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Creatine Phosphate–Creatine Kinase in Enzymatic Synthesis of Glycoconjugates

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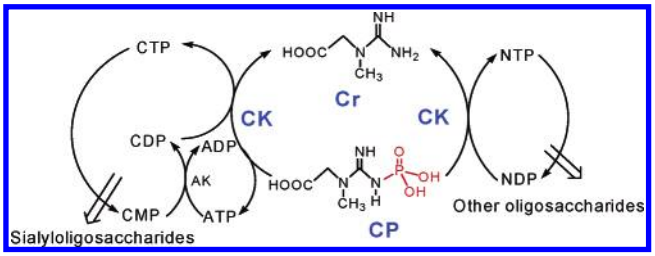
ABSTRACT



Enzymatic production of glycoconjugates is hampered by expensive phosphagens such as acetyl phosphate (AcP) and phosphoenolpyruvate (PEP). Here, we introduce creatine phosphate–creatine kinase system as a novel and practical energy source in carbohydrate synthesis. This system was successfully demonstrated in the production of bioactive oligosaccharides with different sugar nucleotide regeneration systems.

The past twenty years have seen a dramatic intensification in the interest in carbohydrates due to new findings on their biological roles. Glycosylation not only prolongs the half-lives of associated proteins and hormones but also helps many important cellular recognition events in biological systems.[1] Biochemical and biophysical studies of glycoconjugates apparently require large-scale preparations of oligosaccharides of interest.[2] However, the low efficiencies of oligosaccharide synthesis systems have seriously limited progress in glycoscience, especially in the area of glycomedicine. Many attempts to improve the productivity have been made, mainly by exploitation of new glycosyltransferases and novel biosynthetic pathways.[3] Despite that the sugar-nucleotide regeneration systems solved the major obstacle in the carbohydrate production, the expensive sugar nucleotide pool, the technologies are still greatly dependent on high-energy phosphate donors.[4] For example, in our previous studies of "superbeads" technology, the major cost was from phosphoenolpyruvate (PEP).[3f]

Creatine phosphate (phosphocreatine, CP, PCr) is an organic phosphate compound in cellular energy metabolism, in addition to ATP and other nucleoside triphosphates (Figure 1).[5] Because the standard free energy of hydrolysis of CP (−43.1 kJ/mol) is higher than that of ATP (−30.6 kJ/mol), ADP is phosphorylated into ATP in mammalian brain, heart and muscle, via the CP−CK (creatine kinase, creatine phosphokinase, E.C. 2.7.3.2) shuttle for intracellular energy transfer.[6] The CP−CK system has been utilized to regenerate ATP for *de novo* protein syntheses.[7] Though it may seem

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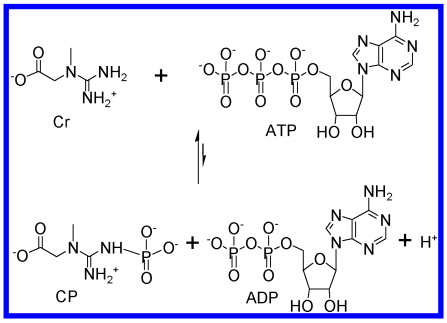


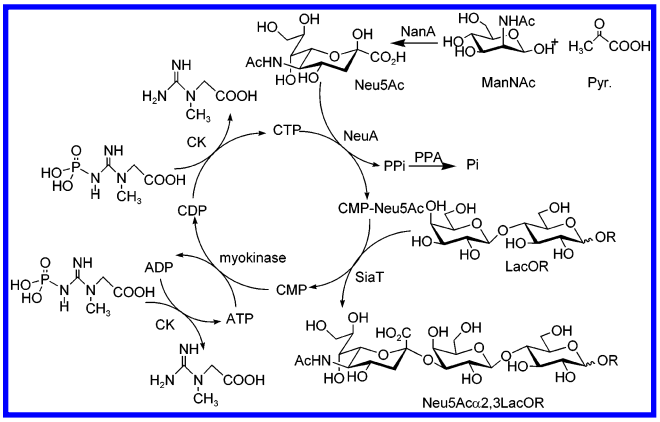
Figure 1. Creatine kinase reaction.

that it is a logical extension to apply this energy supply system to oligosaccharide synthesis, this work is, to our knowledge, the first demonstration of its applicability to systems outside the realm of *in vitro* protein synthesis. To demonstrate its efficiency in carbohydrate synthesis, we applied this phosphagen first in the synthesis of sialyloligosaccharides.

