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Thermodynamics of complexity The live cell[1]

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Abstract

Thermodynamics has always been a remarkable science in that it studies macroscopic properties that are only partially determined by the properties of individual molecules. Entropy and free energy only exist in constellations of more than a single molecule (degree of freedom). They are the so-called emergent properties. Tendency towards increased entropy is an essential determinant for the behaviour of ideal gas mixtures, showing that even in the simplest physical/chemical systems, (dys)organisation of components is crucial for the behaviour of systems.

This presentation aims at illustrating the thesis that the aforesaid holds a fortiori for the living cell: Much of the essence of the live state depends more on the manner in which the molecules are organised than on the properties of single molecules. This is due to the phenomenon of 'Complexity'. BioComplexity is defined here as the phenomenon that the behaviour of two functionally interacting biological components (molecules, protein domains, pathways, organelles) differs from the behaviour these components would exhibit in isolation from one another, where the difference should be essential for the maintenance and growth of the living state. For a true understanding of this BioComplexity, modern thermodynamic concepts and methods (nonequilibrium thermodynamics, metabolic and hierarchical control analysis) will be needed.

We shall propose to redefine nonequilibrium thermodynamics as: The science that aims at understanding the behaviour of nonequilibrium systems by taking into account both the molecular properties and the emergent properties that are due to (dys)organisation. This redefinition will free nonequilibrium thermodynamics from the limitations imposed by earlier near-equilibrium assumptions, resolve the duality with kinetics, and bridge the apparent gap with metabolic control analysis. Subsequently, the complexity of the control of the energy metabolism of *E. coli* will be analysed in detail. New control theorems will be derived for newly defined control coefficients. It will become transparent that molecular genetic experimentation will allow one to penetrate into the mechanisms of the complex regulation of energy metabolism. © 1998 Elsevier Science B.V.

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1. The living cell as a unit of BioComplexity

One of the greatest challenges to biology is to understand how the complex phenomena associated with life ultimately result from interactions between molecules. These interactions must follow the principles of physics and chemistry. Traditionally, physics and chemistry have had a strong preference for simple objects of study, which should be tractable in terms of linear first-order approximations. Only recently, this preference has been superseded by an interest in nonlinear phenomena that led to effects, differing drastically from effects observed in linear systems. Being tied down to one special reality, biology has always been implicitly interested in nonlinear phenomena, because life abounds in them. However, when attempting to *apply* the quantitative methods advocated by chemistry and physics, biology has often been seduced into following the route of linear first-order approximations. Unless executed with care, such linear first-order approximations do away with the phenomena that arise in the nonlinear interactions between the molecules.

In the living cell many processes occur simultaneously. The molecules that are involved react with one another in many different ways, such that these processes are coupled. Free-energy transduction is an example; here, electron transfer through a number of redox components in a membrane is coupled to the transmembrane pumping of protons. The back flux of protons is then coupled to ATP synthesis. ATP hydrolysis is again coupled to many endergonic processes. The enzymes that are involved in most of these reactions would catalyse the reaction differently (e.g. in a different direction) when acting in isolation. For instance, the H^+-ATPase would hydrolyse ATP in vitro, but may synthesise ATP in vivo. Clearly, the organisation of the molecular processes with respect to one another is important here.

2. What is BioComplexity, or what should it be?

When should we consider a system to be complex? The sun contains a gigantic number of molecules, yet the nuclear and chemical reactions that it harbours are comparatively simple vis-à-vis the reactions in a living cell. Clearly, complexity is not synonymous with size, or even with the number of components. Similarly, a table contains many more molecules than a yeast cell, yet is far less complex. A table cannot catalyse a process against its thermodynamically preferred direction, such as the H^+-ATPase can do when presented with a proper electrochemical potential difference for protons. The 10 nm enzyme is clearly more complex than the 1 m sized table.

We define Complexity as the new properties that arise in the interaction of components. These properties should be new, i.e. different from the properties that are already present in the components. The whole of a system of components should differ from the sum of its parts, not merely be more than the sum of its parts. Accordingly, BioComplexity is the difference between a biological system and the sum of its components.

