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Theoretical approaches to the evolutionary optimization of glycolysis
Thermodynamic and kinetic constraints

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It is analyzed whether the structural design of contemporary glycolysis can be explained theoretically on the basis of optimization principles originating from natural selection during evolution. Particular attention is paid to the problem of how the kinetic and thermodynamic properties of the glycolytic pathway are related to its stoichiometry with respect to the number and location of ATP-coupling sites. The mathematical analysis of a minimal model of unbranched energy-converting pathways shows that the requirement of high ATP-production rate favours a structural design that includes not only ATP-producing reactions (P-sites) but also ATP-consuming reactions (C-sites). It is demonstrated that, at fixed overall thermodynamic properties of a chain, the ATP-production rate may be enhanced by kinetic optimization. The ATP-production rate is increased if the C-sites are concentrated at the beginning and all the P-sites at the end of the pathway. An optimum is attained, which is characterized by numbers of coupling sites corresponding to those found in glycolysis. Various extensions of the minimal model are considered, which allow the effects of internal feedback-regulations, variable enzyme concentrations, and the symmetric branching of glycolysis at the aldolase step to be considered.

Keywords: glycolysis; mathematical modeling; evolution; free energy; flux control.

Glycolysis was one of the favoured subjects in the field of mathematical modelling of metabolic systems. Different types of simulation models have been developed: for example, models based on a very detailed kinetic description of the individual enzymes [1–4]; 'skeleton' models [5]; and models that used the quasi-steady-state approximation for simplification [6–8]. Furthermore, glycolysis was an important subject of pioneering theoretical works concerning the elucidation of mechanisms of metabolic oscillations [9–12].

The aim of the present study was substantially different from that of simulation models. Here, we will try to explain certain structural features of glycolysis that are fixed during the life span of an organism but have been changed in an evolutionary time-scale. This study concerns, in particular, the stoichiometry of the pathway and the kinetic parameters of the participating enzymes. In simulation models, these properties are taken into account as given quantities.

There has been no general theory to explain the structural design of metabolic pathways. Various related investigations were based on the hypothesis that, during evolution, systems with certain optimal properties have been selected. Concerning relevant optimization criteria, it is often assumed that metabolic fluxes were important targets of natural selection. Thus, kinetic parameters of contemporary enzymes, that is, elementary rate constants, Michaelis constants and maximal activities, have been calculated on the basis of the assumption that flux maximization played a fundamental role during evolution of metabolism [13–23]. From other possible evolutionary-optimization principles the following have attracted attention: minimization of transient times of metabolic pathways [24]; minimization of the total osmolarity of intermediates [25, 26]; stoichiometric simplicity [27, 28]; and maximization of thermodynamic efficiency (reviewed in [20]).

Because the main biological function of glycolysis is the production of ATP, our interest is focused on the implications of the optimization principle of maximal ATP-production rate on the structural design of glycolysis. In particular we pay attention to the optimal distribution of ATP-producing and ATP-consuming sites with respect to their number and their location along the glycolytic chain.

Our study consists of two parts. This paper concerns the kinetic and thermodynamic features of an optimal ATP-producing metabolic pathway, an analysis of chemical constraints will be published later. To allow general conclusions we do not incorporate in part 1 too many details of present day glycolysis. In a minimal model presented, an unbranched ATP-producing pathway consisting of first-order and pseudo-first-order reactions is

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Abbreviations. A, affinity of a reaction; C^J_{ij}, flux control coefficient for reaction j; $[E]$, concentration of enzyme E; ΔG, change in Gibbs free energy; ΔG^0, change in Gibbs free energy under standard conditions; J, steady-state flux; k_j, k_{-j}, first-order rate constants of reaction j for the forward and backward directions, respectively; κ_j, κ_{-j}, second-order rate constants of reaction j for the forward and backward directions, respectively; q_j, equilibrium constant of reaction j; R, universal gas constant; $[S_A]$, concentration of the metabolite A.

Enzymes. Hexokinase (EC 2.7.1.1); 6-phosphofructokinase (EC 2.7.1.11); pyruvate kinase (EC 2.7.1.40); phosphoglycerate kinase (EC 2.7.2.3); citrate(*si*)-synthase (4.1.3.7); isocitrate dehydrogenase (NAD^+) (1.1.1.41); malate dehydrogenase (1.1.1.37); pyruvate carboxylase (6.4.1.1); phospho*enol*pyruvate carboxykinase (4.1.1.49); L-lactate dehydrogenase (EC 1.1.1.27).

considered. Concentrations of cofactors are assumed to be fixed. In extended versions of this model, the effects of regulatory couplings (feedback inhibitions), variable enzyme concentrations and of the symmetric branching of glycolysis at the aldolase step are taken into account.

The main finding is that optimization of ATP-production rate favours a structural design that includes not only ATP-producing but also ATP-consuming steps and that the exergonic steps should be concentrated at the upper end and endergonic steps at the lower end of the chain. These results are in general agreement with the known design of glycolysis.

The special properties of a pathway with an optimal structural design will become clearer by comparing them with those of non-optimized structures. Of particular theoretical interest are hypothetical pathways that possess an 'antiglycolytic' design, with a location of ATP-consuming and ATP-producing reactions that is the reverse of that found in glycolysis.

A necessary prerequisite of our analysis is that there are different chemical possibilities for ATP production in the conversion of glucose into lactate. This problem is addressed elsewhere (Meléndez-Hevia, E., Waddell, T. G., Heinrich, R. and Montero, F., unpublished results). Examples are given of how ATP could be produced via metabolic routes that differ from those observed in most contemporary cells. However, it will be shown that the number of alternative possibilities is limited due to chemical constraints. In this manuscript, most chemical details will not be taken into account. It is also neglected that glycolysis can have a number of metabolic purposes in addition to ATP production, e.g. to act as a carbon source for serine and alanine, and that in many cells anaerobic glycolysis starts from glycogen instead of from glucose.

THEORY

A minimal model. *Basic assumptions.* A simplistic model for the coupling of an enzymatic chain to ATP production and ATP consumption is considered. We concentrate on the kinetic effects of a change of the equilibrium constants, which are brought about by incorporation of endergonic and exergonic steps.

Let us consider an unbranched chain of n reactions with fixed concentrations of the pathway substrate, $[S_0]$, and the end product, $[S_n]$. For simplicity, we assume that the rate v_i of a reaction interconverting two consecutive intermediates S_{i-1} and S_i is described by the linear kinetic equation

$$v_i = k_i [S_{i-1}] - k_{-i} [S_i] = k_i \left([S_{i-1}] - \frac{[S_i]}{q_i} \right) \qquad (1)$$

where k_i and k_{-i} denote first-order rate constants and q_i is the thermodynamic equilibrium constant of reaction step i. From the steady-state condition

$$J = v_i \qquad (2)$$

(J, steady-state flux) one derives a recursion formula for the steady-state concentration of the metabolites

$$[S_i] = [S_{i-1}]\, q_i - \frac{J}{k_i} q_i . \qquad (3)$$

Applying this formula from $i = 1$ to $1 \leqslant j \leqslant n$ one obtains

$$[S_j] = [S_0] \prod_{i=1}^{j} q_i - J \sum_{i=1}^{j} \frac{1}{k_i} \prod_{m=i}^{j} q_m . \qquad (4)$$

Since $[S_0]$ and $[S_n]$ are considered to be fixed, Eqn (4) leads to the following equation for the steady-state flux

$$J = \frac{1}{D} \left([S_0] \prod_{i=1}^{n} q_i - [S_n] \right), \quad D = \sum_{i=1}^{n} \frac{1}{k_i} \prod_{m=i}^{n} q_m . \qquad (5a,b)$$

An equation for the steady-state flux could also be obtained by using the Michaelis-Menten equation instead of Eqn (1) for reversible reactions. However, the consideration of saturation effects would make the analysis much more complicated, since the resulting equation is of nth order in J [20, 23].

Introducing the overall affinity A of the pathway,

$$A = RT \ln \left(\frac{[S_0]}{[S_n]} \prod_{j=1}^{n} q_j \right) \qquad (6)$$

(R, universal gas constant; T, temperature) Eqn (5) for the steady-state flux may be rewritten as follows

$$J = \frac{[S_n]}{D} \left\{ \exp \left(\frac{A}{RT} \right) - 1 \right\} . \qquad (7)$$

Whilst in this equation the numerator expresses the thermodynamic properties of the chain, the denominator D depends on the details of the kinetic properties of the reactions as well as on the chain length.

