System wide cofactor turnovers can propagate metabolic stability between pathways

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A B S T R A C T

Metabolic homeostasis, or low-level metabolic steady state, has long been taken for granted in metabolic engineering, and research priority has always been given to understand metabolic flux control and regulation of the reaction network. In the past, this has not caused concerns because the metabolic networks studied were invariably associated with living cells. Nowadays, there are needs to reconstruct metabolic networks, and so metabolic homeostasis cannot be taken for granted. For metabolic steady state, enzyme feedback control has been known to explain why metabolites in metabolic pathways can avoid accumulation. However, we reasoned that there are further contributing mechanisms. As a new methodology developed, we separated cofactor intermediates (CIs) from non-cofactor intermediates, and identified an appropriate type of open systems for operating putative reaction topologies. Furthermore, we elaborated the criteria to tell if a multi-enzyme over-all reaction path is of in vivo nature or not at the metabolic level. As new findings, we discovered that there are interactions between the enzyme feedback inhibition and the CI turnover, and such interactions may well lead to metabolic homeostasis, an emergent property of the system. To conclude, this work offers a new perspective for understanding the role of CIs and the presence of metabolic homeostasis in the living cell. In perspective, this work might provide clues for constructing non-natural metabolic networks using multi-enzyme reactions or by degenerating metabolic reaction networks from the living cell.

1. Introduction

Metabolic reaction networks entail thousands of enzymes and connect to a myriad of metabolites, with intermediate number being of the order of 10^3 and average concentration 32 μM for a prokaryote (Atkinson, 1968; Palsson, 2011). A remarkable feature of such networked reaction system is that all the intracellular metabolic intermediates (or internal metabolites) are kept at metabolic homeostasis (in a physiological term) or low level steady state (in an engineering-orientated term). Such networks have been observed to be inherently robust (Stephanopoulos and Vallino, 1991; Ishii et al., 2007; De la Fuente et al., 2014), predominantly operated at thermodynamic non-equilibrium (Prigogine, 1955; Fell, 1997), and are able to accommodate a widely varied metabolic fluxes (Stephanopoulos, 1998; Villadsen, 2016).

Metabolic control and regulation take place notably at metabolic and gene-expression levels (Reich and Sel'kov, 1981; Palsson,

2015). The time constant (or time scale) can be used to differentiate study regimes (Palsson, 2011; Reich and Sel'kov, 1981). Control and regulation at metabolic level is a spontaneous, dynamic reaction process. Representative time constants at this level are in the range of 0.1–10 s (Reich and Sel'kov, 1981; Wiechert, 2002). In contrast, gene-expression and transcriptional control and regulation regimes for remapping metabolic fluxes involve biosynthesis/biodegradation of large molecules (e.g. enzymes), and their typical time constants are > 1 h (Wiechert, 2002; Palsson, 2011). The present work concerns with the control and regulation at the metabolic level with small time constants.

An important milestone in understanding metabolic regulation at metabolic level came from the discovery of enzyme negative feedback control by inhibition (Umbarger, 1956; Yates and Pardee, 1956; Gerhart and Pardee, 1962). Supported by mechanism explorations, such feedback control mechanism largely avoids metabolite build-up (Monod et al., 1963; Savageau, 1969, 1974; Chandra et al., 2011; He et al., 2013). The most efficient feedback control for a linear pathway at metabolic level is end-product inhibition, which may be termed as the optimal design of feedback

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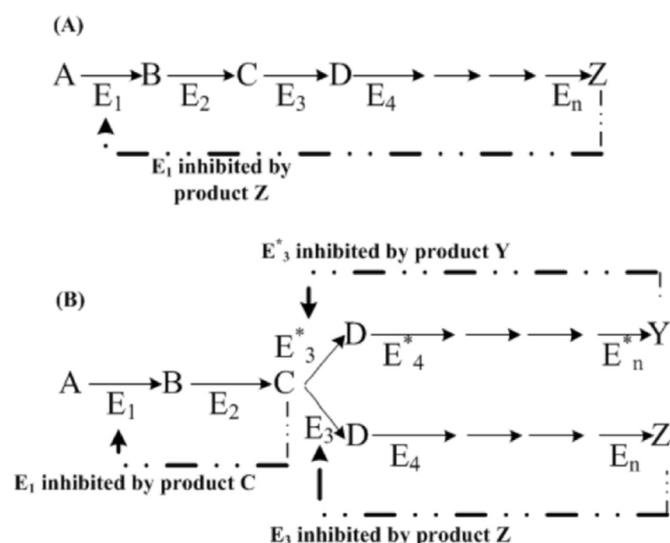
Fig. 1. End-product feedback control by the minimal inhibition loops for (A) linear pathway with only one inhibition loop, and (B) bifurcated pathway with 3 inhibition loops.

inhibition control (Savageau, 1974). It is optimal because such feedback control requires only one inhibition for a linear carbon-backbone pathway (Fig. 1(A)), and at least 3 feedback inhibition loops for a bifurcated pathway system (Fig. 1(B)) (Savageau, 1974, 1976). Even in the case that this mechanism is not functioning, the cell can still use a contingency mechanism to enzymatically hydrolyse the end-product to avoid its build-up (Reaves et al., 2013).

Nevertheless, spontaneous establishment of metabolic homeostasis does not rely only on allosteric enzyme regulation mechanisms. Morowitz et al. were probably the first in reporting the very nature of such autonomous homeostasis – in their wording "passive stability in a metabolic network" (Morowitz et al., 1964). These authors attributed a number of factors to the occurrence of metabolic homeostasis. Among their claims, the role of carbon backbone topology has been well substantiated (Morowitz et al., 2000; Smith and Morowitz, 2004), but the role of the cofactor topology, superimposed onto the carbon backbone topology, has never been evidenced.

As a broad objective, this work is aimed to shed light for understanding why a metabolic network is stable and why all metabolite concentrations are kept at low levels. With low level steady state being taken for granted, up to now the metabolic control and regulation community has given priorities to understanding how metabolic fluxes are amenable to changes in magnitude and distribution (Hofmeyr and Cornish-Bowden, 1991). Such convention had been directed to curate genome-based metabolic reaction networks without involving metabolite concentrations (Palsson, 2015). Understanding of metabolic homeostasis observed for living cells (Savageau, 1976) has long been separated from research on metabolic flux control and regulation (Kacser and Burns, 1973; Heinrich and Rapoport, 1974). In the past, this arbitrary separation had not caused concerns because the metabolic networks investigated were invariably associated with living cells where metabolic homeostasis is out of question. Nowadays, there are challenging needs for reconstructing metabolic networks in synthetic biology or metabolic engineering. In contrast, metabolic homeostasis for multi-enzyme reaction systems under investigation can no longer be taken for granted (Rollin et al., 2015; Keller et al., 2016). In detail, the present work will describe an emergent property resulted from interactions between cofactor intermediate (CIs) turnovers and enzyme feedback inhibition. This property may play a pivotal role to the emergence of metabolic homeostasis.

