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Biophysical Chemistry 55 (1995) 153–165

Biophysical Chemistry

Energy, control and DNA structure in the living cell

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Abstract

Maintenance (let alone growth) of the highly ordered living cell is only possible through the continuous input of free energy. Coupling of energetically downhill processes (such as catabolic reactions) to uphill processes is essential to provide this free energy and is catalyzed by enzymes either directly or via "storage" in an intermediate high energy form, i.e., high ATP/ADP ratio or H^+ ion gradient.

Although maintenance of a sufficiently high ATP/ADP ratio is essential to overcome the thermodynamic burden of uphill processes, it is not clear to what degree enzymes that control this ratio also control cell physiology. Indeed, in the living cell homeostatic control mechanisms might exist for the free-energy transduction pathways so as to prevent perturbation of cellular function when the Gibbs energy supply is compromised. This presentation addresses the extent to which the intracellular ATP level is involved in the control of cell physiology, how the elaborate control of cell function may be analyzed theoretically and quantitatively, and if this can be utilized selectively to affect certain cell types.

Keywords: Metabolic control; DNA supercoiling; Magainins; Multidrug resistance; Glutamine synthetase; Cascade regulation

1. Introduction

Understanding the control and regulation of cell physiology constitutes an immense scientific challenge [1,2]. Cell physiology is the result of kinetic and thermodynamic interactions of a great many molecular components. Because most interactions are complex, for a long time it was impossible to understand regulation of cell function and interaction of cells with their environment. Major problems are the difficulty to assess enzyme kinetic properties in the intact cell, the nonlinearity of most enzyme

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SSDI 0301-4622(94)00148-0

catalyzed reactions and the vast number of enzymes and metabolites involved [1,2]. The sheer complexity of metabolism has long precluded the establishment of strict relationships between the molecular and cellular level. In recent years, concepts and methodologies have been developed that serve to relate the two levels. The relationships between cell and molecule have turned out to be so subtle as to require quantitative analysis for their complete understanding.

Due to several limitations quantitative understanding of cell physiology has been hampered. One such limitation was knowledge about the control of the fluxes through (and control of concentrations in) pathways of intermediary metabolism and free-energy transduction (in which a pathway can be defined as a system that consists of a flux from starting substrates at fixed concentrations to products that are also maintained at constant concentrations [1,3]). Metabolic control analysis (MCA) has advanced an unequivocal definition of the extent to which an enzyme controls a flux and enabled determination of these flux control coefficients. Moreover, it offers explanations for the magnitude of these flux control coefficients [1,4,5] and it provides mathematical theorems pinpointing the molecular properties responsible for the observed distribution of flux control over pathway enzymes. MCA relates the flux control coefficients of all enzymes in a pathway to the so-called elasticity coefficients of these enzymes. Elasticity coefficients are enzyme properties (this in contrast to control coefficients which are system properties) and quantify the extent in which the enzyme activity will change upon a change in an intermediate concentration.

MCA is apt to deal with the non equilibrium processes so characteristic for living systems. Yet, a limitation is that it is a differential analysis, hence only exact for small modulations. Also, it does not directly address the thermodynamics efficiencies. Non equilibrium thermodynamics (NET) dealt with such aspects for near equilibrium processes, but was considered not quite appropriate for the central free-energy transducing processes of the living cell. MNET (mosaic non equilibrium thermodynamics, [1,6]), a merger of NET and biochemical kinetics, is applicable further from equilibrium [1,5]. It contributes to the quantitative understanding of the mechanisms, control and efficiency of cellular free-energy transduction.

MCA and MNET are limited to systems where the concentrations of active enzymes are system parameters subject only to explicit modulations. In actual practice, these concentrations are variable due to variable covalent modification of enzymes, as well as variation in gene expression in actual living systems. Some examples of such variable covalent modification of enzymes are: (i) (in)activation of adenylate cyclase by G-protein and the ensuing effects on the concentration of cAMP (ii) regulatory effects of various growth factors on cells and (iii) modulation of the activity of glutamine synthetase in *Escherichia coli* by the regulatory cascade involving the NR_{II} and NR_{I} proteins.

Consequently, it has become timely to try to remove the limitations of MCA and MNET and (i) develop a theory that deals with hierarchical control systems with variable gene expression [7,8], (ii) employ of molecular genetics methods of modulating gene expression experimentally and (iii) mobilize the enormous increase in efficiency of computer software and hardware, all with the aim of achieving a better understanding of cell physiology. In this paper we shall describe recent progress along these lines.

Our interest is focused on a quantitative understanding of different aspects of cellular physiology, with emphasis on free-energy transduction. We will describe recent progress on theory development in MCA that was necessary to deal with less ideal pathways in which channelling, covalent modification or variable gene expression occurred. Experimentally, channelling (or direct transfer) is studied in group transfer of the bacterial phosphotransferase system, and covalent modification in the cascade control of the glutamine synthetase.

