REVIEWS

Towards a rational approach to the optimization of flux in microbial biotransformations

Douglas B. Kell and Hans V. Westerhoff

Many historical attempts to increase the yield of biotechnological processes have been at best semi-empirical. However, given the availability of modern techniques of genetic and protein engineering, the question arises as to how one might rationally seek to choose the most suitable genes to clone and/or modify for this purpose. The metabolic control theory of Kacser, Burns, Heinrich and Rapoport allows one to decide quantitatively which enzymatic steps are (most) rate-determining to the flux through desired pathways (and why). An extension of these principles allows one rationally to identify optimal strategies for the improvement of microbial processes.

The maximization of the flux through a particular metabolic pathway in a microbial cell suspension represents the primary focus of many areas of biotechnology. Yet the selection, construction and study of commercially important strains has often been at best semi-empirical. In this article we wish to draw attention to the metabolic control theory developed by Kacser, Burns, Heinrich and Rapoport, and to indicate how it offers a *rational* approach to the improvement of microbial biotransformations. Whilst the theory may in fact be applied to virtually any type of system, we shall concentrate on steady-state systems in which a 'cheap' substrate is converted into a value-added 'product'.

It is convenient to consider a generalized, unbranched metabolic pathway of the type $A \rightarrow B \rightarrow C \rightarrow D \rightarrow E \rightarrow F$, where each step is taken to be catalysed by an enzyme (e_1 to e_5). In a typical arrangement, the concentration of A is sufficient to saturate enzyme 1. It is traditional, and indeed natural, to ask the question 'which enzyme is rate-limiting to the flux (and why)?', with the implicit assumption that the next step in a programme of mutation and selection, or (more recently) of strain construction by genetic or protein engineering, might most fruitfully be aimed at that enzyme. Furthermore, numerous informal discussions have indicated to us that the total productivity of a variety of industrial antibiotic fermentations has now approached an apparent asymptote of some 30–50 g l^{-1} in batch cultures, although such data are almost impossible to find in published form. It is therefore reasonable to argue that novel approaches will be required substantially to increase such yields; given also the modern ability to engineer strains genetically for these purposes[1,2], the question of *what* to clone (and/or to modify by protein engineering) takes on a sharper focus.

D. B. Kell is at the Department of Botany and Microbiology, University College of Wales, Aberystwyth, Dyfed SY23 3DA, UK; H. V. Westerhoff is at the Laboratory of Molecular Biology, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA.

We shall see that a consideration of the relationship between (1) the activity of a particular enzyme *in vitro* and (2) the flux through a metabolic pathway of which it is a part *in vivo* leads to the conclusions (a) that the two are in many cases only weakly related and (b) that the concept of 'the rate-limiting step' or of a 'bottleneck enzyme' is only rarely worthwhile. We may illustrate this with reference to Fig. 1, which shows three possible outcomes of an (hypothetical) experiment in which, for various, otherwise isogenic strains, the flux through a particular pathway is plotted versus the amount of one of the constituent enzymes of that pathway, each normalized to the amount in a control, wild-type strain. In case (a), halving or doubling the enzyme concentration hardly affects the pathway flux; our enzyme would not seem to be catalysing the rate-limiting step. In case (b), halving or doubling the concentration of our enzyme changes the flux J in direct proportion; we would then be inclined to identify our enzyme as the rate-limiting step. However, case (c) would, in traditional terms, present us with something of a problem; clearly our enzyme is not as rate-limiting as in case (b) yet it is more rate-limiting than in case (a)! A two-valued logic system (rate-limiting/non-rate-limiting) is quite inappropriate for our problem.