2. Theoretical framework

In order to unfold this research, we first separated CIs from non-cofactor intermediates (NCIs) to reveal the important role of the CIs, and then conceptually connect the systems used to various multi-enzyme reaction systems. We quantitatively illustrated how the metabolic homeostasis of in vivo systems is robust against an environmental perturbation (i.e. substrate concentration variation). This outcome then guided us in validating a suitable type of working open systems.

2.1. CIs versus NCIs

The metabolic network entails 2 types of internal metabolites: (i) a limited number of paired or grouped CIs and (ii) a vast number of standalone NCIs. Enzymes utilise NCIs for stepwise chemical conversion from substrate(s) to desired end products. CIs are usually paired and each paired CI pool size is fixed for global uses at the metabolic level. Common CI pairs/groups include ATP/ADP/AMP (for energy transduction), NAD^+/NADH, $NADP^+$/NADPH, Q/QH_2 (for redox transfer), and CoA-SH/acetyl-CoA/malonyl-CoA/succinyl-CoA (for carbon chain translocation). The energy CI pool should be referred to as $ATP+ADP+AMP+Pi$ at the metabolic reaction time scale (Wiechert, 2002). The catabolic and anabolic redox CIs pool sizes are NAD^++NADH and $NADP^++NADPH$ respectively. The CoA-related CI pool size is the sum of CoASH in free form and covalently bound to certain carbon backbone chains (e.g. acetyl-CoA). Hence, the CoA-bound metabolites are both CIs and NCIs. Besides, metabolism contains further unique reaction topologies comparable to CI turnovers. For instance, cyclic-like reactions for transferring nitrogen-containing groups are different in that there is a net consumption of e.g. NH_2 groups.

Each CI pool size is inherently small (e.g. $ATP+ADP+AMP$ pool typically at 2 mM) (Atkinson, 1977; Palsson, 2011). To simultaneously sustain so many reactions in a homogeneous space, ATP (ADP) has no options but to turn around at globally balanced rates through numerous ATP (ADP)-consuming and generating reactions. Usually, an NCI conversion reaction is realised by utilising a CI as the second reactant. In such network topologies, each CI [e.g. B (Q) out of the B + Q pool, Fig. 2(A)–(C)] can only stay either in steady or oscillating status (Chassagnole, 2002; De la Fuente et al., 2014). At such metabolic homeostasis, the net B-to-Q or Q-to-B flux can be termed as CI turnover flux or CI flux. To make a distinction, the well-understood carbon backbone flux (Stephanopoulos et al., 1998; Villadsen, 2016) can thus be termed as NCI flux. Each reaction stoichiometry connects a CI flux to relevant NCI flux (es). Without metabolic homeostasis, any discussions on fluxes will become groundless and meaningless. This work underpins how metabolic homeostasis may be constructed from scratch, and metabolic flux regulation is not the focus.

2.2. The in-vivo-like system

In the synthetic biology era, the boundary separating an in vivo and an in vitro systems is becoming more and more blurred. In order to discuss this subject systematically, it is useful to define a new concept termed the in-vivo-like system. In the present context, it refers to a multi-enzyme reaction system which possesses CI turnover and metabolic homeostasis. Such a system can be either completely man-made, or derived from native cells. Indeed, new frontiers of metabolic engineering such as cofactor engineering have been researched for years (San et al., 2002; Rollin
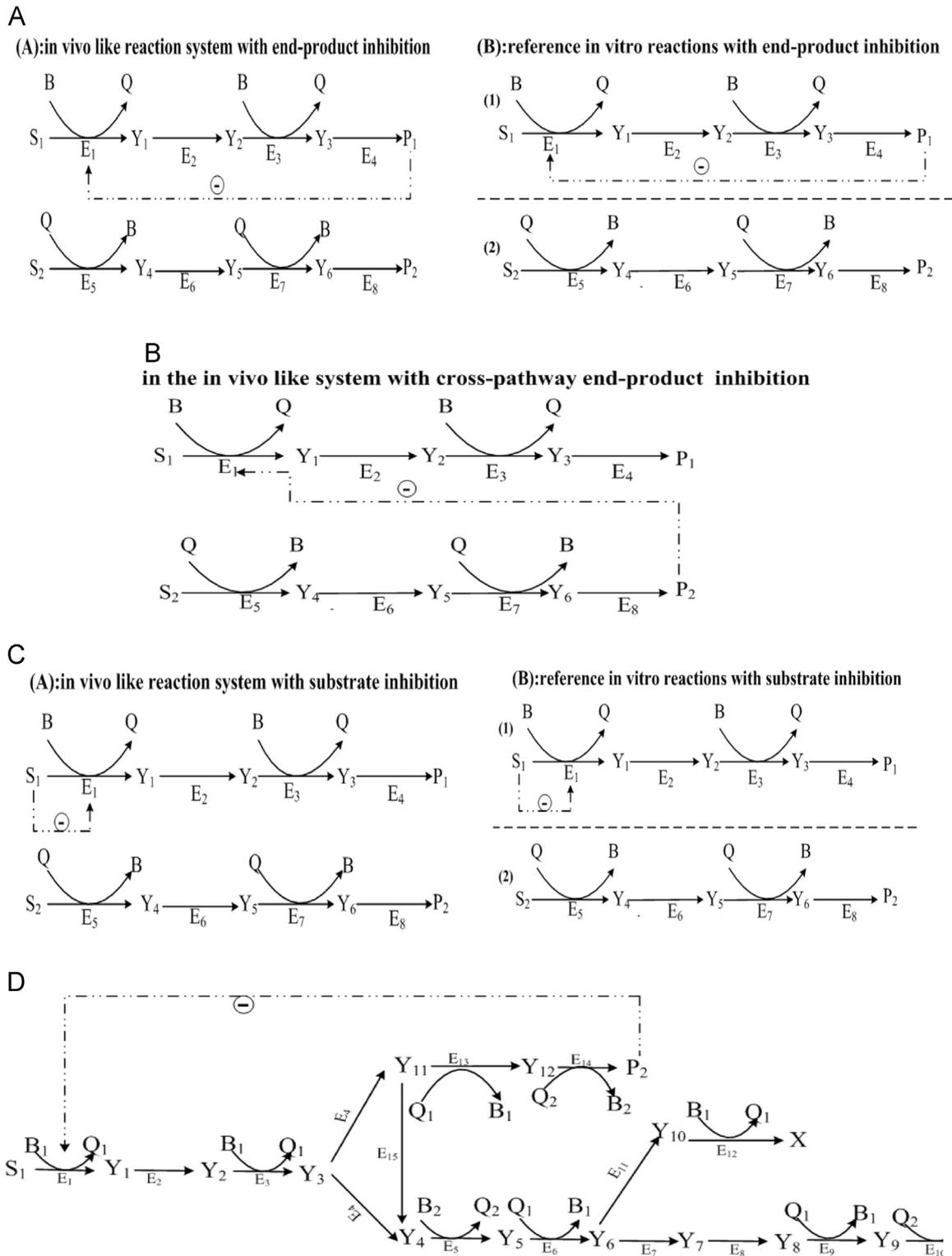
Fig. 2. Exemplified metabolic network topologies used in the present study. The broken arrowed line represents negative feedback enzyme inhibition. **A:** A pair of linear pathways with the end-product inhibition. S_1 & S_2 are substrates, P_1 & P_2 products, Y_1, Y_2,Y_3, Y_4, Y_5, Y_6 NCIs, B & Q CIs in (A). 2 pathways in (B) are disconnected, so B (Q) becomes substrate and product. **B:** A pair of linear pathways with the cross-pathway end-product inhibition. S_1 & S_2 are substrates, P_1 & P_2 products, Y_1, Y_2, Y_3, Y_4, Y_5, Y_6 NCIs, and B & Q CIs. No in vitro reference reactions. **C:** A pair of linear pathways with substrate inhibition. S_1 & S_2 are substrates, P_1 & P_2 products, Y_1, Y_2,Y_3, Y_4, Y_5, Y_6 NCIs, and B & Q CIs in (A). 2 pathways in (B) are disconnected, so B (Q) becomes substrate and product. **D:** Branch network with the end-product inhibition. S_1 is substrate, P_1, P_2 & X products, Y_1, Y_2,Y_3, Y_4, Y_5, Y_6, Y_7, Y_8, Y_9, Y_{10}, Y_{11} & Y_{12} NCIs, B_1/Q_1 and B_2/Q_2 CIs. No in vitro reference reactions.

