Experimental Determination of Control by the H⁺-ATPase in *Escherichia coli*

Peter Ruhdal Jensen,[1] Ole Michelsen,[1] and Hans V. Westerhoff[2]

Strains carrying deletions in the *atp* genes, encoding the H⁺-ATPase, were unable to grow on nonfermentable substrates such as succinate, whereas with glucose as the substrate the growth rate of an *atp* deletion mutant was surprisingly high (some 75–80% of wild-type growth rate). The rate of glucose and oxygen consumption of these mutants was increased compared to the wild-type rates. In order to analyze the importance of the H⁺-ATPase at its physiological level, the cellular concentration of H⁺-ATPase was modulated around the wild-type level, using genetically manipulated strains. The control coefficient by the H⁺-ATPase with respect to growth rate and catabolic fluxes was measured. Control on growth rate was absent at the wild-type concentration of H⁺-ATPase, independent of whether the substrate for growth was glucose or succinate. Control by the H⁺-ATPase on the catabolic fluxes, including respiration, was negative at the wild-type H⁺-ATPase level. Moreover, the turnover number of the individual H⁺-ATPase enzymes increased as the H⁺-ATPase concentration was lowered. The negative control by the H⁺-ATPase on catabolism may thus be involved in a homeostatic control of ATP synthesis and, to some extent, explain the zero control by the H⁺-ATPase on *E. coli* growth rate.

THE H⁺-ATPASE COMPLEX OF *Escherichia coli*

The membrane bound H⁺-ATPase (ATP synthase complex, F_1F_0-ATPase, Mg^{++}-dependent ATPase, proton translocating ATPase) is involved in the free-energy transduction of virtually all living cells, prokaryotic as well as eukaryotic. Depending on the particular species and on the conditions for growth, the enzyme functions either in the direction of ATP synthesis or toward ATP hydrolysis (Futai and Kanazawa, 1983). This review will focus on the importance of the H⁺-ATPase for the aerobically growing *Escherichia coli* cell. Under these conditions, the enzyme complex uses the electrochemical proton potential generated by the respiratory chain to drive the thermodynamically unfavorable reaction of ATP synthesis from ADP and inorganic phosphate.

The H⁺-ATPase complex of *Escherichia coli* consists of eight different subunits and can be divided into two parts that differ in their location and function. One part, the proton translocating, membrane integral part called F_0, is composed of the subunits a, b, and c. The stoichiometry of these subunits is probably 1:2:10 (Foster and Fillingame, 1982a; Sneider and Altendorf, 1987) although as many as 12–15 c subunits have been suggested to be present in the functional H⁺-ATPase (von Meyenburg *et al.*, 1982a). The other part, called F_1, which carries the ATPase catalytic activity, is composed of the 5 subunits α, β, γ, δ, ϵ in the stoichiometry 3:3:1:1:1. The F_1 part is attached to the F_0 part, probably through interactions with the b subunit. A thorough review of structure, function, and catalytic mechanisms of the H⁺-ATPase was given by Senior (1988, 1990).

[1] Department of Microbiology, Technical University of Denmark, Building 221, DK-2800 Lyngby, Denmark.
[2] Department of Microbial Physiology, Free University, De Boelelaan 1087, NL-1081 HV Amsterdam, The Netherlands.

GENETIC ORGANIZATION AND EXPRESSION OF THE GENES CODING FOR THE *E. Coli* H⁺-ATPASE COMPLEX

The *atp* genes (previously called the *unc* genes), which encode the subunits of the H⁺-ATPase, are located close to the origin of replication, at 83 minutes on the *E. coli* chromosome. A total of nine genes, *atpIBEFHAGDC* (Walker *et al.*, 1984; Nielsen *et al.*, 1981; Kanazawa *et al.*, 1981, 1982; Saraste *et al.*, 1981), are transcribed into a polycistronic messenger RNA. *atpBEFHAGDC* encodes structural subunits of the H⁺-ATPase, a, c, b, δ, α, γ, β, ε respectively (Fig. 1). The open reading frame that ends a few base pairs upstream of the first structural gene (*atpB*), i.e., *atpI*, encodes a 14-kD polypeptide (Gay and Walker, 1981; von Meyenburg *et al.*, 1982a). A function has not yet been assigned to this polypeptide, and the *atpI* gene has been shown to be dispensable for normal function and assembly of the H⁺-ATPase (von Meyenburg *et al.*, 1982a; Gay, 1984; Jensen and Michelsen, 1992).

EXPRESSION OF THE *atp* OPERON

In view of the central role of the H⁺-ATPase in cellular free-energy metabolism, it is reasonable to expect that this enzyme complex is expressed under all growth conditions, and this is indeed found to be the case for the conditions tested. A relatively strong promoter (*atpIp*) is located upstream of the *atpI* gene,

and two weak promoters (*atbB1p*, and *atpB2p*) are located within the *atpI* gene (von Meyenburg *et al.*, 1982a; Nielsen *et al.*, 1984). Pedersen *et al.* (1978) and Smith and Neidhardt (1983) analyzed the expression of the two major subunits of the H⁺-ATPase complex (the α and the β subunit), as a function of the growth conditions. They changed the growth rate, either by changing the free-energy substrate for growth or by changing to anaerobic growth conditions, and found that the expression (in percentage of total cellular protein) depended slightly on the growth rate. Since the *atp* operon is located close to the origin of replication, the relative dosage of these genes will depend on the growth rate. When this is taken into account, it appears that the two subunits are almost constitutively expressed, i.e., independent of carbon/energy source and oxygen supply and over a broad range of growth rates. There are some indications that the expression of *lacZ* fusions to the weak *atbB2p* promoter responds to changes in the carbon source (Nielsen, 1985) and to other changes in the growth rate (Jensen, 1991). However, since the overall expression of the H⁺-ATPase appears to be constitutive, the significance of this is unclear.