Sialyloligosaccharides play important roles in a variety of cellular functions.[8] For instance, sialyllactose (SL) can neutralize enterotoxins of various pathogenic microbes, including *Escherichia coli*, *Vibrio cholerae*, and *Salmonella typhimurium*.[9] It has also been reported that 3′-sialyllactose (Neu5Acα2, 3Lac, 3′-SL) interferes with colonization of *Helicobacter pylori* in human stomach and thereby prevents or inhibits gastric and duodenal ulcers.[10] In addition, it has been proposed to inhibit immune complex formation for arthritis treatment by blocking the carbohydrate-binding sites on IgG Fc fragments.[11] To date, commercially available sialyloligosaccharides are still very expensive due to their low abundance in natural sources. For example, 3′-sialyllactose and 6′-sialyllactose isolated from bovine colostrum are sold for $54.75 and $71.50 per 0.5 mg, respectively (Sigma Chemical Co., 2003). Therefore, great efforts have been focused on synthesizing sialyloligosaccharides from cheap and simple starting materials.[3e,12]

Our multienzyme system toward sialyllactosides is based on a new *ex vivo* pathway with the CP−CK energy system.[13] As shown in Scheme 1, the biosynthetic pathway consists of sialyltransferase (SiaT) and five other enzymes for the

Scheme 1. Sialylation Cycle with CP−CK Regeneration System



recycling of cytidine 5′-monophospho-*N*-acetylneuraminic acid (CMP−Neu5Ac). It starts with *N*-acetylmannosamine (ManNAc), pyruvate (Pyr), lactose, CP, and catalytic amounts of ATP and CMP. CMP is initially converted to CDP by myokinase (E.C. 2.7.4.3) in the presence of ATP, which is regenerated from its byproduct ADP in a reaction catalyzed by CK with CP as the phosphate donor. At the same time, *N*-acetylneuraminic acid (Neu5Ac) is synthesized *in vitro* by an aldolase (NanA, E.C. 4.1.3.3)-catalyzed condensation of ManNAc and pyruvate.[14] Neu5Ac is then coupled with CTP to produce CMP−Neu5Ac by NeuA (CMP−sialic acid synthatase, E.C. 2.7.7.43).[15] To drive this reaction forward, inorganic phosphatase (PPA, E.C. 3.6.1.1) is incorporated to hydrolyze the byproduct, pyrophosphate (PPi).[16] An α2,3-SiaT (E.C. 2.4.99.4) will then transfer the sialyl residue from CMP−Neu5Ac to the acceptor.[17] The released CMP can be rephosphorylated by myokinase and creatine kinase with the consumption of 2 equiv of CP. Overall, the production of 1 equiv of sialyloligosaccharide requires 1 equiv each of ManNAc, pyruvate, and acceptor plus 2 equiv of CP.

To optimize the reaction conditions and explore the impacts of different factors on the reaction system, small-scale (0.25 mL) reactions for 3′-SL were carried out at 37 °C for 48 h.[18] The formation of trisaccharide was monitored by high-performance capillary electrophoresis (HPCE) using

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(12) Endo, T.; Koizumi, S.; Tabata, K.; Ozaki, A. *Appl. Microbiol. Biotechnol.* **2000**, *53*, 257−261.

(13) We tried the cycle with ATP as the direct energy source. However, the cycle suffers from a low yield due to the product inhibitions of ATP/ADP and low phosphorylating potential of ATP.