et al., 2015). In this work, we developed a new theory to tell if a multi-enzyme reaction is an in-vivo-like system or not.

To reveal system wide properties of CIs in a multi-enzyme re-action system, the 1st network type for this work is composed of 2 parallel linear carbon backbone pathways (Fig. 2(A)–(C)), and the 2nd branched pathways (Fig. 2(D)). In the living cell, all the carbon backbone pathways are ultimately connected. A pair of linear pathways can thus be viewed to represent those scenarios where

the only link between 2 pathways is via CI turnover. Consequently, focus in Fig. 2(A)–(C) has been given to the CI-related reaction network topologies. To avoid concentrations of the 2 substrates becoming disparate (Fig. 2(A)–(C)), kinetic parameters and enzyme loadings have been arranged symmetrically for the 2 pathways.

Fig. 2(A) is configured so that product of the 1st pathway exerts inhibition on its 1st enzyme whilst the 2nd pathway is free from inhibition. In addition to the end-product inhibition on the same pathway (Fig. 2(A)), it is common that product of another see-mingly unrelated pathway (i.e. the 2nd pathway) can also exert inhibition over that pathway (i.e. the 1st pathway). This scenario is presented by the Fig. 2(B) reaction topology. Fig. 2(C) is configured so that substrate of the 1st pathway exerts inhibition on its own enzyme whilst the 2nd pathway is free from enzyme inhibition. Using these 3 separate enzyme inhibition topologies, we investigated possible roles that CI turnover plays in maintaining metabolic homeostasis. The Fig. 2(D) reaction topology resembles that of glycolysis, but is an intact in-vivo-like system.

2.3. The open system

Metabolism can take place only in open system. As will be shown in the subsequent section, the Fig. 2(A) reaction topology can be classified as an in-vivo-like system (see Section 4). With this pre-knowledge, the effects of substrate feeding on the meta-bolite Y_1 are illustrated (Fig. 3). This "dry" experiment was desig-nated so that the combined amount of substrate fed has been kept the same over the total time duration regardless of the feeding regime. When the substrate is fed at a fixed rate, a low-con-centration quasi-steady state for Y_1 is observed (Fig. 3(A)). When 3 discrete feedings are made, each feeding causes a disturbance, but the Y_1 level reverts back to its quasi-steady state (Fig. 3(B)). When all of the substrate is fed at the very outset, the Y_1 level again reverts back to its quasi-steady state (Fig. 3(C)). Over this time duration, the Fig. 3(C) operation appears to be a batch reac-tion system. As batch reaction is much easy to operate in practice, in the following we will stick to this reaction modality.

Continuous stirred-tank reactor (CSTR) at steady state is an open system, and has often been used for conducting in vivo re-actions (Palsson, 2011; Villadsen et al., 2011, 2016). On one hand, even though the reaction system does not have homeostasis property, use of CSTR may lead to steady states for each inter-mediate. Consequently, CSTR is inappropriate in our quest on the emergence of metabolic homeostasis. On the other hand, if one is using an in vivo system (i.e. cellular reaction system), use of CSTR allows selecting different metabolic states by adjusting dilution rate (another environmental factor). Unlike CSTR, the working open system portrayed above can only reach quasi-steady state owing to the fact that the product is not been removed.

3. Methods

Reaction mechanisms for a two-substrate enzyme reaction are: (i) compulsory-order mechanism, (ii) random-order mechanism, and (iii) double-displacement mechanism (Cornish-Bowden, 2012; Fell, 1997). The mechanism adopted in this work is based on the compulsory-order mechanism. To simplify the calculation, we re-gard only one three-body enzyme complex, rather than two, for each concerned enzyme. Therefore, each 2-substrate reaction en-tails 4 elemental reaction steps. Although arbitrariness is involved in reaction mechanism selection, it is nevertheless so that the outcome kinetic expression formality is expected to be the same or similar regardless of the above mechanisms (Cornish-Bowden, 2012).