Some results outlined below may be easily understood in terms of flux-control coefficients $C^J_{v_i}$ describing the effect of changes of the activities v_i on the steady-state flux.

$$C^J_{v_i} = \frac{v_i}{J} \frac{\partial J / \partial p_i}{\partial v_i / \partial p_i} \qquad (8)$$

where p_i denotes a kinetic parameter which affects only reaction i directly [29–32]. Choosing $p_i = k_i$, one derives from Eqns (1, 5, 8) the following.

$$C^J_{v_i} = \frac{\dfrac{1}{k_i} \displaystyle\prod_{m=i}^{n} q_m}{\displaystyle\sum_{j=1}^{n} \frac{1}{k_j} \prod_{m=j}^{n} q_m} . \qquad (9)$$

In the present context it will be useful to consider the ratio of two control coefficients. With Eqn (9), one obtains for $j > i$

$$\frac{C^J_{v_i}}{C^J_{v_j}} = \frac{k_j}{k_i} \prod_{m=i}^{j-1} q_m . \qquad (10)$$

From this equation it follows that for equilibrium constants larger than unity, $q_m > 1$, the flux-control coefficients of reactions at the upper end of the chain are generally higher than those at the lower end of the chain, i.e. $C^J_{v_i} > C^J_{v_j}$ for $j > i$ provided that k_j is not very much smaller than k_i.

Eqn (1), for the individual rates, and Eqn (5), for the steady-state flux, may be applied for chains with bimolecular reactions involving e.g. cofactors, if they are considered as external reactants. In this case, one has to replace the kinetic constants k_i and k_{-i} by apparent first-order rate constants, which are obtained as products of the corresponding second-order rate constants κ_i and κ_{-i} and the concentrations of those external reactants participating in the corresponding reaction steps. Since we are interested in glycolysis, we consider the case that ATP and ADP may participate in the reaction chain either at ATP-producing sites (P-sites) or at ATP-consuming sites (C-sites). C-sites and P-sites are denoted as coupling sites. Reactions that are involved in neither ATP production nor ATP consumption are denoted as O-sites. The concentrations of ADP, ATP and free inorganic phosphate are considered to be constant.

Coupling of the ith reaction to ATP consumption or ATP production will change the thermodynamic properties. For the equilibrium constant of the uncoupled reaction, q_i, we have $q_i =$

k_i/k_{-i}. The equilibrium constant of the coupled reaction reads $q'_i = \kappa_i/\kappa_{-i}$. The equilibrium constants q'_i and q_i are related to each other by

$$\text{C-sites: } q'_i = q_i K^{-1}, \quad \text{P-sites: } q'_i = q_i K, \qquad (11\,\text{a,b})$$

where K denotes the equilibrium constants for the interconversion of ADP into ATP under physiological condition ($K \ll 1$). It is an apparent equilibrium constant since it includes the fixed concentration of inorganic phosphate.

By necessity, changes in the equilibrium constants as given in Eqns (11 a,b) are brought about by changes in the forward and backward rate constants. We use the following relations between the first-order rate constants $k_{\pm i}$ of the uncoupled reactions and the apparent first-order rate constants $k'_{\pm i}$ of the coupled reactions

$$\text{C-sites: } k'_i = \frac{k_i}{\alpha_i}, \quad k'_{-i} = k_{-i}\beta_i \qquad (12)$$

$$\text{P-sites: } k'_i = k_i \gamma_i, \quad k'_{-i} = \frac{k_{-i}}{\delta_i} \qquad (13)$$

where, for simplicity, we have neglected the effect of the concentrations of ATP and ADP on the apparent first-order rate constants.

Taking into account relations (11 a,b), the coupling parameters α_i, β_i and γ_i, δ_i must fulfil the relations

$$\alpha_i \beta_i = \gamma_i \delta_i = K. \qquad (14)$$

Under special assumptions, the various coupling parameters can be expressed as functions of q_i and K. For that, we use the rate equation proposed for a perfect catalyst. It reads under the condition that only diffusional constraints are operative

$$v_i = \frac{k_d\,[E_i]\,([S_{i-1}]\,q_i - [S_i])}{1 + q_i} \qquad (15)$$

[14, 19]. In this equation, $[E_i]$ denotes the concentration of the enzyme that catalyzes step i and k_d represents the diffusion-controlled upper limit for rate constants characterizing the binding of the substrates or products to the enzyme. A comparison of Eqns (1, 15) yields

$$k_i = \frac{k_d\,[E_i]\,q_i}{1 + q_i}, \quad k_{-i} = \frac{k_d\,[E_i]}{1 + q_i}. \qquad (16\,\text{a,b})$$

Introducing into these equations the equilibrium constants of coupled reactions from Eqn (11), one derives from Eqns (12, 13, 16) for the coupling parameters

$$\alpha_i = \frac{K + q_i}{1 + q_i}, \quad \beta_i = \frac{K(1 + q_i)}{K + q_i}, \qquad (17\,\text{a,b})$$

$$\gamma_i = \frac{K(1 + q_i)}{1 + q_i K}, \quad \delta_i = \frac{1 + q_i K}{1 + q_i}. \qquad (17\,\text{c,d})$$

Since $K < 1$, one obtains from Eqn (17)

$$K < \alpha_i, \beta_i, \gamma_i, \delta_i < 1. \qquad (18)$$

The ATP-production rate is related to the glycolytic flux J in the following way

$$J_{ATP} = (b - a)\,J = d \cdot J, \qquad (19)$$

where a and b denote the number of C-sites and P-sites, respectively. Substituting Eqn (5) in this equation for J, the equilibrium constants q_i and the rate constants k_i for the coupling sites must be replaced by q'_i and k'_i, respectively. In the following, the factor $d = b - a$ is denoted as the excess number of ATP-producing sites.

Concerning the evolutionary optimization of glycolysis, we are mainly interested in the implications of the extremum principle

$$J_{ATP} = \max. \qquad (20)$$

To identify the optimal structural design, the kinetic properties of chains with different numbers and different locations of coupling sites are compared. For this minimal model, no restrictions concerning the number and distribution of coupling sites are made except that

$$a + b \leqslant n, \quad 2b \leqslant n. \qquad (21\,\text{a,b})$$

Relation (21 b) follows from the fact that, for b ATP-producing sites, the chain must contain the same number (b) of sites where substrates are phosphorylated either by ATP or by inorganic phosphate. (A further chemical restriction is that the first step cannot be a P-site and the final step cannot be a C-site, due to the composition of glucose and lactate.). Taking into account these restrictions, different structural designs are obtained by an interchange of the different types of sites within the chain. Furthermore, we will compare pathways of different length (n) under the constraint of a fixed standard free-energy change $\Delta G'^{\circ}_{glyc}$ of the uncoupled interconversion of S_0 into S_n

$$\Delta G'^{\circ}_{glyc} = -RT \ln Q = \text{constant}, \quad Q = \prod_{i=1}^{n} q_i \qquad (22\,\text{a,b})$$

(Q, overall equilibrium constant of the chain in the uncoupled state). By means of these assumptions, alternative pathways are created that differ in the sites where ATP is produced and consumed at the conversion of the initial substrate S_0 into the end product S_n.

Thermodynamically, a chain with a C-sites and b P-sites may be characterized by the overall affinity

$$A = RT \cdot \ln\left\{\frac{[S_0]}{[S_n]}\,K^{(b-a)} \prod_{j=1}^{n} q_j\right\}, \qquad (23)$$

which is independent of the location of C-sites, O- sites, and P-sites along the chain. Since $K < 1$ the overall affinity decreases as $b - a$ increases. A positive glycolytic flux is obtained for positive affinity ($A > 0$), which is fulfilled if the excess number of ATP-producing sites does not exceed a certain maximum, that is, for

$$d = b - a < -\frac{\ln([S_0]\,Q/[S_n])}{\ln K} = d_{max}. \qquad (24)$$

Furthermore, one obtains $J = 0$ for $d = d_{max}$. For $d > d_{max}$ the affinity becomes negative which implies that the reactions proceed in the reverse way, that is from S_n to S_0.

Effect of replacements of O-sites by P-sites or C-sites and their optimal location. The main conclusions concerning the optimal kinetic properties of ATP-producing reaction chains may be derived from the following two theorems.

