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

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The N_ω-phospho-L-arginine energy-buffering system is mainly present in invertebrates for regulating energy requirements when it is highly needed, such as in the flight muscles of an insect or when energy supply fluctuates, as in the medically important protozoa *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania major*. The lack of availability of this important metabolite was due to a tedious chemical procedure, by which N_ω-phospho-L-arginine was prepared in 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 by using the phosphoenolpyruvate/pyruvate kinase system. After standard work-up, the desired product N_ω-phospho-L-arginine was 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 it 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 that are totally different from those pathways found in mammalian host tissues. In a wide variety of invertebrates and certain parasitic protozoa such as *Trypanosoma brucei, Trypanosoma cruzi*, and *Leishmania major*, which cause some of the most debilitating diseases in humans including leishmaniasis, African sleeping sickness, and Chagas disease, phosphoarginine 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] As 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 a 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 catalyzes 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 [Eq. (1):

$$\text{ATP} + \text{L-arginine} \rightleftharpoons \text{phospho-L-arginine} + \text{ADP} \quad (1)$$

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, ArgKs 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 such as phosphocreatine in vertebrates and phosphoarginine in invertebrates has been well studied in physiology since 1927[6] and has also become 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 the physiology, enzymology, and molecular biology of phosphagen synthesis is well understood, the ready availability of phosphoarginine is still lacking. This is

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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 the 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, unstable in hot alkali, and sensitive to heat, rendering its isolation from biological sources difficult, too.[10] Isolation of phosphoarginine, for example, from crayfish tail muscle or freshwater 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 phosphorarginine.[12–15]

A few entirely chemical syntheses involving a number of reaction steps have been published, but each route has its own bottle-necks and challenges. Cramer and co-workers[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 fresh 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. Owing to incomplete 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 preparation of NMR samples of N_ω-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] Owing to the many side products formed, this procedure required a tedious work-up 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* (ArgK-LP) and the biocatalytic synthesis of the important metabolite, N_ω-phospho-L-arginine, on the gram scale (Figure 1).

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

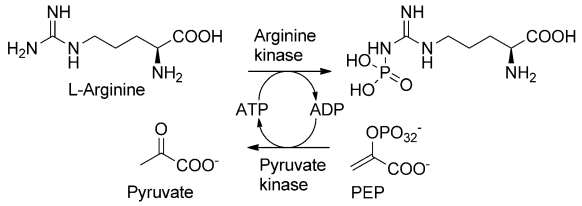

Figure 1. Arginine kinase catalyzed phosphorylation of L-arginine.

ATP regeneration systems have been developed to overcome these problems.[17] For ATP regeneration from ADP, the four systems of acetyl phosphate/acetate kinase,[18] phosphoenolpyruvate (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 typical has total turnover numbers of 100 and the advantage of the stable high-energy donor PEP, which together with PK is commercially available on a large scale and has been successfully used in numerous preparative biocatalytic phosphorylations.[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_ω-phospho-L-arginine on the 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 different crystal structures are also available for this enzyme and its mutants in a substrate-free form (pdb 3M10), in the transition state (pdb 1M15), or bound to different substrate analogs (like pdb 4GW2, 4GWZ, 4GVY). The isolation of ArgK-LP from biological sources such as horseshoe crab sperm or muscle would have been both inefficient and uneconomic compared with the recombinant expression of this enzyme.[7b] Thus, ArgK-LP was overexpressed in *E. coli* BL21 (DE3) as a soluble protein in high yield, that is, 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 (sodium dodecyl sulphate-polyacrylamide gel electrophoresis; see the Supporting Information), which is similar to the sperm ArgK as determined by Strong and Ellington.[23]

As the recombinant protein carried an N-terminal his-tag, ArgK was successfully purified by IMAC purification (immobilized metal affinity chromatography; see the Supporting Information). The obtained yield of the purified ArgK was 24 mg L^{-1} of *E. coli* culture. The specific activity was about 80 U mg^{-1} of purified protein and 834 U mL^{-1} 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^{-1} 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 approximately 28 times higher than that reported by Strong and Ellington.[23] The activity of arginine kinase was determined by the pyruvate kinase/lactate dehydrogenase (LDH) assay as reported by Oliveira et al.[24] using L-arginine as the substrate.

As storage stability could be a cause for loss of activity when working on a 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 apparently had 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 the case where 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).

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 phosphoenolpyruvate 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 spectroscopy and is shown in Figure 3. The reaction was carried out at pH 8.0 and with ATP recycling by using the PEP/pyruvate kinase system with a slightly sub-equimolar amount of PEP. A slight excess of L-arginine compared with 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 3 A) at −3.65 ppm demonstrates the formation of the desired product N_ω-phospho-L-arginine, whereas 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 3 B for a phosphorylation completed within 24 h. 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.

