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To be cited as: *ChemCatChem* 10.1002/cctc.201601080

Link to VoR: http://dx.doi.org/10.1002/cctc.201601080

A Journal of
ChemPubSoc Europe

WILEY-VCH www.chemcatchem.org

Synthesis of N_ω-Phospho-L-arginine by Biocatalytic Phosphorylation of L-Arginine

Bernhard Schoenenberger [a], Agata Wszolek[b], Thomas Milesi[a], Henrike Brundiek[b], Markus Obkircher[a] and Roland Wohlgemuth*[a]

Abstract: The N_ω-Phospho-L-arginine energy-buffering system is mainly present in invertebrates for regulating energy requirements when it is highly needed, as in the flight muscle of an insect or when energy supply fluctuates, as in the medically important protozoa *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania major*. The lacking availability of this important metabolite was due to a tedious chemical procedure, by which N_ω-phospho-L-arginine was prepared up to now over 5 reaction steps in a low yield. Therefore, we aimed at improving the synthetic methodology for the preparation of this important metabolite. As site- and enantioselective kinases have been very useful catalysts for biocatalytic phosphorylations in straightforward syntheses of phosphorylated metabolites, a stable and selective arginine kinase has been selected for the selective phosphorylation of L-arginine. The arg gene has been cloned and expressed in *E. coli* and a highly active arginine kinase has been prepared. A simple synthesis of N_ω-phospho-L-arginine has been developed by arginine kinase-catalyzed phosphorylation of L-arginine combined with the recycling of the phosphorylating agent ATP using the phosphoenolpyruvate/pyruvate kinase system. After standard workup the desired product N_ω-Phospho-L-arginine has been obtained in gram quantities and in one step.

Introduction

N_ω-Phospho-L-arginine was first isolated from the muscle of fresh-water crabs by Meyerhof and Lohmann in 1928[1] and has been shown to play a key role in enzymatic phosphorylations in the invertebrate muscle analogous to the role of phosphocreatine in the vertebrate muscle.[2] It has been found in biosynthetic pathways of parasites totally different from those pathways found in mammalian host tissues. In a wide variety of invertebrates and certain parasitic protozoa like *Trypanosoma brucei, Trypanosoma cruzi* and *Leishmania major*, which cause some of the most debilitating diseases of humankind like leishmaniasis, African sleeping sickness, and Chagas disease, phospho-arginine is the main reserve of high energy phosphate compounds.[3] Therefore, the metabolic pathway of phosphoarginine is an attractive therapeutic target for parasitic diseases.[4] Since the high-energy phosphoarginine is relatively small and a highly diffusible molecule that provides fast energy supply when energy consumption becomes critical, it stabilizes the cellular ATP/ADP ratio and functions as temporal and spatial energy buffer in the cell. It is through this buffering reaction that insect cells can support bursts of nerve or muscle activity that would otherwise drain ATP to levels that would not sustain other essential functions.[5]

The phosphoarginine occurs as a free phosphoamino acid and is associated with a specific arginine kinase (ArgK). This cytoplasmic metabolite kinase catalyses the reversible and ATP-dependent phosphorylation of guanidine acceptor compounds, which act as phosphagen in muscle and nerve cells of invertebrates. Under standard conditions, the reaction: ATP + L-arginine ⇄ phospho-L-arginine + ADP operates close to equilibrium. However, at times of high metabolic activity, when ATP is low, the equilibrium shifts so as to yield net synthesis of ATP. Hence, phosphoarginine acts as an ATP buffer in cells that contain ArgK.[5c] Typically, ArgK are found as monomers targeted to the cytoplasm, but true dimeric and contiguous dimeric ArgKs as well as mitochondrial ArgK activities have been observed, too.[3c] The N-phosphorylation of phosphagens like phosphocreatine in vertebrates and phosphoarginine in invertebrates has been studied well in physiology since 1927[6] and has become also a paradigm for bimolecular enzymatic reactions since the rise of molecular biology 30 years ago.[5b, 7] Detailed solution structure NMR and high-resolution crystallographic analyses of arginine kinase in substrate-free, transition-state and substrate-bound forms have provided mechanistic details and linked substrate-associated motions to intrinsic flexibility.[8]