Why is this important when we are about to engineer a strain for the purpose of increasing the flux through one of the metabolic pathways? Well, the view of metabolic control with which many of us were brought up, was that a metabolic pathway has a single rate-limiting step, usually at the beginning (of the relevant branch). Consequently, we might be tempted to direct all our attention to engineering the first enzyme in the metabolic pathway. More probably, we would be cautious enough first to establish whether the first enzyme (or, perhaps, the second) was the rate-limiting one. We might find an inhibitor of that enzyme and titrate its effect on the pathway flux. If, as in case (b) of Fig. 1, we were to find that inhibition of the enzyme reduced the pathway flux, we would conclude that the enzyme is indeed the rate-limiting enzyme. However, according to this criterion,

© 1986, Elsevier Science Publishers B.V., Amsterdam 0166 – 9430/86/$02.00

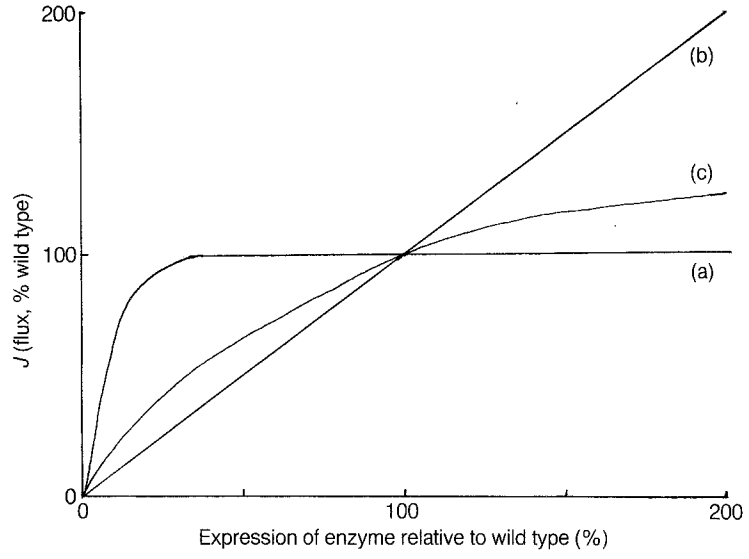
the enzyme in case (c) would also be rate-limiting. Clearly, the difference in behaviour in cases (b) and (c) would suggest that the enzyme in case (c) is less rate-limiting and hence not *the* rate-limiting enzyme. We would, therefore, like to be able to quantify the degree to which an enzyme under study is rate-limiting. The concept of **flux-control coefficients** allows us to do this.

Flux-control coefficients

Kacser and Burns[3,4] and Heinrich and Rapoport[5,6] introduced the flux-control coefficient $C^J_{e_i}$ to express *in quantitative terms* the degree to which an enzyme e_i was controlling the pathway flux. To make $C^J_{e_i}$ dimensionless, it is defined as $(dJ/J)/(d[e]/[e])_{SS}$, that is the fractional change in J (pathway flux) divided by the fractional change in [e] (enzyme concentration or activity) when these changes are made (infinitesimally) small. The subscript SS denotes that these measurements are made under steady-state conditions. Since $(dJ/J)/(d[e]/[e])$ is mathematically identical to $(d \ln J)/(d \ln [e])$, the flux-control coefficient may be obtained as the slope of a plot of J versus [e] on log–log paper, at the value of (J,e) prevailing. As in Fig. 1, $C^J_{e_i}$ will most often take a value between zero (or 0%; case a) and unity (or 100%; case b); in case (c) it turns out to have a value of approximately 0.53.

We may determine values of the flux-control coefficient for each enzyme in a pathway, and it may be proven algebraically that, provided intermediary metabolites (and allosteric modifiers) exhibit pool behaviour, the sum of the flux-control coefficients in a metabolic pathway equals unity. This is known as the **flux-control summation theorem**[3,6]. Thus we may assign to *each* enzyme in a metabolic pathway of interest a *quantitative* measure of the extent to which it takes part in controlling the flux. We may also define flux-control coefficients for pathway substrates and for external effectors (e.g. inhibitors), using exactly the same type of derivation based on fractional changes. However, for reasons of space, we here refer readers to several reviews[7–10] which give a much fuller exposition and which use (as herein) the standardized terminology recently agreed upon[11] by the originators of the metabolic control theory.

Fig. 1



Possible effects of the concentration, modulated by genetic means, of an enzyme on the flux through a pathway of which it is a component. For discussion, see text.

