Electrochemical Recycling of Adenosine Triphosphate in Biocatalytic Reaction Cascades

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Abstract: Adenosine triphosphate (ATP) provides the driving force necessary for critical biological functions in all living organisms. In synthetic biocatalytic reactions, this cofactor is recycled *in situ* using high-energy stoichiometric reagents, an approach that generates waste and poses challenges with enzyme stability and downstream purification. On the other hand, electrons are a cheap and green source of energy. We report a method that uses electricity to turn over enzymes for ATP generation. The method is simple, robust, and scalable, as well as broadly applicable to complex enzymatic processes including a four-enzyme biocatalytic cascade in the synthesis of the antiviral molnupiravir.

Main Text:

The growing need to preserve natural resources has put pressure on the chemical and pharmaceutical industries to decrease the environmental footprint of their manufacturing processes.[1] Biocatalysis has emerged as a viable approach to achieving green, sustainable and cost-effective industrial processes, owing to the high diversity, selectivity and efficiency conferred by enzyme catalysts, combined with mild aqueous reaction conditions.[2,3] Adenosine triphosphate (ATP) produced from photosynthesis or respiration, drives many fundamental metabolic pathways in living organisms.[4] Consequently, many biocatalytic reactions rely on ATP to provide the necessary thermodynamic driving force to access phosphorylated metabolites (Fig. 1A).[5] The prohibitive cost of ATP has led to the development of cofactor recycling systems that enable access to the diverse chemistry catalyzed by ATP-dependent enzymes.[6,7] ATP recycling is typically driven by high-energy stoichiometric sacrificial phosphate donors (i.e. acetyl phosphate or polyphosphate)[5,8] (Fig. 1A), an approach that not only compromises the atom economy and overall greenness of the reaction but also impacts enzyme performance and downstream purification.[9] The cheapest and greenest alternative energy source is electricity, especially when produced from renewable sources (at the time of writing, the average cost of electricity in the United States is 1.1 cents/mol of electrons at 2 V potential).[10] Electrochemical cofactor recycling has been demonstrated for cofactors such as nicotinamide adenosine dinucleotide phosphate (NAD(P))[11] and flavin adenosine diphosphate (FAD) (Fig. 1B).[12,13] Here, we report a robust and scalable method to drive the recycling of the ubiquitous cofactor ATP by replacing sacrificial redox reagents with an electric current (Fig. 1C). We demonstrate the versatility and generality of the electrochemical approach in diverse ATP-dependent kinase-catalyzed transformations, including a complex biocatalytic cascade to access a precursor of the COVID-19 antiviral therapeutic molnupiravir.[14]

The inspiration for bioelectrochemical ATP recycling originated from a previously reported recycling system that relies on the oxidative decarboxylative phosphorylation of pyruvate with O_2. That system was catalyzed by the enzyme pyruvate oxidase (PO) to give acetyl phosphate, which, in turn, was converted to ATP and acetate by acetate kinase (AcK) (Fig. 2A).[14] However, the use of oxygen as a terminal oxidant for PO has several limitations, specifically, the need to add catalase to avoid the accumulation of H_2O_2, the byproduct of O_2 reduction. Furthermore, using O_2 at industrial scale require engineering considerations to ensure efficient mass transfer of O_2 from gas to liquid phase, and, in some cases, poses safety issues.[15,16] We therefore envisioned replacing O_2 with electrons from an electric current in the PO/AcK recycling system. The mechanism of PO has been investigated and consists of a complex sequential mechanism that involves the two cofactors thiamine pyrophosphate (TPP) and FAD. While TPP serves as a nucleophile to activate and decarboxylates pyruvate, FAD serves as a transient electron sink in the reduced form $FADH_2$ before being reoxidized back to FAD by the terminal oxidant O_2.[17] We hypothesized that the 2-electron oxidation of $FADH_2$ in PO could be driven by an electrical current instead, thereby circumventing the need for O_2 (Fig. 2A).