Some examples may help here. Consider two identical television sets. Dismantle one of them into its transistors and resistors, etc. and put the result in a box. Now compare the box with components to the remaining intact television set. On the one hand, the box with components and the television set appear to be the same, because they consist of the same components. On the other hand, the two have virtually nothing to do with one another. The box with resistors and transistors has no relevant functionality whatsoever. Indeed, most of the essence of a television set is not in its transistors and resistors but in the way they have been hooked up to each other. A second example is provided by the enzyme hexokinase, which transfers a phosphoryl group from ATP to glucose, which is an important function for living cells. Now add a protease and compare the intact hexokinase to the set of resultant amino acids. One will readily agree that the intact hexokinase is much more than, and widely different from the amino acids of which it is composed.

It should be noted that the functionality of the object under study is important in the definition of complexity. The intact television set is more complex only if one likes to watch television; the hexokinase is more complex only if it is relevant to couple ATP hydrolysis to glucose phosphorylation.

3. BioComplexity need not be complicated

It is important to note that although a complex system differs from the sum of its components, the

system is not necessarily more complicated that its components. A simple example is that of the H^+-ATPase. Its consists of many amino acids. A molecular-dynamics simulation will show that these amino acid residues and their atoms move about rapidly in a three-dimensional space, leading to a movement that can be fully characterised only by taking refuge to a space with an enormous number of dimensions. Yet, for the H^+-ATPase to function, it is only important that ATP hydrolysis and proton movement are coupled to some extent. For the complex function in the cell, it suffices to know how the two fluxes catalysed by the enzyme depend on the concentrations of ATP, ADP, phosphate and on the electrochemical potential difference for protons.

In general, objects that consist of many components have the tendency to be complicated. The complications often disappear, once one is able to see which properties really matter for functional behaviour and which properties are simple corollaries. Perhaps the simplest example here is the balloon of gas molecules. The molecules in the balloon all have a position and velocity in space at any one moment and this gives rise to a tremendous amount of information. However, all this information is irrelevant for the behaviour of the balloon. It suffices to know a limited number of macroscopic properties, such as pressure, temperature and volume, to understand the functional behaviour of the balloon.

4. Redefining (??) nonequilibrium thermodynamics

Statistical thermodynamics has shown how molecular properties can be related to the macroscopic properties of an object such as a balloon. As it will be important to accomplish the same for the living cell, we think that some sort of thermodynamic approach will also be needed here. Here, it also makes no sense to understand function in terms of the simultaneous detailed behaviour of all the amino acids (including those that are part of the proteins) in the cell. In fact, in most of our analyses of cell function, some sort of thermodynamic approach is already used; we analyse the behaviour of cells in terms of concentrations of the proteins rather than of their constituent amino acids. Low molecular-weight molecules are treated in terms of their concentrations, rather than in terms of their individual space and velocity co-ordinates, as functions of time.

Of course, the thermodynamics needed here should not be limited to equilibrium or near equilibrium systems. It should deal explicitly with the nonequilibrium nature of the living cell, without necessarily moving all the way to the dynamics of systems that exhibit deterministic chaos. We here propose to redefine nonequilibrium thermodynamics as the science that aims at understanding the behaviour of nonequilibrium systems by taking into account both the molecular properties and the emergent properties, which are due to (dys)organisation. Mosaic nonequilibrium thermodynamics [1] and metabolic control analysis [2] are both engulfed by this definition. Surely, with this new definition, biothermodynamics is extended into a new and challenging field.

5. How can one analyse BioComplexity in practice?

Although it has been realised that molecules may function differently in the intact cell, as compared to the test tube, most of the successes of biochemistry have come from precisely the adverse strategy. This strategy forgets about that difference and isolates the molecule of interest and studies it in the test tube because, in such a test-tube experiment, the conditions can be controlled readily and because the analysis is not confounded by actions of other molecules. Hence, this strategy has greatly helped in increasing our understanding of biomolecules. To understand the living cell, however, additional experiments with the intact cell will be needed. Such experiments have been notoriously difficult.

Here, we shall address one particular issue, i.e. to what extent does an enzyme control its own catalytic flux in vivo? To determine this, one should alter the number of active enzymes and, then, measure how this affects the flux through the reaction they catalyse. Clearly, the experiment cannot be carried out with the enzyme in isolation; this would lead to a trivial answer as the flux is proportional to the enzyme concentration. How should one carry out this experiment in vivo? Because of our interest in thermodynamics, we here direct the question to the key

enzyme for biothermodynamics: the H^+-ATPase of free-energy transducing membranes. We ask the question in relation to a simple organism that is accessible to molecular genetic and biochemical experimentation: *E. coli*.