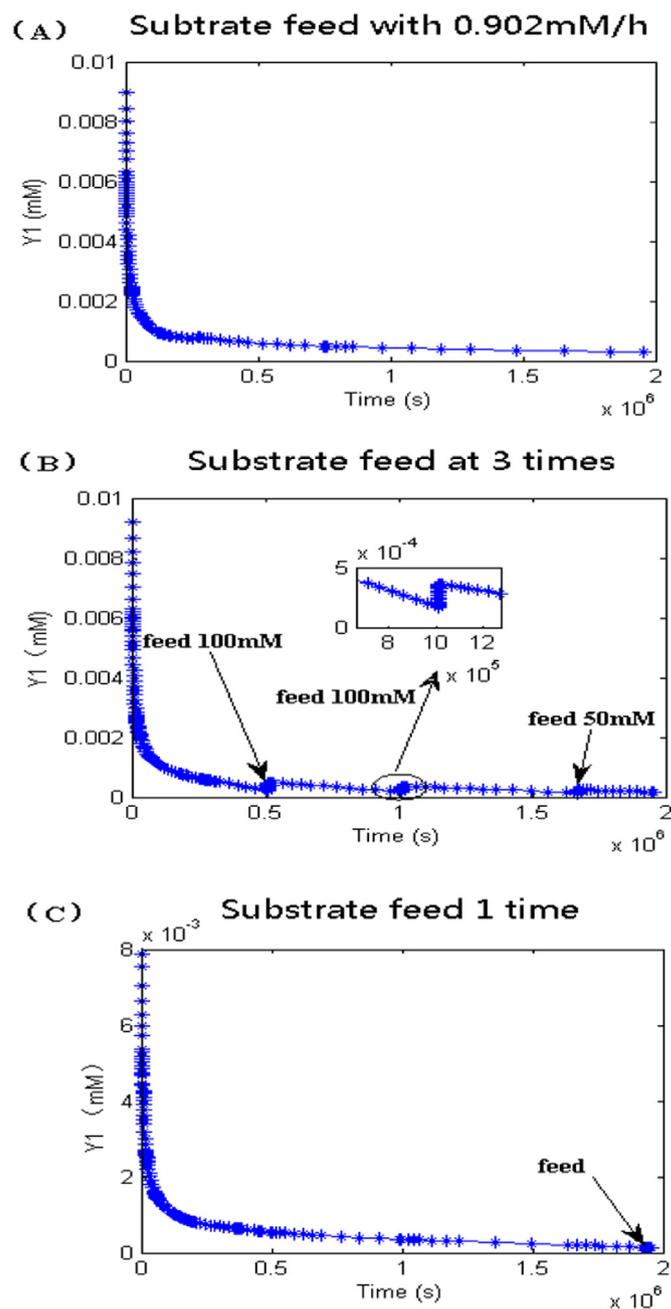Experimentally measured cofactor intermediates (i.e. CI)

Fig. 3. Effects of substrate feeding regimes on the Y_1 (Fig. 2A reaction topology). Three feeding regime are shown, where 500 mM of substrate is added over 542.5 h: (A) continuous feeding at a fixed rate of 0.902 mM/h, (B) initial substrate at 250 mM, followed by 3 discrete feedings (100 mM, 100 mM and 50 mM respec-tively), and (C) all 500 mM substrate starts at the beginning.

concentrations vary to some extents. On average and typically for *E. coli* cells, Palsson (Palsson, 2011) listed the following figures: ATP 1.6 mM, ADP 0.29 mM, AMP 0.087 mM, NAD^+ 0.06 mM, NADH 0.03 mM. Specifically, for *E. coli* cells, Bennett et al. (2009) documented the following experimental results: ATP 9.6 mM, ADP 0.56 mM, AMP 0.28 mM, NAD^+ 0.083 mM, NADH 2.6 mM, $NADP^+$ 0.0028 mM, and NADPH 0.12 mM. For yeast *S. cerevisiae*, Teusink et al. (2000) reported experimental data: ATP 2.5–2.54 mM, ADP 1.31–1.33 mM, AMP 0.18–0.32 mM, NAD^+ 1.07–1.33 mM, and NADH 0.3–0.48 mM. These data have been used as guidance for considering CI pool size in this work.

For the systems presented in Fig. 2, a set of ordinary differ-entiation equations (ODEs) is established based on mass action law

and mass conservation on every metabolite, enzyme and enzyme complex. These equations are simultaneously solved using Matlab with ODE 15 s as the solver. The kinetic parameters, initial conditions, and enzyme loadings used for simulation are given in Tables S1, S2 and S3 of the Supplementary Material.

4. Results and discussion

The central theme of the present work is to testify the presence of a property in the living cell, which emerges by interacting enzyme feedback inhibition with CI turnover. As the starting point, one needs to exclude those systems where the metabolic stability has already been achieved even though the enzyme inhibition and CI turnover are artificially made absent. As a further point, one needs also to exclude those systems where enzyme inhibition alone is sufficient in causing metabolic stability. To the basal system meeting these 2 criteria, the following 3 additional situations were then constructed: (i) the enzyme inhibition is incorporated, (ii) the CI turnover is incorporated, and (iii) both the enzyme inhibition and the CI turnover are incorporated. To introduce CI turnover effects, pertinent in-vivo-like systems were constructed. To remove CI turnover effects, 2 approaches were designed: (i) if present, to turn each pathway into an independent reaction (i.e. an in vitro reference reaction), (ii) to make the CI pool size extraordinarily large. Metabolic stability results (if any) out of these situations were then compared.

4.1. The Fig. 2(A) reaction topology: single end-product inhibition, on its own pathway

Realisation of the optimal end-product inhibition control (i.e. the 1st pathway in Fig. 2(A)) was made decades ago (Umbarger, 1956; Umbarger and Brown, 1957; Monod, 1963). This type of inhibition limits accumulation of metabolic intermediates on the 1st pathway (Savageau, 1976), but we do not have knowledge whether low level steady state can also be established for the 2nd pathway intermediates in Fig. 2(A).

When the only feedback inhibition is taken out, the deficient in-vivo-like system (Row 2 of Fig. 4) and its in vitro reference reactions (Row 4 of Fig. 4) have the same NCI and substrate/product profiles, where the internal metabolites except Y_3 and Y_6 are at steady states. Clearly, in the absence of enzyme inhibition, CI turnover alone cannot lead to metabolic homeostasis.

