Biocatalytic reactions often require supplying chemical energy and phosphate groups in the form of adenosine triphosphate (ATP). Auxiliary enzymes can be used to convert a reaction by-product—adenosine diphosphate (ADP)—back to ATP. By employing real-time mass spectrometry (RTMS), one can gain an insight into inter-conversions of reactants in multi-enzyme reaction systems and optimize the reaction conditions. In this study, temporal traces of ions corresponding to adenosine monophosphate (AMP), ADP and ATP provided vital information that could be used to adjust activities of the ‘buffering enzymes’. Using the RTMS results as a feedback, we also characterized a bienzymatic energy buffer that enables the recovery of ATP in the cases where it is directly hydrolysed to AMP in the main enzymatic reaction. The significance of careful selection of enzyme activities—guided by RTMS—is exemplified in the synthesis of glucose-6-phosphate by hexokinase in the presence of a buffering enzyme, pyruvate kinase. Relative activities of the two enzymes, present in the reaction mixture, influence biosynthetic reaction yields. This observation supports the conclusion that optimization of chemical energy recycling procedures is critical for the biosynthetic reaction economy.

1. Introduction

Native biological systems are characterized with a high level of complexity. Thus, it is appealing to isolate biochemical machinery and reproduce some of the natural processes of interest in controlled laboratory conditions. The term ‘synthetic biology’ appeared in the scientific literature around the year 1980 [1]. Two decades later, it was popularized by Eric Kool and other speakers in the annual meeting of the American Chemical Society...
in San Francisco [2]. ‘Synthetic biology’ was often used as a synonym of ‘biomimetic chemistry’ or with reference to the efforts aiming to understand the origin of life by engineering artificial cells [3,4]. The research efforts in synthetic biology can be divided into two groups. On the one hand, scientists use artificial molecules to reproduce biologically relevant processes. On the other hand, components of biological systems are used to create completely new processes which cannot be found in nature [3,5,6]. Recently, one witnesses a rapid development of related methods and applications of this new discipline. The ability to reconstruct biochemical circuits enhances our cognition of molecular mechanisms and fosters real-world applications [7,8]. Replicating chains of biochemical reactions in vitro or even redesigning biochemical networks—using biomolecular building blocks—is a new avenue of exciting fundamental and applied research in synthetic biology [8].

The supply of chemical energy in biosyntheses is one of the biggest challenges in this field of scientific inquiry. Adenosine triphosphate (ATP) is considered to be a universal chemical energy currency. It is one of the end products of cellular respiration and photophosphorylation [9,10]. Synthetic biologists utilize ATP as the chemical energy source in artificial biocatalytic systems [11–14]. In fact, many common and important enzymes use ATP as the source of chemical energy and phosphate groups. Reactions using ATP normally follow one of the two generic schemes:

\[
\text{ATP} + \text{substrate} \stackrel{\text{enzyme}}{\longrightarrow} \text{ADP} + (P) + \text{product} \tag{1.1}
\]

and

\[
\text{ATP} + \text{substrate} \stackrel{\text{enzyme}}{\longrightarrow} \text{AMP} + (PP) + \text{product}, \tag{1.2}
\]

where (P) and (PP) symbolize phosphate and pyrophosphate residues that can be released to the solution or associate with a reaction product.

Previously, we demonstrated the possibility to adjust the level of ATP in a biocatalytic reaction using a feedback control system facilitated by an electronic microcontroller module and a computer program [15]. However, that approach had various limitations. For instance, it could not remove by-products of reactions, which might inhibit these reactions in longer synthetic runs. It is much more common to employ ancillary enzymes to convert adenosine diphosphate (ADP) to ATP, thus recycling by-products of the main reactions [16]. Several enzymes (pyruvate kinase (PK), creatine kinase (CK) and acetate kinase (AcK)) have been widely used to regenerate ATP from ADP in cell-free systems [16–18].