How could one alter the number of active H^+-ATPases? Until fairly recently, the only tools available were the addition of inhibitors [3], or the use of point mutants with altered activities [4]. Inhibitors often have side effects and have a problem of penetration to their site of action. Point mutants may not only have changed in terms of V_{max}, but also in regulatory properties of the enzyme and this then introduces quite different control aspects [5].

Molecular genetics has not only revolutionised the analysis of individual genes, but has also provided tools to perform in vivo biochemistry. It has provided us with methods to alter the expression level of a gene without changing the kinetic properties of the gene product. The expression levels of enzymes may now be modulated experimentally by substituting a new promoter for the original one, giving rise to a reduced activity. One may also replace the original promoter with a promoter that can be modulated by the use of a repressor system and added inducer [6–8]. Alternatively, one may just mutate the existing promoter by a progressive exonuclease treatment followed by reconstruction. It is important to realise that this methodology now allows one to perform in vivo experiments without destroying cell physiology, especially if the expression is modulated around the wild-type level. It also allows one to compare the effect on cell function to the extent of modulation of the concentration of the enzyme under study. The relative magnitudes then indicate how strongly the enzyme controls the cell function.

Two points need to be addressed in these strategies. Firstly, when one adjusts the concentration of an intracellular enzyme in this way, this cannot be done instantaneously. Hence, the cell is able to readjust the expression of its other genes. Accordingly, one measures potentially the simultaneous effect of varying the concentration of many enzymes. This seems to be in conflict with the standard definition of flux control according to metabolic control analysis. This standard definition of flux control coefficients requires that the concentrations of the other enzymes in the system be held constant while modulating the concentration of interest. Secondly, one might wish to modulate the expression of the gene of interest in trans, (although on the chromosome to maintain a well-defined copy number) therewith maintaining the regulation of the expression of the normal gene. Because this does not allow down modulation of the expression to below the wild-type level, this has not been done in practice yet. What are the implications of this limitation? In the following, we shall analyze the system in detail and then address these two issues.

6. Metabolic control analysis

Metabolic control analysis is able to deal with BioComplexity arising in metabolic pathways. Indeed, one of its tenets is that the control of the flux through a pathway and, hence, through the enzymes involved in the pathway need not be controlled by that enzyme, nor by any single such enzyme. Control of flux and concentrations may be distributed in a subtle manner among the enzymes in the pathway [9,10]. Here, the subtlety refers to the possibility that the control need not be confined to a single rate-limiting step, nor be equally shared by all enzymes, nor reside in the enzyme that catalyses the reactions that is farthest from equilibrium [1]. The distribution of control depends precisely on the extent of nonlinearity of the kinetics of the participating enzymes with respect to the concentrations of their substrates, products and allosteric modifiers. The measure of this nonlinearity is the elasticity coefficient defined by:

$$\varepsilon_X^i = \left(\frac{\partial \ln v_i}{\partial \ln [X]}\right)_{\text{other variables constant}} \quad (1)$$

This coefficient is equal to 1 when the reaction rate varies in direct proportion to the concentration of the metabolite X and -1 when it varies inversely. Its deviation from ± 1 indicates the extent of nonlinearity.

These degrees of nonlinearity determine, for a metabolic pathway, the extent to which enzymes control the flux. We shall first rehearse the argument for the metabolic pathway of Fig. 1. To indicate that the control coefficients discussed here are limited to the metabolic pathway, we shall denote them by a

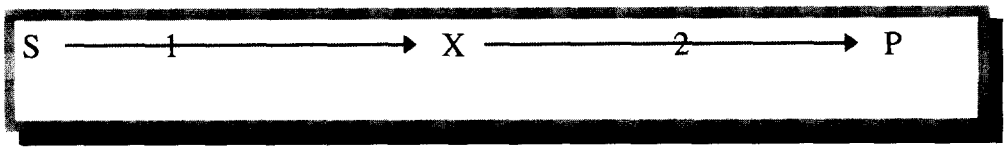

Fig. 1. Metabolic control in a two-step pathway. Enzyme 2 symbolises the H^+-ATPase, and enzyme 1 the electron transfer chain. Both enzymes 1 and 2 may be responsive ("elastic") towards the concentration of metabolite X (e.g. $\Delta\tilde{\mu}_{H^+}$, the proton-motive force).