Control by free-energy metabolism is studied more directly by modulation of one of the most important ATP generating systems in *E. coli*, the H^+-ATPase, and by making free-energy transducing membranes more permeable using magainen peptides. These different aspects will all be described in a larger context so as to understand the links between the metabolic level and the higher hierarchical levels, and to get knowledge about gene regulatory loops connecting the different levels: One known example of control by the metabolic level of gene expression will be

discussed in more detail; how the energy status of the cell as reflected in its ATP/ADP ratio affects DNA supercoiling.

The growing knowledge of regulation of the different levels of the hierarchical system will allow us to understand more complex cell phenomena such as oscillations in glycolytic metabolism and multidrug resistance.

2. Developing control theory

MCA [4,9–13] like biochemical systems analysis (BSA, [13]) and MNET [1,6] devised ways to evaluate control and regulation in complex systems. In MCA the contribution of any enzyme to the control of the metabolic flux (J) is quantified by the enzyme's flux control coefficient (C^J_{Ei}). It relates a fractional change ($\mathrm{d}J/J$) in the steady state flux to the fractional modulation ($\mathrm{d}e_i/e_i$) of the enzyme concentration [4,15]

$$C^J_{E_i} = \frac{(\mathrm{d}J/J)}{(\mathrm{d}e_i/e_i)} = \frac{d\ln|J|}{d\ln e_i} \qquad (1)$$

The metabolic control analysis shows that the contribution of an individual enzyme to the control of flux through a pathway is a systemic property and can be expressed in quantitative terms [3]. A quantitative method based on MCA makes its possible to express the systemic control coefficients into the (molecular) elasticity coefficient. Elasticities and control coefficients describe, in quantitative terms, local and global properties of the metabolic system, respectively. The question arises as to how these may be related to each other, since of course the behavior of the metabolic system of interest does depend upon that of its constituent parts. The MCA formalizes this in terms of so-called flux-control and concentration-control connectivity theorems. Also, corresponding summation and connectivity theorems have been derived.

The understanding of the functioning of the intact cell would be simplified appreciably if it was possible first to analyze particular functional units (modules) of cell physiology separately, and then to integrate the information so as to yield understanding of the control structure in terms of the mutual regulation of the modules. A limitation of the existing (traditional) MCA was that it would be discussed only in terms of kinetic properties of the individual enzymes and not in terms of kinetic properties of these functional metabolic subunits. This limitation has been removed with the development of "overall" [16] or "top-down" [17] control analysis which treated entire groups of reactions as blocks with their own "overall elasticity coefficients" with respect to metabolite concentrations outside such a block. Schuster et al. [5] generalize overall control analysis to cases where modules have several throughput fluxes and/or are connected by more than a single metabolite. This allows one to treat such modules as "superenzymes" catalyzing multiple reactions. By this approach, control may be understood in terms of a few properties of the modules and the interactions between them rather than in terms of the possibly myriad of properties within modules. Not only does this allow for a simpler, i.e., stepwise approach to the understanding of control and regulation, it also allows one to analyze control even if one does not (or cannot) know the details of all the molecular properties of parts of the system. Such parts can remain "black boxes" of which only the input–output characteristics need to be known.

An important special case of modular control analysis is that where flow of compounds between modules can be neglected. A key example is that of metabolism that is regulated at the level of gene expression. The levels of DNA, mRNA, protein and intermediary metabolism are conceived of as independent except for regulatory interactions (Fig. 1). Special properties of such hierarchical control systems have been elaborated mathematically [18]. Depending on whether there is a feedback from the metabolic level to the level of gene expression or not, the control system may be called democratic or dictatorial [8].

Since an enzymatic reaction is composed of elemental steps, it can often be treated as a steady-state module in the sense defined above. This modular control analysis [5] may be considered as a generalization of conventional MCA. In this view, conventional control coefficients are identical to the overall control coefficients pertaining to the catalytic cycle of a particular enzyme. It is therefore not surprising that equations involving overall control coefficients have a similar structure to classical MCA equations.