Because numerous anabolic reactions require ATP, the H⁺-ATPase is expected to be important for the physiology of the normal *E. coli* cell, and as we shall see in the first part, mutations in the genes encoding the H⁺-ATPase do have serious implications for cell function. In the second part we shall ask more quantitative questions such as: *how important* is this
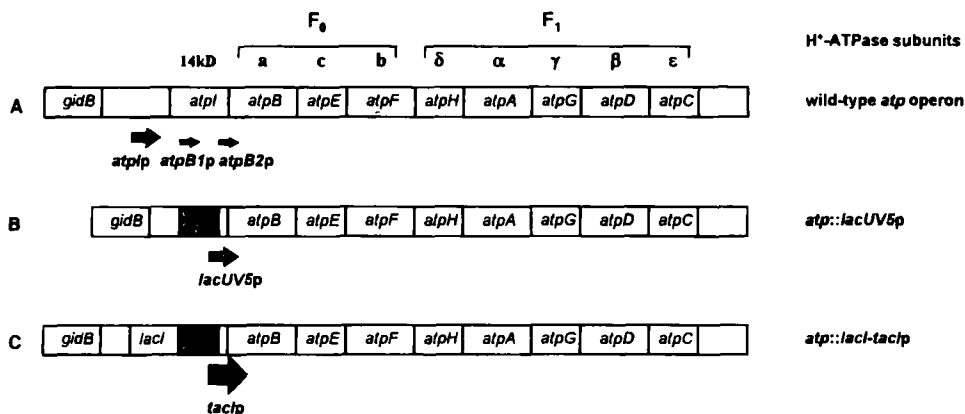

Fig. 1. The *atp* operon of the *E. coli* chromosome. A. The wild-type *atp* operon, the respective subunits of the H⁺-ATPase, and the three normal promoters of the *atp* operon. B. The chromosomal *atp* locus in the strain LM3111, in which the normal *atp* promoters have been replaced by the relatively weak but tight *lacUV5p* promoter. C. The chromosomal *atp* locus in the strain, LM3113, in which the normal *atp* promoters have been replaced by the relatively strong but leaky *tacIp* promoter and an extra copy of the *lacI* gene. See Jensen *et al.* (1993a) for details.

enzyme for maintaining a high growth rate under various conditions, how important is it for the coupling of free-energy transducing processes, and does the enzyme *control* the magnitude of fluxes and metabolite concentrations in the growing *E. coli* cell.

PHYSIOLOGICAL CONSEQUENCES OF MUTATIONS IN THE *E. coli atp* GENES

The first mutations in the *atp* operon were studied by Butlin *et al.* (1971). The selected mutants that were unable to grow on nonfermentable carbon sources, such as succinate. Despite an apparently normal content of respiratory components and a normal respiration rate, the mutants could not synthesize ATP through oxidative phosphorylation, and were named *unc* mutants for *unc*oupled oxidative phosphorylation. Butlin *et al.* showed that the growth rate of these mutants growing in glucose minimal medium is 33% lower than that of the wild-type strain and the growth yield was 50% lower. In addition, they showed that the mutant produced some 50% more acetate than the wild-type strain.

Von Meyenburg *et al.* (1982a) isolated mutants which had *Tn*10 transposon insertions in the promoter region of the *atp* operon. Thus, these *Tn*10 insertions affected the expression of the *atp* genes while the coding sequence for the structural genes were intact. In this way, strains were obtained which had the following expression levels of the *atp* genes: 0.13, 0.17, 0.23, 0.37, 0.47, 1.1, 1.17, and 1.27 times as compared to the wild-type strain. In addition to these strains, a strain which had a part of the *atp* operon deleted, was analyzed. The investigators determined the growth yield of the resulting strains growing in minimal medium with glucose as the carbon source, and found that the growth yield was lower than the wild-type yield for all the strains tested, also the ones which had a slightly enhanced expression of the *atp* genes. From these results it would appear that the optimal expression of the *atp* genes, with respect to growth yield, is closed to the normal level, or somewhere between 0.47 and 1 times the normal level. As discussed below, this is perhaps not quite so.

Later, von Meyenburg and coworkers (1984) examined the growth yield for strains, in which the H⁺-ATPase was being overexpressed from plasmids. Two- to threefold overexpression had a slightly negative effect on the growth yield (to 95%) while 4–5 fold overexpression had a slightly positive effect (to

105%). The growth yield decreased to 67% when the amount of H⁺-ATPase was increased 12 times, as a result of uncoupling possibly; they calculated that under these conditions the membrane area filled by H⁺-ATPase should correspond to twice the total membrane area available, and indeed this resulted in membrane vesicles in the cytoplasm which were covered with H⁺-ATPase. The growth rate of the strains decreased gradually to 36% as the H⁺-ATPase was overexpressed up to 12 times.

Jensen and Michelsen (1992) analyzed the physiology of a strain which had a deletion of all the genes in the *atp* operon. This strain was constructed in order to avoid possible interference from remaining H⁺-ATPase subunits in cells which had either point mutations or partial deletions of the *atp* genes. Indeed, the resulting strain, LM2800, had a slightly higher growth rate and growth yield compared to other point and deletion mutants, which indicates that the later strains might have some uncoupled ATPase activity.

The metabolism of the strain LM2800 growing in minimal glucose medium was then compared to that of the wild-type strain in more detail (Table I). The growth rate was decreased by only 20%, whereas the growth yield was decreased by 42%, which is in fairly good agreement with the previous observations by Butlin *et al.* (1971) and Von Meyenburg *et al.* (1982a, 1984). The fact that the growth yield decreased more than the growth rate implied that the rate of glucose consumption in the *atp* deletion mutant was increased. Because the flux of glucose into anabolism is decreased (the growth rate is decreased), there must be an increased catabolic flux of glucose in these cells, and the formation of other carbon products than biomass should then also be increased. Indeed, the rate of by-product formation for the *atp* deletion mutant was found to be almost twice the rate of the wild-type strain (Table I) and the by-product formed by the mutant consisted of more than 90% of acetate (Jensen and Michelsen, 1992), as has previously been shown for the wild-type *E. coli* strain (Andersen and von Meyenburg, 1980). Acetate formation from glucose generates a surplus of NADH (in contrast to, e.g., lactate formation), and this should confront the cells with a redox problem if the respiration rate was unaffected by the *atp* mutation. However, all the deletion strains, and also some strains carrying point mutations in the *atp* genes, had increased respiration rates, by approximately 40%. The respiration in these mutants is therefore not just uncoupled from ATP synthesis, but even stimulated in these strains. This increase in respiration

Table I. Physiological Consequences of Deleting the *atp* Genes (Data from Jensen and Michelsen, 1992)