lower case "c" (cf. [11]). For the control of enzyme 1 on the concentration of metabolite X one finds:

$$c_1^X \equiv \left\{\frac{d\ln[X]}{d\ln[e_1]}\right\}_{\text{steady state of metabolic pathway}} = -c_2^X \equiv \frac{1}{\varepsilon_X^2 + (-\varepsilon_X^1)} \quad (2)$$

ε_X^2 and $(-\varepsilon_X^1)$ are usually positive.

$$c_1^J \equiv \left\{\frac{d\ln[J]}{d\ln[e_1]}\right\}_{\text{steady state of metabolic pathway}} = \varepsilon_X^2 c_1^X \quad (3)$$

The control by enzyme 2 is equal but of opposite sign. For the flux control coefficients of the two enzymes one finds:

$$c_2^J = -\varepsilon_X^1 c_2^X \quad (4)$$

The control coefficients refer to the local control by the subscripted parameter on the superscripted variable. The ε's are elasticity coefficients quantifying the effect of the subscripted variable on the superscripted rate at constant magnitudes of all other factors that may affect that rate. When reaction 2 is in direct proportion to the concentration of its substrate and reaction 1 inversely proportional to the concentration of its product, then:

$$\varepsilon_X^2 \equiv -\varepsilon_X^1 = 1 \quad (5)$$

In this case the control on flux is shared equally between the two enzymes and the control of either enzyme on the concentration of X is 0.5 in absolute magnitude. In cases of nonlinearity (i.e. of elasticity coefficients deviating from these values), the control is unequally distributed between the enzymes.

From a physics point of view, the equal distribution of control may be most appealing in view of symmetry. However, functionally, depending on the position of the particular pathway in cell metabolism, it may be better to have more control in the demand reaction or more control in the supply reaction. Consequently, in biology one may expect elasticity coefficients to deviate from 1, because evolution has optimised for an unequal distribution of control among the enzymes in the pathway. It is noteworthy to see that the linear case here does not correspond to the paradigm that has dominated discussion of metabolic regulation for a long time, i.e. that there be a single rate limiting step. For such a case, the elasticities should differ drastically, at least one corresponding to a nonlinear concentration dependence of a reaction rate.

Westerhoff et al. [11] have discussed the interpretation of a measurement of the flux control by the H^+-ATPase in *E. coli*, with respect to growth rate. This control coefficient was close to zero [8,12], indicating nonlinearity and BioComplexity.

7. Hierarchical control analysis

That BioComplexity may be operative in *E. coli* energetics is transparent from the fact that, in the intact cell, the concentrations of the enzymes that are involved in the free-energy transduction are subject to control by gene expression. Fig. 2 indicates this for a simple model. The transcription rate of the operon leading to the synthesis of enzyme 1 (in this model transcription and translation have been agglomerated for simplicity; for a more general treatment see [13]) may be elastic (sensitive) to the concentration of metabolite X. In this case, the control hierarchy is "democratic" [14], as indicated by the dashed arrow leading from X to the rate constant of transcription of the first operon, k_{t1}. Similarly, transcription of operon 2, which encodes enzyme 2, may be subject to regulation through the concentration of the metabolic intermediate. Also these influences can be quantified