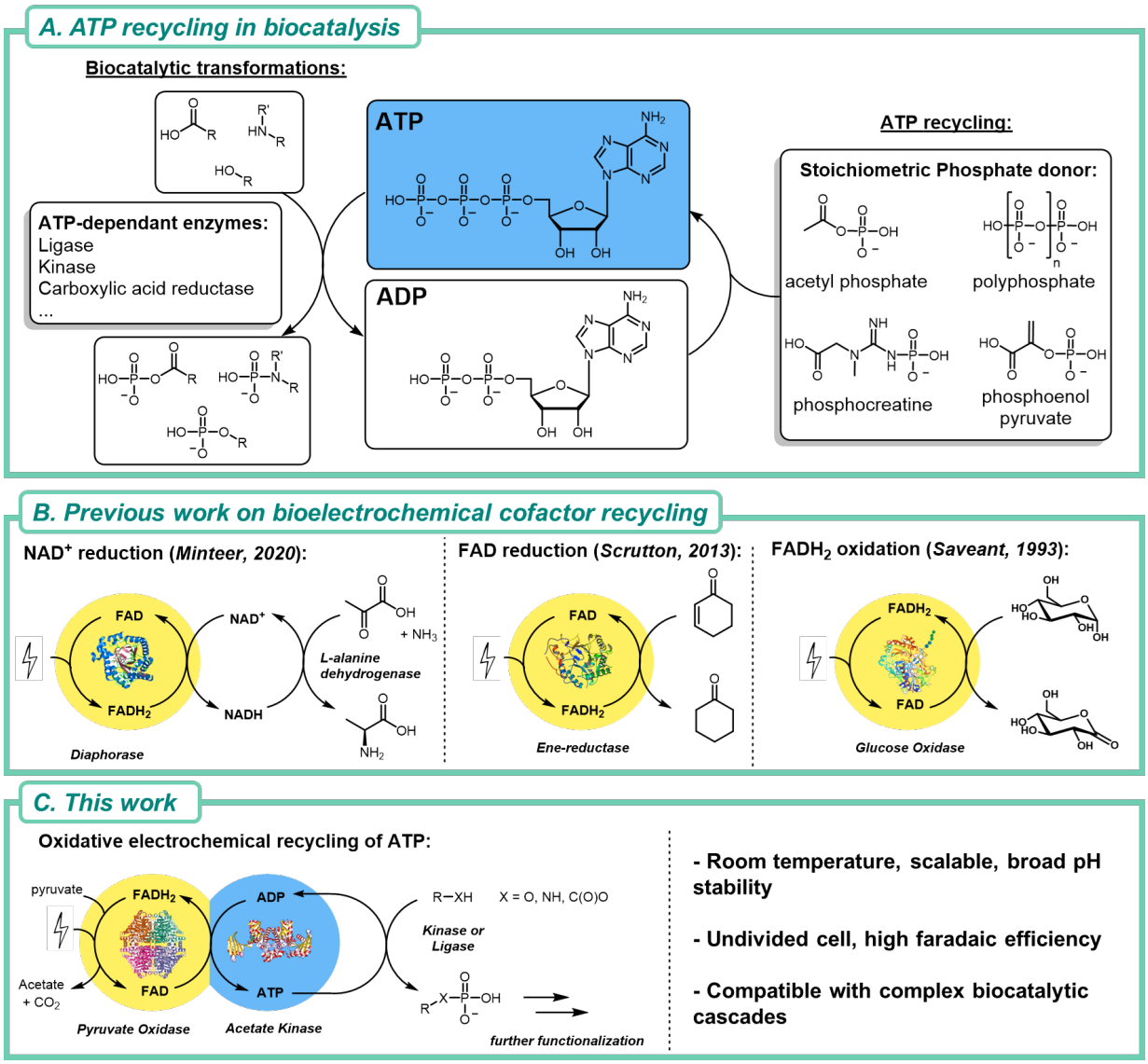

Fig. 1: (**A**) Current ATP recycling systems in various biocatalytic systems and common stoichiometric phosphate donors. (**B**) Previous reported examples of bioelectrochemical cofactor recycling. (**C**) Overview of bioelectrochemical ATP recycling. The lightning icon represent electrochemical input.