PK (E.C. 2.7.1.40) is an omnipresent biocatalyst. It catalyses the last step of the glycolysis process—a metabolic pathway that breaks down glucose to pyruvate [19]. PK catalyses the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP (equation (1.3))

\[
\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{ATP} + \text{pyruvate}. \tag{1.3}
\]

Another wide-spread enzyme—particularly abundant in muscle cells—is adenylate kinase (AK; E.C. 2.7.4.3) [20]. While it belongs to the group of phosphotransferases, it catalyses interconversion of adenine nucleotides (equation (1.4)), playing an important role in the maintenance of cellular energy homeostasis [21]

\[
\text{ATP} + \text{AMP} \rightleftharpoons 2\text{ADP}. \tag{1.4}
\]

Although PK has long been used in ATP regeneration reactions, the usefulness of AK in buffering adenylate forms in biosynthetic processes carried out in vitro [22] has not been very popular.

Mass spectrometry (MS) is a sensitive [23] and fast [24] analytical technique. It enables identification of ions present in complex mixtures by matching mass-to-charge ratios (m/z) and ion fragmentation patterns. It can also be used to follow chemical and biochemical reactions in real time [25]. For example, time-dependent MS signals can reveal variations of the molecules that take part in multi-enzyme reactions [26]. The real-time MS approach can be particularly useful in the optimization of enzyme activities and concentrations of different components to achieve high yields at low expenditure of reagents and catalysts, while decreasing the time required to complete the reactions.
Just as acid/base buffers maintain a relatively constant pH of aqueous solutions, even when reactions consume or release H\(^+\) ions, a ‘chemical energy buffer’ helps to maintain the supply of chemical energy in the form of ATP in order to sustain production of the compounds of interest. Development of chemical energy buffers can facilitate operation of artificial biosystems. They may also improve efficacy of cell-free syntheses. In this report, we propose a generic approach for the development of enzymatic systems that can stabilize ATP levels during \textit{in vitro} syntheses. This approach takes advantage of gas-phase ion abundances as a feedback input in the optimization workflow. It can be applied to develop ATP-recovery systems comprising one or more ancillary enzymes. It also provides insights on the cooperativity of different enzymes in a complex reaction mixture.

2. Results and discussion

(a) Mass spectrometric monitoring

Test reactions were conducted in 1.5 ml microcentrifuge tubes placed in the thermoshaker set to maintain the temperature at 30\(^\circ\)C (scheme 1). Fused silica capillary (ID 150 \(\mu\)m, OD 375 \(\mu\)m), attached to a T-junction (Venturi pump) [27], was dipped in the reaction mixture while the other end of the capillary was positioned approximately 2.5 cm from the inlet of an ion-trap mass spectrometer. The flow rate of the sample solution in the capillary was estimated to be approximately 10 \(\mu\)l min\(^{-1}\). At this flow rate, the volume of the reaction mixture decreased from 500 \(\mu\)l to approximately 300 \(\mu\)l during 20 min monitoring. Please refer to the electronic supplementary material for full description of experimental parameters.

(b) Perturbation with ATP-hydrolysing enzymes

In a typical run, components of the energy buffer were mixed with a perturbing enzyme (apyrase or luciferase). The apyrase enzyme [28] used here (E.C. 3.6.1.5) primarily decomposes ATP to ADP while luciferase (E.C. 1.13.12.7) decomposes ATP to AMP [29]

\[
\text{ATP} \xrightarrow{\text{apyrase}} \text{ADP} + \text{P}_1
\]  

and

\[
\text{ATP} + \text{luciferin} \xrightarrow{\text{luciferase}} \text{AMP} + \text{PP}_1 + hh.
\]  

Developmental work using MS signals as the feedback data for optimization of chemical energy buffers encompassed testing different sets of conditions (scheme 2 and electronic supplementary material, figure S2).

The real-time MS results reveal the positive effect of PK enzyme (40 U ml\(^{-1}\)) on the recovery of the ATP balance disturbed by the presence of an ATP-decomposing enzyme (apyrase, luciferase; figure 1). This effect is dramatic in the case of apyrase, introduced to perturb adenosine nucleotide balance of the reaction mixture. PK can rapidly phosphorylate ADP to produce ATP (scheme 2\(a(i)\)). Nonetheless, because PK uses ADP as a substrate for the synthesis of ATP, it is not capable of regenerating ATP from the excess of AMP, which might have been produced in a biosynthetic reaction.