Despite the fact that physiology, enzymology and molecular biology of phosphagen synthesis has been well understood, the availability of phosphoarginine has still been lacking. This is partly due to the inherent instability of energy-rich phosphoarginine, which makes chemical multistep synthesis tedious. The instability of phosphoarginine results from the fact that in phosphoarginine the phosphate is attached to a nitrogen atom (N-phosphorylation) forming an acid-labile phosphoramidate bond, unlike in case of O-phosphorylation where the phosphate group is attached to a hydroxyl group, yielding a bond of lower acid lability. This is due to the protonation of the bridging nitrogen, which induces considerable lengthening and thereby weakening of the N-P bond [9]. Hence, the high energy N-P bond in phosphoarginine is extremely acid labile, instable in

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hot alkali and sensitive to heat, rendering its isolation from biological sources difficult, too [10]. Isolation of phosphoarginine, e.g. from crayfish tail muscle or fresh water crab muscle (as a barium salt) was low yielding, difficult to reproduce and involved a lengthy and complex extraction procedure with average yields of phosphoarginine less than 1% w/w.[11] Hence, several multistep chemical synthesis routes have been employed for the preparation of phosphoarginine.[12-15]

A few entirely chemical syntheses involving a number of reaction steps have been published, but each route has its own bottlenecks and challenges. Cramer and coworkers[12-13] described the N_ω-phosphorylation of N_α-Z-L-arginine-benzyl ester, the preparation of which requires several synthetic steps. Bis(p-nitrobenzyl) phosphorochloridate was used for phosphorylation. Although crystalline this reagent was not stable in our hands and had to be prepared freshly each time before use. Hydrogenolytic deprotection provided crystalline N_ω-phospho-L-arginine. Another access to this energy rich phosphate described by the same authors was by direct phosphoamidinylation of ornithine by (O-Methyl-isoureido)-phosphonate, which is not available commercially. Due to uncomplete turnover, the work up procedure described is rather lengthy. Direct phosphorylation of arginine by another commercially unavailable reagent, potassium phosphorous amidate, was applied for the peparation of NMR samples of Nω-phosphoarginine.[14] Information on yield and any reaction details were not given and the reaction mixture was not worked up. Yet another synthesis starts from L-arginine and phosphorous oxychloride.[15] Due to the many side products formed this procedure required a tedious workup and gave a very moderate yield.

Phosphorylating enzymes are abundant in nature and kinases have been successfully utilized for selective biocatalytic phosphorylations in straightforward syntheses of phosphorylated metabolites.[16] Therefore, we aimed at implementing an improved and very efficient synthesis of N_ω-Phospho-L-arginine in one reaction step by a simple biocatalytic phosphorylation of L-arginine with a recombinant ArgK. We describe the design, expression, structural and functional characterization of the ArgK from *Limulus polyphemus* and the biocatalytic synthesis of the important metabolite, N_ϖ-phospho-L-arginine, in gram scale.

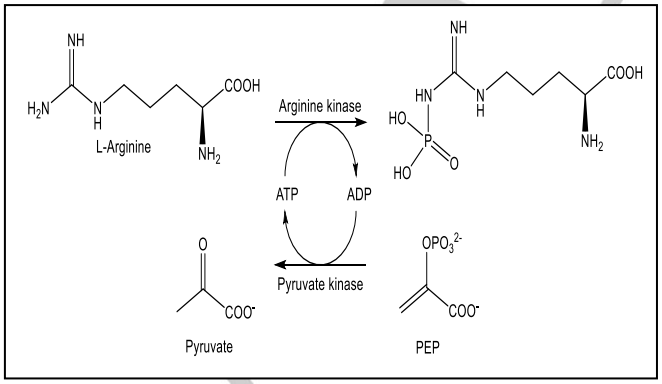


Figure 1. Arginine kinase-catalyzed phosphorylation of L-Arginine

As stoichiometric cofactor addition in ATP-dependent enzymatic phosphorylations can lead to various complications in synthetic applications such as inhibition by cofactor byproducts and challenging product purification, different enzymatic ATP regeneration systems have been developed to overcome these problems.[17] For ATP regeneration from ADP the four systems acetyl phosphate/acetate kinase,[18] phosphoenol pyruvate (PEP)/ pyruvate kinase (PK),[19] creatinphosphate/creatinkinase[20] or polyphosphate/polyphosphate kinase[21] have been most often applied. The PEP/PK-system for ATP regeneration from ADP has typical total turnover numbers of 100 and the advantage of the stable high-energy donor PEP, which together with PK is commercially available at large scale and has been successfully used in numerous preparative biocatalytic phospho-rylations.[22] This is why we have chosen the PEP/PK-system for ATP regeneration.