When enzyme end-product inhibition is brought back, for the in vitro reactions (Row 3 of Fig. 4), low level steady states are attained only for the 1st pathway (i.e. for Y_1, Y_2 and Y_3) but not for the 2nd pathway (i.e. Y_6 accumulates and Y_4 is stabilized at elevated level). However, when the CI turnover is incorporated with end-product inhibition to make the in-vivo-like system, all metabolic intermediates in both pathways are confined at low levels (Row 1 of Fig. 4). Indicatively, interactions between the CI turnover and the end-product inhibition lead to metabolic homeostasis. These interactive effects consequently constitute an emergent property mentioned above. For this in-vivo-like system, NCI stabilized levels increase with either decreasing inhibition strength or
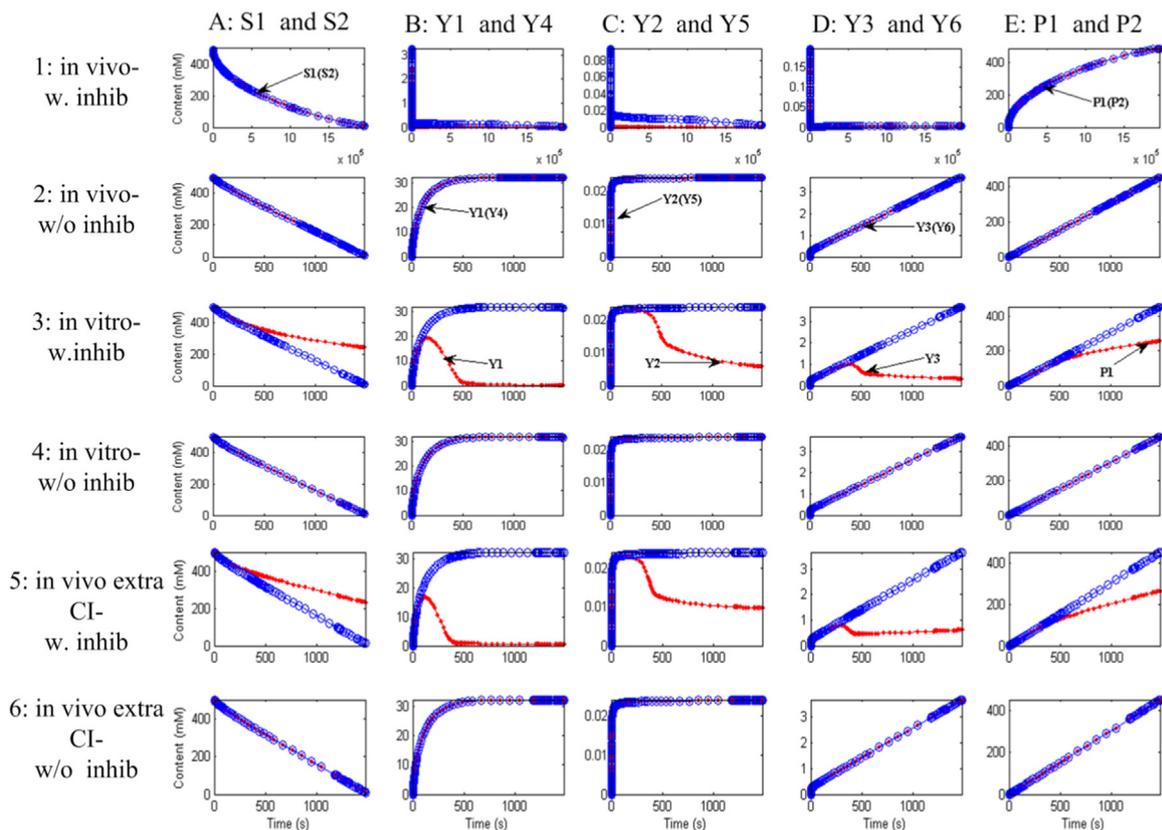

Fig. 4. Reaction time profile comparison for the Fig. 2A network including the in-vivo-like system and its reference in vitro reactions. For in-vivo-like system, B and Q are paired CI and start at 0.75 mM respectively, and B+Q pool of 1000 mM is regarded as extra-large. For the in vitro reference reactions, B/Q is substrate and product. Row 1: in-vivo-like system with end-product inhibition; Row 2: in-vivo-like system without inhibition; Row 3: in vitro reference reactions with end-product inhibition; Row 4: in vitro reference reactions without inhibition; Row 5: in-vivo-like system with end-product inhibition, where CI pool size is extra-large; Row 6: in-vivo-like system without inhibition, where the CI pool size is extra-large. Inhibition strength applied is high ($Ki = 3.8*10^{-5}$).
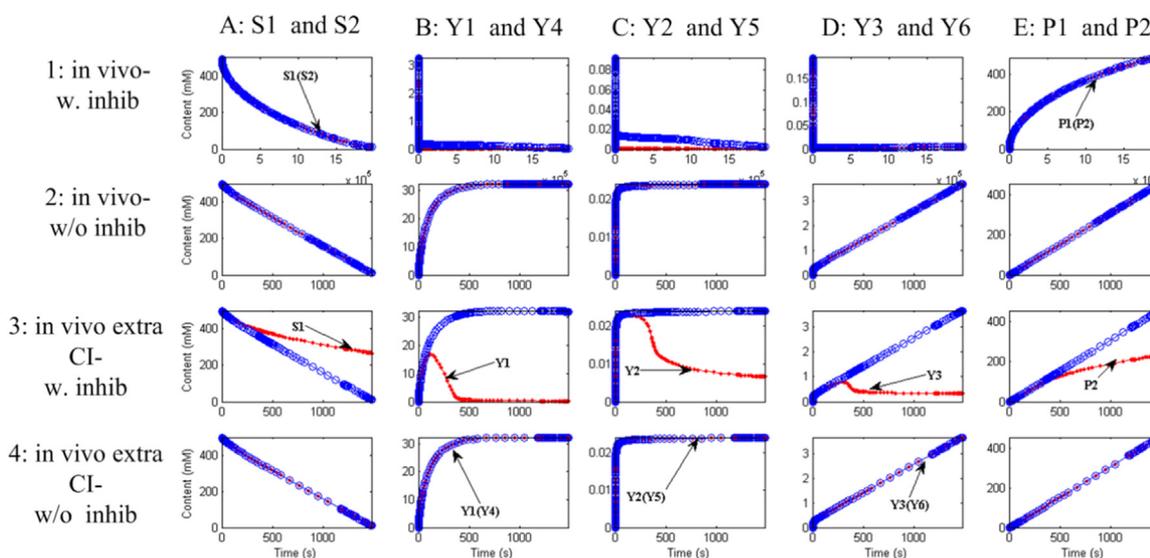
Fig. 5. Reaction time profile comparison for the Fig. 2B network. For in-vivo-like system, B and Q are paired CI and start at 0.75 mM respectively, B+Q pool of 1000 mM is regarded as extra-large. Row 1: in-vivo-like system with cross-pathway end-product inhibition; Row 2: in-vivo-like system without inhibition; Row 3: in-vivo-like system with cross-pathway end-product inhibition, where the CI pool size is extra-large; Row 4: in-vivo-like system without inhibition, where the CI pool size is extra-large. Inhibition strength applied is high ($Ki = 3.8 * 10^{-5}$).

increasing the CI pool size, or both (Fig. S1, Supplementary material). Overall, these NCI concentrations are very low. In terms of the present experimental technologies, measured results may well reject a significant change even though the effect per se is numerically significant.