The importance of the flux control summation theorem

Why is the flux-control summation theorem so important when devising a strategy for strain improvement? Well, if we were to find that enzyme (c) (Fig. 1) has a flux-control coefficient of, say, 0.20 it would make us continue the search for where the rest $(1 - 0.2 = 0.8)$ of the flux control lay. If it were to be in a single enzyme, then that enzyme would be a much better candidate for genetic engineering, simply because the same fractional amplification of its concentration would produce a (0.8/0.2) fourfold greater increase in the pathway flux. In general, one would determine the distribution of the flux control between all enzymes in (or interacting with) the pathway and then concentrate on the enzyme with the highest flux control. (If parameters other than enzyme concentration, for instance K_m or k_{cat}, are to be engineered, the problem becomes somewhat more involved, but remains perfectly tractable, as we have recently shown (Ref. 9 and Westerhoff and Kell, submitted).)

It should be noted that the present type of analysis in no way runs counter to experience. In all the ('natural') metabolic pathways that have been investigated in this way, the flux control is distributed over at least two enzymes. Usually equipartition of the control is not observed[12,13]; some enzymes have more control than others. Especially in metabolic pathways with feedback inhibition, feed-forward stimulation or futile cycles, a rather unexpected candidate may be manifest as the most rate-limiting enzyme[8,10].

Concentration-control coefficients

The steady-state concentrations of metabolic intermediates are also dependent upon the activities of enzymes; the control theory defines concentration-control coefficients for each metabolite of interest. The concentration-control coefficient $C^X_{e_i}$ of enzyme e_i on metabolite X is $(d[X]/[X])/(d[e_i]/[e_i]_{SS}$, where, as in all of the coefficients discussed herein, the subscript refers to the potential *cause* and the superscript to the *effect* (i.e. the part of the system affected). Concentration-control coefficients are also related by a summation theorem; the sum of the concentration-control coefficients of enzymes i ($i = 1$ to n) on metabolite X is in this case zero[6], such that some enzymes will have *negative* concentration-control coefficients. The biotechnological importance of the concentration-control coefficients is most evident in cases[14] where the desired product is not secreted but remains intracellular.

Elasticity coefficients

The rates of turnover of individual enzymes are a function of the concentration of their own substrates, products and allosteric effectors. The metabolic control theory describes these relationships as elasticity coefficients. The elasticity ϵ^i_Y of enzyme e_i towards metabolite (or effector) Y is defined as the fractional change in enzyme turnover divided by the fractional change in the concentration of metabolite (or effector) Y, *under conditions in which the concentrations of all other (parameters and) variables are held constant at their* in-vivo *magnitudes.* Each enzyme will exhibit elasticity coefficients with respect to all substrates, internal or external effectors (e.g. inhibitors) and to other parameters of the system such as Michaelis constants. Elasticities differ from control coefficients in the important respect that they are properties of *individual* enzymes when isolated (either structurally or functionally) from the rest of the metabolic network (but incubated under the conditions prevailing *in vivo*), whilst control coefficients reflect the behaviour of *the whole system* when it is allowed to relax between (asymptotically stable) steady states. Thus elasticities are *local* properties (and therefore defined by partial derivatives) whilst control coefficients are *global* properties (and defined as total derivatives). In a biotechnological context, the flux-control coefficients tell us which enzymes are most rate-determining whilst the elasticities contain the mechanistic information determining *why* they possess such a rate-limitation. Elasticities and control coefficients are, not surprisingly, related to each other.

Connectivities between the control distribution and enzyme properties

The metabolic control theory also delineates the relationships between the control and elasticity coefficients. Qualitatively, enzymes which have low elasticities tend to have high flux-control coefficients: the initial effect of adding a small concentration of an irreversible inhibitor to an enzyme will be an increase in the concentration of its substrate. This will either lead to a significant increase in the enzyme's velocity ('if [S] < K_m'; high elasticity) so as almost to restore the original pathway flux (the enzyme therefore displaying a low flux-control coefficient) or will fail to do so ('if [S] ≫ K_m'; low elasticity and high flux-control coefficient). Quantitatively, we have the flux-control connectivity theorem, which states that the sum of the products of the flux-control coefficients of a series of enzymes and their elasticities towards a given internal effector equals zero, that is $\Sigma C^J_{e_i} \epsilon^i_X = 0$ (Ref. 3). Other connectivity theorems relate metabolite concentration-control coefficients to elasticity coefficients[15].

