Indirect Enzymatic Phosphorylation: Preparation of Dihydroxyacetone Phosphate

Paola D'Arrigo, Valentino Piergianni, Giuseppe Pedrocchi-Fantoni and Stefano Servi*

CNR, Centro di Studio per le Sostanze Organiche Naturali, Dipartimento di Chimica, Politecnico di Milano, Via Mancinelli 7, 20131, Milano, Italy

Starting from natural phosphatidyl choline, the substitution of the choline polar head for an alcohol donor is catalysed by phospholipase D; subsequent hydrolysis of the new phospholipid obtained in the presence of phospholipase C allows the obtainment of the phosphate of the alcohol donor; the overall process is the net transfer of the phosphate moiety from a phospholipid to a primary alcohol and the procedure is verified in the preparation of dihydroxyacetone phosphate

Phosphoric acid monoesters form a class of important compounds relevant for biological studies and as intermediates for the synthesis of complex molecules. For instance glycerol-3-phosphate (G3P) can be used as a starting material for the total synthesis of phospholipids and their analogues,[1] while dihydroxyacetone phosphate (DHAP) is the C_3 unit recognised by several important aldolases and used in the enzymatic synthesis of monosaccharides.[2] The chemical synthesis of phosphate monoesters of multifunctional compounds of low molecular weight, and often water soluble, requires extensive use of protection and deprotection techniques while lowering the efficiency of the process. Enzymatic catalysis seems to offer the solution to the problem. Indeed the enzymatic phosphorylation of glycerol and dihydroxyacetone with glycerol kinase has been efficiently applied to the multigram scale preparation of both phosphates, G3P and DHAP.[3] Extension to other non-natural substrates is also possible. The reaction requires ATP as phosphate donor. The latter compound must be recovered by phosphorylation of the formed ADP using pyruvate kinase and phosphoenol pyruvate (or an equivalent system) which is the ultimate phosphate donor being consumed in a stoichiometric amount. The method thus requires cofactor regeneration and an expensive phosphate donor.

In another enzymatic approach, alkaline phosphatase has been employed in reverse-hydrolysis conditions using various inorganic phosphate donors.[4] Kinetic control in the synthetic direction only allows one to obtain products in limited yields.

Glycerophospholipids are substrates of several phosphodiesterases of broad substrate specificity. Phospholipase D (PLD) and phospholipase C (PLC) have been the subject of extensive studies, both for the transformation of phospholipids for synthetic purposes, and for the investigation of their role in cellular biochemistry.[5-8] Recently PLDs from microbial origin have allowed the easy preparation of a number of non-natural phospholipids modified in their polar head.[9-19] PLC hydrolyses phospholipids giving 1,2-diacyl glycerol (1,2-DAG) and a phosphorylated alcohol. This process has been proposed as a possible access to 1,2-DAG with the natural absolute configuration, and it has been applied for the structural elucidation of the polar head. The absolute configuration of the chiral carbon in the polar head in natural phosphatidyl glycerol has been established by enzymatic analysis of the G3P obtained after hydrolysis catalysed by PLC.[20]

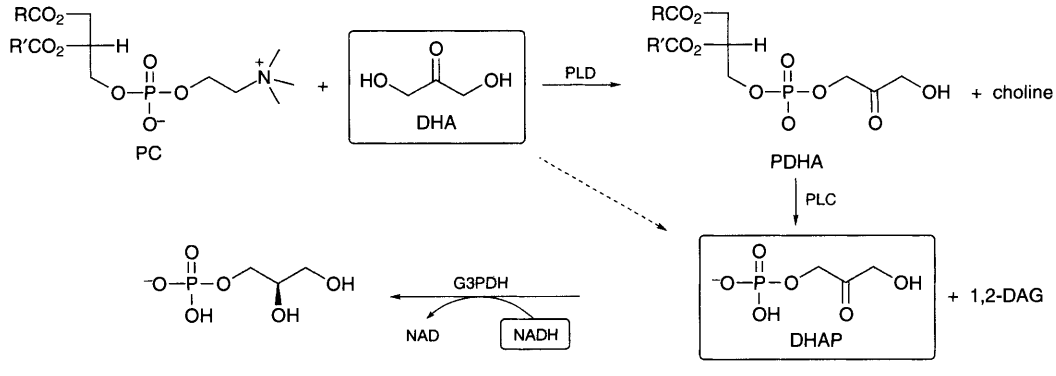
Starting from readily available PC of natural origin it should be possible to prepare a new phospholipid *via* a transphosphatidylation reaction with an alcohol as nucleophile donor. The product thus obtained could then be hydrolysed by PLC to release the corresponding phosphate of the starting alcohol.