We initiated this study by exploring methods to turn over PO electrochemically. Direct electron transfer between enzymes and electrodes is not trivial because most enzymes have their active redox cofactor buried in the protein structure.[18,19] The use of a redox mediator that shuttles electrons between the electrode and the enzyme active site can alleviate some limitations.[20,21] Cyclic voltammetry (CV) of PO in the presence of substrate shows no redox event (Fig. S8), confirming that direct electron transfer between the electrode and the enzyme is not observed under these conditions. CV of redox mediators such as ferrocenemethanol (FcMeOH) show a reversible wave in the presence of pyruvate and phosphate, thereby confirming that the mediator cannot oxidize pyruvate on its own. However, in the presence of PO, a catalytic S-shape curve is observed, indicating turnover of the enzyme by the mediator (Fig. 2B). Using the formalism introduced by Savéant *et al.*[13], the rate constant for the oxidation of PO by the oxidized FcMeOH can be extracted, giving a value of 1.2 x 10^5 $M^{-1}s^{-1}$, similar to the previously reported value for

FcMeOH (1.5 x 10^5 $M^{-1}s^{-1}$). (*16*) Interestingly, this rate is also similar to that previously measured for oxidation of PO with molecular O_2 (1.7 x 10^5 $M^{-1}s^{-1}$),[17,22] suggesting that turnover of PO by a redox mediator is possible at competitive rates.(*21*) Within the ranges studied, the observed oxidation rate is independent of both mediator oxidation potential and pH, since similar rates are observed with ferrocene derivatives with oxidation potentials ranging from 0.25 to 0.48 V vs. Ag/AgCl (Table S1) and from pHs ranging from 6.0 to 7.5 (Table S2). Therefore, the proton-coupled electron transfer between $FADH_2$ and redox mediators is not rate limiting. Instead, formation of a precursor complex between the oxidant and the enzyme is likely rate-limiting, as observed with the electrochemical turnover of glucose oxidase.[13] When PO has a substoichiometric FAD loading, we observe a significant decrease in the oxidation rate (see Table S1), which suggests that the mediator oxidizes $FADH_2$ in the active site and not TPP.

We therefore propose the following mechanism for the electrochemical recycling of ATP: 1) as expected from the known mechanism of PO,[17] pyruvate undergoes oxidative decarboxylative phosphorylation catalyzed by PO to generate acetyl phosphate, while the FAD cofactor in PO is reduced to $FADH_2$; 2) the FcMeOH mediator is oxidized at the anode; 3) two $[FcMeOH]^+$ oxidize $FADH_2$ in PO to give FAD by two single electron transfers, thereby producing two FcMeOH and two protons; 4) at the cathode, two protons are reduced to produce hydrogen, thereby maintaining a constant pH; 5) as in the O_2 system, the acetyl phosphate is used by acetate kinase to phosphorylate ADP, thereby generating ATP and acetate; 6) the ATP is available for an ATP-dependent enzyme for further reactions (Fig. 2C).

