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Excess capacity of H^+-ATPase and inverse respiratory control in *Escherichia coli*

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With succinate as free-energy source, *Escherichia coli* generating virtually all ATP by oxidative phosphorylation might be expected heavily to tax its ATP generating capacity. To examine this the H^+-ATPase (ATP synthase) was modulated over a 30-fold range. Decreasing the amount of H^+-ATPase reduced the growth rate much less than proportionally; the H^+-ATPase controlled growth rate by <10%. This lack of control reflected excess capacity: the rate of ATP synthesis per H^+-ATPase (the turnover number) increased by 60% when the number of enzymes was decreased by 40%. At 15% H^+-ATPase, the enzyme became limiting and its turnover was increased even further, due to an increased driving force caused by a reduction in the total flux through the enzymes. At smaller reductions of [H^+-ATPase] the total flux was not reduced, revealing a second cause for increased turnover number through increased membrane potential: respiration was increased, showing that in *E.coli*, respiration and ATP synthesis are, in part, inversely coupled. Indeed, growth yield per O_2 decreased, suggesting significant leakage or slip at the high respiration rates and membrane potential found at low H^+-ATPase concentrations, and explaining that growth yield may be increased by activating the H^+-ATPase.

Key words: ATP synthase of *E.coli*/*atp* operon/control analysis/growth rate and yield/oxidative phosphorylation and respiration

Introduction

In aerobic cell physiology, much of the free-energy transduction from catabolism to cell processes that require free-energy input, proceeds through the H^+-ATPase of the energy coupling membrane. In eukaryotes this membrane is the inner mitochondrial membrane; in prokaryotes, it is the plasma membrane. When the substrate for catabolism is not fermentable, virtually all ATP synthesis should involve this oxidative phosphorylation. Indeed, deletion mutants of the *atp* operon in *Escherichia coli* did not grow on non-fermentable substrates, such as succinate (von Meyenburg *et al.*, 1984). The H^+-ATPase is also important under anaerobic circumstances. Then it maintains ion and solute gradients across the energy coupling membrane at the expense of the hydrolysis of ATP synthesized by substrate level phosphorylation (reviewed by Maloney, 1987).

Although H^+-ATPase is a key enzyme in the mechanism of cellular free-energy metabolism, it is not known whether under physiological conditions it also controls that metabolism, or the aspects of cell physiology that depend on that mechanism. There could be an excess capacity of the H^+-ATPase activity in the wild-type cell: the individual enzyme may not operate at V_{max} and a decrease in the [ATP]/[ADP] ratio or an increase in membrane potential could still increase the turnover number.

Consequently, it is of interest to determine whether or not, in the wild-type cell, the H^+-ATPase controls cell physiology. The so-called control coefficient is a measure of the extent to which an enzyme controls a physiological process. A good method exists for measuring control coefficients by enzymes in *E.coli*. Basically, one brings the gene encoding the enzyme under the control of a promoter one can modulate from the outside. One then measures the effects on physiological properties and divides these by the measured modulation of the enzyme concentration to obtain the corresponding control coefficients.

Usually the inducible promoters are derived from the lactose operon, which can be induced to various extents by varying the concentration of the inducer IPTG (isopropyl-β-D-thiogalactoside). Until recently, this method had the modulated gene embedded in a plasmid (Walsh and Koshland, 1985; Ruijter *et al.*, 1991). We recently developed a revised method in which we insert an IPTG-inducible promoter upstream of the chromosomal copy of the gene encoding the enzyme of interest (Jensen *et al.*, 1993). This, together with simultaneous inactivation of the lactose permease, has made it easier to modulate accurately the concentration of an enzyme of interest in an *E.coli* cell around the wild-type level and measure its control coefficients.

Here we employ this method to modulate the expression of the *atp* operon and measure the control that the H^+-ATPase exerts on growth rate, growth yield, respiratory rate and substrate consumption. We find that it exerts virtually no control on growth rate, a remarkable result in view of the important role of the H^+-ATPase in cell physiology.