Strain	Specific growth rate (h^{-1})	Growth yield (g dry weight/ mol glucose)	Glucose, rate of consumption (mmol glucose/ h/g dry weight)	By-product, rate of formation (mmol carbon/ h/g dry weight)	Respiration rate (mmol O_2/h/g dry weight)	Membrane potential, $\Delta\Psi$ (mV)	b-Type cytochromes (relative units)	[ATP]/ [ADP] ratio
Wild-type	0.53	80	6.6	7	12.4	154	1	19
atp deletion	0.42	46	9.1	15	17.2	184	1.8	7

rate of the *atp* mutants is surprising, in view of the fact that the *atp* deletion strain had a 20% increased membrane potential (Table I), which should lead to a higher back-pressure on the repiratory proton pumps, and, if anything, should cause the repiration to decrease. A possible explanation for these observations could be that the respiratory pumps are relatively insensitive to the membrane potential and sensitive to the NADH redox potential; an increased catabolic flux and a concomitantly increased NADH production should then lead to an increase in the $NADH/NAD^+$ redox potential which might stimulate respiration. This requires that, in the wild-type cell, the components of the respiratory chain were not working at V_{max}. An alternative explanation could reside in an increase in concentration of the respiratory chain components in the *atp* deletion mutant. Indeed, the expression of b-type cytochromes was increased almost twofold in this mutant (Jensen and Michelsen, 1992).

Jensen and Michelsen also measured the growth rate and respiration rates of some of the strains with *Tn10* insertions in the *atp* promoter region (see above). These strains, which have concentrations of H^+-ATPase in between the deletion and the wild-type strain, also had intermediate growth rates and respiration rates. This indicates that the physiological effect of deleting the *atp* operon is not confined to the deletion mutants only, but is something that happened gradually as the concentration of H^+-ATPase in the cells is decreased from the wild-type level to zero.

CONTROL ANALYSIS OF THE DEPENDENCE OF *E. coli* PHYSIOLOGY ON THE H^+-ATPase

From the experiments with *atp* mutants described above, it is not possible to answer the question about how important the H^+-ATPase enzyme is for the normal function of the *E. coli* cell. In order to answer these questions, it is necessary to modulate the cellular concentration of H^+-ATPase around the normal concentration in the cell, and determine the control by the enzyme on various cellular processes. This approach is known as metabolic control analysis (Kacser and Burns, 1973; Heinrich *et al.*, 1977; Burns *et al.*, 1985; Westerhoff and Van Dam, 1987; Kell and Westerhoff, 1986). We therefore replaced the promoters of the chromosomal *atp* operon by inducible promoter elements of the *lac*-type; see Fig. 1: The weak, but tight, lacUV5p promoter was used for modulating the H^+-ATPase concentration between zero and almost up to the normal expression level. As expected, in the absence of IPTG, it was not possible to discriminate between the physiology of the strain which had the *atp* genes controlled by the *lacUV5* promoter and the strain which had the entire *atp* operon deleted. The strong but leaky *tacI* promoter was employed when expression levels between 0.15 and 4 times the wild-type level were required (Jensen *et al.*, 1993a).

For control analysis it is important to adjust accurately the concentration of the enzyme of interest to any expression level where one wishes to measure control coefficients. However, we observed that changing the IPTG concentration from, e.g., 5 to 5.5 μM caused a change in the expression of the *atp* genes from 0.4 to 1 relative units of H^+-ATPase, which makes it difficult to adjust the enzyme concentration to the right levels (Fig. 2). After introducing a *lacY* mutation that eliminated the lactose (and IPTG) carrier, this problem was alleviated and the experiments could now be performed with any level of H^+-ATPase between 0 and 5 times the normal level.

Another important point for accurate control analysis is that one should try to maintain a steady state with respect to the enzyme concentration that is being modulated, throughout the range of cell concentrations used in the experiment. However, we found that the expression from the lacUV5p promoter increased some 2-fold when the cell concentration increased above an
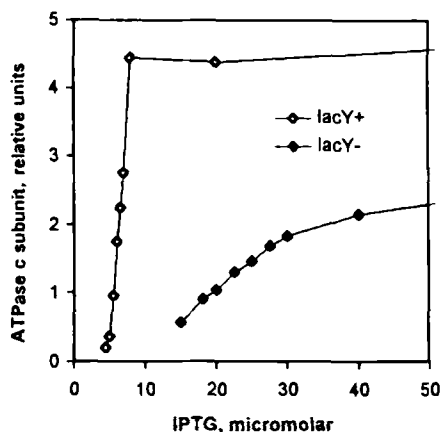
Fig. 2. Expression of the H⁺-ATPase c subunit from the *tac*Ip promoter; dependence on the concentration of IPTG, in either a *lacY⁻* (LM113) or a *lacY⁺* strain (LM3095). (Modified from Jensen *et al.*, 1993a.)

optical density of 0.25 (measured at 450 nm). Interestingly, this problem did not seem to arise when the tacIp promoter was used for modulating the enzyme concentration; here the expression remained constant until optical densities above 0.8 (Jensen *et al.*, 1993a). It is then unlikely that the increased expression from the lacUV5p promoter was due to an increase in intracellular concentration of the inducer IPTG at high cell concentrations, since this should have a similar effect on the tacIp promoter. Regulation through cAMP was also shown to be unlikely (Jensen *et al.*, 1993a).

THE CONTROL BY THE H⁺-ATPase ON ANABOLISM WITH GLUCOSE AS THE SUBSTRATE FOR GROWTH

On glucose, the cells can synthesize ATP by substrate phosphorylation, but the cells could still be limited by the supply of ATP, and the H⁺-ATPase could have some control on growth rate. It was therefore of interest to examine the control by the H⁺-ATPase on *E. coli* physiology during growth on this substrate. The strains with the *atp* genes controlled by *lac*-type promoters, described above, were used to modulate the H⁺-ATPase concentration.