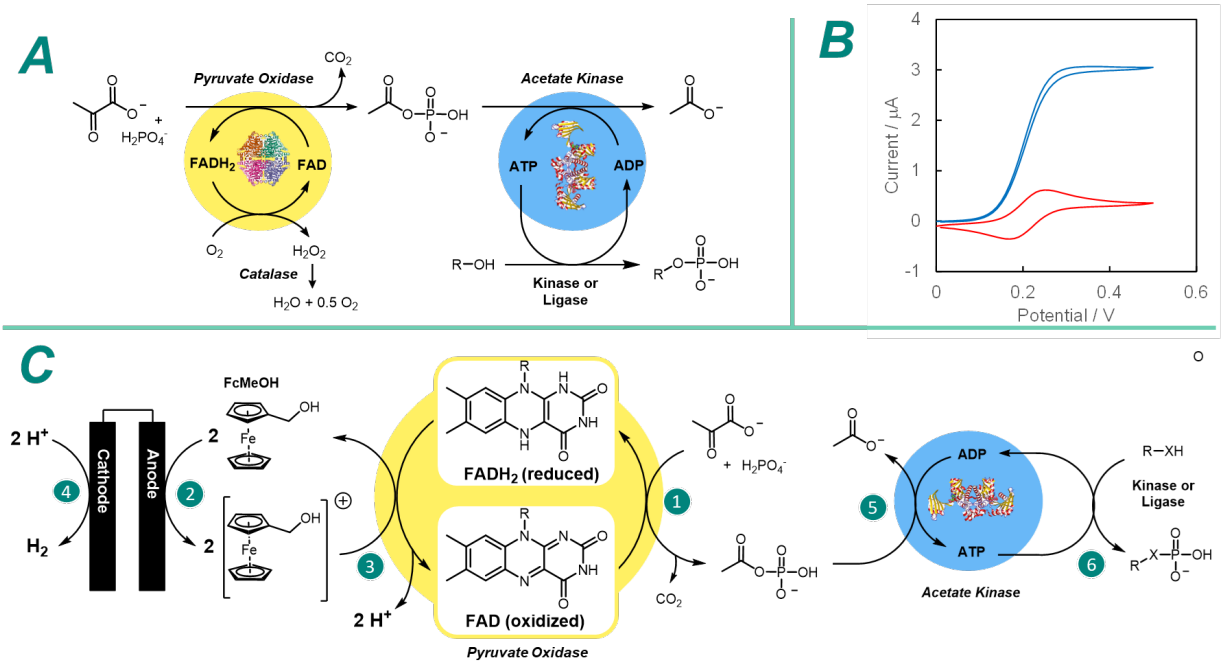

Fig. 2: (**A**) Pyruvate oxidase-dependent ATP recycling system driven by O_2. (**B**) Cyclic voltammetry of FcMeOH with pyruvate and phosphate in the absence (red trace) or in the presence of PO (blue trace) at 5 $mV.s^{-1}$. (**C**) Proposed mechanism for oxygen-free, electrochemical turnover of PO for ATP recycling.

With this analytical data in hand, we began the development of the stoichiometric phosphorylation of ADP to ATP using electrochemical turnover of PO (Fig. 3A). First, redox mediators, including ferrocene derivatives, *N*-oxyls, triphenylamines, quinones and metal

complexes, were screened with the HT*e*-Chem platform.[23] ATP formation was observed in the presence of several mediators, with the highest conversion measured when employing FcMeOH (Fig. S20). Upon scale up, 87% conversion of 8 mmol of ADP to ATP was achieved using FcMeOH as a mediator at a 30 mA current at 1.2 mA/cm^2 for 20 h in an undivided cell consisting of graphite rod anodes and stainless steel cathodes under an N_2 atmosphere (Fig. 3A). A control reaction in the absence of electrochemical current led to 21% conversion to ATP, most likely originating from ADP disproportionation catalyzed by trace amounts of adenylate kinase in the cell lysate. Consistent with this hypothesis, equal amounts of AMP and ATP were produced in the control reaction, unlike for the bioelectrochemical system, which generated <1% AMP. In the absence of mediator, a conversion of only 11% was achieved, while the absence of PO led to 10% conversion, thereby highlighting the necessity of both components to efficiently generate ATP, and demonstrating that the mediator specifically turns over PO and not AcK. When O_2 was used as the oxidant instead of the electrochemical current, a conversion of only 33% was observed (9% AMP), indicating that the conversion seen in the bioelectrochemical method does not originate from either adventitious or electrochemically generated O_2, and that accumulation of H_2O_2 is deleterious to the reaction (Table S3). To mitigate this issue, catalase is required for the oxygen delivered reaction, but is not necessary for the bioelectrochemical system. The highest conversions were achieved at a pH of 6.25 (Table S4), close to the midpoint between the optimal pH of 5.7 for PO[24] and 7.4 for AcK.[25] Real-time measurement of the potential of the anode showed a stable voltage over the course of the constant-current reaction at ~0.35 V vs Ag\AgCl (~1.15 V total cell potential), which is close to the oxidation potential of FcMeOH. Additionally, the pH remained constant during the reaction, as expected from the reaction mechanism (Fig. 2C). When maximum conversion was achieved, the potential rose sharply by ~1 V to reach the water oxidation potential (Fig. S23). The faradaic efficiency was constant throughout the reaction at 74% (Fig. S22), which is remarkably high compared to other mediated bioelectrochemical systems.[26] Notably, the electrodes required for this system are inexpensive, earth-abundant and reusable graphite and stainless steel electrodes, which further contributes to the process sustainability by avoiding the use of precious metals.