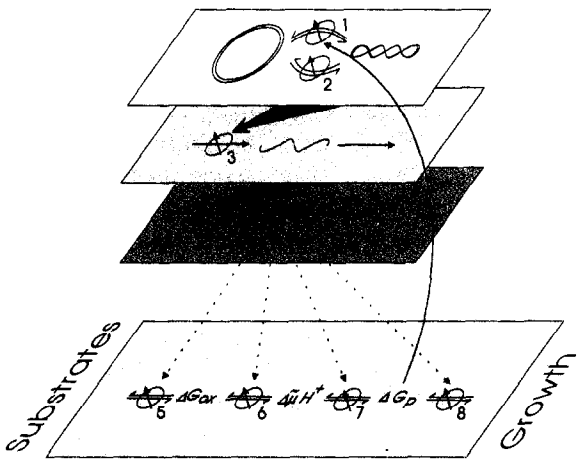

Fig. 1. The hierarchical organization of the regulation of gene expression and free energy transduction in *E. coli*. Four levels of the hierarchical organization are shown in different colors; the DNA level is shown in yellow and depicts the action of gyrase and topoisomerase on DNA structure, the RNA level is shown in blue, and the protein level in pink. As an example at the metabolite level the interactions of the different forms of Gibbs free energy (for aerobically growing cells) are shown in green. ΔG_{ox}, redox potential (of any given couple interacting with the respiratory chain); $\Delta\tilde{\mu}H^+$, electrochemical proton potential; ΔG_p, phosphorylation potential; (Circles with arrow inside) sites of modulation: 1, gyrase; 2, topoisomerase I; 3, transcription; 4, translation; 5, catabolic pathways; 6, respiratory chain; 7, H^+-ATPase; 8, anabolic pathways. (-- →) Dashed lines indicate regulatory interactions.

This generalization may be particularly useful when dealing with slipping enzymes (i.e., enzymes that catalyze several fluxes), a difficult problem in conventional MCA [12,19,20].

Recently Kholodenko and colleagues [21,22,31] developed a new way to analyze control, i.e. by considering the modulation of elemental steps. This approach will allow one to develop control theories for metabolite channelling and group-transfer pathways. The same approach will allow one to analyze the control of signal transduction cascades and develop the control theory for signal transduction in general and for two-way enzymes therein in particular.

3. Control by group transfer and signal transduction

Control of cell physiology reaches beyond metabolic fluxes and metabolic pathways. It includes regulation at the level of gene expression and regulation through cascades of enzymes covalently modifying one another [23]. The analysis of the control and regulation of microbial physiology is greatly facilitated by the application of the well defined concepts of metabolic and hierarchial control theory. One of these is the extent to which a physiological process such as growth is controlled by a biochemical/biophysical reaction such as a transport system. The corresponding flux control coefficient is the percentage by which growth rate increases when the activity of the uptake system is increased by 1% ([20], for complete descriptions see [1,4,25] and Eq. (1)). Hierarchial and modular control analysis has been developed for such systems, again allowing for the definition of flux control coefficients and again proving that for simple pathways the sum of the flux control coefficients over all enzymes in the entire system must be 1 [8,18,19,25].

Another type of regulation is that involving the transfer of a chemical group through a series of proteins, e.g., the bacterial phosphotransferase system transferring a phosphate group from phospho-*enol*pyruvate to a sugar molecule whilst transporting the latter across the membrane [26]. Measurements of the flux control coefficients of the enzymes involved in the *E. coli* phosphotransferase system resulted in control coefficients of the four enzymes involved, only that of II_{CB}^{Glc} differed significantly from zero (0.6, [27]: Van der Vlag, unpublished results). The proteins carrying the transferred group are most often referred to as enzymes. Consequently, one might conjecture that the sum of the control coefficients with respect to the flux of group transfer, over all these enzyme concentrations, equals 1. On the other hand, in these pathways the enzymes do not only act as catalysts but also as substrates. Indeed, Van Dam et al. [24] showed that in contrast to the above, the group-transfer pathways are special in that the enzymes' flux control coefficients add up to values between 1 and 2, rather than to 1. By including all partners in the group transfer and assuming control by unimolecular rate constants to be small ("hit-and-run transfer"), the sum has to be 2.

Pathways in which enzyme concentrations are high with respect to metabolite concentrations have special control properties when the metabolites are subject to moiety conservation [21,28,29,30]). In

cases of enzyme–enzyme interactions as well as in other "non-simple" pathways [31] there is a difference between the control coefficients defined in terms of modulations of activity and those defined in terms of modulations of the enzyme concentration [116]. Both definitions are important since they refer to different experimental methods of determining the control coefficients [32,33]. Furthermore, it was recently deduced that at higher concentrations the effect of protein burden upon manipulation of enzyme concentration and interferes with the determination of control coefficients, becomes more pronounced (Snoep et al., in preparation).

The control theorems developed for a group-transfer pathway can be used for a pathway of dynamic channelling. Van Dam et al. [24] confirm that in the case of dynamic channelling the sum of the flux control coefficients deviates from 1 [34].

Other enzyme systems in which phosphate transfer plays an important role are found in regulatory cascades. The variable gene expression and complexity of some such systems makes it a special challenge for a quantitative description.