Figure 3A, B shows what happened to growth rate and growth yield when the H⁺-ATPase concentration of cells growing on glucose was modulated around the normal concentration. Apparently, the level of H⁺-ATPase c subunit necessary to sustain wild-type growth was higher when the *atp* genes were transcribed

from *lac*-type promoters than in the wild-type cells. Thus, at a level of c subunit close to the wild-type level, the growth rate and the growth yield of those strains were decreased (to 98 and 92%, respectively) and the respiration rate was increased (to 108%) compared to the wild-type strain, LM3118. This could mean that the H⁺-ATPase activity was less than in the wild-type cells, that uncoupled ATPase activity was present, or that the proton gradient was subject to more leakage than usual. However, when the expression of the c subunit was set at 1.4 times the wild-type level, the growth rate, the growth yield, and the rate of respiration (see Fig. 4A) were all very close to the wild-type values. We concluded from this that, at the 1.4-fold concentration of c subunit, the activity of the H⁺-ATPases synthesized was the same in the IPTG modulation strains compared to the wild-type cells, and that the phenomenon was not accompanied by increased uncoupling. We therefore took 1.4 times wild-type c subunit concentration to represent the expression level that is relevant for analysis of the control of H⁺-ATPase at its wild-type level.

Around this "wild-type level" of H⁺-ATPase, the growth rate decreased as the H⁺-ATPase concentration was either decreased or increased, which indicated that the wild-type level of H⁺-ATPase was optimal with respect to growth rate. When the coefficient for control by the H⁺-ATPase on growth rate was calculated at 1.4 times c subunit, it was very close to zero (C = 0.00, Fig. 3C; see also Jensen *et al.*, 1993b). Interestingly, with respect to growth yield, the H⁺-ATPase concentration was not quite optimal; the growth yield could still be increased somewhat by increasing the H⁺-ATPase concentration above the wild-type level. At the wild-type level (1.4 times the wild-type c subunit concentration) the control coefficient with respect to the growth yield was 0.15 (Fig. 3D). At 1.0 times the wild-type c subunit concentration, control on growth rate and growth yield were 0.05 and 0.20, respectively. This result is in contrast to the results of Von Meyenburg *et al.*, (1982, 1984), from which one would conclude that the H⁺-ATPase was optimal with respect to growth yield, i.e., that the control on growth rate and growth yield were both zero.

Figure 3 also shows what happened to growth rate and growth yield at much reduced expression levels. For this purpose we had to use a strain which has the atp operon controlled by the weak but tight *lacUV5* promoter (Jensen *et al.*, 1993). As the concentration of H⁺-ATPase was decreased from the wild-type level to virtually zero, the growth rate decreased gradually
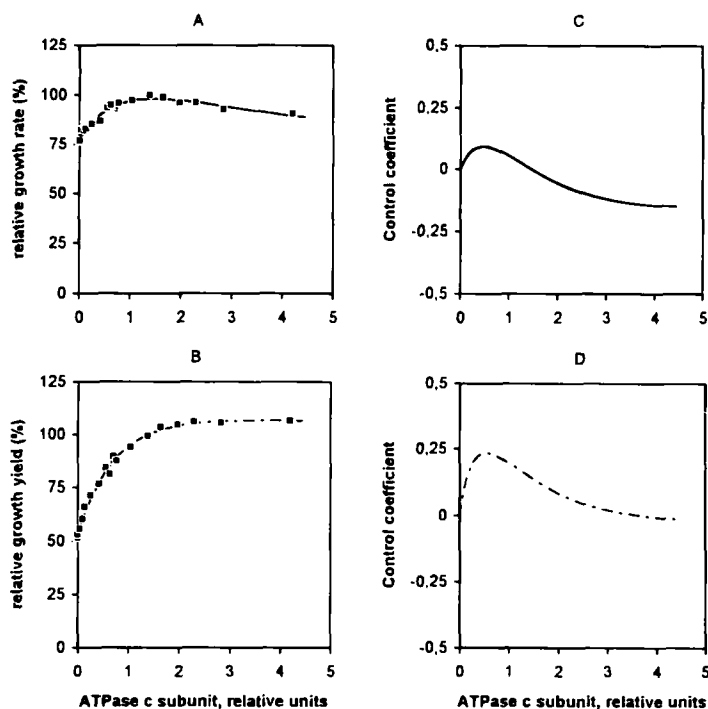
Fig. 3. Dependence of *E. coli* anabolism on the H⁺-ATPase; cells were grown in glucose minimal medium and the cellular concentration of H⁺-ATPase was modulated by changing the concentration of IPTG in cultures of either strain LM3111 (low levels of H⁺-ATPase) or LM3113 (high levels of H⁺-ATPase). Variation of (A) growth rate and (B) growth yield, as a function of the concentration of H⁺-ATPase c subunit. Control by the H⁺-ATPase on *E. coli* growth rate (C) and growth yield (D). (Modified from Jensen *et al.*, 1993b.)

from 100 to 80% and the growth yield decreased from 100 to 50%. The control by the H⁺-ATPase on both growth rate and growth yield reached a maximum around 0.5 times the wild-type H⁺-ATPase level and the control then returned to zero, which reflects the phenomenon that because of substrate level phosphorylation, growth does not completely depend on the H⁺-ATPase.

When the *atp* operon was overexpressed with respect to the wild-type level, the growth rate decreased to 90% and the growth yield increased to 107% as the H⁺-ATPase concentration was increased to 3–4 times the wild-type level.

THE CONTROL BY THE H⁺-ATPase ON CATABOLISM WITH GLUCOSE AS THE SUBSTRATE FOR GROWTH

The dependence of the catabolic processes on the H⁺-ATPase is shown on Fig. 4 for cells grown on glucose. The rate of glucose consumption and the respiration rate both increased gradually as the amount of H⁺-ATPase was decreased compared to the normal level, and at higher than normal levels of H⁺-ATPase these fluxes became lower than in the normal cell. The control by the H⁺-ATPase on respiration passed through a negative minimum close to the wild-type level and then approached zero, whereas the control on the glucose consumption rate remained negative as the H⁺-ATPase was varied (Fig. 4C, D).