With proof of concept that ATP can be generated through electrochemical turnover of PO, we investigated the use of this strategy as a cofactor recycling system in an ATP-dependent biocatalytic cascade, the second step of the recently reported molnupiravir synthesis (Fig. 3B).[14] The reaction consists of a 4-enzyme biocatalytic cascade that installs uracil on 5-isobutylribose **1** to form the nucleoside 5-isobutyluridine **3** via the 5-isobutylribose-1-phosphate intermediate **2**. Specifically, 5-S-methylthioribose kinase (MTRK) uses ATP to phosphorylate **1**, generating intermediate **2**, before uridine phosphorylase (UP) installs uracil on **2** to form **3** and inorganic phosphate, which is then used by the PO/AcK system for ATP recycling (Fig. 3C). A high throughput mediator screen showed that, unlike with the stoichiometric ATP production, *N,N,N*-trimethyltrimethyl-1-ferrocenylmethanaminium chloride ($FcNMe_3$) provided higher conversion than other mediators (Fig. S24) under bioelectrochemical ATP recycling conditions. Upon scale up, 96% conversion of 8.2 mmol of **3** was achieved using 30 mA current at 1.2 mA/cm^2 current density for 20 h in an undivided cell consisting of graphite rod anodes and stainless steel rod cathode under an N_2 atmosphere (Fig. 3A). Running the reaction in the absence of mediator, PO, or electrical current led to conversion of only 1-2%, confirming the need of all three components for productive reaction. When the reaction was performed with O_2 instead of an electrochemical current, a conversion of only 12% was observed, which we attributed to the deactivation of the enzyme by accumulation of H_2O_2 in the absence of catalase (Table S6). This experiment provides further evidence that the conversion in the bioelectrochemical system does not originate

from adventitious or electrochemically generated O_2. The pH profile for the reaction shows that high conversion is observed even at pH 8, although under elevated pH we observed increased levels of impurities such as uridine generated from isobutyryl ester hydrolysis (Table S7). During the electrolysis, the voltage remained constant at ~0.5V vs. Ag/AgCl (~1.4 V total cell potential) until the maximum conversion was reached, after which the potential rose sharply (Fig. S28). Similar to the stoichiometric ADP phosphorylation, the faradaic efficiency during the reaction was stable at an average of 83% (Fig. S27). Finally, it is crucial to maintain current values below the maximum rate of the downstream enzymatic reactions to ensure optimal performance. If the current outpaces the rates of MTRK and/or UP, a shortage of inorganic phosphate will occur, leading to an accumulation of oxidized $FcNMe_3$, rising potential and enzymatic failure due to overoxidation. Indeed, we observed that rates of delivery of electrons equivalents relative to substrate higher than 0.14 $F.mol^{-1}.h^{-1}$ showed significant drops in the conversion (Table S5, S6 and Fig. S25).