4. Cascade control

Recently MCA has been developed so as to analyze the contribution to cell function of the various controlling levels of the hierarchical organization of the living cell. Most explicitly this was done for

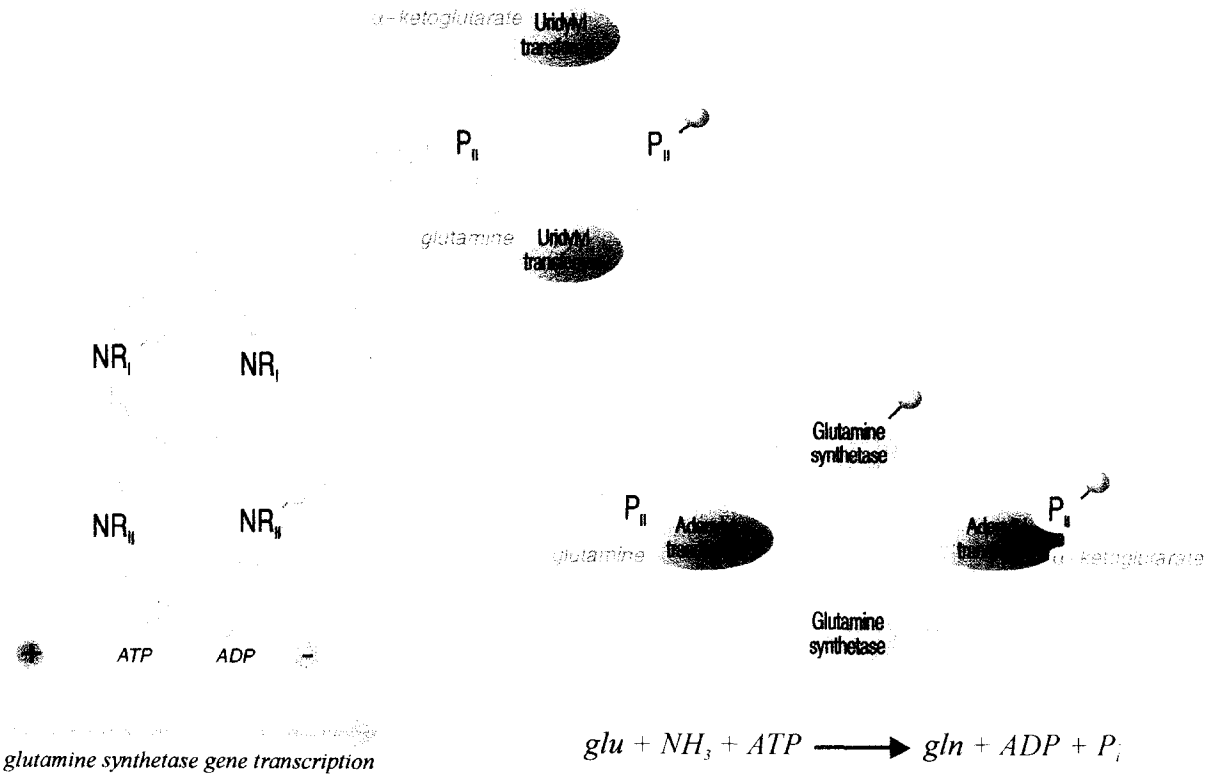

Fig. 2. Regulatory cascades of glutamine synthetase in *E. coli*. At the top of this hierarchical system is the enzyme uridylyltransferase, which can either transfer a UMP group to or detach this group from the protein P_{II}. The activity of uridylyltransferase, and thus the degree of P_{II} modification is determined by the allosteric effectors α-ketoglutarate and glutamine. The concentration ratio of these two metabolites reflects the nitrogen status of the cell. P_{II} and α-ketoglutarate and glutamine affect by allosteric interaction the activity of the enzyme adenylyltransferase, which can either transfer an AMP group to or detach this group from glutamine synthetase. Adenylylated glutamine synthetase is virtually inactive in the cell. P_{II} also affects the dephosphorylation of the protein NR_I, which is a modulator of transcription of the gene encoding glutamine synthetase. These mechanisms serve to downregulate glutamine synthetase activity and synthesis at high glutamine/α-ketoglutarate ratios, and upregulate this activity and synthesis at low ratios.

systems with variable gene expression and for regulatory cascades: the "hierarchical" or "modular" metabolic control theory [8,18]. The approach started from the general formalism of Reder [35] and has the advantage of providing a better understanding of the control of the hierarchical levels of a cascade. Here, the modular control theory will be made explicit for the glutamine synthetase regulatory cascade, which is a complex and interesting system controlling the assimilation of NH_4^+ in enteric bacteria ([36], review, see Fig. 2).