ATP SYNTHESIS WITH GLUCOSE AS THE SUBSTRATE FOR GROWTH

With succinate as the sole substrate for growth, the relative rate of ATP synthesis by oxidative phosphorylation can be estimated from the growth rate, because substrate level phosphorylation on this substrate is low. With glucose, however, a significant amount of ATP may be synthesized by substrate level
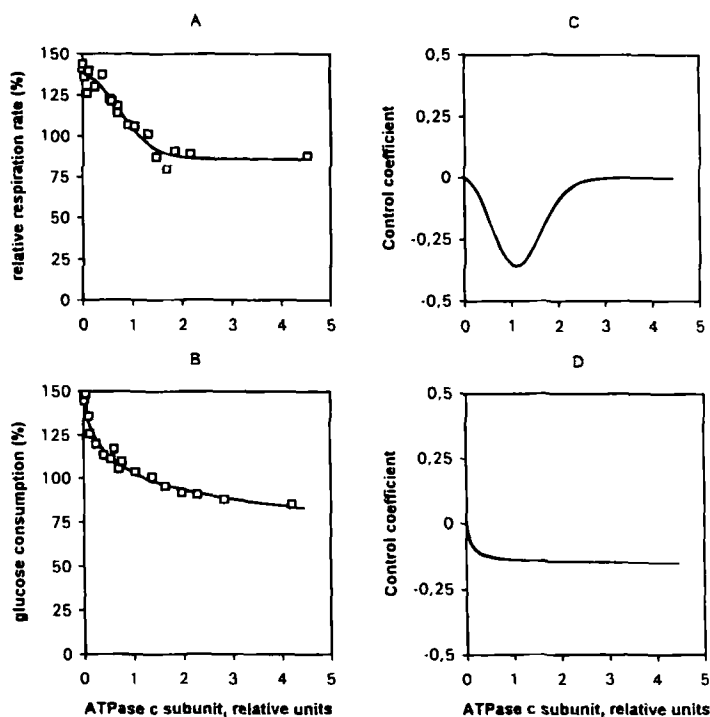
Fig. 4. Dependence of *E. coli* catabolism on the H⁺-ATPase; cells grown in glucose minimal medium, see legend to Fig. 3. Variation of (A) respiration rate and (B) glucose consumption rate, as a function of the concentration of H⁺-ATPase c subunit. Control by the H⁺-ATPase on *E. coli* respiration rate (C) and glucose consumption rate (D). (Modified from Jensen *et al.*, 1993b.)

phosphorylation, and it becomes necessary to correct for this in order to estimate the flux through oxidative phosphorylation (and through the H⁺-ATPase).

An *atp* deletion mutant only synthesizes ATP by substrate level phosphorylation, and the total rate of ATP generation can therefore be calculated from the metabolic fluxes in these cells. Both the anabolic and the catabolic fluxes contribute to ATP synthesis. In the anabolic reactions that lead to the precursors for the building blocks, there is a net production of 5.6 mmol ATP/g dry weight (see Neidhardt *et al.*, 1993) or 0.25 mol ATP/mol glucose consumed. This corresponds to a flux of 2.6 mmol ATP/h/g dry weight, because the *atp* mutant has a specific growth rate of 0.47 h⁻¹ and a growth yield of 44.3 g dry weight/mol glucose (see Jensen and Michelsen, 1992). The calculation of the amount of ATP produced in the catabolic processes is simplified by the fact that the by-product excreted by both the *atp* mutant (Jensen and Michelsen, 1992) and the wild-type strain (Andersen and von Meyenburg, 1980) growing on glucose was found to be almost exclusively acetate and CO_2. Catabolism of glucose should therefore yield 4 ATP molecules per glucose

molecule, and the amount of ATP produced in the catabolic processes per mmol glucose consumed (ATP_{catab}) can then be calculated as

$$ATP_{catab} = (6 - 1.17 \times \text{cell carbon})/6$$
$$\times 4 \text{ mmol ATP/mmol glucose}$$

i.e., the input carbon (6 mmol carbon/mmol glucose) minus the carbon found in cell material (the factor 1.17 includes a correction for CO_2 produced in anabolism; see Jensen and Michelsen, 1992), and multiplied by the factor 4/6 to convert the numbers into mmol ATP/mmol glucose. Using the growth yield measured for the *atp* deletion strain, i.e., 1.77 mmol cell carbon/mmol glucose, we find that $ATP_{catab} = 2.62$ mmol ATP/mmol glucose consumed. With the growth rate and growth yield data given above, this implies that the catabolic rate of ATP production is 27.9 mmol ATP/h/g dry weight. The total ATP production for the *atp* mutant is therefore estimated at 2.6 + 27.9 = 30.5 mmol ATP/h/g dry weight.

In the same way substrate-level phosphorylation was calculated for the strains that do contain H⁺-

ATPase. Figure 5A shows the calculated rates of sub-
strate-level phosphorylation for cells with different
contents of H$^+$-ATPase, divided into the anabolic and
catabolic routes. We see that the estimated rate of
substrate-level phosphorylation decreased rapidly as
the amount of H$^+$-ATPase increased and that the contri-
bution by the anabolic route became larger. In the wild-
type cells (at 1.4 relative units of H$^+$-ATPase), 30%
of the cell's substrate phosphorylation goes through
the anabolic route.

Once the total rate of ATP synthesis is known for
the *atp* deletion mutant, we may estimate the total rate
of ATP synthesis in the wild-type strain, or strains
with other levels of H$^+$-ATPase, by correcting for the
difference in growth rate. This extrapolation assumes
that the total rate of ATP synthesis is proportional to
the growth rate, which may cause us to overestimate
the rate of ATP synthesis if the difference in growth
rate is large. However, in this case the difference in
growth rate is merely 20% so this assumption should
be fairly safe here. Figure 5B shows the rate of total
ATP synthesis estimated following this argumentation
for cells with different content of H$^+$-ATPase. If we
subtract the two contributions by substrate level phos-
phorylation (Fig. 5A) from the total rate of ATP synthe-
sis (Fig. 5B), we get the difference curve (Fig. 5C)

which represents the rate of ATP synthesis by oxidative
phosphorylation. The last curve (Fig. D) shows the
rate of ATP consumption by the known anabolic pro-
cesses in the cell, which can be calculated from the
molecular composition of the cells, the biosynthetic
pathways, and mRNA turnover. The numbers used in
these calculations are: synthesis of building blocks
from the precursors, 18.5 mmol ATP/g dry weight;
polymerization reactions, 22.7 mmol ATP/g dry weight
(Neidhardt *et al.*, 1993). The cell thus synthesizes
approximately 1.5 times the amount of ATP that one
can account for in the cell material (cf. Stouthammer,
1973). The rate of ATP consumption by some of the
(minor) cellular ATP consuming processes in the grow-
ing cell have been estimated, e.g., mRNA turnover.
However, some fluxes are more difficult to measure,
e.g., ATP consumption by the DNA gyrase and topo-
isomerase IV activities, and by chaperones.