In the majority of cases, it is not possible to obtain the in vitro reference reactions out of an in-vivo-like system (e.g. the Fig. 2 (B) and (D) reaction topologies). In such cases, use of an extra-large CI pool size (e.g. B+Q=1000 mM) is the only alternative for dampening or even removing the CI turnover effect. Validity of this approach is confirmed by comparing Row 2 with Row 6 (Fig. 4), and Row 3 with Row 5 (Fig. 4). The difference between Row 1 and Row 5 (Fig. 4) indicates that metabolic homeostasis is more likely to be associated with small CI pool size.

4.2. The Fig. 2(B) reaction topology: single end-product inhibition, cross pathways

In the absence of enzyme inhibition, all the displayed metabolites behave identically between the deficient in-vivo-like system (Row 2 in Fig. 5) and that with extra-large CI pool size (Row 4 in Fig. 5). Y_1 and Y_4 are stabilized but at an unacceptably high level (i.e. 30 mM), Y_2 and Y_5 stabilized at a low level (i.e. 0.02 mM), and Y_3 and Y_6 simply accumulate almost in a linear fashion. This outcome confirms that metabolic homeostasis cannot be achieved without the enzyme inhibition. For the in-vivo-like system where both the enzyme inhibition and the CI turnover are present, metabolic homeostasis is satisfactorily reached (Row 1 of Fig. 5). Quantitatively, NCI steady state concentrations for this in-vivo-like system can be increased by reducing inhibition strength (Fig. S2A in Supplementary material) or increasing the CI pool size (Fig. S2B, Supplementary material), or a combination of both. In the presence of the enzyme inhibition and with the extra-large CI pool size (Row 3 of Fig. 5), NCI concentrations on the 1st pathway (i.e. Y_1, Y_2 and Y_3) can be stabilized to low levels, but those on the 2nd pathway (i.e. Y_4, Y_5 and Y_6) cannot. In detail, Y_4 is stabilized yet its level is too high, Y_5 is stabilized to a low level, and Y_6 simply keeps accumulating. Indicatively, low-level NCI steady states for the 1st pathway are independent of the CI turnover when the CI pool size becomes extra-large. Note that the 1st pathway has inhibition whereas the 2nd pathway does not have. Again, the enzyme

inhibition is essential for metabolic homeostasis, which can be propagated to another inhibition free pathway with the aid of small size CI turnover.

4.3. The Fig. 2(C) reaction topology: single substrate inhibition, on its own pathway

Substrate inhibition is also important for enzyme regulation at both gene and metabolic levels (Cornish-Bowden, 2012; Yoshino and Murakami, 2015; Reed et al., 2010). It is therefore worth providing evidences at the metabolic level that the emergent property out of the CI turnover and the enzyme inhibition is also present for this in-vivo-like system (Fig. 2(C)).

When the enzyme inhibition is absent, the corresponding NCI profiles are identical between the deficient in-vivo-like system, the in vitro reference reactions, and the deficient in-vivo-like system with extra-large CI pool size (Row 2, Row 4 and Row 6 of Fig. 6). When the substrate inhibition is introduced, (i) the in-vivo-like system instantaneously reaches metabolic homeostasis (Row 1 of Fig. 6), (ii) for the in vitro reference reactions (Row 3 of Fig. 6), all NCIs on the 1st pathway (Y_1, Y_2 and Y_3) and 2 NCIs on the 2nd pathway (Y_4 and Y_5) reach low-level steady states whereas Y_6 keeps accumulating, (iii) the NCI profiles between the (deficient) in-vivo-like system with extra-large CI pool size and the in vitro reference reactions (Row 5 and Row 3 of Fig. 6) are very similar. These observations corroborate with conclusions made on the 2 previous in-vivo-like systems that an emergent property is exhibited following interactions between the enzyme inhibition and small pool size CI turnover. Initial substrate concentration in Fig. 6 is reduced to 5 mM (rather than 500 mM) due to the substrate inhibition nature. This alteration however is avoided for the 2nd in vitro reference reaction and for the 2nd pathway of the (deficient) in-vivo-like system with extra-large CI pool size (Row 3 and Row 5 of Fig. 6).