In enteric bacteria under nitrogen limiting conditions, assimilation of ammonia occurs primarily through the functioning of glutamine synthetase (GS)/glutamine oxoglutarate aminotransferase (GOGAT) [36,37]. This process is regulated at three levels: (i) at the level of gene expression, transcription of the GS gene proceeds from two tandemly arranged promoters. It was found that during nitrogen limitation, expression of the major transcript is dependent on the RNA polymerase sigma factor 54 together with phosphorylated NR_I [37]. NR_I phosphorylation is catalyzed by NR_{II} which also has NR_{II}-phosphatase activity [39–41]. This activity is stimulated by a protein, P_{II} [40]. P_{II} is subject to modification by uridylylation on a tyrosine residue [42]. Uridylylation and deuridylylation of P_{II} depend on the nitrogen state of the cell; (ii) at the metabolic level, the activity of GS is inhibited allosterically by a number of nitrogen-containing metabolites [43]; (iii) at the level of covalent GS modification. The GS activity is reduced upon adenylylation of a tyrosine residue [44,45]. Adenylylation and deadenylylation of GS are differentially modulated by P_{II} [46].

Thus nitrogen assimilation is regulated by two divergent cascades (bicyclic): one at the level of gene expression and one affecting the GS activity by covalent modification of the enzyme. In P_{II}, the two share at least one common factor. The activity of adenylyl transferase is regulated by the ratio P_{II}/P_{II}-*UMP*, thus uridylyl transferase serves as a sensor of the nitrogen status of the cell.

As GS is genetically regulated, the question arose whether the proteins involved in the adenylylation cascade are also genetically regulated. If so, the two cascades would share two properties: P_{II} (see above) and the regulation at the transcription level. Van Heeswijk et al. [38] studied the three genes from *E. coli* coding for uridylyl-transferase, P_{II} and adenylyl-transferase. They found out that the functioning of the GS adenylylation cascade is regulated by modulation of the activities, rather than by changes in the expression of their genes [37,38].

5. Control by free-energy metabolism

The H^+-ATPase is a key enzyme in the mechanism of cellular free-energy metabolism and is important under aerobic as well as under anaerobic conditions. In aerobic cell physiology, much of the free-energy transduction from catabolism to endergonic cell processes is catalyzed by H^+-ATPase of the energy-coupling membrane (for eukaryotes the inner mitochondrial membrane; for prokaryotes the plasma membrane) [47–49]. Under anaerobic circumstances it maintains ion and solute gradients across the energy coupling membrane at the expense of the hydrolysis of ATP synthesized by substrate level phosphorylation [50].

It is not known whether under physiological conditions the H^+-ATPase also controls the cellular free-energy metabolism, or the aspects of cell physiology that depend on that mechanism. To investigate the extent to which this enzyme (also) controls the growth rate, growth yield and respiratory rate we modulated the expression of the *atp* operon, determined the effect on said properties and quantified them in terms of (flux) control coefficients. (The "control coefficient" is a measure of the extent to which an enzyme controls a physiological process (a steady-state flux or concentration)).

Although the methods which have been used to modulate enzyme activity in order to determine measure control coefficients [51–54] have been successful, they still suffer from limitations. Jensen et al. [55] developed a (revised) method, in which an IPTG-inducible promoter was inserted upstream of the chromosal copy of the gene encoding the enzyme of interest; together with simultaneous inactivation of the lactose permease it has become easier to modulate accurately the enzyme concentration of interest in *E. coli* cells around the wild-type level and measure its control coefficients. This method has been used to modulate the expression of the *atp* operon and measure control of the H^+-ATPase on growth rate, growth yield, respiratory rate and sub-

strate consumption [24,48,56]. The H^+-ATPase was found to exert virtually no control on growth rate which is remarkable in view of the important role of the H^+-ATPase in cell physiology. Because the sum of the flux control coefficients is limited to 1, this may reflect a shift of control to where it should be: at the substrate input. It may have been achieved by a stimulative effect on respiration due to a reduced cellular ATP level in the presence of an increased membrane potential [56]. Interestingly, this extra regulation requires the coupling between respiration and phosphorylation to be loose, hence yield and thermodynamic efficiency to be smaller than maximal.

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; Jensen et al. [48] suggested that the turnover number of the individual H^+-ATPase molecules changes in response to changes in the concentration of the enzyme. There are at least two possible 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 of $\Delta\tilde{\mu}_{H^+}$, just because of a decreased influx of protons. 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]). 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.

In the *E. coli* cells, the control exerted by the H^+-ATPase on respiration is negative. This is a surprising finding, as a decrease in the amount of H^+-ATPase in the cell should be expected to lead to an increase in membrane potential. At increased membrane potential, the respiration rate is expected to decrease, and the control on respiration exerted by the H^+-ATPase is, therefore, expected to be positive.

The phenomena of incomplete coupling and inverse respiratory control may explain that whereas the H^+-ATPase hardly controls the growth rate of wild-type *E. coli*, it does control its growth yield somewhat. A slight increase in the P/O ratio as the amount of H^+-ATPase was increased indicates that the degree of coupling between oxidative phosphorylation and respiration could be enhanced by increasing the concentration of H^+-ATPase above the wild-type level, resulting in an increased growth yield. The finding that the control on yield exceeds the control on growth rate is surprising since the sum of the flux control coefficients is greater (equals 1) than the sum of control coefficients with respect to yield (equals 0). The positive control of the H^+-ATPase on yield is due to the negative control on substrate consumption rate and the absence of control on growth rate.