The average turnover number of the ATP synthe-
sis by the H$^+$-ATPase enzymes during growth on glu-
cose can now be calculated from the flux of ATP
through oxidative phosphorylation (in ATP/second/g
dry weight), by normalizing with the H$^+$-ATPase con-
tent (the wild-type cells contain 4.9×10^{15} H$^+$-ATPase
molecules/g dry weight; von Meyenburg *et al.*, 1984).
For the wild-type strain, a turnover number of 650
ATP/second was obtained, or one ATP per 1.5 millisec-
ond. As the cellular content of H$^+$-ATPase is decreased
below the wild-type level, the turnover number
increased at least twofold (Fig. 6A).

The P/O ratio reflects the degree of coupling in
oxidative phosphorylation, i.e., how many ATP mole-
cules are formed per oxygen atom used by the cells.
By dividing the curve in Fig. 5C with the one in Figure
4A one should obtain the P/O ratio for the whole
range of H$^+$-ATPase concentrations tested: Fig. 6B.
Interestingly, the P/O ratio for the wild-type cell (1.4
relative units of H$^+$-ATPase here corresponds to the
wild-type level of H$^+$-ATPase, see above) appears to
be rather low, approximately 1.1, when calculated in
this way. The P/O ratio decreased with decreasing
amounts of H$^+$-ATPase enzyme in the cells, and
increased above the wild-type P/O ratio when the H$^+$-
ATPase was overexpressed. Thus, it seems that the
H$^+$-ATPase concentration in the normal *E. coli* cell
is not quite optimal with respect to the coupling of
oxidative phosphorylation. Moreover, the P/O ratio
varies quite extensively, which suggests that coupling
is, or can be quite, loose. In a parallel paper (van
Spanning *et al.*, 1995) causes for variable H$^+$/O ratio
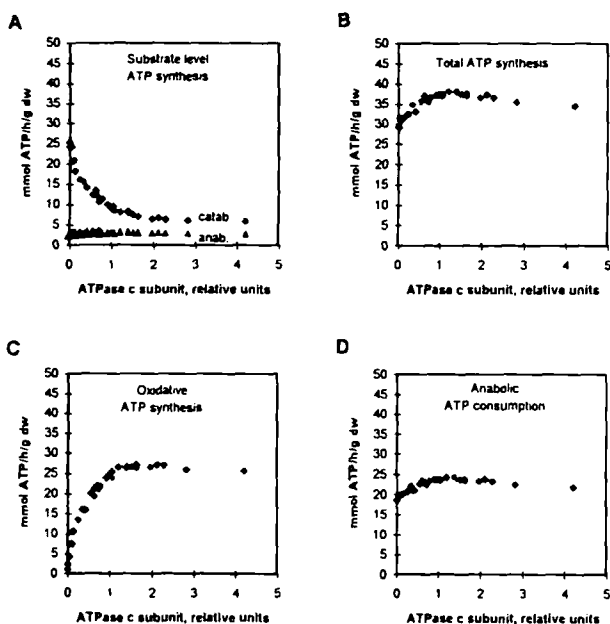are discussed.

Fig. 5. Estimated rates of ATP fluxes through various routes in *E. coli* growing in glucose minimal medium. The rates were calculated as described in the text, using the growth rate and growth yield data from Fig. 3 for the various concentrations of H$^+$-ATPase.
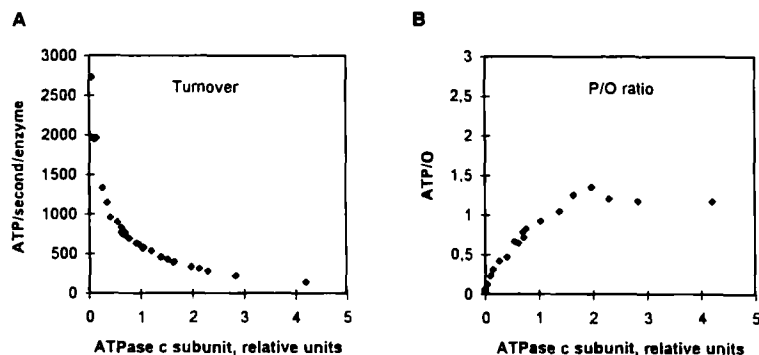
Fig. 6. Turnover number of the H⁺-ATPase (A) and degree of coupling of oxidative phosphorylation (B) as a function of the concentration of H⁺-ATPase c subunit, for cells grown with glucose. The turnover number and the P/O ratio was calculated by dividing the estimated rate of oxidative ATP synthesis (Fig. 5) by the concentration of H⁺-ATPase c subunit and the measured respiration rates, respectively (see Fig. 4 and Jensen et al., 1993b). See also text.

CONTROL BY THE H⁺-ATPase ON E. coli GROWTH IN SUCCINATE MINIMAL MEDIUM

As described above, atp mutants fail to grow with succinate as the sole carbon and free energy source. Intuitively, one therefore expects that the control by the H⁺-ATPase on growth rate should be higher when the growth substrate is succinate as compared to glucose. Figure 7A, B shows what happened to the growth rate and growth yield as the concentration of H⁺-ATPase was changed around the normal level (Jensen et al., 1993c). With this substrate the physiological variables (growth rate, yield) all came close to the normal values when the expression of H⁺-ATPase was close to the wild-type expression. Also with this substrate it was found that around the normal concentration of H⁺-ATPase, the growth rate did not change much as the H⁺-ATPase concentration was varied around the normal level; the control coefficient by H⁺-ATPase on growth rate, at the wild-type level of c subunit, was very close to zero ($C = 0.0$; Jensen et al., 1993c; see also Jensen et al., 1993d). Even taking out as much as 50% of the H⁺-ATPase content had only a minor effect on the growth rate. As the H⁺-ATPase concentration was decreased further, the enzyme did become limiting for the growth rate, and its control coefficient with respect to growth rate approached 1 (Fig. 7C). Overexpression of the H⁺-ATPase had a negative effect on the growth rate and we may therefore conclude that also for growth on succinate the wild-type H⁺-ATPase concentration is optimal with respect to growth rate.

The growth yield depended somewhat on the H⁺-ATPase concentration during growth on succinate, and the control on growth yield was 0.2 at the wild-type level (Fig. 7D). As the concentration of H⁺-ATPase was increased above 2.5 times the wild-type level, the growth yield was slightly decreased. Below the normal level of enzyme, the control on growth yield increased toward 0.5.