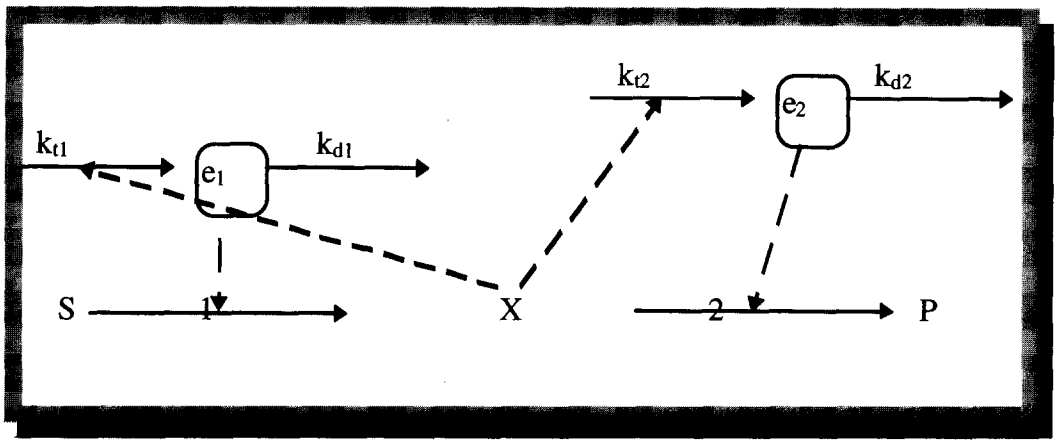

Fig. 2. Scheme of hierarchical control system. Enzymes 1 and 2 catalysing reactions 1 and 2 of the bottom metabolic pathway are each produced by transcription and subject to degradation. Flows are indicated by full arrows, influences by dashed arrows.

in terms of elasticity coefficients, e.g.:

$$\varepsilon_X^{t1} = \left(\frac{\partial \ln v_{t1}}{\partial \ln[X]}\right)_{\text{other variables constant}} \tag{6}$$

v_{t1} refers to the transcription rate of operon 1.

Whenever the network of interactions is known, control and co-response coefficients can be expressed into the kinetic properties of the enzymes in the system. We shall here do this for the simplified case of Fig. 2 and then analyse co-response and control in a democratically controlled system. In Fig. 2, three levels may be recognised. These are defined as parts of the network that are autonomous in the sense that there is no flux between them, although they may influence each other by allosteric interactions. These levels correspond to the metabolic level around X, the gene expression level around enzyme e_1 and the gene expression level around enzyme e_2. Each of these levels may first be treated individually, leaving the regulatory interaction with the other levels out of consideration. This will lead to control coefficients that are local with respect to these levels [13]. We shall continue to denote these control coefficients by the lower case "c". The control coefficients that do take into account the effects of the regulatory interactions between the three levels will be denoted by capital "C"2 (In [15], the local control coefficients were denoted by capital C, the global control coefficients by capital C′). The local control coefficients can be expressed readily into elasticity coefficients by standard methods [1]. For the control by the transcription rate constants on the concentrations of the two enzymes one finds:

$$c_{k_{t1}}^{e_1} = \frac{1}{\varepsilon_{e_1}^{d_1} + (-\varepsilon_{e_1}^{t_1})} \tag{7}$$

Assuming that transcription/translation is not product sensitive in the sense of being directly inhibited by the mRNA or protein produced, implies that the latter elasticity coefficient equals zero. We shall also assume that the protein is degraded in a first-order process, which implies that the former elasticity equals 1. Hence:

$$c_{k_{t1}}^{e_1} = c_{k_{t2}}^{e_2} = -c_{k_{d1}}^{e_1} = -c_{k_{d2}}^{e_2} = 1 \tag{8}$$

For the metabolic level, the values for the elasticity coefficients may be different. The local control coefficients are the same as indicated above (Eqs. (2)–(4)).

The above equations apply to the case in which control is dictatorial, i.e. in which there is no back pressure effect from the metabolic level on transcription. In this case, the transcription rate constant completely controls the concentration of the corresponding enzyme; the enzyme concentration should increase proportionally with that rate constant. The rate constant of degradation should have a similarly complete but negative control; the enzyme concentration should be inversely proportional to the magnitude of that rate constant.

In a democratic control hierarchy, however, X affects the rate of transcription, and because the concentrations of the enzymes affect the steady-state concentration of X, this modulates the effect of an increase in the transcription rate constant on the concentration of the enzymes. We shall now examine the effect of this modulation. Focussing on the level of enzyme 1, we note that the transcription rate is modulated in two ways. First, by the direct activation of the transcription rate constant and, second, by the variation in X that results from the altered transcription rate and altered concentration of enzyme 1. Hence:

$$C^{e_1}_{k_{t1}} = \frac{\mathrm{d}\ln[e_1]}{\mathrm{d}\ln k_{t1}} = \frac{\partial \ln[e_1]}{\partial \ln k_{t1}} + \frac{\partial \ln[e_1]}{\partial \ln X}\frac{\mathrm{d}\ln[X]}{\mathrm{d}\ln k_{t1}} = \frac{\mathrm{d}\ln[e_1]}{\mathrm{d}\ln k_{t1}} + \frac{\partial \ln[e_1]}{\partial \ln k_{t1}}\frac{\partial \ln v_{t1}}{\partial \ln X}\frac{\mathrm{d}\ln[X]}{\mathrm{d}\ln k_{t1}} = 1 + \varepsilon^{t_1}_X C^X_{k_{t1}} \quad (9)$$