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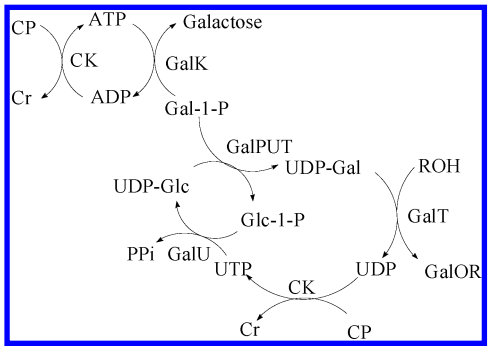
a UV detector ($\lambda = 245$ nm) after PMP (1-phenyl-3-methyl-5-pyrazolone) derivatization. The PMP method constitutes a great advantage over the common reductive−amination derivatization method, where it avoids acidic desialylation during the process.[19] Although the reported pH optimum of sialyltransferase is 6.5[17] and it is suggested that acidic media is favorable for the creatine phosphate-cleavage reaction (Figure 1),[20] the multienzyme sialylation reaction was performed at pH 7.5 to prevent a decomposition of activated neuraminic acid at lower pH. This pH was well tolerated by NanA (pH optimum 7.7),[14] NeuA (pH optimum 9.5),[15] myokinase (pH optimum 8.0),[21] and PPA (pH optimum 9.1).[22] DTT was used to stabilize NanA, NeuA, and SiaT in the reaction. The ManNAc can be epimerized chemically from cheaper GlcNAc to reduce the cost in large-scale production.[23]

Besides the sialylation discussed above, the new energy system can be utilized in other sugar nucleotide regeneration systems.[24] Herein, we coupled this energy source to the known UDP−Gal regeneration cycles, the KTCU cycle (Scheme 2) and the CUPE cycle (Scheme 3), for the synthesis

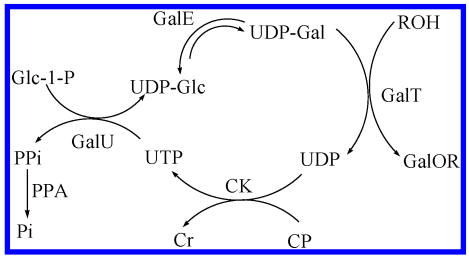
Scheme 2. KTCU Cycle with CP−CK Regeneration System[a]



[a] Abbreviations: GalK, galactokinase, GalPUT, galactose-1-phosphate uridylyltransferase; GalU, glucose-1-phosphate uridylyltransferase; GalT, galactosyltransferase; Glc-1-P, glucose-1-phosphate; Gal-1-P, galactose-1-phosphate; UDP-Glc, uridine 5′-diphospho-α-D-glucose; UDP-Gal, uridine 5′-diphospho-α-D-galactose.

of biomedically important galactosides such as α-Gal epitope and globotriose.[25] In our previous studies, PEP was coupled as a direct energy source to drive the glycosylation. When

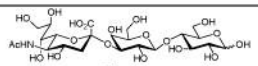
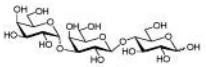
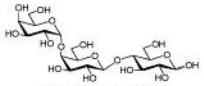
Scheme 3. CUPE Cycle with CP−CK Regeneration System[a]



[a] Abbreviation: GalE, UDP-Gal 4′-epimerase.

PEP was replaced with CP, the galactoside yields were comparable with the cycles supplied with the old PEP−PK system (Table 1). These new cycles, especially the CUPE

Table 1. Yields of Glycoconjugate Synthesis with Different Sugar Nucleotide Regeneration Systems

enzyme systems	Products	PEP-PK (%)	CP-CK (%)
NNPSMC	 3'-SL	33	35 (30)[a]
αKTCU	 α-Gal	20	23
CKTCU	 Globotriose	30	35
αCUPE	α-Gal	25	32 (30)
CCUPE	Globotriose	40	45 (43)

[a] Yields in parentheses are isolated yields based on 100 mg-scale preparative synthesis. Others are milligram-scale reactions and were obtained by HPCE or HPLC analyses.

cycle, are much more cost effective, because only 1 equiv of CP was needed in the reaction and another phosphate can be provided by inexpensive Glc-1-P. Optimization of reaction conditions was conducted with a chromophore-labeled lactose [(4,5-dimethoxy-2-nitrophenyl)methyl *O*-(*β*-D-galac-

(18) Optimal condition for the multienzyme sialylation was as follows: acceptor (25 mM), ManNAc (25 mM), pyruvate (125 mM), CP (50 mM), $MnCl_2$ (10 mM), $MgCl_2$ (5mM), DTT (2mM), NanA (4 U/mL), NeuA (4 U/mL), PPA (4 U/mL), SiaT (0.4 U/mL), CK (20 U/mL), myokinase (20 U/mL), catalytic amounts (2.5 mM) of ATP and CMP in 50 mM HEPES buffer (pH 7.5).

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(20) pH optimum is ~6.5 for CK, see: Wyss, M.; Smeitink, J.; Wevers, R. A.; Wallimann, T. *Biochim. Biophys. Acta* **1992**, *1102*, 119−166.