We hypothesized that the combination of PK with AK may enable the recovery of ATP from AMP in two steps. The effect of PK (in the absence of AK) was negligible in the case of luciferase incubated with low concentration of ATP (0.032 mM). This observation is explained in the following way: luciferase rapidly decomposed a large fraction of ATP to AMP, while PK is unable to phosphorylate AMP (scheme 2\(b(i)\)). In the case of a higher concentration of ATP (0.320 mM), the energy balance of the reaction mixture was not markedly perturbed by luciferase (which was supplied at a relatively low activity). When the activity of apyrase was particularly high, PK could only preserve energy balance for a few hundred seconds (figure 1 and electronic
Scheme 1. Real-time mass spectrometry system used to track the action of chemical energy buffers. Enzymatic reactions were conducted in a microcentrifuge tube placed in a thermoshaker (30°C, 300 r.p.m.). They were sampled and automatically transferred to the ion source of mass spectrometer. (Online version in colour.)

supplementary material figure, S3). In the absence of constant supply of AMP in the apyrase-perturbed system, the reaction catalysed by AK (20 U ml⁻¹) had no effect on the energy balance (scheme 2a(ii)). However, the introduction of AK to the reaction mixture did help to maintain the energy balance in the case of luciferase (figure 1). In this case, AK phosphorylated AMP to produce ADP thus enabling re-entry of the adenylate moiety to the (AMP ↔ ADP ↔ ATP) cycle (scheme 2b(ii)). ADP could further be phosphorylated to regain ATP. It is noteworthy that AK uses one molecule of AMP as well as one molecule of ATP to produce two molecules of ADP (equation (1.4)). Therefore, ATP is initially consumed in order to re-use AMP. However, in the following reaction catalysed by PK, two molecules of ATP would be restored from two molecules of ADP. Overall, the two-enzyme system enables complete restoration of adenylate—even when the main product of the biocatalytic process is AMP—the molecule that cannot be directly phosphorylated by PK. Nonetheless, at low ATP concentrations with luciferase, the buffering efficiency is not as good as in the case of the PK system with apyrase. This is because two biocatalytic steps (AMP → ADP and ADP → ATP) are involved in the recovery of ATP from AMP, while the reaction rates may be lower at low concentrations of substrates (due to the Michaelis–Menten kinetic characteristics; see also §2d).

Please note that the effect of a chemical energy buffering enzyme (PK, AK) on ATP level can also be evaluated using chemiluminescence spectroscopy (cf. electronic supplementary material, figure S1). However, that approach requires the use of a reporter system—for example, luciferin/luciferase reaction—sensitive to ATP. Only one reactant can be monitored at a time and the reporter system can be the source of non-negligible confounding factors (e.g. nonlinear response, kinetic deceleration due to consumption of luciferin, inhibition by unrelated solutes present in the reaction mixture). Although direct-infusion MS is known to be vulnerable to interferences (e.g. ion suppression) (e.g. [23,30]), these drawbacks appear to be less problematic than those of the chemiluminescence monitoring approach (compare electronic supplementary material, figure S1b, blue line, with figure 1c, luciferase, 0.032 mM ATP, +AK, red line). In fact, chemiluminescence always decreases abruptly following few tens of seconds of incubation. The nonlinearity between chemiluminescence and ATP concentration is in part due to the nonlinear dependence behaviour of enzyme–substrate interactions.