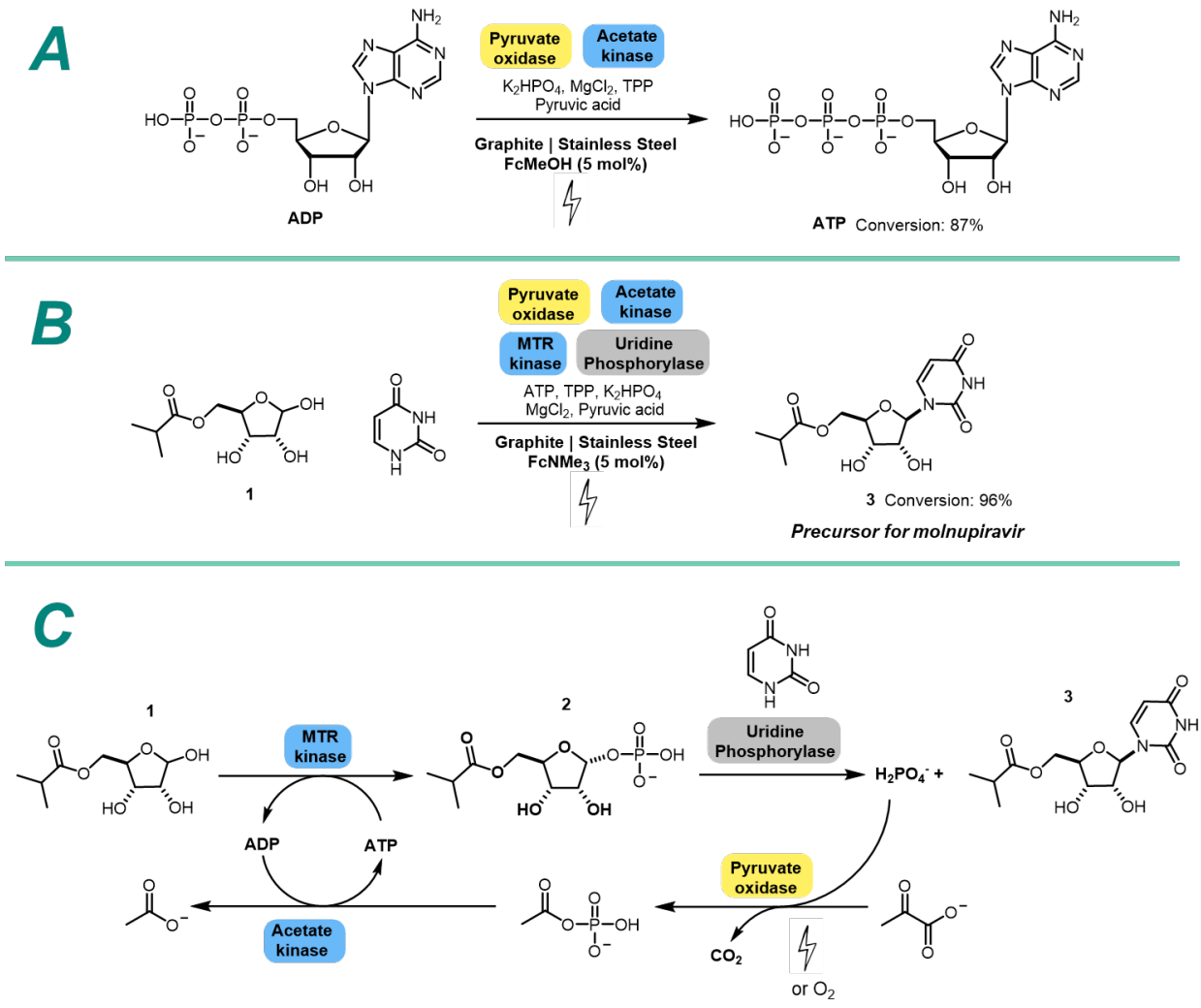

Fig. 3: (**A**) Bioelectrochemical stoichiometric ATP formation. For details on conditions, see supporting information (**B**) Bioelectrochemical glycosylation using electrochemical ATP recycling For details on conditions, see supporting information (**C**) Reaction scheme for the bioelectrochemical glycosylation.