Results

Dependence of E.coli physiology on the H^+-ATPase

To allow the modulation of the concentration of H^+-ATPase around its wild-type level, we replaced the three wild-type *atp* promoters with the IPTG-inducible *tacI* promoter. This was done upstream of the chromosomal *atp* operon in a *lacY* mutant to allow precise control of the expression of the *atp* operon. We grew the resulting strain, LM3113, for at least 10 generations at an OD_{450} of <0.1

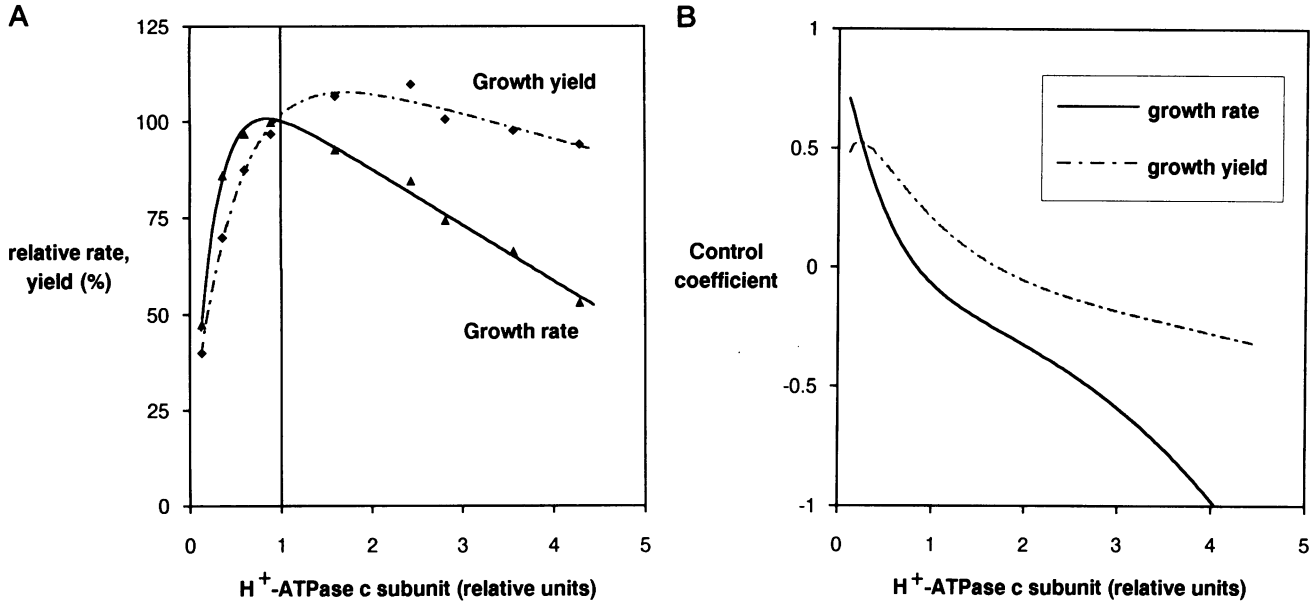

Fig. 1. Dependence of *E.coli* physiology on H^+-ATPase. (**A**) The growth rates and growth yields (relative to the wild-type starin, LM3118) of cultures grown in succinate minimal medium, were plotted as a function of the expression of H^+-ATPase *c* subunit. *E.coli* cells were used, in which the expression of the *atp* operon could be controlled by the *tacI* promoter (strain LM3113) through the addition of different concentrations of IPTG. Smooth curves were drawn through the experimental data points as described in Materials and methods. (**B**) Control of H^+-ATPase on *E.coli* physiology, during growth on succinate. The log-log derivatives of the smooth curves on Figure 3 were plotted as a function of the level of H^+-ATPase (see text for details). 100% and 1 refer to wild-type magnitudes.

in the presence of various concentrations of IPTG, before determining the growth rate of the cells (from OD_{450}) in the exponential phase. The extent of the modulation of the concentration of the H^+-ATPase was followed by measuring the concentration of the *c* subunit of the ATPase relative to the wild-type concentration by densitometry of autoradiograms from SDS–polyacrylamide gels of total cell preparation. Thus, 1 relative unit of H^+-ATPase *c* subunit refers to the normal concentration in the wild-type cells. The growth yield was determined from the final OD for a complete culture initiated at a known limiting concentration of growth substrate.

Figure 1 shows how the growth rate and growth yield responded, as the concentration of H^+-ATPase in the cells was changed over a 30-fold range, from 0.15 to 4.5 times the wild-type concentration. When the expression of H^+-ATPase was decreased from 1 to 0.6 relative units, the growth rate was only very slightly affected (by 3%). In the same range of expression, the growth yield decreased to 85% of the wild-type yield.

Strains which carry deletions in the *atp* operon fail to grow on succinate (von Meyenburg *et al.*, 1984), which indicates that the rate of substrate phosphorylation during growth on this substrate is low. We should therefore expect a much stronger dependence of growth on the H^+-ATPase at highly reduced expression of the *atp* operon. Indeed, when the expression was reduced to 0.15 relative units, this resulted in a decrease of growth rate to 48% and the growth yield was reduced to 40%.