The control coefficient $C^X_{k_{t1}}$ quantifies the extent to which the transcription rate constant for enzyme 1 controls the steady-state concentration of X. This control follows two routes. The first is the most obvious one: the concentration of enzyme 1 increases and affects $[X]$ through the control exerted by reaction 1 on that concentration. Less obvious is the fact that, because the concentration of X may also change, the transcription of the gene encoding enzyme 2 may change. The concomitant change in concentration of enzyme 2 may affect $[X]$, because reaction 2 also controls the latter concentration at steady state:

$$C^X_{k_{t1}} = \frac{\mathrm{d}\ln[X]}{\mathrm{d}\ln k_{t1}} = c^X_1 C^{e_1}_{k_{t1}} + c^X_2 C^{e_2}_{k_{t1}} \quad (10)$$

The rate constant for transcription of the operon encoding enzyme 1 can only affect the concentration of enzyme 2 through its effect on the concentration of X. Hence:

$$C^{e_2}_{k_{t1}} = \frac{\mathrm{d}\ln[e_2]}{\mathrm{d}\ln k_{t1}} = \varepsilon^{t2}_X C^X_{k_{t1}} \quad (11)$$

Counting the elasticity coefficients and the lower-case control coefficients as knowns, the above constitute three equations in terms of three unknowns. Solving these for the control exerted by the transcription rate constant of operon 1 on the concentration of enzyme 1, one obtains:

$$C^{e_1}_{k_{t1}} = \frac{1 - \varepsilon^{t_2}_X c^X_2}{1 - \varepsilon^{t_2}_X c^X_2 - \varepsilon^{t_1}_X c^X_1} = 1 - \frac{-\varepsilon^{t_1}_X}{-\varepsilon^{t_1}_X + \varepsilon^{t_2}_X - \varepsilon^1_X + \varepsilon^2_X} \quad (12)$$

It is of interest to compare this to the control of 100% (1) exerted by the transcription rate constant on the concentration of the enzyme in the dictatorial control system, where transcription is insensitive to the events at the metabolic level. Indeed, if we equate the effect of $[X]$ on transcription ($\varepsilon^{t_2}_X$) to zero, the above control becomes equal to 1. When X affects the rate of transcription, the transcription control of the concentration of enzyme 2 is mollified. In the homeostatic case, $\varepsilon^{t_1}_X$ and c^X_2 are negative, whilst $\varepsilon^{t_2}_X$ and c^X_1 are positive. Consequently, the control by transcription on the concentration of enzyme 1 is complete (i.e. equals 1) save the regulation of transcription of that gene by X (quantified as $-\varepsilon^{t_1}_X$), relative to all regulations by X in the system (the denominator in the expression).

For the control by the transcription rate constant on the concentration of metabolite X one finds:

$$C^X_{k_{t1}} = \frac{1}{-\varepsilon^{t_1}_X + \varepsilon^{t_2}_X - \varepsilon^1_X + \varepsilon^2_X} \quad (13)$$

Here, one recognises the phenomenon that all elasticities in the system contribute to the dynamic buffering of metabolite concentrations: as any of the elasticities increases, the control of the metabolite concentration by the transcription rate constants decreases. Comparison with the control exerted by enzyme 1 on $[X]$ in the metabolic system (as quantified by Eq. (2)) shows that the control is smaller in the case of the democratic hierarchy, because now also the regulation of transcription by X has a homeostatic effect.