To separate the effects of enzyme inhibition and CI pool size for the in-vivo-like system, it is observed that (i) high enzyme inhibition strength does not noticeably reduce steady state NCI concentrations (Fig. S3A, Supplementary material), (ii) alteration of CI pool size does not noticeably affect NCIs levels on the 1st pathway whereas a reduction in CI pool size can result in a further decrease of NCI levels on the 2nd pathway (Fig. S3B,
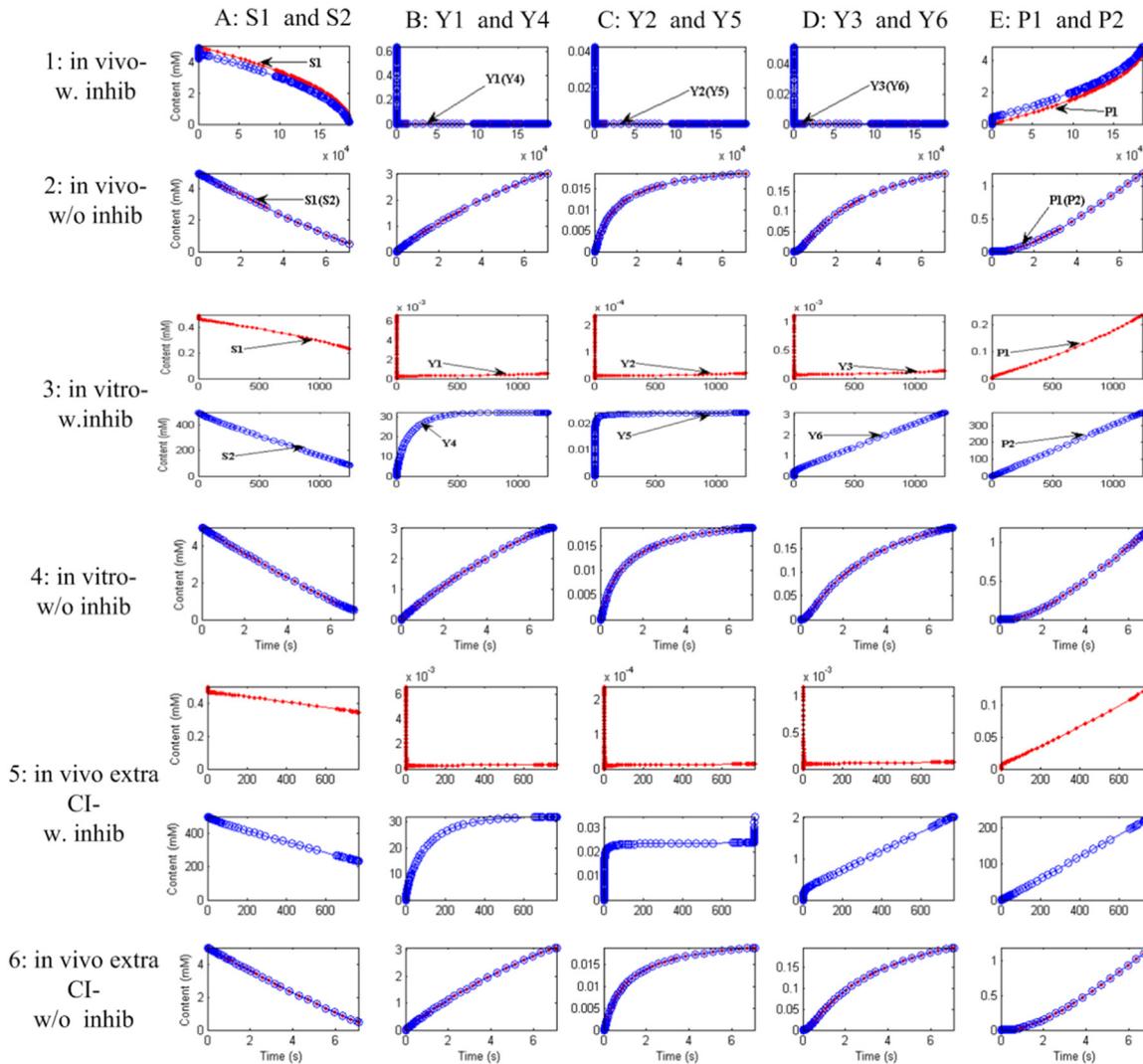
Fig. 6. Reaction time profile comparison for the Fig. 2C network including the in-vivo-like system and its reference in vitro reactions. For in-vivo-like system, B and Q are paired CI and start at 0.75 mM respectively, B+Q pool of 1000 mM is regarded as extra-large. For the in vitro reference reaction, B/Q is substrate and product. Row 1: in-vivo-like system with substrate inhibition; Row 2: in-vivo-like system without inhibition; Row 3: in vitro reactions with substrate inhibition; Row 4: in vitro reactions without inhibition; Row 5: in-vivo-like system with substrate inhibition, where CI pool size is extra-large; Row 6: in-vivo-like system without inhibition, where CI pool size is extra-large. Inhibition strength applied is high ($Ki=3.8*10^{-5}$).

Supplementary material). These properties for this in-vivo-like system (Fig. 2(C)) are different to those for the other 2 in-vivo-like systems (i.e. Fig. 2(A) and (B), in Supplementary material). As the NCI levels in these 3 in-vivo-like systems are unanimously low, effects of enzyme inhibition and CI pool size on NCI concentrations are likely to be practically insignificant in response to environmental changes. These results might underpin the well-observed metabolic robustness (Kitano, 2007; Stephanopoulos and Vallino, 1991).

4.4. Fig. 2(A)–(C): metabolic stability propagation from one pathway to the other

When the difference is only on inhibition topology (Fig. 2: A, B and C), the 3 in-vivo-like systems achieve metabolic homeostasis all based on the interaction between enzyme inhibition and CI turnover. When the enzyme inhibition is taken away, NCIs on both pathways of any system lose low-level steady states. When one significantly dampens the CI turnover effect, only NCIs on the 1st pathway of any system remain to have low-level steady states. This specific outcome is consistent with the existing knowledge that enzyme feedback control can avoid metabolite build-up (see

Section 1).

4.5. The Fig. 2(D) reaction topology: branched network

When considering in-vivo-like systems containing branched backbone pathways, the Fig. 2(D) reaction topology has been used to explain establishment of spontaneous metabolic homeostasis. This example entails B_1/Q_1 and B_2/Q_2 to resemble ATP/ADP and NAD^+/NADH in glycolysis. To globally balance the CI turnovers, an overall biomass (X) production "pathway" is introduced. This in-vivo-like system does not have reference in vitro reactions, and so extra-large CI pool size is used to remove the CI turnover effects.