When we increased the expression of the *atp* operon above the wild-type level, the growth rate diminished gradually, and at 4.3 times the wild-type expression level, the growth rate was 55% of the wild-type rate. In the same expression range, the growth yield first increased to 110% (at 2.5 relative units) and then decreased to 94%.

Quantification of the observed physiological effects of changing the [H^+-ATPase]: the control coefficient

In the above discussion of the dependence of *E.coli* physiology on the H^+-ATPase, we were limited to rather qualitative terms, such as 'slightly affected' or 'strongly dependent'. However, there is a tool which allows for accurate quantification of these effects, in terms of so-called flux control coefficients (Kacser and Burns, 1973; Burns *et al.*, 1985). This control coefficient describes by how many percent the flux through a metabolic pathway changes, when the concentration of one of the enzymes of the pathway is changed by e.g. 10%. If the resulting change in flux is also 10%, then the control coefficient is equal to 1, and the enzyme is then said to be in strict control of the flux. Mathematically the control coefficient is equal to the log-log derivative of the response curves shown in Figure 1A.

To determine the coefficients of control for the H^+-ATPase, one has to change the concentration of this enzyme by a small fraction and measure the effect on the steady state properties of interest. In theory the fractional change should be infinitely small, but it is not practicable to make such a change. Instead, we chose to modulate the H^+-ATPase concentration over a small but finite range around its wild-type level. This then allowed us to determine a smooth curve for the dependence of the property (*P*) under study on the H^+-ATPase concentration. The slope of this curve at the wild-type level of H^+-ATPase was then used to estimate the control coefficient, using the following equation:

$$C^P_{[H^+\text{-ATPase}]} = (dP/P)/(d[H^+\text{-ATPase}]/[H^+\text{-ATPase}])$$

In this way we found that control on growth rate was virtually absent ($C^{\mu}_{[H^+\text{-ATPase}]} = 0.0$) and the H^+-ATPase exerted a small positive control on the growth yield ($C^{Y}_{[H^+\text{-ATPase}]} = +0.2$). In other words, changing the

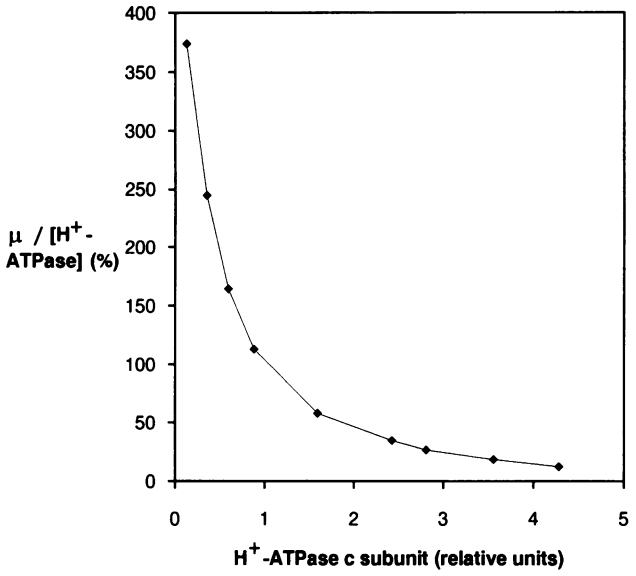

Fig. 2. Growth rate per unit of H^+-ATPase. The specific growth rate divided by the concentration of the H^+-ATPase was plotted as a function of the concentration of H^+-ATPase *c* subunit. Assuming that the rate of ATP synthesis by oxidative phosphorylation is proportional to the growth rate, this plot gives an indication of the variation of the relative turnover number of the H^+-ATPase.

concentration of the H^+-ATPase to 10% above or below the wild-type level did not affect the growth rate significantly, whereas a 10% change in the concentration of H^+-ATPase resulted in a 2% change in the growth yield.

To estimate the control efficients for levels of H^+-ATPase other than the wild-type level, we fitted a smooth curve through the experimental date points (Figure 1A) and then plotted the log-log derivative of that curve as a function of the level of H^+ATPase (Figure 1B). We found that the control on both the growth rate and the growth yield increased as the expression of the *atp* operon was reduced far below the wild-type expression level. At 0.15 relative units, the control on growth rate was 0.7 and the control on growth yield was 0.5. At over-expression of the *atp* operon, the control on growth rate became highly negative ($C^{\mu}_{[H^+\text{-ATPase}]} = -1$ at [H^+ATPase] = 4.3 U). The control on growth yield became slightly negative at high expression levels.