Results and Discussion

The objective of this study was to develop a simple and straightforward biocatalytic procedure for the phosphorylation of L-arginine to N_ϖ-phospho-L-arginine in gram-scale. Hence, we chose a recombinant overexpression strategy for ArgK employing the *argK* gene from *Limulus polyphemus* (Uniprot P51541, KARG_LIMPO). This ArgK is one of the best characterized ones and also different crystal structures are available for this enzyme and its mutants in a substrate free form (pdb 3M10) in transition state (pdb 1M15) or bound to different substrate analogues (like pdb 4GW2, 4GWZ, 4GVY). The isolation of ArgK-LP from biological sources like horseshoe crab sperm or muscle would have been both inefficient and uneconomic as compared to the recombinant expression of this enzyme [7b]. Thus, ArgK-LP was overexpressed in *E. coli* BL21 (DE3) as a soluble protein in high yield, i.e. around 30% of the protein was localized in the soluble protein fraction of *E. coli*. The size of the cytoplasmatic expressed monomer was approximately 40 kDa, as determined by SDS-PAGE analysis (see supporting information), which is similar to the sperm ArgK as determined by Strong and Ellington.[23]

Since the recombinant protein carried an N-terminal his-tag, ArgK was successfully purified by IMAC purification (see supporting information). The obtained yield of the purified ArgK was 24 mg/L of *E. coli* culture. The specific activity was about 80 U/mg of purified protein and 834 U/ml in the crude extract.

In a previous study by Strong et al.,[23] in which no codon-optimized gene was expressed, the concentration of soluble ArgK-LP was much lower (~3 mg/L of culture) and most of the recombinant protein aggregated as insoluble and inactive protein.[15] In addition to that, the specific activity of the recombinant ArgK-LP in our study was also ~28 times higher than reported by Strong and Ellington.[23] The activity of arginine kinase was determined by the pyruvate kinase/LDH assay according to Oliveira et al.[24] using L-arginine as substrate.

Since storage stability could be a cause for loss of activity when working in large scale, we studied the activity of arginine kinase over one month in different formulations and storage temperatures (4°C and -20°C). The storage temperature and the addition of 30% glycerol had apparently a positive influence on the enzyme activity. Without any precautions a maximum loss of

40% was observed, when ArgK-LP was stored at 4°C. In case the crude extract of ArgK was freeze-dried, a maximum loss of 50% was determined. When glycerol was added and Arg-LP was stored at -20°C, only 13 % loss of activity could be detected (Figure 2).

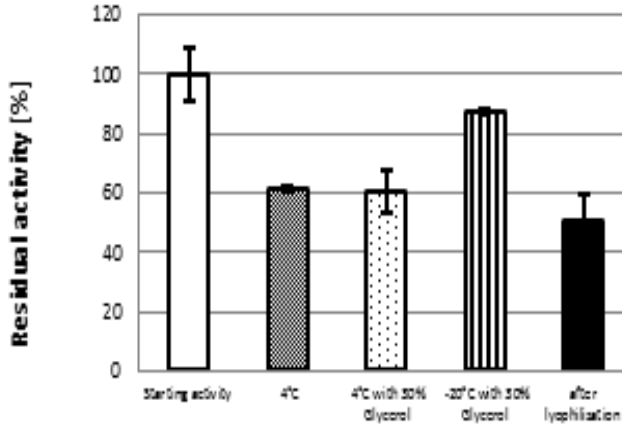


Figure 2. Storage stability investigation of recombinant arginine kinase in different formulation
Arginine kinase activity measured in different enzyme formulations are shown as relative activities compared to the activity of the freshly prepared crude enzyme (white bar). From left to right: Activity of soluble enzyme stored over one month at 4°C (grey bar), soluble enzyme stored over one month at 4°C with additional of 30% glycerol (dotted bar), soluble enzyme stored over one month at -20°C with additional of 30% glycerol (dashed bar), freeze-dried arginine kinase (black bar).The activity results ± standard deviations are the means of three independent experiments, each set measured in triplicate.