Since growth rate must always be limited by some process(es) the observation that the growth rate is not depending on the [H^+-ATPase] cannot be interpreted so as to indicate that the *E. coli* K-12 strain has been optimized for growth rate. Rather, it may be useful for the cell to have the control on growth rate residing in other processes. The high control of growth rate by substrate uptake as suggested before remains to be tested for growth on succinate or glucose, but has been observed for growth on lactose [57]. In addition, an increased protein synthesis burden ensuing from overexpression may reduce the magnitude of control coefficients (Snoep et al., in preparation).

Hierarchical control theory [8,18] predicts that in systems where regulation may involve variable gene expression in addition to metabolic regulation, the elasticity coefficient should effectively also include interaction through the gene expression. Consequently, the low control found for the H^+-ATPase could be due to a strong dependence of the expression of the genes encoding component of the respiratory chain on the intracellular level of ATP or on membrane potential. The level of b-type cytochromes was found to be increased in an *atp* deletion strain [57a].

In yeast the plasma membrane H^+-ATPase exerts a strong control on growth rate [58]. The metabolic role of this H^+-ATPase, however, is quite different, not lying on the main route of free-energy transduction but serving to control the intracellular pH.

6. Interfering with cellular free-energy metabolism: magainins

Magainins are positively charged peptides in the skin of *Xenopus laevis*. They were proposed to be

cytotoxic because they permeabilize free-energy transducing membranes for ions [59–61]. The hypothetical channel-forming properties of magainins were (further) studied in synthetic bilayers, using patch clamp techniques [62–64].

Most studies aiming at the functional basis of the broad spectrum antibiotic activity of magainins have been performed in complex proteinaceous systems, such as whole cells or isolated mitochondria [59–61,65]. The effects of magainins suggested positive cooperativity in their mechanisms of action. This cooperativity was revealed by a supralinear concentration dependence in the stimulation of respiration of isolated rat liver mitochondria, cytochrome c oxidase reconstituted liposomes, and hamster spermatozoa [59–61,65] or by the inhibition of the mobility of hamster spermatozoa [65]. Such cooperativity suggests the formation of a complex constituting the active form of magainins [59,60]. Due to their size and amphophilic properties, it is conceivable that magainins form transmembrane α-helices.

In the model and study of Cruciani et al. [63,64], the ion specificities would constitute experimental evidence for a channel. Several studies [66–68] showed increased helical content for magainins in hydrophobic environments but also in lipid vesicles. A first structural model for a magainin "channel" was proposed by Guy and Raghatan [69]. Bechinger et al. [70] supported the idea that magainins lie in the plane of the bilayer.

The molecular mechanism of action of peptides has not yet been elucidated because in particular there is a contradiction between structural studies in protein-free systems and functional studies with proteinaceous membranes. One possible resolution of this would be that in proteinaceous membranes magainins form a complex, whereas in protein-free membranes they do not [71].

In liposomes constituted by zwitterionic and acidic phospholipids, Vaz Gomes et al. [71] observed that magainin-induced membrane permeability did exhibit positive cooperativity. Vaz Gomes et al. [71] observed that synergism between magainin 2 and PGLa (1:1) occurred also in a system devoid of a transmembrane electric potential (as opposed to other model systems). Synergism seemed also to be potentiated by increasing amounts of negative membrane charge, i.e., at higher mole fractions of DCP (dicetylphosphate). This suggested that, for cooperativity and synergism and hence for the functional unit to be oligomeric, neither the presence of membrane proteins nor the presence of catalytic activities such as those causing a transmembrane electric potential difference are essential.

Vaz Gomes et al. [71] also investigated how a transmembrane electric potential, negative inside, affected the activity of the peptides. They concluded that electric potentials near and across the target membrane affect the activity of magainins and that the more active magainin forms are oligomers. They did not exclude the possibility that monomers also have some membrane-permeabilizing activity. In fact, most of the concentration dependencies could be explained by superposition of a monomeric and a stronger oligomeric membrane-permeabilizing magainin form.

In view of the investigations of Vaz Gomes et al. [71] and considering the physicochemical properties of magainins, the following tentative mechanism of action is proposed. Magainins are known to acquire an α-helical conformation in a hydrophobic/membrane environment. When bound to the surface of a membrane (electrostatic interactions playing an important role), the peptides may change their conformation from random coil (in solution) to α-helix. Transmembrane reorientation and formation of a complex can in principle occur in the absence of a transmembrane electric potential, although the observations substantiate a more effective or faster formation of the membrane-disturbing structure when an electric potential is present [71]. To clarify whether a faster binding process occurs, or a more efficient (maybe larger) pore is formed, further studies are required.