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topyranosyl)-(1→4)-β-D-glucopyranoside, LacChr][26] as the galactosyl acceptor so that RP-HPLC with UV detection (λ = 348 nm) could be used to monitor the reaction conveniently and quantitatively.[27]

The capacity of the CP−CK energy system was explored in practical synthesis of glycoconjugates. Using recombinant enzymes expressed in *E. coli*, we synthesized 3′-SL and several galactosides in preparative scale (about 0.1∼1 g). In large-scale synthesis (2.5 mmol, 100 mL) of α-Gal, only 4% (mol/mol) (1 mM) UDP was applied to decrease the cost further without significantly affecting the yield (25%). The enzymatic product of LacChr with α1,3-GalT is a novel α-Gal derivative, α-GalChr. It can also be regarded as a potential assay substrate for other transferases.[28] Time course studies indicated that the reaction reached saturation after 36 h. α-GalChr accumulated to 17 mM (72% yield) in the reaction mixture. The purified enzymatic products were characterized by NMR and mass spectrometry and confirmed by comparison with the authentic compounds.[3f,25a,c,29]

The acetyl phosphate−acetyl kinase (AcP−AK) system as a practical energy source was first applied in carbohydrate synthesis by G. M. Whitesides and his colleagues in 1977.[30] PEP−PK system was then introduced in carbohydrate chemistry by the same group in 1982.[31] Our approach based on CP−CK has both advantages and disadvantages relative to the schemes based on PEP−PK and AcP−AK systems.[32] (1) PEP is a stronger phosphorylating agent than PCr and AcP, so it can be used to drive more thermodynamically unfavorable reactions.[5a] (2) The stabilities of PEP and PCr in solution are much higher than that of AcP. They can be added in one portion at the beginning of the reaction, while the AcP must be added continuously.[30,33] (3) Since the K_m of ADP for CK is lower than that for PK and AK, it is possible to use lower concentration of A(T,D)P to reduce the product inhibition and achieve higher turnover numbers with CK than with PK or AK.[34] (4) The cost of AK is much higher than that of CK and PK, and PEP is the most expensive reagent among all three regeneration systems (Sigma Chemical Co., 2003). (5) Inhibition of PK by pyruvate requires that more kinase be used in the reaction.[35] Therefore, the balance of all these factors, in our experience, is that CP−CK is a more convenient and useful system than PEP−PK and AcP−AK systems for carbohydrate synthesis, either on laboratory or industrial scale (Table 2).

Table 2. Comparison of ATP Regeneration Systems: AcP−AK, PEP−PK, and CP−CK Systems

entry	AcP-AK	PEP−PK	CP−CK
free energy of reagent ($\Delta G°'$, kJ/mol)	−43.1	−61.9	−43.1
half-life of reagent (days)	0.34	98	12
K_m value of enzyme (mM, MgADP)	1.5	0.3	0.051
cost of enzyme/reagent ($/kU and $/mmol)	90/7.9	11.6/38	9.8/9.7
product inhibition on enzyme (K_I, mM)	−	1	−

In summary, we report here a novel and versatile phosphate donor in glycoconjugate synthesis and demonstrated its efficiency in syntheses of natural and unnatural oligosaccharides. In addition, the CP−CK energy system is cost effective and of great utility in the large-scale production of carbohydrates. Work to apply this new energy system in our superbeads technology is in progress.

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Supporting Information Available: Abbreviations, experimental procedures and full characterization for oligosaccharides 3′-sialyllactose, α-Gal trisaccharide, globotriose, and α-GalChr and ^{1}H NMR and ^{13}C NMR spectra for the compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

OL034319A

(26) Cohen, S. B.; Halcomb, R. L. *J. Org. Chem.* **2000**, *65*, 6145−6152.
(27) Optimal condition for the enzymatic galactosylation was as follows: acceptor (25 mM), Glc-1-P (25 mM), CP (25 mM), $MnCl_2$ (10 mM), $MgCl_2$ (5mM), DTT (2mM), CK (20 U/mL), GalU (4 U/mL), PPA (4 U/mL), GalT (0.4 U/mL), myokinase (20 U/mL), catalytic amounts (2.5 mM) of UDP-Glc or UDP in 50 mM HEPES buffer (pH 7.5).
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