The development of biocatalytic phosphorylations is greatly facilitated by direct ^{31}P-NMR-analysis of product formation in the reaction mixture.[25] The simultaneous consumption of the phosphoenol pyruvate and the formation of the correct product is thereby a great advantage and suitable reaction conditions can be easily developed. The time course of a biocatalytic N_ω-phosphorylation of L-arginine was therefore followed by ^{31}P-NMR and is shown in figure 3. The reaction was carried out at pH 8.0 and with ATP recycling using the PEP pyruvate kinase-system with a slight sub-equimolar amount of PEP. A slight excess of L-arginine compared to PEP was chosen to avoid incomplete transformation of the latter which in our hands turned out to be difficult to separate from the arginine-phosphate. In contrast to ^{1}H-NMR-spectra the ^{31}P-NMR spectra are very clear and the signal increase with increasing time (from bottom lane 1 to the top lane 7 in figure 3A) at -3.65 ppm demonstrates the formation of the desired product N_ω-phospho-L-arginine, while the decreasing signal at 0.80 ppm shows the consumption of PEP, in line with ATP turnover. The time required for complete conversion can be optimized by selecting the total activity of the arginine kinase utilized for the reaction, shown in figure 3B for a phosphorylation completed within 24 hours. The method turned out to be suitable for the synthesis of preparative quantities of N_ω-Phospho-L-arginine, whereby the use of crude extract of ArgK and purified ArgK gave similar results.

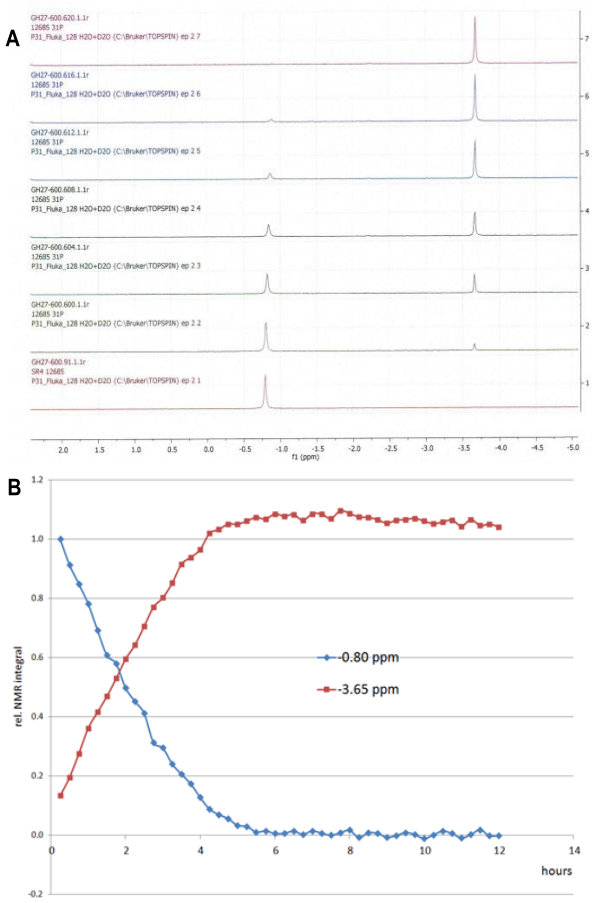


Figure 3. A) Reaction kinetics of biocatalytic phosphorylation of L-arginine; ^{31}P-NMR of reaction mixture from t=0 (lane 1) to t=5h (lane 7); signal at 0.80 ppm: PEP; signal at -3.65 ppm: N_ω-Phospho-L-arginine B) Time course of N_ω-Phospho-L-arginine product formation and PEP consumption during biocatalytic phosphorylation of L-arginine

Conclusions

The objective of this study was to develop a simple and straightforward biocatalytic procedure for the phosphorylation of L-arginine to N_ω-phospho-L-arginine in gram-scale. Tedious multi-step chemical procedures of N_ω-phospho-L-arginine described with 5 reactions steps have been replaced by a straightforward and highly efficient one-step biocatalytic phosphorylation of L-arginine using a recombinant arginine kinase. A highly active arginine kinase has been prepared by cloning and expressing the gene for ArgK from *Limulus polyphemus* in *E. coli*. ^{31}P-NMR enabled the rapid development of the arginine kinase-catalyzed phosphorylation of L-arginine combined with the recycling of the phosphorylating agent ATP using the phosphoenolpyruvate/ pyruvate kinase system. The biocatalytic procedure and the subsequent workup to the pure product N_ω-Phospho-L-arginine has been successfully demonstrated and opens up new opportunities for the selective biocatalytic N-phosphorylation of interesting small-molecular weight compounds and metabolites.[26]

Experimental Section

Unless otherwise stated, all chemicals were of analytical grade and were purchased from Sigma-Aldrich.