7. Control of DNA structure

In the control hierarchy of the living cell, transcription of the genetic information is an important aspect. The question whether the control of cellular energetics is a democratic or a dictatorial hierarchy paraphrases the question if the cell's energy state affects the rate of transcription of genes encoding free-energy transducing enzymes.

On paper, there are at least two possible mecha-

nisms for such control. The trivial one of these runs through the requirement of nucleoside-tri-phosphates for the synthesis of mRNA. A more interesting mechanism involves the high energy structure of DNA observed in prokaryotes: DNA gyrase reduces the linking number of topologically constrained double- stranded DNA molecules, even when the decrease in linking number increases the elastic energy of the DNA molecules [72]. The enzyme obtains the necessary Gibbs energy from the concomitant hydrolysis of ATP. This results in a high energy structure of the DNA that depends on the continuous dissipation of Gibbs energy of ATP hydrolysis. A second enzyme, topoisomerase I, changes the linking number in the direction of relaxation.

The transcription rate of many genes is sensitive to the extent of supercoiling of their promoter region [72]. Moreover, in vitro the extent of supercoiling achieved by DNA gyrase depends strongly on the hydrolytic Gibbs energy of ATP hydrolysis [73]. These observations have led to the hypothesis that the cellular energy state may control transcription through DNA gyrase and DNA supercoiling [73].

Indeed, decreasing the intracellular ATP/ADP concentration ratio resulted in a relaxation of DNA supercoiling [8,56,74–76,76a]. Ensuing research will focus on the effect of supercoiling on the expression of specific genes involved in the free energy metabolism, and their subsequent effect on the ATP/ADP ratio.

8. Control of dynamics

In recent years, it has been recognized that signal transduction has major impact on the glycolytic flux under various conditions and that there is a hitherto overlooked key regulator of PFK-1 (phosphofructokinase-1) involved, i.e., fructose 2,6-bisphosphate. PFK-1 can be synthesized and degraded by the two-way enzyme PFK-2 (phosphofructokinase-2-fructose-2,6-biphosphatase). The expression level of PFK-2 has little effect on steady-state flux, but strong implications for the rate at which the glycolytic flux changes upon transitions in signalling.

Oscillations in glycolytic metabolism occur in a variety of organisms and conditions [77–79]. After glucose has been added to intact starved cells of yeast and a steady state has been reached, addition of cyanide leads to oscillations in the intracellular NADH concentration [80]. These oscillations have mainly been monitored "macroscopically", i.e. through the NAD(P)H fluorescence of populations of yeast cells. Only limited information is available concerning oscillations of individual cells [81,82]. Populations of intact cells exhibit a temperature dependent frequency [83].

Yeast cells exhibit different dynamic behavior depending on the growth phase in which they were harvested. When the cells were harvested around the growth phase transition (diauxic shift) from using glucose to using ethanol as a substrate, limit-cycle (long-lasting) oscillations were observed, whereas when they were harvested at another growth phase they showed damped oscillations [84,85]. At high cell densities the oscillations last longer than at low cell densities [82,86]. Only at high cell concentrations, due to a synchronizing mechanism, do the cells may remain in phase [87]. A short train of oscillations is observed when the cells are harvested during growth on glucose or during growth on ethanol [85,88]. These differences most probably reflect differences in the cellular make-up, rather than differences in metabolite concentrations, since this behavior was conserved over hours of starvation. Richard et al. [83] report that with respect to the concentrations of the C_6 and C_3 compounds of glycolysis the sustained oscillations are confined to the upper part of glycolysis. The oscillations involve NADH, inorganic phosphate, the phosphorylation potential, energy charge and redox potential. Fructose-2,6-bisphosphate does not oscillate suggesting that in this time-dependent phenomenon PFK-2 does not play a role.

One role of cyanide in the oscillations is to simulate anaerobiosis at the level of cytochrome oxidase. However, Richard et al. [89] found that under anaerobic conditions in the absence of cyanide, oscillations were less pronounced. Moreover, other inhibitors of respiration or anaerobiosis led to a much shorter train of oscillations than cyanide. Richard et al. [89] examined the possible side effects of cyanide. The known reactivity of cyanide with acetaldehyde [90] is important for the persistence of the oscillations. Cyanide addition yielded longer series of oscillations [91]. This suggested that cyanide does not only act as an inhibitor of respiration. Richard et al.

[89] found limit-cycle oscillations only in a certain concentration range, further suggesting that there is another effect of cyanide. This effect was identified as the removal of acetaldehyde. These and other results suggested that for sustained glycolytic oscillations to occur it is not only required that the cells are anaerobic but also that acetaldehyde is continuously removed at a certain rate. In addition, a specific cellular make-up is required which includes an increased activity of hexokinase.