Excess capacity of H^+-ATPase in the wild-type E.coli cell

The low level of control exerted by the H^+-ATPase on the growth rate when the enzyme was expressed at the wild-type level indicated that there was an excess capacity of this enzyme present in the normal *E.coli* cells with respect to the growth rate. Assuming that the rate of ATP synthesis is proportional to the growth rate of the cells, then we may obtain an estimate of the rate of ATP synthesis per H^+-ATPase (the turnover number), as the ratio between the growth rate and the concentration of H^+-ATPase, since oxidative phosphorylation dominates during growth on succinate. It appears that the turnover number calculated in this way changed quite extensively when the amount of H^+-ATPase in the cells varied (Figure 2). Thus, when the amount of H^+-ATPase was decreased from the wild-type level to 0.6 relative units, the turnover number appeared to increase to 160%; further reduction of the H^+-ATPase

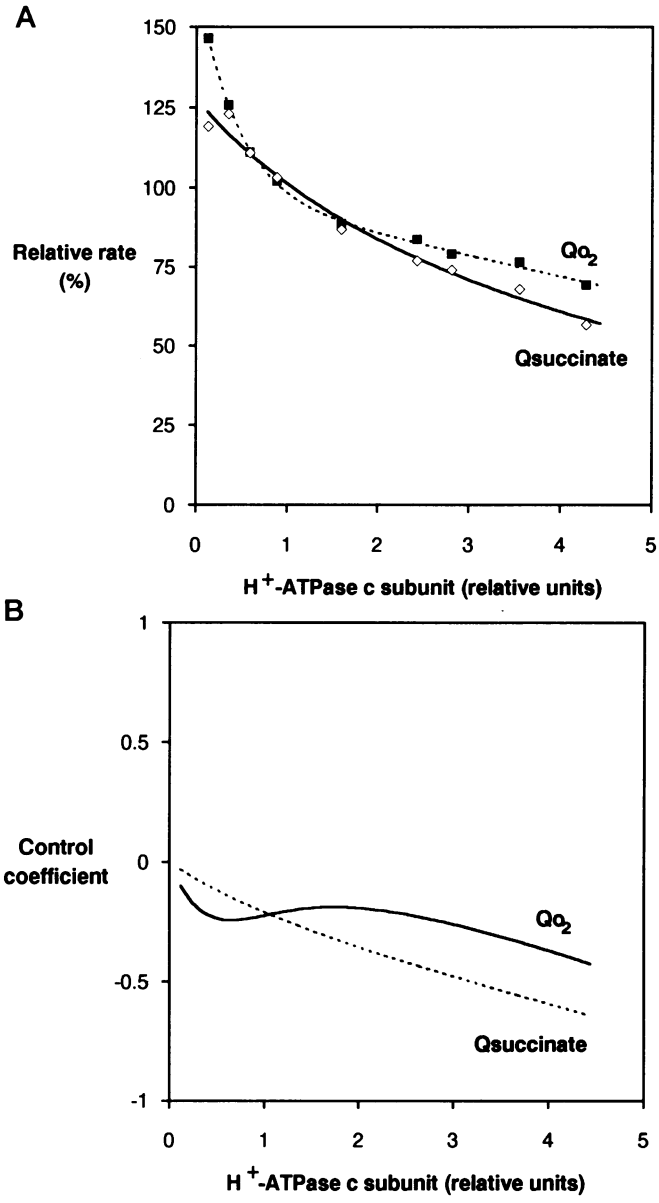

Fig. 3. Rate of oxygen and succinate consumption as a function of the level of H^+-ATPase. (**A**) The respiration rates and substrate consumption rates of cultures which expressed the *atp* genes to various extents were plotted as a function of the relative content of H^+-ATPase. The substrate consumption rates were calculated from experimental measurements of growth rate and growth yield. (**B**) Control of H^+-ATPase on the rate of succinate and oxygen consumption. The log-log derivatives of the smooth curves on Figure 3A were plotted as a function of the level of H^+-ATPase (see text for explanation).

concentration resulted in an even further increase. On the other hand, increasing the amount of H^+-ATPase resulted in a decreased turnover number, i.e. an inverse relationship between the level of H^+-ATPase and its turnover number (v_{ATP}) becomes apparent.