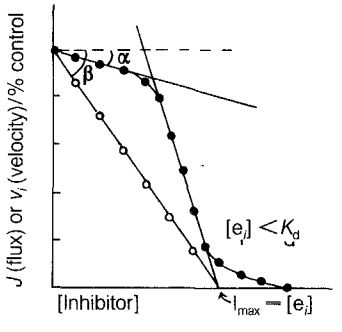
The connectivities between control coefficients and elasticity coefficients are important as they allow one to find the reason (in terms of enzymatic properties, i.e. the elasticities) *why* certain enzymes have high or low flux-control coefficients; in unbranched pathways the connectivity theorems allow one to express the control coefficients in terms of the elasticities. In branched pathways additional 'branching' theorems are needed and the flux ratio at the branch points enters the equations (Refs 6, 9, 10, 16 and Westerhoff and Kell, submitted). The algebra has become relatively trivial due to the recent elaboration of a new set of algorithms (Refs 10, 16 and Westerhoff and Kell, submitted).

There is an especially important and experimentally useful relationship between the effect of a reasonably specific and tight-binding external effector (e.g. inhibitor I) on a given 'target' enzyme and on the pathway flux: $C^J_I = C^J_{e_i} \epsilon^i_I$. This equation provides one means for estimating the values of flux-control coefficients.

Experimental estimation of flux-control coefficients

If one plots (the initial stages of) a titration curve of both the steady-state pathway flux and the 'isolated' activity of a 'target' enzyme versus the inhibitor concentration (on the same scale), the ratio of the tangents of the angles between the titration curve and the horizontal line passing through the flux (velocity) at an inhibitor concentration of zero is equal to the flux-control coefficient (Fig. 2; Refs 7, 9 and Westerhoff and Kell, submitted). Other means of obtaining experimental values for the flux-control coefficients include: (a) the use of a series of mutant strains expressing different levels of the enzyme of interest[17], (b) addition of exogenous enzymes (or analogous catalysts) to metabolic pathways reconstituted partially or wholly *in vitro*[7,18], and (c) the construction of strains in which the structural gene for the enzyme of interest is placed under the control of an appropriately inducible promoter such as the *tac* promoter[19]. (The pioneering experiments of Groen *et al.*[7] and of Walsh and Koshland[19] also serve to stress the important point that the flux-control coefficients are not constants but are very likely strongly to vary with the environmental conditions.) In addition, it is possible to express the flux-control coefficients in terms of the

Fig. 2



Estimation of the flux-control coefficient $C^J_{e_i}$ of an enzyme e_i by the use of a tight-binding and specific inhibitor of that enzyme[7,9,12]. In this approach, both the normalized pathway flux (J, •) and the normalized velocity of the (functionally) isolated enzyme (v_i, ○) are titrated using a tight-binding, stoichiometric and specific inhibitor of the enzyme. In this case, the initial concentration of enzyme may be obtained from the extrapolated titration curve (allowing for the fact that at very low enzyme concentrations the dissociation constant of the enzyme/inhibitor complex must be taken into account). The flux-control coefficient of the enzyme is given by tan α/tan β.

elasticity coefficients, and Fell and Sauro[16] and the present authors (submitted) have presented a matrix method which allows one to derive flux-control and concentration-control coeffcients solely from the known structure of, and the elasticities of the enzymes in, the metabolic pathway(s) of interest. It should be noted that enzymes located after a branch point will tend to have negative flux-control coefficients with respect to the flux towards the other branch(es), in that stimulating their activity will tend to decrease the flux through the other branch(es).

Measurement of elasticity coefficients

If the enzymic rate equation is known one may obtain the elasticity coefficient by differentiating this equation with respect to the effector of interest[6,7]. Experimentally, one should best attempt to measure the elasticities directly *in vivo* or, more conveniently[18], by incubating part or all of the system under the appropriate conditions *in vitro*. Given the recent development of cell-free systems for the study of secondary metabolite production[20,21], we may assume that this latter approach will become increasingly applicable in biotechnology.