NMR spectroscopy
NMR-spectra were measured in D_2O at room temperature on a Bruker Avance III 600 MHz spectrometer equipped with a BBO probe head with z-gradient using 600.2 MHz for ^{1}H-NMR and 150.9 MHz for ^{13}C-NMR.

Gene synthesis and subcloning of ArgK
The arginine kinase gene ArgK was derived from *Limulus polyphemus* (UniProtKB: P51541) as described by Strong and Ellington [15]. The synthetic gene was codon optimized for *E. coli* and equipped with an N-terminal 6x-his-tag and a TEV protease cleavage site for optional removal of the affinity tag. The gene was synthesized and subcloned into pET24a(+) via NdeI and NotI by Genscript (USA), giving vector pET24-His(TV)-Karg-LimPo(op).

Recombinant expression of ArgK in *E. coli*
E. coli strain BL21 (DE3) was transformed with the derived plasmid using standard procedures.[27] Selected *E. coli* transformants were used for expression tests, in which different media, induction times and temperatures were optimized. The selected conditions were later used for the overexpression of ArgK in *E.coli* BL21 (DE3). Firsty, an *E.coli* overnight culture was prepared in 5 ml Luria Bertani (LB) medium.[28] supplemented with 50 µg ml−1 kanamycin and incubated at 30 °C and 180 rpm. Next a 50 mL pre-culture was prepared at 37 °C and 150 rpm in 250 mL Erlenmayer flask with baffles. Therefore, 50 ml of Terrific Broth (TB) medium supplemented with kanamycin (50 µg mL^{-1})[28] was inoculated with 500 µL of the corresponding overnight culture. When the pre-culture reached the mid-log phase, the main culture was started. Hence, 400ml of fresh TB medium in a 2 L Erlenmeyer flask was inoculated with 20 ml of the *E. coli* pre-culture. After the culture reached an OD600 of 1.3 (optical density), protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The main culture was shaken at 110 rpm for 20 h at 25 °C. Cells were harvested by centrifugation at 5000rpm for 30 min at 4°C and washed once with an ice-cold 50 mM sodium phosphate buffer at pH 7.5. The *E. coli* cell pellets containing the overexpressed enzyme (rec. ArgK) were frozen at -20°C.

Cell disruption
For cell disruption, frozen cells were adjusted to an OD_{600} of 40 in potasium phosphate buffer (TrisHCl-buffer: 50 mM, pH 7.5) and mixed with glass beads (Ø 0,25-05µm) at an approximate ratio of 1:1. Then, the cells were disintegrated mechanically by a cell disruptor (Retsch Mixer Mill MM 200: two cycles for 5 min at 600 rpm). The samples were cooled on ice and insoluble cell debris was removed by centrifugation (5000 rpm, 30 min, 4°C). The supernatant, which contained the soluble protein fraction, was immediately used for activity determination.

IMAC-purification and protein content determination of rec. ArgK
In order to purify the recombinant ArgK by IMAC (immobilized metal ion chromatography), the soluble protein fraction was incubated with Talon® resin on an orbital shaker (30 min, 4° C). Then, a standard gravity flow protocol was applied to purify the target protein. Therefore, the resin was washed at 4°C with 10 column volumes (CV) of TrisHCl-buffer (50 mM, pH 7.5) containing 10 mM imidazole in order to get rid of unspecifically bound protein. The target protein ArgK was eluted with 1.5 CV of elution buffer (50mM TrisHCl-buffer, pH 7.5, 150 mM imidazole). Samples were desalted on desalting columns in order to remove imidazole from the purified protein samples (Zeba Spin columns, Pierce). The purified protein was immediately used for activity determination.
The protein content of the purified protein was determined according to the BCA method according to the manufacturer's protocol (Pierce Protein Research Products, Thermo Scientific) using bovine serum albumin (BSA) as a standard. The analysis of ArgK overexpressionn and the purity was performed using 12, 5% SDS-polyacrylamide gels. Gels were stained with Coomassie Blue. Gels were analyzed using software of GEL ANALYZER (Version 2010a).