The mechanistic basis for changing the turnover number of the H^+-ATPase

The increased turnover number of the individual H^+-ATPase enzyme explains why the growth rate was not decreased in proportion to the reduction of the H^+-ATPase enzyme concentration. What is the basis for this change in

the turnover number? One possible explanation is that, as the concentration of H^+-ATPase was reduced, the back-flow of protons through the cytoplasmic membrane decreased, resulting in an increased build-up of membrane potential and therefore a higher turnover number of the remaining H^+-ATPases. Indeed, we have previously shown that a strain that was depleted H^+-ATPase had an increased membrane potential (Jensen and Michelsen, 1992). However, we also found that the level of *b* cytochromes in this deletion strain was reduced, suggesting that part of the increase in membrane potential may have been due to activation of the respiration driven proton pumps.

The observation that with decreasing concentrations of the H^+-ATPase, the growth yield (Y) decreased whilst the growth rate (μ) remained virtually constant, implied that the rate of succinate consumption ($Q_{succinate}$) increased, since $Q_{succinate} = \mu/Y$. In Figure 3, we show how $Q_{succinate}$ changed with the amount of H^+-ATPase in the cells. Reducing the H^+-ATPase from the wild-type level (= 1 U) to 0.6 relative units, resulted in a 10% increase in $Q_{succinate}$, and when the H^+-ATPase was over-expressed, $Q_{succinate}$ decreased below the wild-type rate. Over the entire range of H^+-ATPase studied, $Q_{succinate}$ changed by a factor of two. Since the growth rate, and therefore the flow of succinate to cell material remained almost constant when the amount of H^+-ATPase was decreased from the wild-type level to 0.6 U, then an increased rate of succinate consumption must reflect an increased catabolic flow. Indeed, we found an enhanced respiration rate in the cells which contained lowered amounts of H^+-ATPase (Figure 3), and this may also be expected to result in an increased membrane potential.

The controls exerted by the H^+-ATPase on the succinate consumption rate and the respiration rate were also quantified in terms of control coefficients. The control on succinate consumption was -0.25 and the control on respiration rate was -0.24, at the wild-type enzyme level. The controls on both fluxes were negative throughout the entire range of H^+-ATPase analysed, see Figure 3B.

Another factor that may be expected to affect the turnover number of the H^+-ATPase is the intracellular ratio of [ATP]/[ADP]. We therefore measured this ratio in cells which expressed the *atp* genes to various extents. In wild-type cells, we found that this ratio was close to 12. Cells which contained lower amounts of H^+-ATPase (0.15 relative units) also showed a lower ratio of [ATP]/[ADP] (equal to 3), whereas cells which over-expressed the H^+-ATPase (4.5 relative units) had a higher ratio than in the wild-type cells (close to 20). If, in normal cells, the H^+-ATPase is inhibited by a high concentration of ATP, then a reduced [ATP]/[ADP] ratio would be expected to release this back-pressure; these results would then provide an additional explanation for the variable turnover number of the H^+-ATPase.

Discussion

We have examined the extent to which the H^+-ATPase controls the physiology of *E.coli* growing on the non-fermentable substrate succinate, by modulating the expression of the *atp* operon. We observed that the control by the H^+-ATPase on growth rate at the wild-type level was close to zero. This demonstrates that an essential enzyme (for growth on succinate) need not exert much control under normal conditions.

A control coefficient of zero for growth could imply that the concentration of the enzyme in wild-type *E.coli* is optimal for growth rate. Indeed, the variation of growth rate with H^+-ATPase concentration exhibited a maximum close to the wild-type level of the enzyme (Figure 1). This result is in line with the results obtained by von Meyenburg *et al.* (1982, 1984). These investigators measured the growth rate of *E.coli* strains, growing with glucose as a substrate, which either had a deletion in the *atp* operon or over-produced the H^+-ATPase from plasmids. Both the strain that had a deletion in the *atp* operon and a strain that expressed the H^+-ATPase at double the wild-type level had decreased growth rates, which indicates that the optimal concentration of this enzyme is less than two relative units. From those experiments, however it was not possible to conclude if the H^+-ATPase concentration was optimal with respect to growth *yield*. Our results suggest that it is not quite optimal; only at higher concentrations is the H^+-ATPase optimal with respect to growth yield. Around the wild-type level, the growth yield still increased with the concentration of H^+-ATPase. This reflects the phenomenon that at the wild-type level the enzyme exerts negative control on the rate of substrate consumption.