Two correlates of the metabolic control theory

The metabolic control theory gives a fundamental and satisfying explanation for at least two phenomena commonly encountered in biotechnological work: (a) the fact that a great many rounds of mutation and selection are required to obtain productive strains, and (b) that the (total) productivity of many antibiotic fermentations correlates much more with the activity (*in vitro*) of the enzymes of secondary metabolism than with the concentration of appropriate precursors and intermediates[21].

The first finding follows from the flux-control summation theorem: since the sum of the flux-control coefficients equals one, and control is shared among many enzymes, the flux-control coefficient of any given enzyme, especially in a long pathway, will tend to be small[22]. (By contrast, short pathways or sections thereof are likely to contain enzymes exhibiting high flux-control coefficients; in such a case fermentor productivity may be rather directly related to the activity of an individual enzyme, as appears to be the case with IMP dehydrogenase in the guanosine fermentation, for instance[23].) Thus, not only will the rate control by any given enzyme in general be small, but control will continually be shifting between different enzymes as the programme of mutation and selection proceeds. The second finding follows from the fact that (1) the concentrations of most metabolites are variables and not parameters (i.e. they are set by the complement and properties of the cellular enzymes) and (2) from the definition of the flux-control coefficient, flux control is in all cases related to the absolute amount of enzyme present.

Alterations of enzymic properties other than their concentrations

When the goal is to increase the flux through a given metabolic pathway of an organism, one has in principle the possibilities not only of increasing the intracellular *concentration* of enzymes in the pathway but also to alter the *kinetic properties* of those enzymes. In the ideal case, one would know which K_m values and enzyme concentrations might be engineered and by what percentage. With respect to the enzyme concentrations, the choice between the alternatives will be determined by the mathematical product of the percentage change in the enzyme concentration achievable and the flux-control coefficient of that enzyme. The enzyme for which this product takes the highest value should be the one that is actually engineered[9,10].

With respect to the engineering of the values of K_m (or K_i if the enzyme has inhibitor-binding sites), one should multiply the achievable fractional change in K_m by the elasticity coefficient of the enzyme towards the relevant metabolite and then multiply the result by the flux-control coefficient of the enzyme. Again, the K_m for which this overall product is the greatest is the best candidate for the engineering. In this way one can assess the relative merits of changing the values of the inhibitor constants versus changing the concentration of an enzyme. These methods are described in detail elsewhere[9,10].

Absolute fluxes

Metabolic control theory considers (small) *changes* in fluxes, not the magnitudes of the fluxes themselves. The magnitudes of the fluxes are addressed by system theories which have rate equations in the integral form. Examples are the mosaic non-equilibrium thermodynamic method[24] and the biological system theory[25]. Because of the enormous kinetic complexity of even the smallest biological systems, such system theories are bound to be approximate (in contrast to the metabolic control theory, which, by limiting itself to small changes, can remain exact). For our present purpose, the absolute magnitudes of the metabolic fluxes are not fundamentally relevant, since we just consider the question of how they may be increased, regardless of their absolute magnitudes. There is one point, however, where the absolute magnitudes of the fluxes become important; that is when asking the question of whether they can be increased *at all*, in view of the possibility that they may already be taking place at their diffusion-controlled rates.

As mentioned above, a 'good' value for the total yield of a secondary metabolite fermentation might be some 40 g/l. However, as also pointed out by Suckling[20], if this takes five days to accomplish, the rate of production may only be about 1 μmol (litre of culture s)$^{-1}$, corresponding to a value probably not exceeding 10 nmol min^{-1} (mg protein)$^{-1}$. Even for pathways of primary metabolism, such as the production of ethanol by *Zymomonas mobilis*[26], the best (largest) rates are only some 3 μmol min^{-1} (mg protein)$^{-1}$. This corresponds (if glycolytic enzymes *in toto* constitute 10% of the cell protein and each has a M_r of 20 kDa) to a turnover number of some 100 s^{-1}, a number similar to those exhibited by other, even membranous, enzymes of primary metabolism such as the respiratory chain[27] and ATP synthase[28]. How do such values compare with the maximum values possible *in prin-*

ciple if the flux is limited by the rate at which metabolites can diffuse from one enzyme to the next?