Theorem 1. The replacement of an O-site by a C-site, that is, $a \rightarrow a + 1$, at any reaction increases the glycolytic rate J, and the replacement of an O-site by a P-site, i.e. $b \rightarrow b + 1$, decreases J. This theorem points to the kinetic effects of a change of the number of coupling sites.

The kinetic effects of a variation of the location of coupling sites at fixed numbers a and b are described by

Theorem 2. J as well as J_{ATP} are increased by an exchange of a P-site at reaction i for an O-site at reaction m with $i < m$, and by an exchange of a C-site at reaction j for an O-site at reaction m with $m < j$, provided that the affinity A and the excess number, d, of ATP-producing sites are positive.

Proof of Theorem 1. Let us consider the replacement of an O-site by a C-site at reaction i (Part 1 of Theorem 1). According

to Eqns (5, 11, 12), the flux $J(O_i)$ with an O-site at reaction i and the flux $J(C_i)$ with a C-site at reaction i read

$$J(O_i) = \frac{[S_0]\,Q - [S_n]}{\sum_{j=1}^{i-1} \frac{Q_{j,n}}{k_j} + \frac{Q_{i,n}}{k_i} + \sum_{j=i+1}^{n} \frac{Q_{j,n}}{k_j}}, \qquad (25a)$$

$$J(C_i) = \frac{[S_0]\,K^{-1}\,Q - [S_n]}{K^{-1}\sum_{j=1}^{i-1} \frac{Q_{j,n}}{k_j} + K^{-1}\,\alpha_i \frac{Q_{i,n}}{k_i} + \sum_{j=i+1}^{n} \frac{Q_{j,n}}{k_j}}, \qquad (25b)$$

with Q from Eqn (22b) and $Q_{j,n} = \prod_{m=j}^{n} q_m$. Strictly speaking, Eqn (25a) applies to the situation that all reactions are O-sites. Both equations can, however, also be applied to the case $a, b > 0$, when all earlier replacements have already been taken into account by inclusion of the values K, α_j, β_j, γ_j and δ_j in the values of (apparent) kinetic and equilibrium constants. From Eqns (25a, b) it follows directly that $J(C_i) > J(O_i)$ only if

$$[S_0]\,Q\left\{\frac{Q_{i,n}}{k_i}K^{-1}(1-\alpha_i) + \sum_{j=i+1}^{n}\frac{Q_{j,n}}{k_j}(K^{-1}-1)\right\} \qquad (26)$$

$$+ [S_n]\sum_{j=1}^{i-1}\frac{Q_{j,n}}{k_j}(K^{-1}-1) + [S_n]\frac{Q_{i,n}}{k_i}(K^{-1}\alpha_i - 1) > 0\,.$$

Eqn (26) holds true under consideration of Eqn (18), which completes the proof. Part 2 of Theorem 1 can be proved in an analogous way.

Proof of Theorem 2. For fixed numbers of P-sites and C-sites, the numerator of Eqn (5a) is independent of the distribution of these sites along the chain. To investigate the influence of the location of P-sites on J and J_{ATP} (first statement of Theorem 2), we compare, therefore, the denominators D of Eqn (5) for the following two situations: P-site at reaction i and O-site at reaction m [denominator $D(P_i, O_m)$], and O-site at reaction i and P-site at reaction m (denominator $D(O_i, P_m)$], where in both cases $i < m$. One obtains

$$D(P_i, O_m) = K\sum_{j=1}^{i-1}\frac{Q_{j,n}}{k_j} + K\gamma_i^{-1}\frac{Q_{i,n}}{k_i} + \sum_{j=i+1}^{n}\frac{Q_{j,n}}{k_j}, \qquad (27a)$$

$$D(O_i, P_m) = K\sum_{j=1}^{m-1}\frac{Q_{j,n}}{k_j} + K\gamma_m^{-1}\frac{Q_{m,n}}{k_m} + \sum_{j=m+1}^{n}\frac{Q_{j,n}}{k_j}. \qquad (27b)$$

From these equations it follows

$$D(P_i, O_m) - D(O_i, P_m) = \frac{Q_{i,n}}{k_i}K(\gamma_i^{-1} - 1) \qquad (28)$$

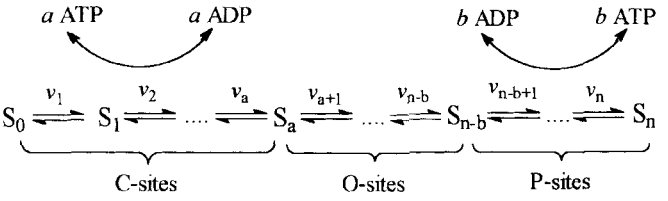
$$+ \sum_{j=i+1}^{m-1}\frac{Q_{j,n}}{k_j}(1-K) + \frac{Q_{m,n}}{k_m}(1 - K\gamma_i^{-1}) > 0\,,$$

where Eqn (18) has been taken into account. For a positive overall affinity (A), Eqn (28) implies $J(O_i, P_m) > J(P_i, O_m)$ and, for $b - a > 0$, $J_{ATP}(O_i, P_m) > J_{ATP}(P_i, O_m)$, which completes the proof. The second statement of Theorem 2 can be proved in an analogous way.

RESULTS

It follows from theorem 2 that J_{ATP} becomes maximum when all P-sites are located at the lower end of the chain and all C-sites are located at the upper end of the chain (Scheme 1).

Scheme 1.



Therefore, the optimal ATP-production rate reads

$$J_{ATP}(a,b) = \frac{b-a}{D(a,b)}\left\{[S_0]\,K^{(b-a)}\prod_{j=1}^{n} q_j - [S_n]\right\}, \qquad (29a,b)$$

$$D(a,b) = D_a + D_0 + D_b$$

where

$$D_a = \prod_{j=1}^{a}\frac{1}{k_j}\,\alpha_j\,Q_{j,n}\,K^{(b-a+j-1)}, \qquad (30a,b)$$

$$D_0 = K^b\sum_{j=a+1}^{n-b}\frac{Q_{j,n}}{k_j},$$

$$D_b = \sum_{j=n-b+1}^{n}\frac{1}{k_j\,\gamma_j}\,Q_{j,n}\,K^{(n-j+1)}. \qquad (30c)$$

By means of Eqns (29, 30), it is shown that an optimum for the ATP-production rate J_{ATP} is not only obtained by the proper localization of P-sites and C-sites at the beginning and the end of the chain, respectively, but also by variation of their numbers, a and b. For this, we consider the case of equal values for all thermodynamic equilibrium constants ($q_j = q$) and equal values for enzyme concentrations ($[E_j] = [E]$). Under these conditions, Eqn (30a–c) permit an explicit evaluation by means of the formula for geometric progressions. Taking into consideration Eqns (12, 13, 17) one obtains

$$k_d[E]\cdot D_a = (K+q)\,q^{n-a}\,K^{b-1}\left[\frac{(q/K)^a - 1}{q/K - 1}\right] \qquad (31a)$$

$$k_d[E]\cdot D_0 = (1+q)\,q^b K^b\left(\frac{q^{n-b-a}-1}{q-1}\right) \qquad (31b)$$

$$k_d[E]\cdot D_b = (1+qK)\left(\frac{(qK)^b - 1}{qK - 1}\right). \qquad (31c)$$

Fig. 1A, B show the glycolytic rate J and the ATP-production rate J_{ATP}, respectively, as functions of the number of coupling sites for a chain with 10 reactions for special values of the thermodynamic parameters Q and K. The fluxes are normalized with respect to the rate $v_{normal} = k_d[S_0][E]$ which, according to Eqn (15), represents the forward reaction rate of the first reaction for $q_1 \to \infty$ and $[E_1] = [E]$. (The same normalization is used in all subsequent figures, except of Fig. 6.) The curves in Fig. 1A, B are calculated on the basis of Eqns (29, 31) by taking into account Eqn (19). The starting points of the curves at low b values are given by the condition that we only consider chains where the number of P-sites exceeds the number of C-sites (i.e. $b \geq a$). The end points at high b values are determined by the limited total number of sites (i.e. $a + b \leq n$). Broken lines connect points that are physically unrealistic since Eqn (21b) is violated.