The fact that the H^+-ATPase did not control the growth rate of the wild-type *E.coli* cell, indicates that there is an excess capacity of this enzyme present in these cells; our analysis suggests that the turnover number of the individual H^+-ATPase molecules changes in response to changes in the concentration of the enzyme. In order to be able to calculate the turnover number of the H^+-ATPase, we made the assumption that the rate of oxidative phosphorylation was proportional to the growth rate, which assumes both that (i) ATP consumption and growth rate are strictly coupled, and that (ii) ATP generated by substrate level phosphorylation is negligible. The former assumption is probably true for low [ATP]/[ADP] ratios, i.e. at low concentrations of H^+-ATPase; at high [ATP]/[ADP] ratios, however, uncoupled ATP hydrolysis might take place, in which case the turnover number derived from Figure 2 would be underestimated.

Although the assumption of strict coupling between growth rate and ATP consumption may not be quite correct, the main conclusion of excess capacity of the H^+-ATPase in the wild-type cells does not critically depend on it. However, if the turnover number did not change when the amount of H^+-ATPase was reduced from 1 to 0.6 relative units, then the rate of ATP synthesis would be decreased by 40%, practically without affecting the growth rate of the cells (Figure 1). This would not only mean that 40% of the ATP actually synthesized in the wild-type cells growing on succinate normally would have to be hydrolysed without coupling to growth, but also that all such hypothetical uncoupled hydrolysis of ATP disappeared when the amount of enzyme was reduced by 40%.

The second part of the assumption, that ATP synthesis by substrate level phosphorylation is negligible during growth on succinate, seems reasonable considering the fact that *E.coli* fails to grow on succinate in the absence of the H^+-ATPase (von Meyenburg *et al.*, 1984). When succinate is catabolized to CO_2, one molecule of ATP is formed by substrate level phosphorylation per molecule of succinate.

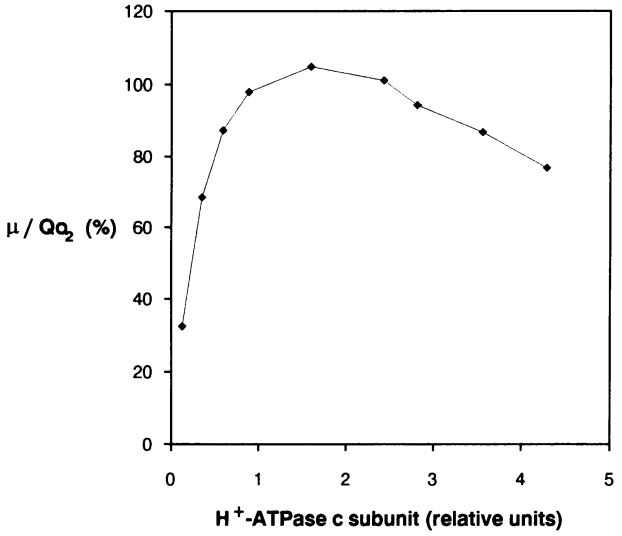

Fig. 4. Implications for coupling. The specific growth rate divided by the respiration rate was used as an indicator of the change in P/O ratio, resulting from changes in cellular H^+-ATPase content.

Since this is accompanied by the generation of five molecules of NADH and one of $FADH_2$, the contribution from substrate level phosphorylation would be expected to be very low in the wild-type cells. In cells that contain 0.6 units of H^+-ATPase, the increase in the catabolic flow was merely 10% and the assumption should still be valid. It is only when the amount of H^+-ATPase is reduced to a large extent that the relative contribution from substrate level phosphorylation to overall ATP synthesis may become significant.

There are at least two mechanisms that may explain the increased turnover number of the H^+-ATPase. One is that the decreased concentration of H^+-ATPase leads to an increase in $\Delta\tilde{\mu}H^+$, just because of a decreased influx of protons. Since the H^+-ATPases clearly do not function at V_{max} under physiological conditions, we may expect an increased $\Delta\tilde{\mu}H^+$ to increase the turnover number of the remaining enzymes. The increase in respiration rate in response to a reduced concentration of H^+-ATPase should then have enhanced this effect. Secondly, under physiological conditions, the H^+-ATPases may not function at maximal rate as a result of product inhibition (at high [ATP]) and/or substrate limitation (at low [ADP]); for instance because it functions not too far from the 'static head', where $\Delta\tilde{\mu}H^+$ balances the free energy of ATP hydrolysis. We found that the [ATP]/[ADP] ratio was decreased in cells which contained low amounts of H^+-ATPase and increased in cells which contained high amounts, compared with the wild-type cells. Therefore, if the H^+-ATPase is subject to ATP inhibition or ADP limitation under normal physiological conditions, then these findings may also explain the variable turnover number of the H^+-ATPase.