The turnover number for a simple, irreversible ($K_{eq} \gg 1$) enzymatic reaction is $v/[E]_O = [S]_P \cdot k_{cat}/(K_m + [S]_P)$. Here $[E]_O$ is the total enzyme concentration, $[S]_P$ the substrate concentration *in vivo* and K_m the Michaelis constant, equal to $(k_{-1} + k_{cat}/k_1$. An upper limit for k_1 (the rate constant for formation of the enzyme–substrate complex) is the diffusion-limited rate constant k_D, that is the rate at which substrate and enzyme (S and E) would collide by free diffusion. Since the 'viscosity' of the aqueous cytoplasm is some tenfold greater than that of pure water[29,30] and the reduction in effectiveness due to collisions in the wrong orientation is reduced by the caging effect[31], it is resonable to take a value for k_D of $10^9\ M^{-1}\ s^{-1}$ (Ref. 32). Whereas k_1 is bounded by the physical limit imposed by diffusion, k_{-1} (the dissociation rate constant for the E–S complex) and k_{cat} (the rate constant for formation of product) are in principle subjected to unlimited optimization by evolutionary changes in the enzyme. The question then arises as to whether k_{-1} and k_{cat} are actually already optimized (maximized) in typical microorganisms, so that further optimization is impossible. Optimization of k_{-1} would imply that this rate constant is small relative to $(k_{cat} + k_1\ [S]_P)$. In this case k_{cat}/K_m becomes equal to k_D. For a few enzymes, such as carbonic anhydrase, catalase and triose phosphate isomerase[33], values for k_{cat}/K_m of approximately $10^8\ M^{-1}\ s^{-1}$ have indeed been found, indicating that at least in those cases k_{-1} is close to its optimal value and not, therefore, a candidate for further optimization.

Let us consider the situation that would exist if k_{cat} were already to have an extremely high, optimal value. For very high values of k_{cat} the above turnover number reduces to $v/[E]_O = k_D \cdot [S]P$. Typically, metabolite concentrations range from 1 μM to 10 mM. With the above value for k_D, this suggests an upper limit to the turnover number ranging from 10^3 to $10^7\ s^{-1}$. The higher range should be characteristic for the majority of mainstream enzymes, and is clearly higher than the number calculated above for the actual turnover number of typical enzymes. We therefore conclude: (1) that metabolic fluxes tend to be orders of magnitude slower than they could be relative to the limitations imposed by diffusion, (2) that the question of how to increase metabolic fluxes and the answers suggested by metabolic control theory are more than relevant, and (3) that k_{cat} is an important candidate for further optimization. The latter suggests that further advances based upon protein engineering[34] should not focus exclusively on decreasing values of K_m, and are likely to require modifications of amino acids far removed from the active site[35].

The cases where metabolite concentrations are low are of some interest. An example is 1,3-diphosphoglycerate, which, possibly to avoid the problem of diffusion limitation, is transferred directly from glyceraldehyde-3-phosphate dehydrogenase to phosphoglycerate kinase[36]. This suggests possibilities for optimizing microbial metabolic fluxes even beyond the diffusion-controlled limit by causing the formation of super-complexes, or even fusion proteins, of the enzymes constituting the pathway(s) of interest.

In conclusion, the metabolic control theory developed by Kacser, Burns, Heinrich and Rapoport provides an appropriate framework for devising rational approaches to the improvement of biotechnological processes. The low values of k_{cat} exhibited by most enzymes tend to fix the absolute fluxes that may be observed when substrate concentrations are maintained (by evolutionary pressures) at a modest value. Consideration of each of these points will allow the fullest exploitation to be made of modern techniques of genetic and protein engineering in the optimization of the rate of microbial biotransformations.

Acknowledgements

DBK thanks the Biotechnology Directorate of the Science and Engineering Research Council, UK for financial support, and Professor Gareth Morris and Dr Rick Welch for many stimulating discussions.