While figure 1 illustrates changes in chemical energy balance reflected in the ratio ATP/(ATP + ADP + AMP), it is also instructive to consider the ratios ADP/(ATP + ADP + AMP) and AMP/(ATP + ADP + AMP) (figure 2). These results provide an insight on the role of AK in the two-enzyme energy buffer. In the case of the perturbation with apyrase, AK played a minor
role. However, in the case of the perturbation with luciferase, the level of ADP increased in the presence of AK. The surplus pool of ADP could be used by PK to restore the initial level of ATP (cf. figure 2, +AK and −PK versus +AK and +PK). Notably, AK also effectively removed AMP from the reaction mixture (cf. figure 2, orange traces). In fact, AMP is known to inhibit some enzymes [31]. Moreover, a related intermediate product, dehydroluciferyl-adenylate (L-AMP), is
Figure 1. Changes to ATP balance (expressed as the ratio of ATP signal to the sum of the signals of all adenylate forms; cf. electronic supplementary material, equation S3) over time during perturbation with ATP-consuming enzymes—apyrase and luciferase. Red line, reaction cocktail contains PK (40 U ml$^{-1}$). Grey line, reaction cocktail does not contain PK. Nominal AK activity (in the variants labelled as ‘+AK’): 20 U ml$^{-1}$. Initial ATP concentrations: (left sub-columns) 0.032 mM; (right sub-columns) 0.320 mM. Nominal enzyme activities: (a) apyrase, 0.000 units per millilitre (U ml$^{-1}$); luciferase, 0.00 light units per millilitre (LU ml$^{-1}$); (b) apyrase, 0.005 U ml$^{-1}$; luciferase, 1.52 $\times$ 10$^9$ LU ml$^{-1}$; (c) apyrase, 0.010 U ml$^{-1}$; luciferase, 3.04 $\times$ 10$^9$ LU ml$^{-1}$; (d) apyrase, 0.100 U ml$^{-1}$; luciferase, 6.07 $\times$ 10$^9$ LU ml$^{-1}$. ATP was injected after 60 s from the start of MS monitoring; thus, the ratio values obtained for the time 0–60 s are not meaningful. (Online version in colour.)
Influence of 40 U ml\(^{-1}\) PK and 20 U ml\(^{-1}\) AK on the ratios of ATP, ADP and AMP (to the sum of all adenylate forms; cf. electronic supplementary material, equations S3–S5). Nominal apyrase activity: 0.010 U ml\(^{-1}\); nominal luciferase activity: \(3.04 \times 10^{9}\) LU ml\(^{-1}\). Two different ATP concentrations were tested: 0.032 mM and 0.32 mM. Violet, blue and orange traces represent ATP, ADP and AMP, respectively. This figure has been constructed using the data from the same experiment as the one depicted in figure 1; the traces for ATP are identical with those shown in figure 1c and they are included here for comparison. ATP was injected after 60 s from the start of MS monitoring; thus, the ratio values obtained for the time 0–60 s are not meaningful. (Online version in colour.)

Figure 2. Influence of 40 U ml\(^{-1}\) PK and 20 U ml\(^{-1}\) AK on the ratios of ATP, ADP and AMP (to the sum of all adenylate forms; cf. electronic supplementary material, equations S3–S5). Nominal apyrase activity: 0.010 U ml\(^{-1}\); nominal luciferase activity: \(3.04 \times 10^{9}\) LU ml\(^{-1}\). Two different ATP concentrations were tested: 0.032 mM and 0.32 mM. Violet, blue and orange traces represent ATP, ADP and AMP, respectively. This figure has been constructed using the data from the same experiment as the one depicted in figure 1; the traces for ATP are identical with those shown in figure 1c and they are included here for comparison. ATP was injected after 60 s from the start of MS monitoring; thus, the ratio values obtained for the time 0–60 s are not meaningful. (Online version in colour.)

a strong inhibitor in luciferin/luciferase reaction. Hence, it is beneficial to remove AMP from the enzymatic reaction mixture.