It may be useful to compare the control exerted by the H^+-ATPase in *E.coli* with the control of the enzyme on oxidative phosphorylation in rat liver mitochondria. In the latter system, the control exerted by the adenine nucleotide translocator may have to be added to that exerted by the H^+-ATPase. Since the bacteria in our experiments are growing actively, we should compare them with actively phosphorylating mitochondria. In the latter case the control by the enzyme on respiration is also rather small but positive (Moreno-Sanchez, 1985). The translocator for adenine nucleotides usually carries a significant, positive fraction (some 30%) of the control (Groen *et al.*, 1982). In contrast to these positive controls, in the *E.coli* cells the control exerted by the H^+-ATPase on respiration is negative, i.e. reducing [H^+-ATPase] leads to a higher respiration rate, a phenomenon we call inverse respiratory control. At first this may be a surprising result, as a decrease in the amount of H^+-ATPase in the cell would be expected to lead to an increase in membrane potential, and we have previously shown that this actually occurs (Jensen and Michelsen, 1992). If the *E.coli* situation were analogous to that of mitochondria, at increased membrane potential the respiration rate should decrease as a result of increased back-pressure, and the control on respiration exerted by the H^+-ATPase should be positive. An important difference, however, between isolated mitochondria and intact cells is that in the former case the redox pressure at which electrons are fed into the respiratory chain is kept constant experimentally, whereas in the later case, that redox pressure may vary, as a consequence of changes in the catabolic flow.

If we assume that the rate of ATP generation is proportional to the growth rate, then we may obtain an indication of how the (relative) P/O ratio and hence the degree of coupling of oxidative phosphorylation varied as the concentration of the H^+-ATPase was changed, by plotting the growth rate divided by the respiration rate versus the concentration of H^+-ATPase (Figure 4). The P/O ratio calculated in this way decreased dramatically when the amount of H^+-ATPase was reduced, demonstrating that leakage or slippage increased in parallel with the increase in turnover number. These phenomena of incomplete coupling and inverse respiratory control may therefore explain that, whereas the H^+-ATPase hardly controls the growth rate of wild-type *E.coli*, it does control its growth yield somewhat ($C = 0.2$). Furthermore, the P/O ratio seemed to increase slightly as the amount of H^+-ATPase was increased above the wild-type level; it then decreased again. This indicates that the degree of coupling between oxidative phosphorylation and respiration could be enhanced by increasing the amount of H^+-ATPase to above the wild-type level, resulting in an increased growth yield.

An intriguing observation is that as the expression of the *atp* operon was increased above the wild-type level, the control on growth rate became negative. This negative effect of H^+-ATPase over-production has been reported before (von Meyenburg *et al.*, 1984); the present experiments suggest that it is not an effect of excessive over-production but a tendency that already arises just above the wild-type level. Explanations of this phenomenon could include an inhibitory effect on growth of high concentrations of ATP or high [ATP]/[ADP] ratios. Furthermore, as the concentration of H^+-ATPase increases, $\Delta\tilde{\mu}H^+$ is expected to drop, and succinate transport, which is energized by $\Delta\tilde{\mu}H^+$ (Gutowski and Rosenberg, 1975), may thus slow down, resulting in carbon limitation of the cells. Other membrane processes essential for growth may also be affected.

The observed dependence of growth rate on the concentration of H^+-ATPase demonstrates that the wild-type level of the enzyme is optimal for growth rate when succinate (Figure 1) or glucose (Jensen,P.R., Michelsen,O. and Westerhoff,H.V., in preparation) is the growth substrate. With respect to growth yield, the H^+-ATPase level seems

sub-optimal. Since growth rate must always be limited by some process(es) (Westerhoff and Van Dam, 1987), this observation cannot just be interpreted so as to indicate that the *E.coli* K-12 strain used in our studies has been optimized for growth rate: the optimization with respect to the H^+-ATPase activity can only have shifted the limitation of growth rate to other processes. One could argue that it may be useful for *E.coli* to have a highly flexible ATP generating system, which would respond rather elastically to changes in $\Delta\tilde{\mu}H^+$, such as those resulting from minor, mechanical membrane damage, activation of substrate uptake systems, or cell division. Alternatively, or in addition, it may be useful for the cell to have the control on growth rate residing in other processes, such as those that sense the availability of growth substrate. The high control of growth rate by substrate uptake predicted by the latter possibility remains to be tested for growth on succinate or glucose, but has been observed for growth on lactose (Dijkhuizen *et al.*, 1987).