References

1 Malpartida, F. and Hopwood, D. A. (1984) *Nature* 309, 462–464
2 Stanzak, R., Matsushima, P., Saltz, R. H. and Rao, R. N. (1985) *Bio/Technology* 4, 229–232
3 Kacser, H. and Burns, J. A. (1973) *Symp. Soc. Exp. Biol.* 27, 65–104
4 Kacser, H. and Burns, J. A. (1979) *Biochem. Soc. Trans.* 7, 1149–1160
5 Heinrich, R. and Rapoport, T. A. (1974) *Eur. J. Biochem.* 42, 89–95
6 Heinrich, R., Rapoport, S. A. and Rapoport, T. (1977) *Prog. Biophys. Mol. Biol.* 32, 1–83
7 Groen, A. K., van der Meer, R., Westerhoff, H. V., Wanders, R. J. A., Akerboom, T. P. M. and Tager, J. M. (1982) in *Metabolic Compartmentation* (Sies, H., ed.), pp. 9–37, Academic
8 Westerhoff, H. V., Groen, A. K. and Wanders, R. J. A. (1984) *Biosci. Rep.* 4, 1–22
9 Kell D. B. and Westerhoff, H. V. *FEMS Microbiol. Rev.* (in press)
10 Westerhoff, H. V. and van Dam, K. (1986) *Mosaic Non-Equilibrium Thermodynamics and the Control of Biological Free Energy Transduction*, Elsevier
11 Burns, J. A., Cornish-Bowden, A., Groen, A. K., Heinrich, R., Kacser, H., Porteous, J. W., Rapoport, S. M., Rapoport, T. A., Stucki, J. W., Tager, J. M., Wanders, R. J. A. and Westerhoff, H. V. (1985) *Trends Biochem. Sci.* 10, 16
12 Groen, A. K., Wanders, R. J. A., Westerhoff, H. V., van der Meer, R. and Tager, J. M. (1982) *J. Biol. Chem.* 257, 2754–2757
13 Salter, M., Knowles, R. G. and Pogson, C. I. (1986) *Biochem. J.* 234, 635–647
14 Chisti, Y. and Moo-Young, M. (1986) *Enzyme Microb. Technol.* 8, 194–204
15 Westerhoff, H. V. and Chen, Y. (1984) *Eur. J. Biochem.* 142, 425–430
16 Fell, D. A. and Sauro, H. M. (1985) *Eur. J. Biochem.* 148, 555–561
17 Flint, H. J., Tateson, R. W., Barthelmess, I. B., Porteous, D. J., Donachie, W. D. and Kacser, H. (1981) *Biochem. J.* 200, 231–246
18 Torres, N. V., Mateo, F., Meléndez-Hevia, E. and Kacser, H. (1986) *Biochem. J.* 234, 169–174
19 Walsh, M. and Koshland, D. E., Jr (1985) *Proc. Natl Acad. Sci. USA* 82, 3577–3581
20 Suckling, C. J., ed. (1984) *Enzyme Chemistry; Impact and Applications*, Chapman & Hall
21 Behal, V. (1986) *Trends Biochem. Sci.* 11, 88–91
22 Kacser, H. and Burns J. A. (1981) *Genetics* 97, 639–666
23 Miyagawa, K., Kimura, H., Nakahama,

K., Kikuchi, M., Doi, M. Akiyama, S. and Nakao, Y. (1985) *Bio/Technology* 4, 225–228
24 Westerhoff, H. V., Lolkema, J. S., Otto, R. and Hellingwerf, K. J. (1982) *Biochim. Biophys. Acta* 683, 181–220
25 Savageau, M. A. (1976) *Biochemical Systems Analysis; A Study of Function and Design in Molecular Biology*, Addison-Wesley
26 Rogers, P. L. and Lee, K. J. (1980) *Adv. Biochem. Eng.* 23, 37–84
27 Hackenbrock, C. R. (1981) *Trends Biochem. Sci.* 6, 151–154
28 Kell, D. B. and Westerhoff, H. V. (1985) in *Organized Multienzyme Systems; Catalytic Properties* (Welch, G. R., ed.), pp. 63–139, Academic Press
29 Mastro, A. M. and Keith, A. D. (1984) *J. Cell. Biol.* 99, 180S–187S
30 Jacobson, K. and Wojcieszyn, J. (1984) *Proc. Natl Acad. Sci. USA* 81, 6747–6751
31 Astumian, R. D. and Chock, P. B. (1985) *J. Phys. Chem.* 89, 3477–3482
32 Brocklehurst, K. (1977) *Biochem. J.* 163, 111–116
33 Fersht, A. R. (1985) *Enzyme Structure and Mechanism*, 2nd edn, W. H. Freeman
34 Fersht, A. R., Shi, J-P., Wilkinson, A. J., Blow, D. M., Carter, P., Waye, M. M. Y. and Winter, G. P. (1985) *Angew. Chem. Int. Ed.* 23, 467–473
35 Somogyi, B., Welch, G. R. and Damjanovich, S. (1984) *Biochim. Biophys. Acta* 768, 81–112
36 Srivasta, S. and Bernhard, S. *Curr. Top. Cell. Regul.* 27 (in press)