(c) Influence of abrupt alterations to energy balance

In complex biosynthetic reaction systems, fluctuations of energy-carrying nucleotides may be more spontaneous than in one-enzyme model reactions. Thus, it is also necessary to verify the influence of possible abrupt changes of ATP, ADP and AMP on the energy balance. In the following test, we challenged both energy buffers (PK and PK + AK) by spiking the reaction mixture with 16 nanomoles of ATP, ADP or AMP, which corresponds to an approximately 0.032 mM increase of concentration. In most cases, this concentration spike—induced 10 min after the start of the measurement (indicated in figure 3 with red arrows)—did not have a major effect on the ratios of nucleotides (computed with electronic supplementary material, equations S3–S5). Lack of any major influence of the concentration spike on the nucleotide traces is—to some extent—related to the way of computing the data: while numerator increases, denominator also increases, and the ratio value is not affected significantly (following few seconds of shaking the reaction cocktail). On the other hand, increasing ATP concentration must affect the signal ratio ADP/(ATP + ADP + AMP) because of the increase of ATP signal (in the denominator).
It is also easy to accept that spiking the reaction cocktail with ADP can readily increase the ADP level (figure 3, frames B1 and B2, blue lines) while adding AMP can increase the AMP level (figure 3, frames A1, C1 and C2, orange lines). However, some of the features in figure 3 cannot be explained with these intrinsic limitations of the data processing algorithm (electronic supplementary material, equations S3–S5) because they are related to specific functions of the individual components of the multi-enzyme reaction system. For example, in the presence of AK, AMP level was quickly brought down after the AMP spike (figure 3, frames A1 and A2, orange lines) and a surplus amount of ADP was promptly produced (figure 3, frame A2, blue line). In the variant with luciferase, following an AMP spike, AMP was quickly removed when AK was present in the mixture (figure 3, frame C2, orange line). This observation can be explained in the following way: AMP was first phosphorylated producing ADP (figure 3, frame C2, blue line), that in turn was phosphorylated to ATP by PK.

(d) ATP recovery during phosphorylation of glucose

The above results emphasize the usefulness of real-time MS traces in the optimization and mechanistic studies of enzymatic energy buffers. We further aimed to show that the approach is practicable while optimizing conditions for biosynthetic reactions that lead to the compounds of interest. Glucose-6-phosphate (G6P) is the first intermediate of glycolysis and is also an important reagent used in biotechnology [14]. It can be produced by phosphorylating glucose in the presence
of hexokinase (HK) and using ATP as co-substrate

\[
glucose + ATP \xrightarrow{\text{hexokinase}} G6P + ADP. \tag{2.3}
\]

Because the by-product of that reaction is ADP, in this case, it was not necessary to introduce AK as an ancillary enzyme.

Application of the alternating polarity mode in the ion-trap mass spectrometer enabled simultaneous recording of positively and negatively ionizable species, including glucose, G6P, ATP, ADP and AMP. Scaling individual signals with the sum of the signals of related species (i.e. glucose/(glucose + G6P) and G6P/(glucose + G6P)) provided a convenient way of semi-quantitative evaluation of the progress of the reaction. The end of the reaction is marked with the beginning of plateau.

First, the HK activity was fixed at 40 U ml\(^{-1}\), whereas PK activity was varied from 0 to 80 U ml\(^{-1}\) (figure 4\(a\)). While 80 U ml\(^{-1}\) PK led to fastest completion of synthesis, we further limited PK to 40 U ml\(^{-1}\) (due to the high cost of the enzyme). Subsequently, we varied HK activity from 0 to 5 U ml\(^{-1}\) (figure 5\(a\)). Apart from tracking the progress of glucose phosphorylation, it is of particular interest to analyse relative changes of ATP and ADP nucleotides (figures 4\(b\) and 5\(b\)). At the start of the reaction, ATP was quickly converted to ADP. The initial level of ATP did not recover until the reaction was complete (violet line in figure 4\(b\)). Naturally, an increased activity of PK promoted fast back-conversion of ADP to ATP (figure 4\(b\)).

While inspecting the traces of glucose and G6P (figure 5\(a\)), we noted that increasing HK activity initially accelerates conversion of glucose to G6P. However, it also leads to prompt depletion of ATP, which cannot be recovered quickly (figures 5\(b\) and 6). Considering the Michaelis–Menten kinetics \[32\], low level of ATP can slow down HK-catalysed reaction

\[
E + S \rightleftharpoons v_0 = \frac{v_{\text{max}}[S]}{K_M + [S]}, \tag{2.5}
\]

where \(E, S, P\) and \(P\) represent enzyme, substrate and product, respectively; while \(v_0, v_{\text{max}}\) and \(K_M\) symbolize initial reaction rate, maximum reaction rate and the substrate concentration at which the reaction rate is at half-maximum. The \(K_M\) value of HK from \textit{Saccharomyces cerevisiae} for ATP is

\[
E + S \rightleftharpoons ES \rightarrow E + P \tag{2.4}
\]
Figure 5. Synthesis of glucose-6-phosphate (G6P) by phosphorylation of glucose at different activities of HK. Nominal PK activity: 40 U ml\(^{-1}\). Initial ATP concentration: 0.320 mM. Initial glucose concentration: 1 mM. (a) Ratios of glucose (dark green) and G6P (light green) to the sum of glucose and G6P. (b) Ratios of ATP (violet) and ADP (blue) to the sum of all adenylate forms. For representative mass spectra, see figure 6. All experiments for this figure were conducted during 1 day (different from figure 4). Note that, due to instability of enzyme preparations, the actual activities of PK and/or HK activities were slightly lower than the nominal values; thus, the yields were lower than those observed in the experiment depicted in figure 4. (Online version in colour.)