The fact that the growth rate remained constant while the H⁺-ATPase concentration was halved indicated that also with this substrate the turnover number of individual H⁺-ATPase enzyme was changing (Jensen et al., 1993c). With succinate as the substrate, however, it is not possible to estimate the flux through oxidative phosphorylation, as was done above for glucose as the substrate. But an estimate of the turnover number could still be obtained by assuming that the flux through oxidative phosphorylation is proportional to the growth rate of the cells (see Jensen et al. 1993c). Figure 8 shows how the turnover number of ATP synthesis by the individual H⁺-ATPase enzyme, as estimated from the growth rate, varied with the concentration of H⁺-ATPase. Apparently there is an excess capacity of H⁺-ATPase in the normal E. coli cell: when less enzyme is present, the remaining enzymes work faster, giving rise to some homeostatic control of ATP synthesis. An interesting question then emerges: how is the increase in driving force, which is necessary for increasing the turnover number of the enzymes, established? Figure 9 shows that also with succinate as the substrate for growth, there was a gradual increase in the respiration rate and the rate of
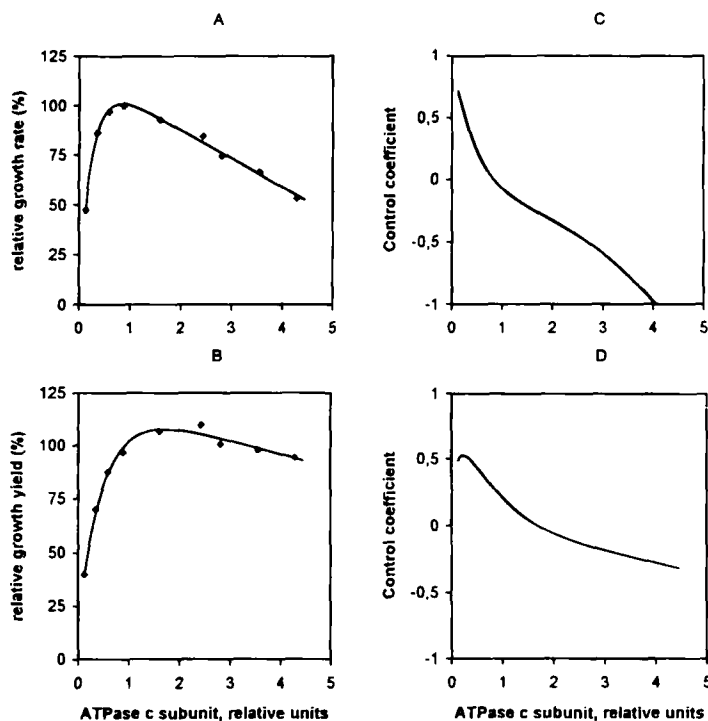
Fig. 7. Dependence of *E. coli* anabolism on the H⁺-ATPase; cells were grown in succinate minimal medium and the cellular concentration of H⁺-ATPase was modulated by growing cultures of strain LM3113 with various concentrations of IPTG in cultures. Variation of (A) growth rate and (B) growth yield, as a function of the concentration of H⁺-ATPase c subunit. Control by the H⁺-ATPase on *E. coli* growth rate (C) and growth yield (D). (Modified from Jensen *et al.*, 1993c).
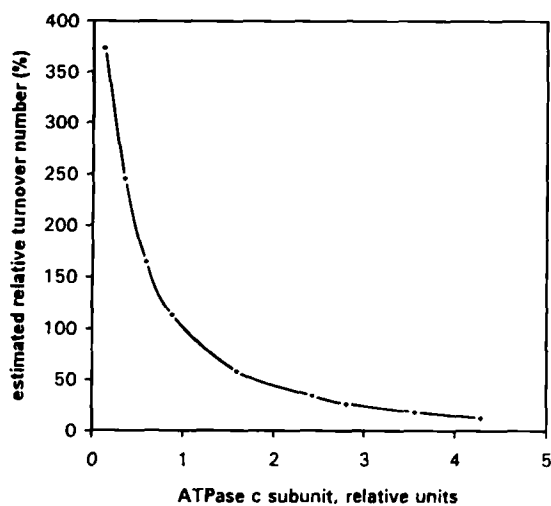

Fig. 8. Estimated relative turnover number of the H⁺-ATPase as a function of the concentration of H⁺-ATPase c subunit, for cell grown with succinate. This turnover number is given as percentage of the wild-type turnover number, and was calculated by dividing the relative growth rate (Fig. 7) by the respective concentrations of H⁺-ATPase c subunit. (from Jensen *et al.*, 1993c).

succinate consumption, as the amount of H⁺-ATPase was lowered. Figure 9C, D shows the corresponding control coefficients; the control by the H⁺-ATPase on these catabolic fluxes was negative throughout the range of H⁺-ATPase analyzed. The phenomenon that the H⁺-ATPase has a negative control on respiration was called inverse respiratory control (Jensen *et al.*, 1993c). "Inverse" refers to the fact that the H⁺-ATPase in mitochondria is normally found to have a positive control on respiration; oligomycin inhibits mitochondrial respiration.

