **35**, 103-119.
Sanger,F., Coulson,A.R., Hong,G.F., Hill,D.F. and Petersen,G.B. (1982) *J. Mol. Biol.*, **162**, 729-773.
Schneider,E. and Altendorf,K. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 7279-7283.
Schneider,E. and Altendorf,K. (1985) *EMBO J.*, **4**, 515-518.
Sebald,W., Hoppe,J. and Wachter,E. (1979) in Quagliariello,E., Palmieri,E., Papa,S. and Klingenberg,M. (eds.), *Function and Molecular Aspects of Biomembrane Transport*, Vol. **313**, Elsevier, Amsterdam, pp. 63-74.
Sebald,W., Friedl,P., Schairer,H.U. and Hoppe,J. (1982) *Ann. N.Y. Acad. Sci.*, **402**, 28-44.
Sekiguchi,M. and Iida,S. (1967) *Proc. Natl. Acad. Sci. USA*, **58**, 2315-2320.
Shioi,J.-I., Matsuura,S. and Imae,Y. (1980) *J. Bacteriol.*, **144**, 891-897.
Steffens,K., Schneider,E., Herkenhoff,B., Schmid,R. and Altendorf,K. (1984) *Eur. J. Biochem.*, **138**, 617-622.
von Meyenburg,K. (1971) *J. Bacteriol.*, **107**, 878-888.
von Meyenburg,K. and Hansen,F.G. (1980) *ICN-UCLA Symp. Mol. Cell. Biol.*, **19**, 137-159.
von Meyenburg,K., Hansen,F.G., Nielsen,L.D. and Riise,E. (1978) *Mol. Gen. Genet.*, **160**, 287-295.
von Meyenburg,K., Hansen,F.G., Riise,E., Bergmans,H.E.N., Meijer,M. and Messer,W. (1979) *Cold Spring Harbor Symp. Quant. Biol.*, **43**, 121-128.
von Meyenburg,K., Jørgensen,B.B., Nielsen,J. and Hansen,F.G. (1982a) *Mol. Gen. Genet.*, **188**, 240-248.
von Meyenburg,K., Jørgensen,B.B., Nielsen,J., Hansen,F.G. and Michelsen,O. (1982b) *Tokai J. Exp. Clin. Med.*, **7**, 12-31.
von Meyenburg,K., Jørgensen,B.B. and van Deurs,B. (1984) *EMBO J.*, **3**, 1791-1797.
von Meyenburg,K., Hansen,F.G., Atlung,T., Boe,L., Clausen,I.G., van Deurs,B., Hansen,E.B., Jørgensen,B.B., Jørgensen,F., Koppes,L., Michelsen, O., Nielsen,J., Pedersen,P.E., Rasmussen,K.V., Riise,E. and Skovgaard,O. (1985) in Schaechter,M., Neidhardt,F.G., Ingraham,J. and Kjeldgaard,N.O. (eds.), *Molecular Biology of Bacterial Growth*, Jones and Bartlett Publishers Inc., pp. 260-281.
West,I.C. (1970) *Biochem. Biophys. Res. Commun.*, **41**, 655-661.
West,I.C. and Mitchell,P. (1972) *J. Bioenerg.*, **3**, 445-462.
Yamaguchi,K. and Yamaguchi,M. (1983) *J. Bacteriol.*, **153**, 500-554.

Received on 3 May 1985; revised on 1 July 1985