The glycolytic rate J decreases for all possible values of a monotonically with the number (b) of P-sites (Fig. 1A). This property follows directly from theorem 1, part 2. For low values

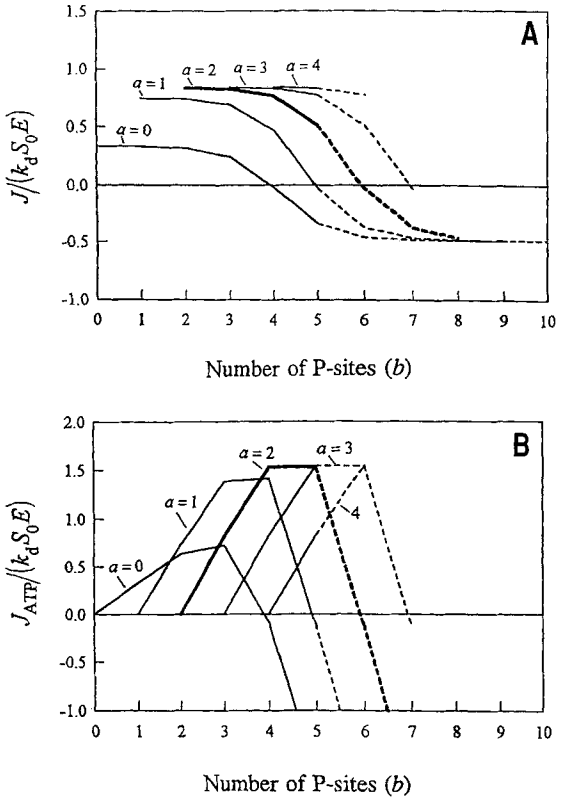


Fig. 1. Glycolytic rate and the ATP-production rate for a pathway of ten reactions. Normalized values of J (A) and J_{ATP} (B) are represented as functions of b located at the lower end of the chain for various values of a at the upper end of the chain according to Eqns (5, 19, 31). Parameter values: $Q = 1024$ ($q_i = q = 2$), $K = 0.17$, $[E_i] = [E]$, $[S_0] = [S_n]$. The thermodynamic limit according to Eqn (24) is $d_{max} = 3.94$. The thick lines connect points for $a = 2$, which corresponds to the situation of glycolysis (two C-sites in the first part of the pathway). Broken lines connect points for $2b > n$, which physically cannot be realized due to the constraint described by Eqn (21 b).

of a ($a \leq 3$) the flux J may become negative at high numbers of P-sites ($b - a > d_{max}$) (Eqn 24). For small values of b with $b > a$, the flux J is rather insensitive to variations of b. This is in accordance with the result that, for $q_i > 1$, flux control in unbranched chains is mainly exerted by the first enzymic steps; that is, a change of the kinetic properties of reactions at the end of the chain (resulting from the incorporation of P-sites) has little effect on the steady-state flux (Eqn 10). The ATP-production rate (J_{ATP}) (Fig. 1 B) displays a maximum at variations of the number (b) of P-sites. This is explained by the fact that the two factors in Eqn (19), $b - a$ and J, change in opposite directions with variations of b. In particular, the increase of J_{ATP} results from the insensitivity of J to variations of b for low b values. At higher values of b the decrease of J overcompensates the increase of $b - a$. The flux J (Fig. 1 A) increases with the number (a) of ATP-consuming sites at the upper end of the chain. This results from theorem 1, part 1. This effect is most pronounced at the transition from $a = 0$ to $a = 1$ which makes the first reaction more irreversible due to $K < 1$. Since steps behind quasi-irreversible reactions in unbranched chains exert minor flux control (Eqn 10), further replacements of O-sites by C-sites at subsequent reactions yield less effect. The maximum for the ATP-production rate is higher for $a = 1$ (located at $b = 4$ at the chosen parameter values) than for $a = 0$ ($b = 3$).

That J_{ATP} is optimized if the chain contains C-sites at the upper end of the chain and P-sites at the lower end of the chain is qualitatively in accordance with the stoichiometric structure

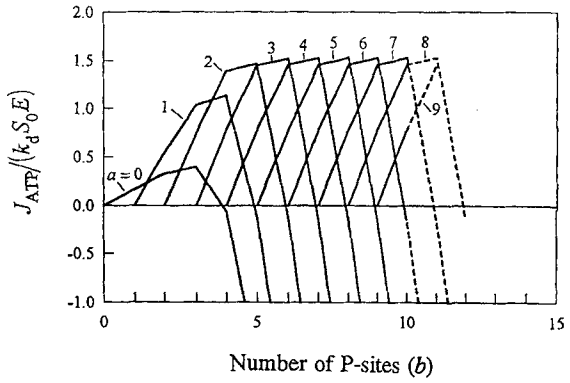


Fig. 2. ATP-production rate for a pathway of 20 reactions. J_{ATP} is represented as a function of b located at the lower end of the chain for various values of a at the upper end of the chain. The parameter values are the same as used in Fig. 1, with the exception that the overall equilibrium constant $Q = 1024$ results for $n = 20$ in $q_i = q = 1.414$. The thick line connect points for $a = 2$. Broken lines connect points for $2b > n$.

of glycolysis. There, the ATP-consuming reactions catalyzed by hexokinase and phosphofructokinase are located within the first part while the ATP-producing reactions catalyzed by the phosphoglycerate kinase and pyruvate kinase belong to the last part of glycolysis.

Dependence on chain length. Fig. 2 shows the ATP-production rate as a function of the number of coupling sites for $n = 20$. Compared with the results for $n = 10$ (Fig. 1 B), the main results concerning optimization of ATP-production are unaffected by an increase of the chain length. The case $n = 20$ allows the consideration of more cases for the number of C-sites and P-sites. From analysis of this case, it becomes clearer that optimization of the ATP-production rate may result in a number of nearly equivalent solutions characterized by the same excess number (d) of ATP-producing sites at differing values of a and b. In particular, for the given parameter values the optima for J_{ATP} for $b - a = 3$ with $a \geq 2$ are all nearly the same.

This results from the fact that, for higher a values, many reactions at the beginning of the chain become quasi-irreversible due to the coupling to ATP consumption. Thus, the control of the glycolytic flux is fully exerted by these reactions, and further changes of the kinetic parameters of reactions located downstream will have only a negligible effect on J at constant d (Eqn 10).

Antiglycolytic design. It may be interesting to compare the kinetic properties of an optimal ATP-producing pathway with those of non-optimized pathways. In particular, one may study unbranched chains, where the P-sites are located at the upper end of the chain while the C-sites are located at the lower end. Since, for such a pathway, the order of P-site and C-sites is reversed compared with that of glycolysis, the corresponding design is denoted as anti-glycolytic. With glucose as the starting substance, it is chemically impossible to have ATP-producing steps at the very beginning. However, in the present section, we are interested only in the kinetic effect of the number and location of exergonic and energonic reactions. Thus, we make the crude assumption that the kinetic equation for the flux $J^{anti}(b,a)$ for the antiglycolytic design is obtained by introducing into the general flux equation (Eqn 5) apparent first-order rate constants and equilibrium constants of P-sites for reactions j ($1 \leq j \leq b$) and apparent first-order rate constants and equilibrium constants of C-sites for reactions j ($n - a + 1 \leq j \leq n$) according to Eqns (11–13, 16, 17).

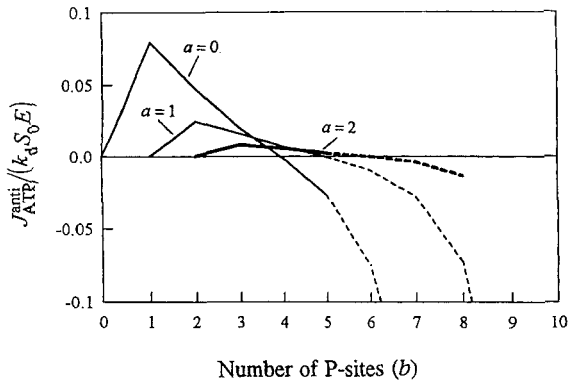


Fig. 3. ATP-production rate for the antiglycolytic design. J_{ATP}^{anti} is represented as function of b located at the upper end of the chain for various values of a at the lower end of the chain for $n = 10$. The parameter values are the same as used in Fig. 1.