CONCLUDING REMARKS

Control by the H⁺-ATPase on *E. coli* growth rate appears to be virtually absent, independent of whether the substrate is succinate or glucose. The cells appear to have a special control feature that is responsible for this phenomena: when the availability of ATP is restricted, substrate catabolism is stimulated and the result is a homeostatic control of the total rate of ATP
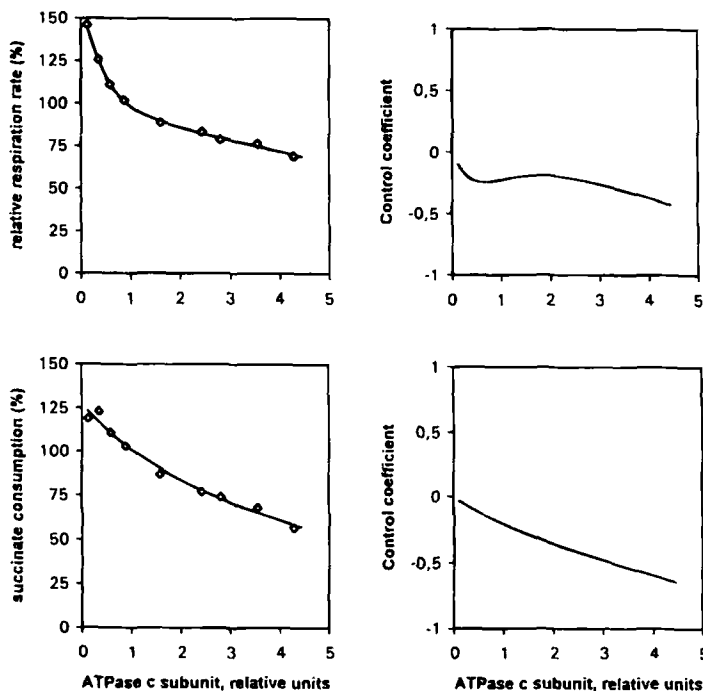
Fig. 9. Dependence of *E. coli* catabolism on the H⁺-ATPase; cells grown in succinate minimal medium, see legend to Fig. 7. Variation of (A) respiration rate and (B) succinate consumption rate, as a function of the concentration of H⁺-ATPase c subunit. Control by the H⁺-ATPase on *E. coli* respiration rate (C) and succinate consumption rate (D). (Modified from Jensen *et al.*, 1993c).

synthesis, so that the anabolic flux can be kept constant. The mechanism for this homeostatic control could involve changes in the cellular energy state, as reflected in the intracellular ATP/ADP ratio, which may in turn affect the expression of genes coding for the respiratory chain components.

REFERENCES

Andersen, K. B., and von Meyenburg, K. (1980). *J. Bacteriol.* **144**, 114–123.

Burns, J. A., Cornish-Bowden, A., Groen, A. K., Heinrich, R., Kacser, H., Porteous, J. W., Rapoport, S. M., Rapoport, T. A., Stucki, J. W., Tager, J. M., Wanders, R. J. A., and Westerhoff, H. V. (1985). *Trends Biochem. Sci.* **10**, 16.

Butlin, J. D., Cox, G. B., and Gibson, F. (1971). *Biochem. J.* **124**, 75–81.

Foster, L. D., and Fillingame, R. H. (1982). *J. Biol. Chem.* **257**, 2009–2015.

Futai, M., and Kanazawa, H. (1983). *Microbial. Rev.* **47**, 285–312.

Gay, N. J. (1984). *J. Bacteriol.* **158**, 820–825.

Gay, N., and Walker, J. E. (1981). *Nucleic Acids Res.* **9**, 3919–3926.

Heinrich, R., Rapoport, S. M., and Rapoport, T. A. (1977). *Prog. Biophys. Mol. Biol.* **32**, 1–83.

Jensen, P. R. (1991). Ph.D. thesis, Department of Microbiology, Technical University of Denmark.

Jensen, P. R., and Michelsen, O. (1992). *J. Bacteriol.* **174**, 7635–7641.

Jensen, P. R., Westerhoff, H. V., and Michelsen, O. (1993a). *Eur. J. Biochem.* **211**, 181–191.

Jensen, P. R., Michelsen, O., and Westerhoff, H. V. (1993b). *Proc. Natl. Acad. Sci. USA* **90**, 8068–8072.

Jensen, P. R., Westerhoff, H. V., and Michelsen, O. (1993c). *EMBO J.* **12**, 1277–1282.

Jensen, P. R., Oldenburg, N., Petra, B., Michelsen, O., and Westerhoff, H. V. (1993d). *Modern Trends in Biothermokinetics* (Schuster *et al.*, eds.), Plenum Press, New York, 391–396.

Kacser, H., and Burns, J. (1973). *Rate Control of Biological Processes* (Davies, D. D., ed.), Cambridge University Press, London, pp. 65–104.

Kanazawa, H., Kayano, T., Mabuchi, K., and Futai, M. (1981) *Biochem. Biophys. Res. Commun.* **103**, 604–612.

Kanazawa, H., Mabuchi, K., and Futai, M. (1982). *Biochem. Biophys. Res. Commun.* **107**, 568–575.

Kell, D. B., and Westerhoff, H. V. (1986). *FEMS Microbiol. Rev.* **39**, 305–320.

Neidhardt, F. C., Ingraham, J. L., and Schaechter, M. (1993). *Physiology of the Bacterial Cell*, Sinauer Associates, Massachusetts.

Nielsen, J. (1985). Ph.D. thesis, Department of Microbiology Technical University of Denmark.

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.

Pedersen, S., Bloch, P. L., Reeh, S., and Neidhardt, F. C. (1978). *Cell* **14**, 979–990.

Saraste, M., Gay, N. J., Eberle, A., Runswick, M. J., and Walker, J. E. (1981). *Nucleic Acids Res.* **9**, 5287–5296.

Senior, A. E. (1988). *Physiol. Rev.* **68**, 177–231.

Senior, A. E. (1990). *Annu. Rev. Biophys. Biophys. Chem.* **19**, 7–41.

Smith, M. W., and Neidhardt, F. C. (1983). *J. Bacteriol.* **154**, 344–350.

Sneider, E., and Altendorf, K. (1987). *Microbiol. Rev.* **51**, 477–497.

Stouthammer, A. H. (1973). *Antonie van Leeuwenhoek* **39**, 545–565.

Van Spanning, R. J. M., de Boer, A. P. N., Reijnders, W. N. M., de Gier, J.-W. L., Delorme, C. O., Stouthamer, A. H., Westerhoff, H. V., Harms, N., and van der Oost, J. (1995). *J. Bioenerg. Biomembr.* **27**, 499–512.

Von Meyenburg, K., Jørgensen, B. B., Nielsen, J., Hansen, F. G., and Michelsen, O. (1982a). *Tokai J. Exp. Clin. Med.* **7**, supplement: 23–31.

Von Meyenburg, K., Jørgensen, B. B., Nielsen, J., and Hansen, F. G. (1982b). *Mol. Gen. Genet.* **188**, 240–248.

Von Meyenburg, K., Jørgensen, B. B., and van Deurs, B. (1984). *EMBO J.* **3**, 1791–1797.

Walker, J. E., Saraste, M., and Gay, N. J. (1984). *Biochim. Biophys. Acta* **768**, 164–200.

Westerhoff, H. V., and Van Dam, K. (1987). *Thermodynamics and Control of Biological Free Energy Transduction*, Elsevier, Amsterdam.