Because of the complexity of the system, the rate constant of transcription of operon 1 also controls, to some extent, the concentration of enzyme 2. This influence runs through the concentration of metabolite X and its effect on the transcription rate constant of operon 2:

$$C^{e_2}_{k_{t1}} = \frac{\varepsilon^{t_2}_X}{-\varepsilon^{t_1}_X + \varepsilon^{t_2}_X - \varepsilon^1_X + \varepsilon^2_X} \quad (14)$$

By controlling the concentration of enzyme 1, the

transcription rate constant k_{t1} will also affect the metabolic flux. The magnitude of this effect is given by the control coefficient of the flux with respect to that transcription rate constant:

$$C^J_{k_{t1}} = \frac{\mathrm{d}\ln J}{\mathrm{d}\ln k_{t1}} = \frac{\partial \ln J}{\partial \ln e_1}\frac{\mathrm{d}\ln[e_1]}{\mathrm{d}\ln k_{t1}} + \frac{\partial \ln J}{\partial \ln e_2}\frac{\mathrm{d}\ln[e_2]}{\mathrm{d}\ln k_{t1}}$$
$$= c^J_1 C^{e_1}_{k_{t1}} + c^J_2 C^{e_2}_{k_{t1}}$$
$$= c^J_1 C^{e_1}_{k_{t1}}\left(1 + \frac{-\varepsilon^1_X}{\varepsilon^2_X}\frac{\varepsilon^{t_2}_X}{\varepsilon^2_X - \varepsilon^1_X + \varepsilon^{t_2}_X}\right) \quad (15)$$

This equation shows that the control exerted by the transcription rate constant on the metabolic flux is equal to the metabolic control coefficient of that enzyme, multiplied by the control of the transcription rate constant on the enzyme level in the entire system and then again modified by a factor. Indeed, the regulation and control is complex.

A combination of the foregoing equations leads to an expression for the co-response coefficient of the metabolic flux with respect to enzyme 1 under modulation of the transcription rate constant of enzyme 1 (cf. Ref. [11]:

$${}^{t1}O^J_{e_1} = \frac{C^J_{k_{t1}}}{C^{e_1}_{k_{t1}}} = c^J_1\left(1 + \frac{-\varepsilon^1_X}{\varepsilon^2_X}\frac{\varepsilon^{t_2}_X}{\varepsilon^2_X - \varepsilon^1_X + \varepsilon^{t_2}_X}\right) \quad (16)$$

The co-response coefficient [16] gives the relative change in one variable divided by the relative change in a second variable as induced by the modulation of a parameter (indicated at the left hand top). As discussed by Westerhoff et al. [11], this is the property that has actually been measured by Jensen et al. [8]. If the operon of the other enzyme in the metabolic system is controlled dictatorially, or if enzyme 1 has most of the control, then this co-response coefficient is quite a good measure of the metabolic control coefficient c^J_1.

Here, we may address the second issue raised above. What are the consequences for the experiments if one eliminates the endogenous promoter of the operon one modulates. The above equation for the co-response coefficient lacks the transcription elasticity of operon 1. Consequently, there should be no implication for the value of the co-response coefficient one measures. From the expression for the control coefficient of the transcription rate constant (and for the control coefficient of the metabolic process itself), one can see that these will be affected by the elasticity of the corresponding operon for X. Hence, if that elasticity is removed, the hierarchical control coefficients will be affected.

Having derived the expressions for the control exerted by the transcription rate constant, we may do the same for the rate constant characterising either of the metabolic reactions. As described by Westerhoff et al. [11], the control by reaction 1 on any system variable may be defined in terms of the steady-state effect of a proportional modulation of both the forward and the reverse V_{max} of this step, without modulating the enzyme concentration (which is a variable in the case of a democratic hierarchy, hence cannot be modulated by the experimenter). Denoting these control coefficients by subscript 1, one finds the following relations between them:

$$C^X_1 = c^X_1(1 + C^{e_1}_1) + c^X_2 C^{e_2}_1 \quad (17)$$
$$C^{e_1}_1 = \varepsilon^{t_1}_X C^X_1 \quad (18)$$
$$C^{e_2}_1 = \varepsilon^{t_2}_X C^X_1 \quad (19)$$

Solving these equations one obtains:

$$C^X_1 = C^X_{k_{t1}} \quad (20)$$
$$C^{e_1}_1 = C^{e_1}_{k_{t1}} - 1 \quad (21)$$
$$C^{e_2}_1 = C^{e_2}_{k_{t1}} \quad (22)$$