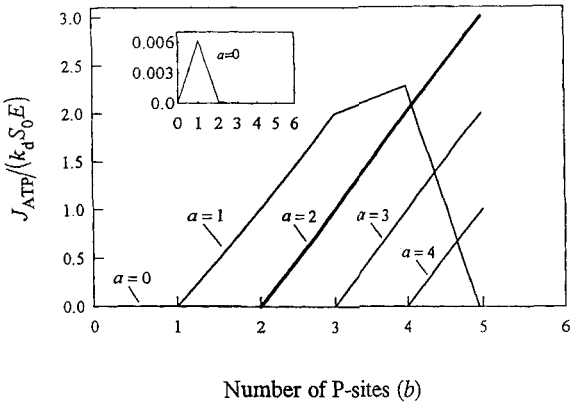


Fig. 4. ATP-production rate for realistic values of thermodynamic parameters. J_{ATP} is represented as a function of b located at the end of the chain at various values of a at the beginning of the chain according to Eqns (29, 30) for $n = 10$. The equilibrium constants are $q_O = 2089.23$, $q_{PO} = 0.0125$, $q_C = 3.323\times10^6$, $q_P = 1.312$ which correspond to the realistic ΔG values given in the text for the various types of sites. Other parameter values: $[E_i] = [E]$, $[S_0] = [S_n]$, $d_{max} = 3.94$. The thick lines connect points for $a = 2$.

Fig. 3 shows J_{ATP}^{anti} as function of the number (b) of P-sites for various values of the number (a) of C-sites, where all the other parameters are the same as used in Fig. 1. A comparison of Fig. 1 B and Fig. 3 shows that for given a and b, $J_{ATP} > J_{ATP}^{anti}$ holds true. While in a pathway with a glycolytic design, an increase of the number of C-sites may lead to an increase of the ATP-production rate (Figs 1 B and 2), the opposite effect is obtained for a pathway with an antiglycolytic design. An increase of a generally lowers the ATP-production rate, J_{ATP}^{anti}.

Towards realistic values of the thermodynamic parameters. By means of free-energy changes, the maximal excess number (d_{max}) of ATP-producing sites (Eqn 24) may be expressed as

$$d_{max} = -\frac{RT \cdot \ln([S_0]/[S_n])}{\Delta G_{ATP}} + \frac{\Delta G'^{\circ}_{glyc}}{\Delta G_{ATP}} \qquad (32)$$

where ΔG_{ATP} denotes the free-energy change of ATP hydrolysis under physiological conditions. Since

$$\Delta G'^{\circ}_{glyc} \approx -197 \text{ kJ/mol}, \quad \Delta G_{ATP} \approx -50 \text{ kJ/mol} \qquad (33\,a,b)$$

[33], one obtains for $[S_0] \approx [S_n]$ the value $d_{max} \approx 3.94$. This number has been used to calculate the curves shown in Fig. 1. However, the overall equilibrium constant Q and the equilibrium constant K resulting from the realistic thermodynamic parameters by use of the formulae $Q = \exp(-\Delta G_{glyc}/RT)$ and $1/K = \exp(-\Delta G_{ATP}/RT)$ are much higher and much lower, respectively, than those used in Fig. 1.

To introduce into Eqns (29–31) realistic equilibrium constants q_i and the corresponding rate constants k_i and coupling parameters α_j and γ_j that result from Eqn (17 a,c) we have to take into account that the existence of b ATP-producing steps necessitates that the chain contains the same number of phosphorylation steps. If there are a ATP-consuming reactions, the remaining $b-a$ phosphorylations have to be carried out with inorganic phosphate as the substrate. In the following, these reactions are called O_P-sites. As a consequence, the chain contains $n-2b$ O-sites with no phosphorylation or dephosphorylation. To correct the total number of reactions n at variations of b and a, the two consecutive reactions $S_{j-1} + P_i \rightarrow (S_{j-1} - P)$ and $(S_{j-1} - P) \rightarrow (S_j - P)$ are combined into one reaction

$$S_{j-1} + P_i \xrightarrow{\sigma_j} (S_j - P).$$

Analogously, the C-sites

$$S_{j-1} + ATP \xrightarrow{\sigma_j} (S_j - P) + ADP$$

result from combination of the reactions

$S_{j-1} + ATP \rightarrow (S_{j-1} - P) + ADP$ and $(S_{j-1} - P) \rightarrow (S_j - P)$

and the P-sites

$$(S_{j-1} - P) + ADP \xrightarrow{\sigma_j} S_j + ATP$$

from combination of

$(S_{j-1} - P) \rightarrow (S_j - P)$ and $(S_j - P) + ADP \rightarrow S_j + ATP$.

Furthermore, by means of the simplifying assumptions that the standard free-energy change of the reaction

$(S_{j-1} - P) \rightarrow (S_j - P)$ equals that of $S_{j-1} \rightarrow S_j$

and that it is independent of j, the overall reactions are characterized by the following free-energy changes.

$$\text{O-sites:} \quad \Delta G_O = \frac{1}{n}\Delta G_{glcy},$$

$$O_P\text{-sites:} \quad \Delta G_{OP} = \Delta G_O - \Delta G_{hydr}, \qquad (34\,a,b)$$

$$\text{C-sites:} \quad \Delta G_C = \Delta G_{ATP} + \Delta G_O - \Delta G_{hydr}, \qquad (34\,c)$$

$$\text{P-sites:} \quad \Delta G_P = \Delta G_O + \Delta G_{hydr} - \Delta G_{ATP} \qquad (34\,d)$$

where ΔG_{hydr} denotes the free-energy change of the hydrolysis of a phosphorylated compound $(S_j - P)$. We use the value $\Delta G_{hydr} = -13$ kJ/mol, which corresponds approximately to the free-energy change of the splitting of glucose 6-phosphate into glucose and inorganic phosphate under physiological conditions [34]. For all steps, the corresponding equilibrium constants are calculated according to the formula $q = \exp(-\Delta G/RT)$ with the ΔG-values taken from Eqn (34).

Fig. 4 shows the ATP-production rate for the thermodynamic parameters given in Eqn (33), which yield, according to Eqns (24, 32) with $[S_0] \approx [S_n]$, the same d_{max} value as used in Fig. 1. The thick solid line represents the case $a = 2$, that is, the situation observed in glycolysis. It is seen that the difference between the curves for $a = 0$ and $a \neq 0$ is much more pronounced than for lower Q values (inset to Fig. 4 shows, for $a = 0$, the ATP-production rate on an expanded scale). For $a = 0$ and $a = 1$, the property is retained that J_{ATP} exhibits a maximum at variation of the number (b) of P-sites. For $a > 1$, there is also a maximum but the descending part of the curve at high b values is missing. This is explained by the restriction $2b \leq n$ (Eqn 21 b) which, for $n = 10$, does not permit that at increasing d values the factor $K^{(b-a)}$ becomes low enough to pro-

duce a significant decrease of the overall affinity A and of the numerator in Eqn (29) for J_{ATP}.

A striking feature of the curves shown in Fig. 4 is that for $a \neq 0$ the curves on the left side of the maximum are almost straight lines. This property is due to the fact that, for realistic thermodynamic parameters, the first reaction, which is a C-site for $a \neq 0$, becomes nearly irreversible. This implies full control of the glycolytic flux by reaction 1 (Eqn 10). Since $[S_0]$ = constant, one obtains $J \approx$ constant which means that, below the thermodynamic limit, J_{ATP} increases nearly proportionally with $d = b - a$.

From the curves shown in Fig. 4, it is seen that the J_{ATP} optimum for $a = 2$ is not very much higher than that for $a = 1$. One could conclude, therefore, that for thermodynamic and kinetic reasons a high ATP production would be guaranteed by only one ATP-consuming site at the first step of the chain. However, as shown below and elsewhere (Meléndez-Hevia, E., Waddell, T. G., Heinrich, R. and Montero, F., unpublished results) the existence of two ATP-consuming sites at the beginning of glycolysis is favoured on the basis that $a = 2$ allows for a symmetric pathway in the degradation of the triose phosphates in the lower part of glycolysis.