Without enzyme inhibition, most NCIs (i.e. Y_3, Y_4, Y_7, Y_8, Y_{10}, Y_{11} and Y_{12}) cannot be stabilized to low levels in this deficient in-vivo-like system, and so the system is unable to reach metabolic homeostasis (Fig. 7(B)). In contrast, the in-vivo-like system with the end-product inhibition acquires instantaneous metabolic homeostasis (Fig. 7(A)). When the B_1+Q_1 pool size for the in-vivo-like system is increased to extra-large, the NCI profiles show decent similarities to the deficient in-vivo-like system where the enzyme inhibition is absent (Fig. 7(B) vs (C)). As before, the in-vivo-like system with extra-large CI pool size (Fig. 7(C)) cannot
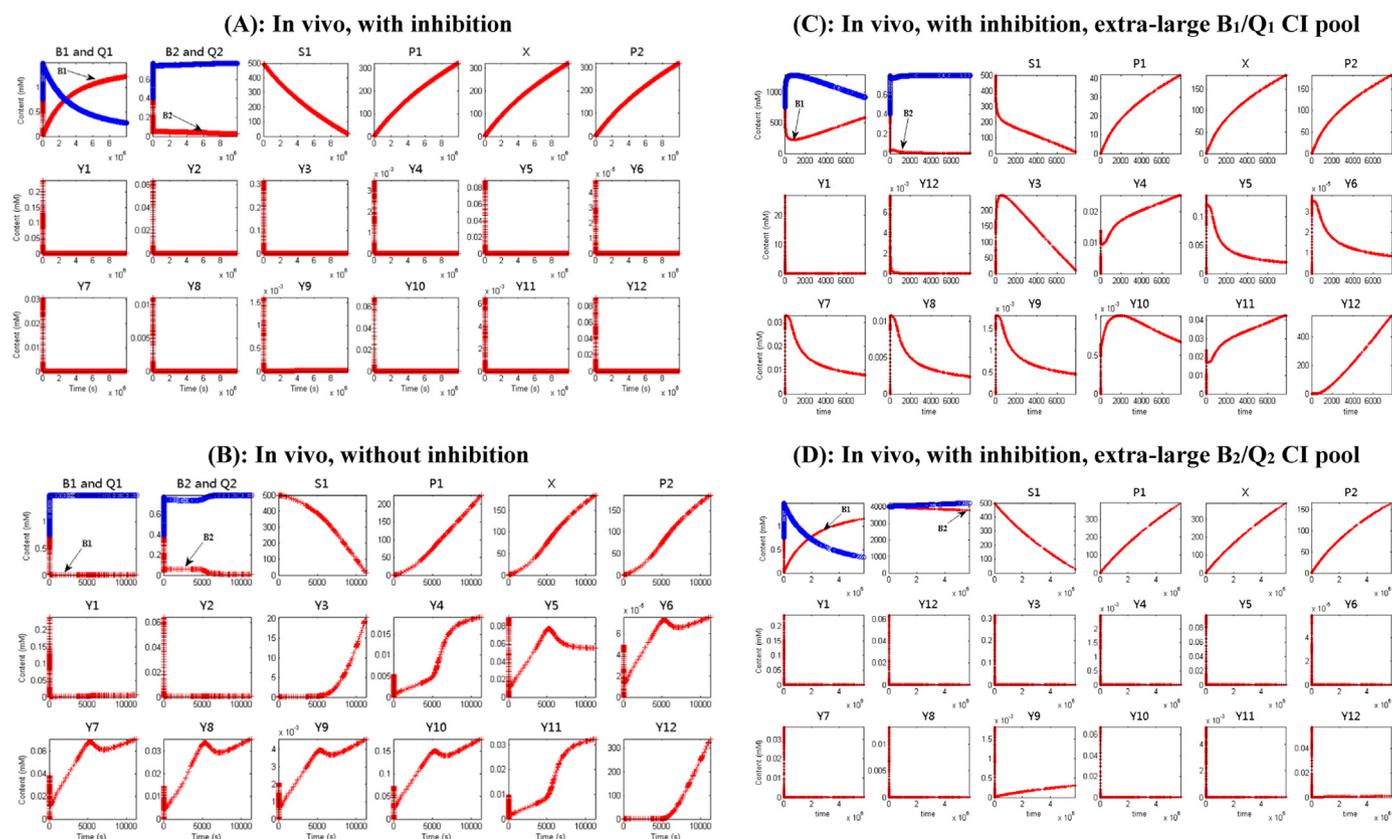
Fig. 7. Reaction time profile comparison for the Fig. 2D network topology. B1 and Q1 are paired and each starts at 0.75 mM for their normal pool size. B2 and Q2 are paired and each starts at 0.4 mM for their normal pool size. B1+Q1 pool of 1500 mM, and B2+Q2 pool of 8000 mM, are regarded as extra-large. Inhibition strength applied is high (Ki=3.8*10^{-4}).

spontaneously reach metabolic homeostasis. However, metabolic homeostasis for the in-vivo-like system is not affected when the B$_2$+Q$_2$ pool size is increased to extra-large (Fig. 7(D) vs (A)). On one hand, it is the B$_1$/Q$_1$ turnover with the end-product inhibition that contributes to homeostasis establishment. On the other hand, this homeostasis-forming property does not emerge from those CI turnover topologies in which the concerned NCI flux(es) has(have) no influence on the overall CI balance. For instance, B$_2$/Q$_2$ in the Fig. 2(D) topology has been shown to balance their turnover unconditionally. Except linear pathways, it can be difficult to visually identify such CI turnover topologies.

With regards to the influence on NCIs in this in-vivo-like system, an increase of the end-product inhibition strength (Fig. S4A, Supporting material) or a decrease of the B$_1$+Q$_1$ pool size (Fig. S4B, Supporting material) slightly suppresses their steady-state concentrations. However, a change in the B$_2$+Q$_2$ pool size has little effects on their steady-state concentrations (Fig. S4C, Supporting material). This is consistent with the above analysis that no emergent property is present between the end-product inhibition and the B$_2$/Q$_2$ turnover.

4.6. Comments on in vivo, in-vivo-like, and in vitro systems

A clear intention of the present work is to uncover reaction topologies used by living cells to maintain their intracellular metabolic reactions at homeostasis. Understanding of such mechanisms would allow us to construct perhaps simplified multi-enzyme reaction systems to possess at least certain cellular reaction properties such as steady state or low-level steady state for NCIs. It is important to stress that not all multi-enzyme reaction systems with CI turnover are in-vivo-like systems. Indeed, most of the experimentally explored remain to be in vitro systems rather than

in-vivo-like systems (e.g. Rollin et al., 2015). We will compare both pros and cons of in vitro and in-vivo-like systems in the forthcoming publications.

A foundation in reasoning in-vivo-like systems is based upon the spontaneous robustness of any such systems in response to drastic environmental changes. The results we used for illustration (Fig. 3) may seem to be specific, but it is obvious to us that such property is generic for any in vivo and thus in-vivo-like systems. Further work is under way in our group to provide evidence in a more systematic fashion.

5. Conclusion

Based on simple but characteristic metabolic reaction systems, this work has identified a fundamental mechanism that contributes to the establishment of metabolic stability at metabolic level (i.e. typical time scale of 0.1–1 s). This mechanism has been phrased as follows: located rather evenly in the metabolic network, CIs participate in most reactions and turnover globally using a fixed and small pool size. Working with enzyme feedback inhibition for the in vivo metabolic reaction topology, omnipresence of CIs and their balanced turnovers contribute to establish metabolic homeostasis.

By separating global turnover of the paired CIs from the carbon backbone metabolic flux for each reaction topology, we have identified that low-concentration steady states for NCIs are able to propagate from one pathway to the other. By contrast, metabolic flux analysis and the likes have always been based on the prior assumption that metabolic homeostasis had already been there. In our view, this is the reason why this unique property concerning CI turnover had not been discovered before. Broadly speaking, this

work offers a new perspective for understanding the role of co-factor intermediates [e.g. the energy metabolism (Atkinson, 1968)] and the presence of metabolic homeostasis in the living cell. Specifically, this work might provide clues for constructing non-natural metabolic networks either using multi-enzyme reactions or by degenerating metabolic reaction networks from the living cell.

Author contributions

Y.H.G. designed the research, Y.Y. and Y.H.G. worked together for all the work, and J.V. gave advice throughout this work and ensured the paper quality.

Acknowledgments

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.meteno.2016.06.002.

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