For the control of enzyme 1 on the metabolic flux, one finds:

$$C^J_1 = c^J_1(1 + C^{e_1}_1) + c^J_2 C^{e_2}_1 = C^J_{k_{t1}} \quad (23)$$

Clearly for all but one of these four, the control coefficient by the metabolic reaction rate equals that by the corresponding transcription rate constant. This is because the model assumes the transcription rate to have a proportional effect on protein concentration in the absence of back regulation by X, and because the regulatory effect of an increased concentration of an enzyme only runs through the altered metabolite concentration it generates. There is no direct protein burden effect in this model (cf. Ref. [17].

We are now in a position to answer the question concerning the validity of performing control analysis in a system with varying gene expression. The control coefficient measured in such a case, i.e. C^J_1. differs from the metabolic control coefficient c^J_1, as indicated

by the above equations. Yet it is a valid control coefficient in its own right. It quantifies what is relevant to the cell, the regulation at the metabolic level plus the regulation at the gene expression level. Indeed, the difference between this hierarchical control coefficient and the metabolic control coefficient (which might be measured by considering the rapid effect of an inhibitor) should shed light on the importance of regulated gene expression.

8. Theorems

Metabolic control analysis contains laws that govern the control of fluxes and concentrations. These can be formulated as relationships that need to be fulfilled by the control and the elasticity coefficients. Kahn and Westerhoff [13] have derived similar laws for hierarchical systems. For the system under study here, these can be checked on the basis of the above equations. First, one finds that the summation theorem for concentration control coefficients applies both to the local and to the hierarchical control coefficients. For the control of the concentration of the metabolic intermediates these read:

$$C^X_{k_{t1}} + C^X_{k_{t2}} = C^X_1 + C^X_2 = 0 = c^X_1 + c^X_2 \qquad (24)$$

For the control on the concentration of enzyme 1:

$$C^{e_1}_{k_{t1}} + C^{e_1}_{k_{t2}} - 1 = C^{e_1}_1 + C^{e_1}_2 = 0 \qquad (25)$$

For the control on the metabolic flux:

$$C^J_{k_{t1}} + C^J_{k_{t2}} = C^J_1 + C^J_2 = 1 = c^J_1 + c^J_2 \qquad (26)$$

In terms of the control by the degradation rate constants, there are additional summation theorems.

The connectivity theorems also apply. Interestingly, for the hierarchical control coefficients, not only the metabolic elasticity coefficients appear in these theorems, but also the elasticity coefficients with respect to the corresponding gene expression regulation:

$$C^X_1(\varepsilon^1_X + \varepsilon^{t_1}_X) + C^X_2(\varepsilon^2_X + \varepsilon^{t_2}_X) = -1 = c^X_1\varepsilon^1_X + c^X_2\varepsilon^2_X \qquad (27)$$

$$C^J_1(\varepsilon^1_X + \varepsilon^{t_1}_X) + C^J_2(\varepsilon^2_X + \varepsilon^{t_2}_X) = 0 = c^J_1\varepsilon^1_X + c^J_2\varepsilon^2_X \qquad (28)$$

As a consequence, the expressions for the hierarchical control coefficients in terms of the elasticity coefficients are quite similar to the expression for the metabolic control coefficients in terms of the elasticity coefficients. The only difference is an extra elasticity quantifying the extra regulation through gene expression. Thus, for the control exerted by enzyme 2 on the metabolic flux, one finds:

$$C^J_2 = \frac{-\varepsilon^1_X - \varepsilon^{t_1}_X}{\varepsilon^2_X + \varepsilon^{t_2}_X - \varepsilon^1_X - \varepsilon^{t_1}_X} \qquad (29)$$

This is a rather simple expression. Apparently, the effects of a hierarchy of regulation processes of cellular free-energy transduction can be analysed in fairly simple terms by an appropriate generalised nonequilibrium thermodynamic approach, which we have called hierarchical control analysis.

9. Concluding remarks

We have shown that the BioComplexity of the living cell can be analysed by a combination of noninvasive, molecular genetic experiments with a quantitative interpretation using methods deriving from metabolic control analysis. The equations derived for an example show how the various regulatory interaction together yield a subtly controlled cell function.

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