Extensions of the model. *Regulatory feedbacks.* Contemporary glycolysis is characterized by a large number of internal regulators (enzyme activation or inhibition by substances other than substrates or products). Examples are the inhibition of hexokinase by glucose 6-phosphate and (in erythrocytes) by 2,3-bisphosphoglycerate [7] or the inhibition of phosphofructokinase by phospho*enol*pyruvate [35]. Such regulatory couplings have been neglected in the minimal model presented above. As a consequence of this simplification, there is only a very weak feedback from the lower part of the chain to the upper part, that is, alterations of the kinetic parameters of reactions at the end of the chain may change the glycolytic flux only by affecting the balance between forward and reverse reactions. Therefore, the absence of regulatory mechanisms becomes crucial under circumstances where the first steps of the chain are quasi-irreversible, which is the case for ATP-consuming sites located at the upper end of the chain ($a \neq 0$). Then flux control is exerted exclusively by the first reaction and the rate J_{ATP} increases linearly with increasing b (Fig. 4).

Let us consider a situation where there is feedback inhibition on reaction i which is exerted by an internal metabolite S_m located downstream of reaction i, that is, $i \leqslant m < n$. For reaction i, we use the rate equation

$$v_i = \frac{k_i([S_{i-1}] - [S_i]/q_i)}{1 + [S_m]/K_i}, \qquad (36a)$$

where K_i is the inhibition constant which describes a non-competitive inhibition. An equation for the steady-state flux may be derived as follows. Eqn (4) allows the expression of the concentrations $[S_{i-1}]$, $[S_i]$ and $[S_m]$ as functions of J, $[S_0]$ and $[S_n]$. Inserting the resulting expressions into equation $v_i = J$, with v_i taken from Eqn (35), one arrives at the following quadratic equation for the steady-state flux

$$a_2 J^2 + a_1 J + a_0 = 0 \qquad (36a)$$

where

$$a_0 = k_i Q_{1,i-1}([S_0] - [S_n]/Q_{1,n}), \qquad (36b,c)$$

$$a_1 = 1 + \frac{[S_n]}{K_i Q_{m+1,n}} + k_i A_{1,i-1} + \frac{k_i A_{i+1,n}}{Q_{i,n}}$$

$$a_2 = \frac{A_{m+1,n}}{K_i Q_{m+1,n}}, \quad A_{j,k} = \sum_{s=j}^{k} \frac{Q_{s,k}}{k_s}, \quad Q_{j,k} = \prod_{s=j}^{k} q_s. \qquad (36d-f)$$

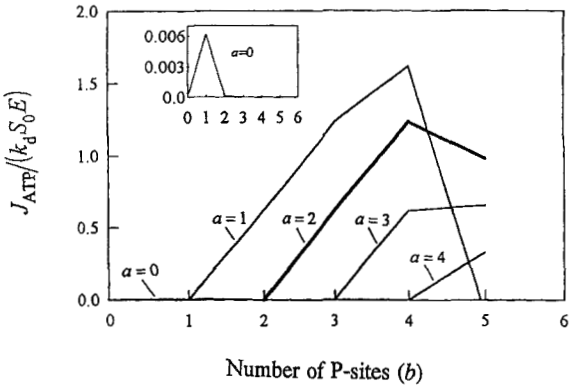


Fig. 5. Effect of feedback inhibition on the ATP-production rate. J_{ATP} is represented as a function of a and b for the model including the inhibition of the first reaction of the pathway by an internal metabolite S_m with $m = 5$ and $K_i = 1$. The other parameters are the same as used in Fig. 4. The curves are calculated by means of Eqns (19, 36) for a chain of ten reactions.

The effect of a feedback-inhibition on the ATP-production rate is shown in Fig. 5 for $m = 5$ and $i = 1$, that is, the metabolite located in the middle of the chain inhibits the first reaction. The thermodynamic parameters are the same as given in the legend to Fig. 4. In contrast to the case without feedback inhibition, the maximum values for J_{ATP} are decreased, and for $a = 2$ and $a = 3$ the functions $J_{ATP}(b)$ are not straight lines. This is explained by the observation that an increase of the number of ATP-producing sites, which slows down the reactions at the lower end of the chain, is accompanied by an accumulation of the metabolite concentrations in the middle part, and in this way by a stronger inhibition of the first reaction.

Fig. 5 resembles in some respects Fig. 1 B since in both cases most of the curves $J_{ATP}(b)$ are non-monotonic. Whilst in Fig. 1, this behaviour is due to product inhibitions resulting from (unrealistically) low values of the equilibrium constants, in Fig. 5 it results mainly from the non-competitive inhibition of the first reaction by the metabolite S_m ($m = 5$), which is distant from that reaction. In the latter case, reactions at the lower end have positive flux-control coefficients despite that for $a \neq 0$ the first reaction is quasi-irrevsible. One may speculate that the evolution of regulatory feedback mechanisms was driven by the demand to circumvent the accumulation of flux control in a low number of irreversible reactions [36, 37].

Effect of enzyme concentrations. In the minimal model outlined above, the enzyme concentrations $[E_i]$ enter the expressions (16a,b) for the rate constants as fixed quantities. The glycolytic flux J and the ATP-production rate J_{ATP} is changed by a variation of the distribution of the enzyme concentrations along the pathway. In the present section, we analyze how the results of the minimal model are modified if those enzyme concentrations are considered which maximize the ATP-production rate. Since Eqn (5) for J is a homogeneous function of first degree of the enzyme concentration, optima for J and J_{ATP} may only be found if upper limits for $[E_i]$ are taken into account. In the following we use the constraint

$$\sum_{j=1}^{n} [E_j] = [E]_t \leqslant [E]_t^0, \qquad (37)$$

which expresses the fact that the total enzyme concentration $[E]_t$ for a metabolic pathway is limited by the capacity of the living cell to synthesize proteins [13, 20]. For any optimal solution, the total sum of enzyme concentrations must be equal to its maximum value $[E]_t^0$. By means of the method of Lagrange multipli-

ers, the spectrum of optimal enzyme concentrations is, therefore, determined by the condition

$$\frac{\partial}{\partial [E_i]}\left\{J - \lambda\left(\sum_{j=1}^{n} [E_j] - [E]_t^0\right)\right\} = \frac{\partial J}{\partial [E_i]} - \lambda = 0\,. \qquad (38)$$

where λ denotes the Lagrange multiplier. Introducing Eqn (5) into Eqn (38) yields, under consideration of $[E]_t = [E]_t^0$,

$$[E_i] = \frac{[E]_t^0 \sqrt{(1 + q_i)\, Q_{i+1,n}}}{\sum_{j=1}^{n} \sqrt{(1 + q_j)\, Q_{j+1,n}}}\,. \qquad (39)$$

It is seen that the optimal distribution of enzyme concentrations depends on the equilibrium constants q_i, which, in the context of our model, depend on the location of the coupling sites. Introducing the optimal enzyme concentrations given in Eqn (39) into Eqns (5, 16a), one arrives at the following expression for the optimal flux

$$J = \frac{k_d [E]_t ([S_0]\, Q - [S_n])}{\left[\sum_{j=1}^{n} \sqrt{(1 + q_j)\, Q_{j+1,n}}\right]^2}\,. \qquad (40)$$

This equation may also be applied to the case that some reactions are coupled to ATP consumption and others to ATP production by replacing the equilibrium constants q_i by q_i' according to Eqn (11 a, b). The resulting expression has, compared with the flux equation used in the minimal model, the advantage that it is independent of the coupling parameters α_i, β_i, γ_i and δ_i transforming changes of the equilibrium constants into changes of the forward and backward rate constants (Eqns (12, 13).

Theorems 1 and 2 remain valid by applying Eqn (40) for the glycolytic flux and Eqn (19) for the relation between J and J_{ATP}. In particular, we arrive at the conclusion that J_{ATP} becomes maximum when all P-sites are located at the lower end and all C-sites are located at the upper end of the chain (Scheme 1).

For $q_i = q$, the sums which enter the denominator of the flux equation can be evaluated by means of the formula for geometric progressions. One obtains

$$J_{ATP} = \frac{(b - a)\,([S_0]\, K^{b-a}\, Q - [S_n])}{(D_a + D_o + D_b)^2} \qquad (41\,a)$$

with

$$D_a = \sqrt{\left(1 + \frac{q}{K}\right) q^{n-a} K^b}\left[\frac{(q/K)^{a/2} - 1}{(q/K) - 1}\right] \qquad (41\,b)$$

$$D_o = \sqrt{(1 + q)\, q^a K^b}\left[\frac{q^{(n-a-b)/2} - 1}{q^{1/2} - 1}\right] \qquad (41\,c)$$

$$D_b = \sqrt{1 + q K}\left[\frac{(q K)^{b/2} - 1}{(q K)^{1/2} - 1}\right]. \qquad (41\,d)$$

Fig. 6A shows J_{ATP} as a function of the number of coupling sites for a chain with ten reactions and for the same thermodynamic parameters as used in Fig. 1. The flux is normalized with respect to $v_{normal} = k_d[S_0]\,[E]_t^0/n$. The main conclusion derived for the minimal model remains valid by considering states with optimal distribution of enzyme concentrations. However, the proper adjustment of enzyme concentrations along the chain in Fig. 6A leads to ATP-production rates that are generally higher than those depicted in Fig. 1B.