Materials and methods

Bacterial strains

The *E.coli* K12 strain used as the wild-type strain in this study, LM3118, has the genotype, F^+, *asnB32*, *thi-1*, *relA1*, spoT1, lacUV4 and *lacY*^{am}. In the strain LM3113, the *atp* promoters have been exchanged with the *tacI* promoter (Jensen *et al.*, 1993).

Growth of bacterial cultures

The cells were grown in minimal MOPS medium (Neidhardt *et al.*, 1974), supplemented with 1 μg/ml thiamine and 0.05% succinate. In order to pregrow the cells for more than 10 generations, we inoculated the cultures the day before sampling, using previously determined generation times of the individual cultures to calculate the dilution required. As inoculation material, we used cells that were already growing exponentially in the growth medium of interest, in order to avoid a lag phase. During the period of pre-cultivation, the optical density never exceeded 0.1 OD_{450} units. All the experiments were performed at 30°C. The wild-type strain, LM3118, had an actual growth rate of $\mu = 0.33\ h^{-1}$ (0.48 doublings/h) and the growth yield was 36.5 g dry weight/mol succinate.

Measurement of respiration rate

Respiratory rates were determined by transferring 15 ml of exponentially growing culture ($OD_{450} = 0.1-0.2$) to a reaction tube, and recording the decrease in dissolved oxygen concentration by a Clark-type electrode. The respiration rate of the wild-type strain, LM3118, was 16.3 mmol O_2/h/g dry weight.

Measurement of cellular ATP/ADP content

Samples were withdrawn from the cultures and immediately mixed with 1 vol phenol [80°C, equilibrated with TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and containing 0.1% 8-hydroxyquinoline] by vortexing for 10 s. After 5 min at room temperature, the samples were mixed for 10 s, cooled on ice and centrifuged for 5 min at 16 000 *g*. The water phase was removed and extracted with chloroform to remove phenol. The ATP assay was carried out at room temperature, using a luciferin-luciferase ATP monitoring kit, obtained from and used as recommended by LKB. [ADP] was determined in the same sample, after the determination of [ATP], by addition of phosphoenolpyruvate (3 mM) and pyruvate kinase and recording the increase in [ATP].

Quantification of c subunit

This was done as described by von Meyenburg *et al.* (1982). Briefly, the total protein content of the cells was labelled with [^{35}S]methionine and separated by SDS–PAGE. The content of H^+-ATPase *c* subunit was quantified relative to lipoprotein (lpp) after optical scanning of the autoradiograms.

Calculation of control coefficients from the experimental data points

In order to calculate the H^+-ATPase at the wild-type enzyme level, we used data points from 0.5 to 2 relative units of H^+-ATPase. The data points referred to growth rate, growth yield, respiratory rate or substrate consumption rate as a function of the concentration of the *c* subunit. Through corresponding data points a second order polynomium was drawn, using a non-linear, least squares fitting procedure (using MLAB, Civilized Software Inc., Bethesda, MD, USA). The control coefficient was then calculated as described below, at the level of *c* subunit corresponding to the wild-type H^+-ATPase concentration. In order to calculate the control of the H^+-ATPase on the growth variables for the entire range of H^+-ATPase expression, we drew smooth curves through the experimental data. The growth rate curve was drawn using the function, $\mu(c) = [14.4(7.9 - c)(1 - e^{-4c}) + 3,4]$, the growth yield curve was drawn using the function, $Y(c) = [6.7(15.7 - c)(1 - e^{-2c}) + 17.7]$, the respiration rate using the function, $Q_{O_2}(c) = [6.6(-9.9 - c)(1 - e^{-2.4c}) + 164]$ and the rate of succinate consumption was drawn using the function, $Q_{succinate}(c) = [159(c + 0.72)^{-0.3} - 36)$, where *c* is the concentration of H^+-ATPase *c* subunit. The functional forms used led to the best fits out of a number of functional forms tried. The parameters were estimated through a non-linear least squares fit. We checked that other fitting functional forms did not lead to results differing by more than the experimental error from what we obtained by these functional forms. The control coefficients were calculated from the functions above, using the equation, $C_c^{F(c)} = [dF(c)/F(c)/d(c)/c] = d\log[F(c)]/d\log(c)$, i.e. the percentage change in variable $[F(c)]$, divided by the percentage modulation of the H^+-ATPase concentration (*c*) in the limit of the latter percentage to zero (Westerhoff and van Dam, 1987).

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