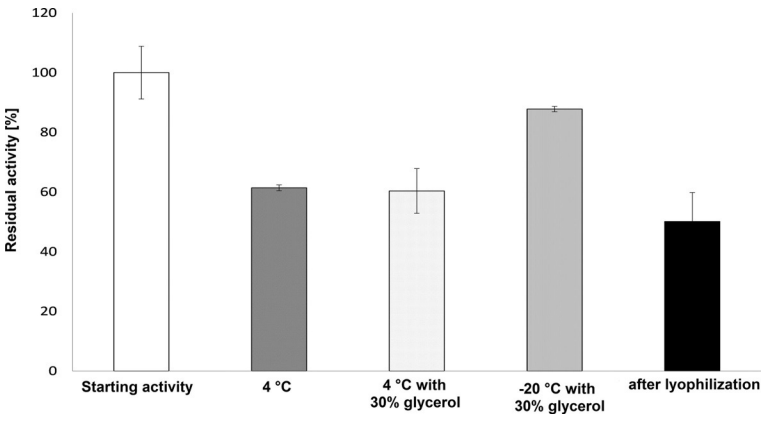

Figure 2. Storage stability investigation of recombinant arginine kinase in different formulations. Arginine kinase activity measured in different enzyme formulations are shown as relative activities compared to the activity of the freshly prepared crude enzyme (white bar). From left to right: Activity of soluble enzyme stored over one month at 4 °C (grey bar), soluble enzyme stored over one month at 4 °C with addition 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.

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 on a gram scale. Tedious multistep chemical procedures of N_ω-phospho-L-arginine described with five reactions steps have been replaced by a straightforward and highly efficient one-step biocatalytic phosphorylation of L-arginine by 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 spectroscopy enabled the rapid development of the arginine kinase catalyzed phosphorylation of L-arginine combined with the recycling of the phosphorylating agent ATP by using the phosphoenolpyruvate/pyruvate kinase system. The biocatalytic procedure and the subsequent work-up 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 low-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 with 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 spectroscopy.

Gene synthesis and subcloning of ArgK

The arginine kinase gene ArgK was derived from *Limulus polyphemus* (ArgK-LP; UniProtKB: P51541) as described by Strong and Ellington.[15] The synthetic gene was codon optimized for *E. coli* and equipped with an N-terminal 6×-his-tag and a TEV (tobacco etch virus) 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).