Similar conversions and efficiencies were observed in batch-type reactors with multiple rod electrodes directly dipping in the reaction medium and in flow reactors with continuous parallel plates electrodes at identical current densities. Using a flow cell with a working electrode surface area of 256 cm^2 allowed the execution of the reaction at a 22.5 g scale with respect to **1** at a current of 373 mA, which reached 94% conversion (Fig S30). The productivity for this reaction is 1.1g/L/h with an average faradaic efficiency of 76% (Fig. S31) at a current density of 1.5 mA/cm^2.

We further explored the application of the bioelectrochemical ATP recycling in preparative biotransformations that rely on an ATP-dependent kinase or ligase enzymes (Fig. 4). First, we investigated the phosphorylation of 2-ethynylglycerol using pantothenate kinase (PanK), a key step in the synthesis of the HIV therapeutic islatravir, which uses a stoichiometric amount of propionyl phosphate to recycle ATP.[9] Under bioelectrochemical conditions and in the presence of stoichiometric phosphate, 2-ethynylglycerol was phosphorylated to produce (*S*)-1-phosphate-2-ethynylglycerol in 82% conversion using $FcNMe_3$ as mediator (Fig. 4, entry 1). Second, we explored the bioelectrochemical phosphorylation of creatine to phosphocreatine using creatine phosphokinase. This transformation is thermodynamically higher by 3 kcal/mol with ATP as the phosphate source,[27] and is typically used in the reverse direction, with phosphocreatine providing the driving force for ATP formation. We were therefore pleased to observe a 28% yield of phosphocreatine with FcMeOH as mediator (Fig. 4, entry 2) for this challenging uphill phosphorylation. Then, we probed ATP-grasp ligases, a promising class of enzymes which catalyze amide couplings between two amino acids and have been used to synthesize complex peptides in the drug discovery space.[28] Under bioelectrochemical conditions, a member of this enzyme family, namely YwfE,[29] formed the L-serine-L-phenylalanine dipeptide in 35% conversion using FcMeOH as mediator (Fig. 4, entry 3). Finally, we demonstrated that the PO/AcK bioelectrochemical recycling system is not limited to adenine-based nucleotide recycling, as guanosine-5'-diphosphate (GDP) was successfully phosphorylated into guanosine-5'-triphosphate (GTP) in 87% yield using FcMeOH as mediator (Fig. 4, entry 4). This further expands the scope of biocatalytic transformations possible with this method since several biological reactions rely solely on GTP hydrolysis as a driving force rather than ATP, the most notable example being protein synthesis in ribosomes.[30] Interestingly, in all cases, the bioelectrochemical recycling systems performs either similarly or better than O_2 and catalase as the driving force for PO turnover.