Determination of arginine kinase activity
Arginine kinase activity was measured spectrophotometrically at λ=340 nm based on ADP release by coupled assay with pyruvate kinase and LDH, according to Strong and Ellington,[23] Oliveira et al.[24] ArgK was assayed at room temperature by recording at λ= 340 nm the oxidation of NADH (ε =6.22 x10^3 M^{-1} cm^{-1}). The assay mixture contained 100 mM Tris–HCl buffer pH 7.5, 1 mM phosphoenol pyruvate, 5 mM $MgCl_2$, 50 mM KCl, 0.15 mM NADH, 4 units of pyruvate kinase, 8 units of lactate dehydrogenase, 2.5 mM ATP and 10 mM L-arginine in a total volume of 1ml. The reaction was started by the addition of recombinant protein. One unit of enzyme activity is defined as the amount of enzyme catalyzing the conversion of 1 µmol of substrate per minute at pH 7.5 and 25 ° C.

Determination of storage stability
The storage stability was investigated by determination of the catalytic activity of arginine kinase for three different formulations and different temperature. The aliquot in TrisHCl-buffer: 50 mM, pH 7.5 was transferred into test tubes. One aliquot of the enzyme was stored as a liquid at 4°C and -20°C for one month, to the other 30% of glycerol was added as cryoprotectant to protect enzyme from freezing damage ant the enzyme was stored as well as a liquid at 4°C and -20°C, another one was freeze-dried (CHRIST beta 1-8 freeze dryer, Martin Christ Freeze Dryers, Osterode am Harz, Germany) where the liquid was frozen at -80°C. Dehydration step lasted for 20h at temperature of -53°C until dried powder formed. The resulting lyophilized powders were rehydrated to its original volume at room temperature with 50mM TrisHCl-buffer pH 7.5. Then the samples were subjected to the subsequent activity tests. The residual activity of the enzyme was measured on the same day.

Lab-scale preparation of Nω-Phospho-L-arginine
To 85ml of a 67mM solution of L-arginine in water, adjusted to pH 8.0 with diluted acetic acid, magnesiumchloride (20mM), ATP (0.05 equ.) and PEP (0.95 equ.) were added and the pH again adjusted to 8.0. Then 200U pyruvate kinase and 200µl arginine kinase solution (see above) were added and the reaction mixture again adjusted to pH 8 by addition of a small amount of 0.1M acetic acid while stirring. The reaction progress was monitored by ^{31}P-NMR (fig.4), consumption of PEP was complete after ~5h. After 2 days the reaction mixture was concentrated, dried and worked up by column NP chromatography on silica gel (methanol/water=1:1) to yield after conversion to the lithium salt 410mg (28%) N_ω-phospho-L-arginine.
Analytical Data: Single spot on TLC (silicagel, H_2O/n-PrOH/NH_4OH = 11:6:3); ^{1}H-NMR: (D_2O, 400MHz, δ) 3.45 (dd, 1H, J1≈J2≈6); 3.17 (t, 2H, J=6.8); 1.82 (m, 2H); 1.60 (m, 2H); ^{31}P-NMR: (D_2O, 162MHz, δ, CPD) 3.26

Gram scale peparation of N_ω-Phospho-L-arginine
Based on the NMR experiments (Figures 4A and 4B) a lab scale procedure for the gram scale preparation of N_ω-Phospho-L-arginine was performed. The amount of required kinase was determined in order to convert 1g of L-Arginine within aprox. 6 hours. After complete consumption of PEP as indicated by ^{1}H- and ^{31}P-NMR the reaction mixture was purified by chromatography on silica gel as described in the lab-scale preparation above. As considerable loss of the sensitive N-phosphate occurred, there is room for further yield improvements by optimizing the work up and isolation procedure.

Acknowledgements

We thank Dr.Christine Hellriegel for the NMR kinetic data aquisition and analysis. We also would like to thank the German Federal Ministry of Education and Research (BMBF) for the support of project P28 under the cluster of Biocatalysis2021.