Figure 6. Mass spectra of ATP, ADP and G6P recorded at different times of HK reaction. Nominal HK activity: 1 U ml\(^{-1}\). Nominal PK activity: 40 U ml\(^{-1}\). Red frames highlight the signals of G6P (m/z: 259), ADP (m/z: 426) and ATP (m/z: 506). (Online version in colour.)
approximately 0.15 mM (median of six literature values [33]), while the initial ATP concentration in the present reaction was 0.320 mM, i.e. approximately two times higher than the $K_M$ value. Thus, prompt depletion of ATP may slow down the HK-catalysed reaction (equation (2.3)). If the consumption of ATP is much faster than its synthesis (due to the high abundance of HK enzyme), the rate of G6P synthesis is limited by ATP. This problem can be rectified by raising the activity of PK at an increased cost (more biocatalyst used). For instance, it is helpful to compare the reaction catalysed by 40 U ml$^{-1}$ PK and 40 U ml$^{-1}$ HK with the reaction catalysed by 80 U ml$^{-1}$ PK and 40 U ml$^{-1}$ HK (figure 4). Here, the increase of PK activity shortened the whole reaction (from approx. 1000 to approx. 600 s; cf. inflection points of the corresponding ATP traces). We should also note that a decrease in G6P synthesis rate can additionally be due to inactivation of the enzymes over time.

Overall, the above analysis, enabled by the real-time MS results, highlights the need to carefully select enzyme activities for multi-enzyme reactions. When the activities of the two catalysts are not balanced, the ‘cooperation’ between them does not lead to high yields. Moreover, many enzymes are expensive and their expenditure needs to be limited. Real-time MS enables balancing activities of the biocatalysts to prevent their excessive use and to reduce the time required to complete the reaction. The example described in §2d shows a biosynthetic reaction producing ADP. In future, it would be interesting to apply the two-enzyme buffer (PK + AK; described in §2b) along with other model enzymes, which catalyse synthesis of a product of commercial interest while converting ATP to AMP.

3. Conclusion

We have demonstrated the utility of real-time mass spectrometry (RTMS) signals in the development and refinement of energy buffers to sustain biosynthetic reactions. Careful adjustment of enzyme activities is important for assuring high reaction yields, short reaction times and substrate/catalyst economy. The method enables long-term monitoring of all major components in such complex reactions. The significance of careful selection of enzyme activities—enabled by the RTMS signals—is exemplified in the case of the synthesis of glucose-6-phosphate by hexokinase in the presence of a buffering enzyme, PK. Here, the reaction yield is partly limited by the activity of the ATP regenerating enzyme—PK. As mass spectrometers are nowadays less expensive and smaller than before, it is likely that they will soon become an important part of synthetic biology toolkit—especially when using reactant recovery reactions such as those discussed in this report. In this work, mass spectrometric monitoring was applied to a homogeneous multi-enzyme system. However, biological cells are highly compartmentalized and different enzymes are localized in different compartments. Thus, it is appealing to miniaturize the RTMS approach to enable enzyme activity studies in such microscopic and spatially heterogeneous systems. The follow-up work should also include kinetic simulations of the multi-enzyme reaction systems. Such numerical models would enable cross-validation of the real-time datasets provided by MS.

Data accessibility. The electronic supplementary material enclosed with this paper contains the original datasets (Origin files).

Authors’ contributions. T.-R.C. conducted the experiments, acquired and analysed the data, and drafted the manuscript. P.L.U. conceived of the study, coordinated the study and helped draft the manuscript. All authors gave final approval for publication.

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References