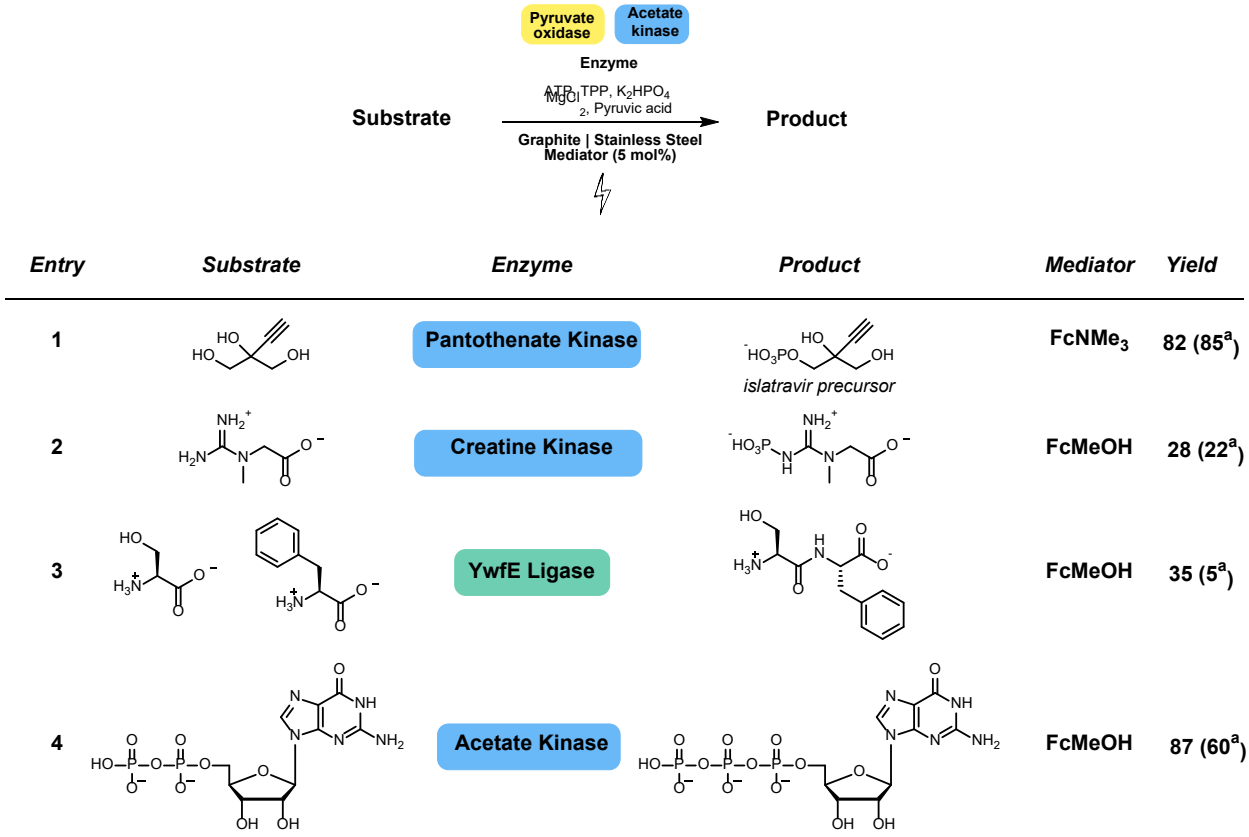

Fig. 4: Reaction scope for the bioelectrochemical ATP recycling. For details on conditions for individual reactions, see supporting information. [a] conversion for the reaction performed with O_2 and catalase and in the absence of current.

In conclusion, our bioelectrochemical system can be used as the driving force for recycling of ATP, an energy source that is ubiquitous in living organisms, instead of high energy phosphate sources or stoichiometric oxidants. Optimal conversions are achieved if the current is kept sufficiently high to drive catalysis, but sufficiently low to not outpace downstream enzymatic processes, highlighting the delicate interplay of these multi-enzyme systems and the corresponding tuneability achievable using electrochemistry. This method was demonstrated on a >20 g scale and was used to successfully drive biocatalytic cascades based on applications of phosphorylation chemistry. Looking forward, we believe the use of bioelectrochemistry is poised to expand, owing to the wealth and diversity of redox biotransformation that could theoretically be driven electrochemically. To that end, achieving higher performance will be crucial, and will require optimization of electrochemical cell design as well as improvements in enzymatic activity through directed evolution, opening the door for exciting applications such as green production of commodity chemicals, bioplastics, biofuels and pharmaceuticals alike.[7]

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Investigation: SR, GB, MC, KM, KS, AS

Supervision: NAS

Writing – original draft: SR

Writing – review & editing: all

Competing interests: the authors declare that they have no competing interests.

Data and materials availability: The data supporting the findings of this study are available within the paper and its Supplementary Information

Supplementary Information

Materials and Methods

Supplementary Text

Figs. S1 to S41

Tables S1 to S8

References