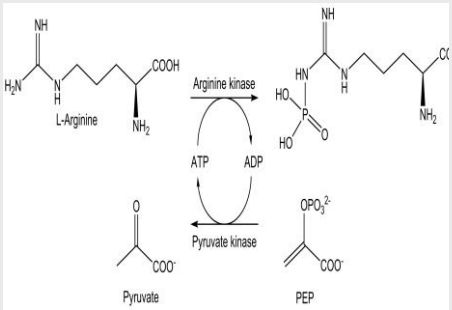
Keywords: N_ω-Phospho-L-arginine • Arginine kinase • Phosphagen • Biocatalysis • Enzymatic Phosphorylation

[1] O.Meyerhof, K. Lohmann, *Biochem. Z.* **1928**, *196*, 22-49 and 49-72.
[2] a) K. Lohmann, *Biochem. Z.* **1934**, *271*, 264-277; b) K.Lohmann, *Biochem. Z.* **1935**, 282, 109-119; c) K.Lohmann, *Biochem. Z.* **1936**, *286*, 28-44; d) S. Ochoa, *Biochem J.* **1938**, *32*, 237-242; e) J. K. Parnas, *Annu. Rev. Biochem.* **1932**, *1*, 431-457.
[3] a) M. Parsons, E. A. Worthey, P. N. Ward, J. C. Mottram, *BMC Genomics* **2005**, *6:127*, 1-19; b) C. A. Pereira, G. D. Alonso, M. C. Paveto, A. Iribarren, M. L. Cabanas, H. N. Torres, M. M. Flawia, *J. Biol. Chem.* **2000**, *275*, 1495-1501; c) K. Uda, N. Fujimoto, Y. Akiyama, K. Mizuta, K. Tanaka, W. R. Ellington, T. Suzuki, *Comp. Biochem. Physiol., Part D: Genomics Proteomics* **2006**, *1*, 209-218.
[4] C. A. Pereira, *Infectious Disorders-Drug Targets* **2014**, *14(1)*, 30-36.
[5] a) A. Schneider, R. Wiesner, M. Grieshaber, *Insect Biochem.* **1989**, *19*, 471-480; b) G. Zhou, T. Somasundaram, E. Blanc, G. Parthasarathy, W. R. Ellington, M. S. Chapman, *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 8449-8454; c) R. Kucharski, R. Maleszka, *Gene* **1998**, *211*, 343-349.
[6] P. Eggleton, G. P. Eggleton, *J. Physiol.* **1928**, *65*, 15-24.
[7] a) J. Bolte, G. M. Whitesides, *Bioorg. Chem.* **1984**, *12*, 170-175; b) S. J. Strong, W. R. Ellington, *J. Exp. Zool.* **1993**, *267*, 563-571; c) W. E. Teague, G. P. Dobson, *J. Biol. Chem.* **1999**, *274*, 22459-22463; d) G. Zhou, W. R. Ellington, M. S. Chapman, *Biophys. J.* **2000**, *78*, 1541-1550; e) G. Zhou, G. Parthasarathy, T. Somasundaram, A. Ables, L. Roy, S. J. Strong, W. R. Ellington, M. S. Chapman, *Protein Sci.* **1997**, *6*, 444-449.
[8] a) M. S. Yousef, F. Fabiola, J. L. Gattis, T. Somasundaram, M. S. Chapman, *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2002**, *58(12)*, 2009-2017; b) X. Niu, L. Bruschweiler-Li, O. Davulcu, J. J. Skalicky, R. Brüschweiler, M. S. Chapman, *J. Mol. Biol.* **2011**, *405*, 479-496.
[9] a) J. Fuhrmann, V. Subramanian, P. R. Thompson, *Angew. Chem. Int. Ed.* **2015**, *127(49)*, 14928-14931; b) E. A. Ruben, M. S. Chapman, J. D. Evanseck, *J. Phys. Chem. A* **2007**, *111*, 10804-10814.
[10] H. Ouyang, C. Fu, S. Fu, Z. Ji, Y. Sun, P. Deng, Y. Zhao, *Org. Biomol. Chem.* **2016**, *14*, 1925-1929.
[11] F. Marcus, J. Morrison, *Biochem. J.* **1964**, *92*, 429-435.
[12] F. Cramer, E. Scheiffele, A. Vollmar, *Chem. Ber* **1962**, *95*, 1670-1682.
[13] F. Cramer, A. Vollmar, E. Scheiffele, *Angew. Chem.* **1960**, *72(6)*, 211-211.
[14] T. Ruman, K. Długopolska, A. Jurkiewicz, D. Rut, T. Frączyk, J. Cieśla, A. Leś, Z. Szewczuk, W. Rode, *Bioorg. Chem.* **2010**, *38*, 74-80.
[15] T. Nguyen Van, T. Nguyen Van, J. Roche, *Bull. Soc. Chim. Biol.* **1962**, *44*, 285-290.