Fig. 6B shows the optimal enzyme concentrations for $a = 0$ and $b = 0$ (curve a) and for $a = 2$ and $b = 4$ (curve b). Curve a confirms that for $q_i > 0$, the enzyme concentrations giving rise to an optimal flux decrease monotonically from the beginning

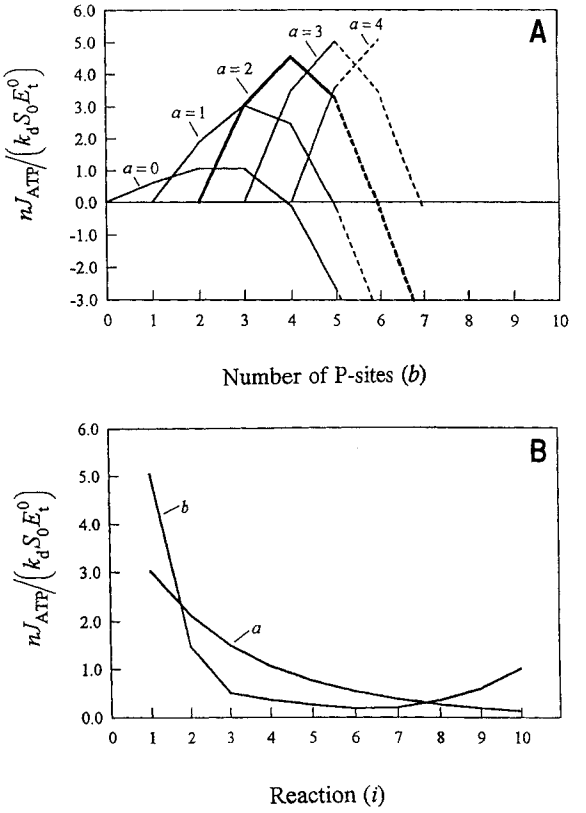
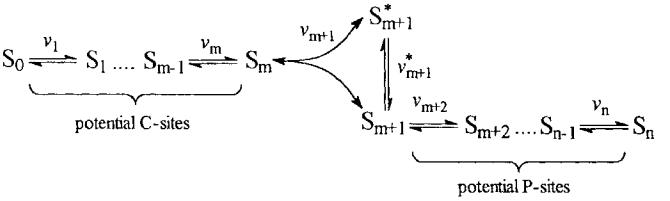


Fig. 6. ATP-production rate and optimal distribution of enzyme concentrations. (A) J_{ATP} at optimal enzyme concentrations. The curves are calculated by means of Eqn (41) for $n = 10$. (B) Optimal distribution of enzyme concentrations according to Eqn (39), (a) $a = b = 0$; (b) $a = 2$, $b = 4$. $[E]_t^0 = 10$. The other parameter values are the same as used in Fig. 1.

Scheme 2.



to the end of the chain [15, 20]. When the chain contains ATP-producing sites at its lower end, the effect of the low rate constants of these reactions is counterbalanced by higher enzyme concentrations. Accordingly, there is a non-monotonous distribution of enzyme concentrations in states of maximal steady-state flux.

Effect of branching. In the above analysis it has been neglected that glycolysis is characterized by a splitting of C_6 compounds into two C_3 compounds at the aldolase reaction. To introduce this feature the branching model depicted in Scheme 2 will be considered.

The mathematical treatment can be generalized to scheme 2, where at step $m+1$, with $m + 1 < n$, a splitting of the compound S_m into the compounds S_{m+1} and S_{m+1}^* occurs, and where the latter two compounds can be interconverted in an isomerization reaction (see Appendix).

According to the results derived for the unbranched chain, it is meaningful to assume that all C-sites may be located only in

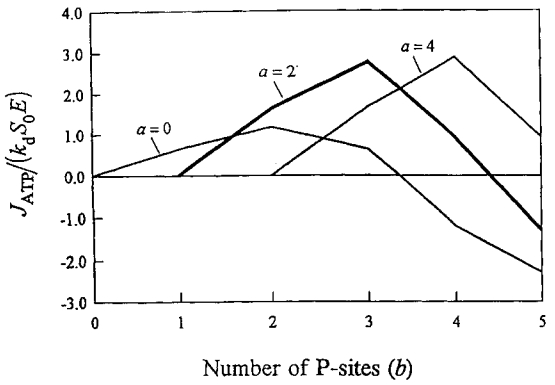


Fig. 7. ATP-production rate for the branching model. The curves for J_{ATP} are calculated by means of Eqn (A5a–g) for $m = 5$ and $n = 11$. The other parameter values are the same as used in Fig. 1. The P-sites are located at the end of the chain and the C-sites at the beginning of the chain. Reactions $m+1$ and $(m+1)^*$ are considered to be in equilibrium.

the upper part of the chain (reactions 1 to m) and all P-sites only in the lower part of the chain (reactions $m+2$ to n). The steady state of the chain is characterized by $J_2 = 2\,J_1$, where J_1 and J_2 are the steady-state fluxes of reactions 1 to $m+1$ and $m+2$ to n, respectively. Therefore, we have $d = 2b - a$ for the excess number. Because the reaction $m+1$ is bimolecular in the backward direction, a quadratic equation results for the glycolytic flux [Eqn (A5) in the Appendix]. By solving this equation, one can express the glycolytic flux and the ATP-production flux as functions of a and b. The results are depicted in Fig. 7 for fixed and equal enzyme concentrations and for the same parameter values as used in Fig. 1 for the linear model. Only even numbers of C-sites may be considered since otherwise the degradation pathways of S_{m+1} and S^*_{m+1} could not be the same. The conclusions concerning the optimal number of ATP-consuming and ATP-producing steps derived for the linear model remain valid by consideration of branching. In particular, replacement of an O-site by a C-site in the first part of the chain results in an increase of the ATP-production rate. One may conclude that there is no essential kinetic difference in the linear and branching models with respect to the efficiency of ATP production. However, the branched system seems to be more effective from a chemical point of view. For example, an excess number $d = 2$, as observed in glycolysis, can be realized in the branched model with $a = 2$ and $b = 2$, while in the linear model there are the possibilities $a = 1$, $b = 3$ and $a = 2$, $b = 4$, that is, in the linear model more ATP-producing reactions are necessary than in the branching model (Meléndez-Hevia, E., Waddell, T. G., Heinrich, R. and Montero, F., unpublished results).

DISCUSSION

In the present report, we analyzed whether the design of contemporary glycolysis may be understood on the basis of optimization principles. Since the main metabolic function of glycolysis consists of ATP production, particular attention is paid to the kinetic effects of a change in the location and the number of ATP-producing and ATP-consuming steps. The main result of our investigation is that the optimization of kinetic properties favours pathways where the steps at the upper end of the chain are exergonic or coupled to exergonic processes (such as ATP hydrolysis) and steps at the lower end of the chain are endergonic or coupled to endergonic processes (such as ATP production). This result is in accordance with glycolysis but also with other metabolic systems. For example, in the citric-acid cycle, there are two exergonic reactions located at the beginning: the citrate-synthase reaction, which involves hydrolysis of the energy-rich thioester-binding of acetyl-CoA; and the isocitrate-dehydrogenase reaction. The following reactions yield the energy-rich compounds GTP, NADH and $FADH_2$. The last reaction of the cycle, the malate-dehydrogenase reaction, is very endergonic. Another example is gluconeogenesis, which becomes possible by circumventing the pyruvate kinase reaction by means of two steps, catalyzed by pyruvate carboxylase and phospho*enol*pyruvate carboxykinase, which involve hydrolysis of either ATP or GTP. Similary, fatty-acid oxidation is initiated by fatty-acid activation in an ATP-dependent acylation reaction to form fatty-acyl-CoA. Further fatty acid oxidation yields NADH and $FADH_2$, which are oxidized through oxidative phosphorylation to form ATP.