Temperature-induced synthesis of recombinant proteins

Celia A. Caulcott and Malcolm Rhodes

It is possible to switch on the synthesis of recombinant proteins in *E. coli* simply by increasing the temperature of the fermentation. How this is achieved is one of the success stories of recombinant DNA technology.

The bacterium, *Escherichia coli* has been studied in considerable detail by geneticists and molecular biologists and was chosen early on as an appropriate host organism for the expression of recombinant proteins. It has remained suitable for commercial applications since it is possible to grow *E. coli* expressing heterologous (foreign) proteins at very high cell densities. This review will consider the development of systems for expressing recombinant proteins in *E. coli*, and in particular, the choice of fermentation temperature as a method for controlling the expression systems will be discussed.

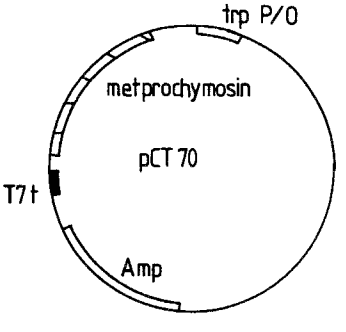
Development of constitutive expression systems

The first systems for expressing heterologous proteins in *E. coli* involved inserting the desired gene into one of various expression plasmids capable of replicating in the bacterium[1]. It was soon observed that in order to accumulate sufficient product, certain conditions had to be satisfied. For example, it was found that the presence of multiple copies of the heterologous gene was necessary[2,3], and that transcription of the gene was best initiated by a strong promoter[1]. Furthermore, to culture expressing microorganisms, a host that could grow rapidly and use the supplied carbon source efficiently was desirable. (Such host strains were also more useful if they had no unusual nutritional requirements.)

To fulfil these criteria, expression vectors were developed from natural *E. coli* plasmids such as Col E1 which are found in multiple copies. Powerful transcriptional promoters such as the *trp* or *lac* promoter/operator regions were inserted into the plasmid with the desired gene immediately after the promoter region (Fig. 1). Such expression systems might be expected to be controlled by the system of metabolic regulation found in the appropriate host operons. However, it was discovered that many of the systems were not adequately controlled, but gave constitutive expression of the recombinant protein[4]. This constitutive expression of heterologous product was not initially perceived as a problem, and many groups worked with such plasmids[5–8].

Although capable of accumulating proteins to 5% or greater of the total cellular protein[5,7], various problems with the constitutive expression systems emerged. It was found that in the absence of any selection for plasmids expressing the desired gene product, plasmid-free cells frequently appeared[9–11]. Freed from the plasmid burden, such cells proved to have a growth advantage over those carrying the expressing plasmid[4,12] and could overgrow plasmid-bearing cells during a fermentation. As a result it was quite possible, during a production process involving several inoculum stages, for the proportion of the cellular population carrying

Fig. 1



Simple map of pCT70, a plasmid derived from pAT153, expressing the met-prochymosin gene.

C. A. Caulcott and M. Rhodes are at Celltech Ltd, 244 Bath Road, Slough, Berkshire SL1 4DY, UK.

© 1986, Elsevier Science Publishers B.V., Amsterdam 0166 – 9430/86/$02.00