[16] D. Gauss, B. Schoenenberger, G.S. Molla, B.M. Kinfu, J. Chow, A. Liese, W. Streit, Biocatalytic Phosphorylation of Metabolites, in: Applied Biocatalysis – From Fundamental Science to Industrial Applications, Eds. A.Liese, L.Hilterhaus, U. Kettling, G.Antranikian, Wiley-VCH, Weinheim, Germany, **2016**.
[17] a) D. C. Crans, R. J. Kazlauskas, B. L. Hirschbein, C. H. Wong, O. Abril, G. M. Whitesides, *Methods Enzymol.* **1987**, *136*, 263-280; b) J. N. Andexer, M. Richter, *ChemBioChem*, **2015** , *16(3)*, 380-386.
[18] a) J. M. Lewis, S. L. Haynie, G. M. Whitesides, *J. Org. Chem.* **1979**, *44*, 864-865; b) C.-H. Wong, A. Pollak, S. D. McCurry, M. M. Sue, J. R. Knowles, G. M. Whitesides, *Methods Enzymol.* **1982**, *89*, 108-121; c) D. C. Crans, G. M. Whitesides, *J. Org. Chem.* **1983**, *26*, 3130-3132; d) D. M. Kim, J. R. Swartz, *Biotechnol. Bioeng.* **2001**, *74*, 309-316.
[19] B. L. Hirschbein, F. P. Mazenod, G. M. Whitesides, *J. Org. Chem.* **1982**, *47*, 3765-3766; b)
[20] J. Zhang, B. Wu, Y. Zhang, P. Kowal, P.G. Wang, *Org. Lett.* **2003**, *5*, 2583-2586.
[21] a) K. Murata, T. Uchida, J. Kato, I. Chibata, *Agric. Biol. Chem.* **1988**, *52*, 1471–1477; b) S. M. Resnick, A. J. B. Zehnder, *Appl. Environ. Microbiol.***2000**, *66(5)*, 2045-2051; c) A. Kameda, T. Shiba, Y. Kawazoe, Y. Satoh, Y. Ihara, M. Munekata, K. Ishige, T. Noguchi, *J. Biosci. Bioeng.* **2001**, *91(6)*, 557-563.
[22] a) A. Gross, O. Abril, J. M. Lewis, S. Geresh, G. M. Whitesides, *J. Am. Chem. Soc.***1983**, *105*, 7428-7435; b) M. D. Bednarski, D. C. Crans, R. DiCosimo, E. S. Simon, P. D. Stein, G. M. Whitesides, M. J. Schneider, *Tetrahedron Lett.* **1988**, *29*, 427-430;
[23] S. J. Strong, W. R. Ellington, *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **1996**, *113*, 809-816.
[24] J. S. Oliveira, C. A. Pinto, L. A. Basso, D. S. Santos, *Protein Expression Purif.* **2001**, *22*, 430-435.
[25] a) R. Matsumi, C. Hellriegel, B. Schoenenberger, T. Milesi, J. van der Oost, R. Wohlgemuth, *RSC Adv.* **2014**, *4*, 12989-12994; b) D. Gauss, B. Schoenenberger, R. Wohlgemuth, *Carbohydr. Res.* **2014**, *389*, 18-24.
[26] R. Wohlgemuth, *Biotechnol. J.* **2009**, *4*, 1253-1265.
[27] D.Hanahan, *J. Mol. Biol.* **1983** , *166*, 557-580.
[28] J.Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning*, Vol. 2, New York: Cold Spring Harbor Laboratory Press, **1989**.

Entry for the Table of Contents

FULL PAPER

A straightforward one-step synthesis of N_ω-phospho-L-arginine has been developed by arginine kinase-catalyzed phosphorylation of L-arginine using the phosphorylating agent ATP, which was recycled with the phosphoenolpyruvate/pyruvate kinase regeneration system for the selective phosphorylation of L-arginine. A highly active and stable arginine kinase has been prepared by cloning and expressing the *argK* gene in *E.coli* .



*Bernhard Schoenenberger, Agata Wszolek, Thomas Milesi, Henrike Brundiek, Markus Obkircher and Roland Wohlgemuth**

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Synthesis of N_ω-Phospho-L-arginine by Biocatalytic Phosphorylation of L-Arginine