In the present reports many chemical constraints which may favour special structural designs have been neglected (Meléndez-Hevia, E., Waddell, T. G., Heinrich, R. and Montero, F., unpublished results). Several other simplifications of the present model deserve special consideration:

The concentrations of the adenine nucleotides ATP and ADP are considered to be fixed, that is, Eqn (5) for the glycolytic flux is derived from the steady-state assumption for the carbohydrates but not for the adenine nucleotides. In a more-detailed consideration, it would be possible to treat the concentrations of ADP and ATP as system variables. Since glycolysis is characterized by a net production of ATP, this would necessitate incorporation of non-glycolytic ATP-consuming processes. This has been done in several simulation models of glycolysis [2–5, 7, 8]. Variable concentrations of the adenine nucleotides would result in non-linearities in the steady-state equations, due to the consideration of bimolecular reactions, as well as in further feedbacks from the lower end to the upper end of the chain. Preliminary analysis reveals that the variable concentrations of cofactors do not affect the main conclusions derived in the present paper (Stephani, A. and Heinrich, R., unpublished results).

In the present analysis, it was assumed that the last step of the pathway may be an ATP-production site. Since in anaerobic organisms, the redox cycle must be completed, glycolysis ends with the lactate-dehydrogenase reaction, which is highly exergonic but cannot be used to make ATP (Meléndez-Hevia, E., Waddell, T. G., Heinrich, R. and Montero, F., unpublished results). Therefore, it seems to be necessary to modify the model by introducing one uncoupled step (O-site) with a high equilibrium constant at the end of the pathway. Analysis shows that this modification has a minor kinetic effect.

In defining the antiglycolytic design, it was assumed that ATP-producing sites may be located at the first reactions of the pathway. This cannot be the case in a pathway with glucose as the initial substrate. It is possible, however, to give the concept of antiglycolytic design a more precise meaning by taking into account phosphorylation steps with inorganic phosphate as the substrate at the very beginning of the pathway, i.e. preceding those of the first ATP-producing steps (Meléndez-Hevia, E., Waddell, T. G., Heinrich, R. and Montero, F., unpublished results). This modification does not affect our general conclusion that a reverse order of C-sites and P-sites, compared with glycolysis, gives a poor kinetic design.

Recently, we have proposed the function

$$\eta = d \cdot (\Delta G'^{\circ}_{glyc} - d \cdot \Delta G'^{\circ}_{ATP}) = d \cdot \Delta G'^{\circ}_{net} \qquad (42)$$

as a measure for the efficiency of glycolysis [38]. In contrast to the criteria based on the ATP-production rate, the function η only depends on thermodynamic quantities and not on the rate constants of the reactions that characterize the kinetic properties of the chain. The principle η = min proposed in [38] quantifies

the hypothesis that cellular evolution of glycolysis may have sought to maximize the number of ATP molecules produced and maintain the maximally negative net-free-energy change. η displays a minimum with variations of ΔG_{ATP} ranging from -25 kJ/mol to -70 kJ/mol. Taking into account the value for $\Delta G'^{0}_{glyc}$ given in Eqn (33a), the minimum value of η is attained for $d \approx 2$ and $\Delta G_{ATP} = -50$ kJ/mol. As mentioned above, a ΔG_{ATP} value of -50 kJ/mol is the accepted value for phosphorylation under cellular conditions. Therefore, this result is in good accordance with the design of glycolysis ($a = 2$, $b = 4$).

There is some relationship between the principles J_{ATP} =max and η = min. Neglecting the denominator D in Eqn (5) for the steady-state flux, one gets, under consideration of Eqn (29), for $[S_0] = [S_n]$

$$J_{ATP} = d\,\{\exp[(d \cdot \Delta G_{ATP} - \Delta G'^{0}_{glyc})/RT] - 1\} \qquad (43)$$

where the equilibrium constants Q and K have been related to the free energy changes $\Delta G'^{0}_{glyc}$ and ΔG_{ATP} respectively. Expanding the exponential function in Eqn (43) in a Taylor series, and taking into consideration only the linear terms, one arrives immediately at $J_{ATP} = (-\eta)$. However, it is also seen that this approximation would necessitate that the term $d \cdot \Delta G_{ATP} - \Delta G'^{0}_{glyc}$ is small compared to $RT = 2.577$ kJ/mol (at $T = 310$°K) which, for glycolysis, is generally not the case (Eqn 33).

Our theoretical approach to explain the structural design of glycolysis in terms of thermodynamic and kinetic parameters is based on the hypothesis that optimization of the net flux through this pathway, in particular, the ATP-production rate, was of utmost importance during evolution. We are aware that this may be not sufficient to explain all details of the design of glycolysis. Other systemic properties should be taken into account in future optimization studies, such as stability of steady states, transition times and regulation by internal effectors. An attempt to include the effects of feedback inhibitions is presented above.

Furthermore, our analysis on glycolysis optimization is based on the assumption that the system works under conditions of constant chemical affinity, that is, at constant substrate and product concentrations. This assumption is valid, for example, in red blood cells where the concentrations of glucose and lactate remain almost unaffected at variations of the kinetic parameters and of the rate of glycolysis [7]. Therefore, the ATP-production rate (J_{ATP}) or the glycolytic rate J may be considered as targets of optimization. However, a different case can also be considered, where the system works under the constraint of a constant input flux (J_{in}). In such systems, the steady state is externally imposed, that is, it is not a variable which may be affected by kinetic optimization. However, optimization of the stoichiometry is meaningful in this case. This concerns, in particular, the maximization of the number of ATP molecules which can be produced/molecule of glucose.

APPENDIX

Let us consider the reaction chain depicted in Scheme 2 where at the inner step $m+1$ a splitting of the compound S_m into the compounds S_{m+1} and S^*_{m+1} occurs. The latter compounds can be interconverted in a reaction with a rate v^*_{m+1}. The steady state of the chain is characterized by $J_2 = 2J_1$ where J_1 and J_2 are the steady-state fluxes of the reactions 1 to $m+1$ and $m+2$ to n, respectively. Taking into account Eqn (4), the metabolite concentrations $[S_m]$ and $[S_{m+1}]$ can be expressed as

$$[S_m] = [S_0]\,Q_{1,m} - J_1 \sum_{j=1}^{m} Q_{j,m}\,k_j^{-1} \qquad (A1)$$

$$[S_{m+1}] = Q^{-1}_{(m+2),n}\left\{[S_n] + 2J_1 \sum_{j=1}^{n-m-1} Q_{(m+j+1),n}\,k^{-1}_{m+j+1}\right\}. \qquad (A2)$$

Taking into account the steady state conditions for $[S_m]$ and $[S^*_{m+1}]$, respectively

$$J_1 = v_{m+1} = k_{m+1}[S_m] - k_{-(m+1)}[S^*_{m+1}][S_{m+1}] \qquad (A3)$$

$$J_1 = v^*_{m+1} = k^*_{m+1}[S^*_{m+1}] - k^*_{-(m+1)}[S_{m+1}] \qquad (A4)$$

one finds for the determination of the glycolytic flux the quadratic equation $a_2 J_1^2 + a_1 J_1 + a_0 = 0$ with

$$a_0 = k_{-(m+1)}k^*_{-(m+1)}b_3^2 - k_{m+1}k^*_{m+1}b_1 \qquad (A5a)$$

$$a_1 = k_{m+1}k^*_{m+1}b_2 + 2k_{-(m+1)}k^*_{-(m+1)}b_3b_4 + k^*_{m+1} + k_{-(m+1)}b_3 \qquad (A5b)$$

$$a_2 = k_{-(m+1)}b_4[1 + k^*_{-(m+1)}b_4] \qquad (A5c)$$

and

$$b_1 = [S_0]\,Q_{1,m}, \quad b_2 = \sum_{j=1}^{m}\frac{Q_{j,m}}{k_j}, \quad b_3 = [S_n]\,Q^{-1}_{(m+2),n},$$
$$b_4 = 2\,Q^{-1}_{(m+2),n}\sum_{j=1}^{n-m-1}\frac{Q_{(m+j+1),n}}{k_{m+j+1}}. \qquad (A5d-g)$$

From Eqn (A5a to g), the ATP-production rate is obtained by use of the formula $J_{ATP} = (2b - a)J_1$.

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