9. Failing to control the uncontrolled: multidrug resistance (MDR)

The cellular pharmacological basis for multidrug resistance (resistance of (in vitro) cells to certain anticancer drugs (e.g. anthracyclines and vinca alkaloids) [92]) often appears to be a reduced accumulation of drugs [93–95]. The ability of tumor cells to become multidrug resistant (MDR) by exposure to cytostatic drugs has often been correlated to the overexpression of the *MDR1* gene. This gene codes for an M_r 170000 protein that is associated with the plasma membrane and has been termed P-glycoprotein (Pgp, [93,94,96]). In normal human tissues and human cancers, the Pgp has been shown to be present. The amount of Pgp proved to vary between tissues [97,98]. A widely accepted model of its function proposes Pgp to be an ATP-dependent drug efflux pump [99,100]: One of the mechanisms responsible for MDR is thought to be an energy-dependent transport system for natural-product cytotoxic drugs such as doxorubicin and daunorubicin [93,94]. However, several MDR cell lines have been described that do not contain Pgp and yet show reduced accumulation of drugs in the cells [101–103]. Some of these cell lines are postulated to have drug efflux pumps other than Pgp [104]. To what degree Pgp and the other (MRP and as yet putative) drug efflux proteins have a homologous structure and activity remains to be resolved. Doxorubicin and daunorubicin, which are frequently used as anticancer drugs [105], are lipophilic weak bases and passive diffusion of the non-charged molecules across membranes is much faster than of the protonated forms [106–109] and therefore, not only presence of an efflux pump, but also the pH gradient across the membrane influences drug accumulation [110].

Spoelstra et al. [111] evaluated both passive membrane transport for daunorubicin and the Pgp-mediated active efflux of daunorubicin in cancer cell lines with different levels of Pgp. They found a positive cooperativity between the variation of the pump rates and the intracellular daunorubicin concentration which provides evidence that at least two binding sites for daunorubicin are present on the active transport system of daunorubicin. This positive cooperativity has since been confirmed in studies with plasma membrane vesicles [112]. In cell lines with the highest amount of Pgp, the passive efflux rate of daunorubicin proved to be a substantial part of the total daunorubicin efflux rate for the cell lines used. In cell lines with relatively low levels of Pgp, passive daunorubicin efflux was even the main route of daunorubicin transport from the cells, determining the intracellular steady-state concentrations of daunorubicin.

Mülder et al. [113] studied the effects of drug efflux pumps on the amount of daunorubicin associated with the plasma membrane. They found that in Pgp-containing 2R160 cells daunorubicin is pumped from the cytosol rather than from the membrane. This is in apparent conflict with the suggestion that Pgp pumps primarily from the membrane [114]. However, it should be noted that the distinction between pumping from the membrane and pumping from the cytosol is less absolute than it may seem. Most likely, the drug pumps reside in the plasma membrane, hence pump from a drug-binding pocket in or at the membrane (drug-binding site of the pump). The distinction between "vacuum cleaning" and "non-vacuum cleaning" is whether the exchange of drug between this pocket and the membrane compartment as a whole is faster than the exchange with cytosol (vacuum cleaner) or vice versa (non-vacuum cleaner). This distinction is only of importance when drug pumping is faster than exchange between membrane and cytosol. In these terms, Mülder et al. [113] found that in 2R120 cells (which do not contain Pgp, but are multidrug resistant) the drug-binding site of the pump exchanges more slowly with the cytosol, whereas in 2R160 cells it exchanges more slowly with the membrane. So it could be quite conceivable that in different cells Pgp exchanges more readily with the membrane phase and has more of a vacuum-cleaning character,

as Gottesman [114] suggested. Mülder et al. [113] developed a method which may serve to quantify further the extent to which various drug efflux pumps are in contact with membrane rather than cytosol.

An important question is what controls the extracellular concentration of drug that leads to a certain toxicity for the cancer cell. The quantitative model developed in conjunction with experimental studies ([92]; Westerhoff et al., in preparation; Jongsma et al., in preparation), was subjected to a control analysis. It turned out [115] that the P-glycoprotein exerted a control close to 1 on the isotoxic drug dose and the passive membrane permeability a control close to -1.

10. Discussion

In this paper we have presented recent work on the pleiotropic control function of free-energy metabolism in the living cell. Among the important developments are accurate theoretical (such as modular control theory) and experimental (such as fine tuning of chromosomal gene expression) tools. In addition, it has been possible to examine the high free-energy structure of the DNA and the free-energy state of the cell in vivo.

Peptides may be used to interfere with cellular energy metabolism, but more work on increasing their specificity will be appropriate. The kinetics of free energy dissipating drug transport out of tumor cells can now be analyzed rigidly and this may open up possibilities for further rationalizing drug treatment of tumors.

Acknowledgements

These studies are supported by the Netherlands Organization for Scientific Research (NWO). We are much indebted to our generous colleagues Karel van Dam and Jan Lankelma.

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