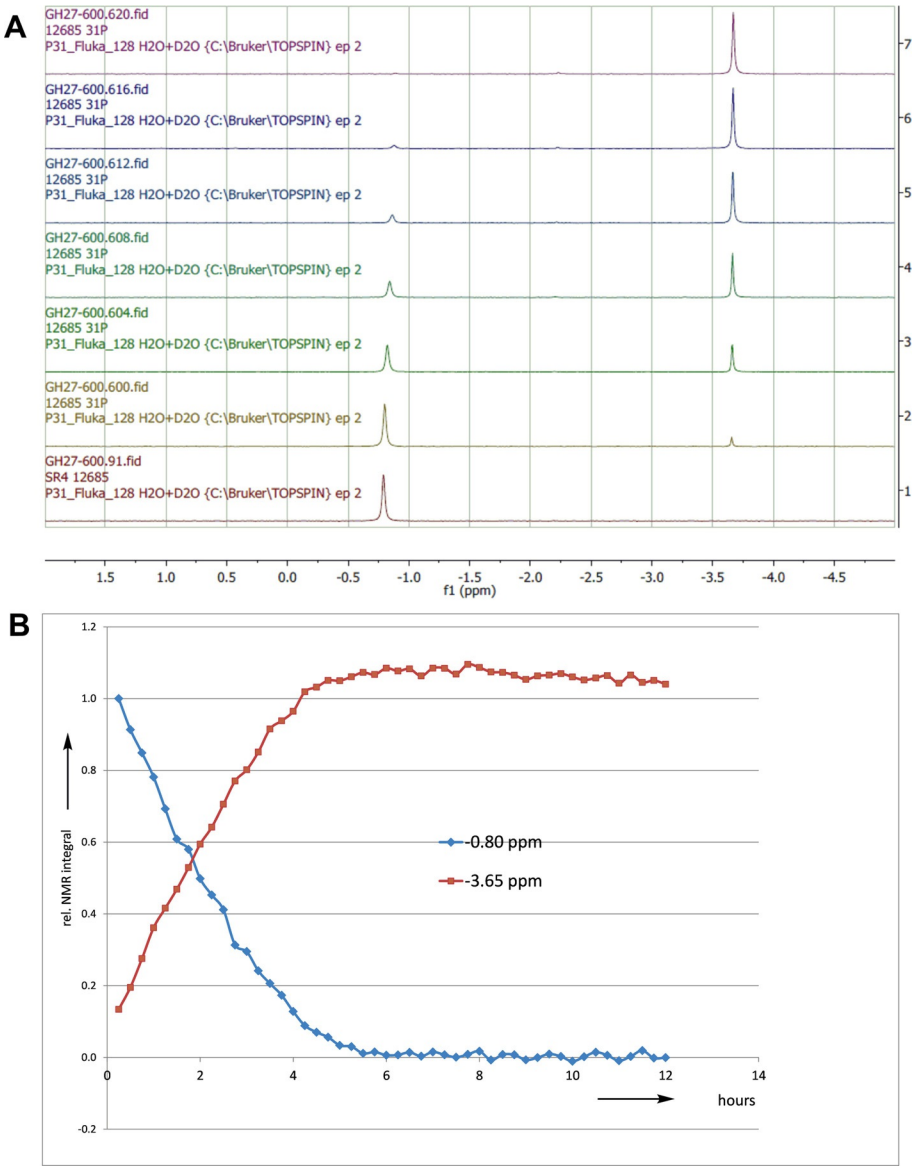

Figure 3. A) Reaction kinetics of biocatalytic phosphorylation of L-arginine; ^{31}P NMR spectra of a reaction mixture from $t=0$ (lane 1) to $t=5$ h (lane 7); signal at 0.80 ppm: PEP; signal at −3.65 ppm: N_ω-phospho-L-arginine. B) Time course of N_ω-phospho-L-arginine product formation and PEP consumption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

Recombinant expression of ArgK in *E. coli*

E. coli strain BL21 (DE3) was transformed with the derived plasmid by 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). First, 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 a 250 mL Erlenmayer flask with baffles. Thereafter, 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, 400 mL 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 OD_{600} 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 5000 rpm for 30 min at 4 °C and washed once with 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 potassium phosphate buffer (TrisHCl buffer: 50 mM, pH 7.5) and mixed with glass beads (Ø = 0.25–0.5 μm) 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

To purify the recombinant ArgK by IMAC (immobilized metal ion chromatography), the soluble protein fraction was incubated with Talon® resin in 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 ten column volumes (CV) of TrisHCl buffer (50 mM, pH 7.5) containing 10 mM imidazole to get rid of unspecifically bound protein. The target protein ArgK was eluted with 1.5 CV of elution buffer (50 mM TrisHCl buffer, pH 7.5, 150 mM imidazole). Samples were desalted on desalting columns 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 bicinchoninic acid assay (BCA) method according to the manufacturer's protocol (Pierce Protein Research Products, Thermo Scientific) by using bovine serum albumin (BSA) as a standard. The analysis of ArgK overexpression and purity was performed by using 12.5% SDS-polyacrylamide gels. Gels were stained with Coomassie Blue. Gels were analyzed by using the software GEL ANALYZER (Version 2010a).

Determination of arginine kinase activity

Arginine kinase activity was measured spectrophotometrically at $\lambda = 340$ nm based on ADP release by coupled assay with pyruvate kinase and LDH, according to Strong and Ellington,[23] and Oliveira et al.[24] ArgK was assayed at room temperature by recording the oxidation of NADH ($\lambda = 340$ nm, $\varepsilon = 6.22 \times 10^3\ M^{-1} cm^{-1}$). The assay mixture contained 100 mM Tris-HCl buffer pH 7.5, 1 mM phosphoenolpyruvate, 5 mM $MgCl_2$, 50 mM KCl, 0.15 mM NADH, four units of pyruvate kinase, eight units of lactate dehydrogenase, 2.5 mM ATP, and 10 mM L-arginine in a total volume of 1 mL. 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 temperatures. Aliquots in TrisHCl buffer (50 mM, pH 7.5) were transferred into test tubes. One aliquot of the enzyme was stored as a liquid at 4 °C and another at −20 °C for one month. Another two had 30% glycerol added as cryoprotectant to protect the enzyme from freezing damage and the enzyme was stored, also 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. The dehydration step lasted for 20 h at a temperature of −53 °C until dried powder formed. The resulting lyophilized powders were rehydrated to its original volume at room temperature with 50 mM 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 N_ω-phospho-L-arginine

Magnesium chloride (20 mM), ATP (0.05 equiv), and PEP (0.95 equiv) were added to a solution of L-arginine in water (85 mL, 67 mM), adjusted to pH 8.0 with diluted acetic acid, and the pH again adjusted to 8.0. Then, 200U pyruvate kinase and arginine kinase solution (200 μL, see above) were added and the reaction mixture again adjusted to pH 8 by addition of a small amount of 0.1 M acetic acid with stirring. The reaction progress was monitored by ^{31}P NMR (Figure 3); consumption of PEP was complete after approximately 5 h. 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, 410 mg (28%) N_ω-phospho-L-arginine.

Analytical data: Single spot on TLC (silicagel, H_2O/*n*-PrOH/NH_4OH = 11:6:3); ^1H NMR (D_2O, 400 MHz): $\delta = 3.45$ (dd, 1H, $J_1, J_2 \approx 6$ Hz), 3.17 (t, 2H, $J = 6.8$ Hz), 1.82 (m, 2H), 1.60 ppm (m, 2H); ^{31}P NMR (D_2O, 162 MHz, CPD): $\delta = 3.26$ ppm.

Gram-scale peparation of N_ω-phospho-L-arginine

Based on the NMR experiments (Figure 3A and B), a procedure for the gram-scale preparation of N_ω-phospho-L-arginine was developed. The amount of required kinase was determined based on that needed to convert 1 g of L-arginine within approximately 6 h. After complete consumption of PEP as indicated by ^1H and ^{31}P NMR spectroscopy, 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.

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Keywords: arginine kinase · biocatalysis · enzymatic phosphorylation · N_ω-phospho-L-arginine · phosphagen

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