We propose this method as a general way of indirect enzymatic phosphorylation using only accessible hydrolytic enzymes and not requiring added cofactors. This should be possible by considering three factors: (*a*) in the first step, working in the synthetic direction, the formation of the ester is largely favoured over the hydrolytic product (phosphatidic acid, PA) allowing the obtainment of the product of kinetic control in high yield; this comes from a peculiarity of the PLD of bacterial origin.[21] (*b*) In the second reaction, performed in a biphasic system, the reaction product will pass into the aqueous phase from which it can be recovered easily as the only water-soluble organic molecule of low molecular weight.[3] (*c*) Both enzymes show a broad substrate specificity. It is therefore expected that a wide range of new phospholipids can act as substrates for the coupled action of the two phospholipases.

While the principles of this sequence have been evident previously, its feasibility arises from the availability of almost any desired new phospholipid modified in the polar head in good yields and purity, from the transphosphatidylation reaction catalysed by PLD from bacterial origin.

We have applied this concept to the phosphorylation of dihydroxyacetone. In a first set of experiments we checked the possibility of obtaining DHAP through the outlined sequence using commercial PLC. Subsequently we tried to apply the reaction on a larger scale, using as source of PLC a crude preparate from a culture broth of *Bacillus cereus* for the direct isolation of the product.

The reaction sequence is illustrated in Scheme 1. Phosphatidyldihydroxyacetone (PDHA) was prepared starting from PC from soy beans† of 95% purity in a biphasic system according to a general procedure previously described by us.[14]‡ When the concentration of DHA dimer was kept at 4 mol dm^{-3}, the formation of PA was minimised and the product precipitated as



Scheme 1 Sequential use of PLD and PLC in the introduction of a phosphate group into DHA

a waxy solid of >85% purity (HPLC). If PA is present in the crude preparate, further purification is necessary since the presence of PA severely inhibits the hydrolysis.§ PDHA (200 mg) was dissolved in methyl *tert*-butyl ether (4 ml) and mixed vigorously with an aqueous solution (5 ml, pH 7.3, 0.1 mol dm^{-3} dimethylglutarate buffer, 37 °C, 0.05 mol dm^{-3} Zn^{2+}) of the PLC preparate (Sigma type IV, 50 U). The reaction was followed by TLC (disappearance of the starting material, formation of the 1,2-DAG). When most of the phospholipid had disappeared (24 h), the aqueous phase was assayed for DHAP by an enzymatic method.[22] Conversion was estimated to be >80%. The apparent K_m of DHAP was evaluated to be 0.1 mol dm^{-3} (PC K_m is 2.0 x 10^{-2} mol dm^{-3}). Due to the rather large K_m of the substrate, a preparative biotransformation requires an accessible source of enzyme. We found that from the culture broth of *Bacillus cereus* IPV 288,¶ with standard procedures,[23] a crude preparate containing 2 U mg^{-1} of solid was obtained. This corresponds to a productivity of 2 kU l^{-1} of culture broth. The availability of this enzyme thus allows the biotransformation to be performed on a larger scale. Thus starting from 2 g of PDHA, 980 mg of the barium salt of DHAP (65% purity as determined by enzymatic assay), was obtained in the following manner: the aqueous phase was separated from the organic layer; this was washed with an equal amount of buffer and the aqueous phases were pooled. This was treated with 650 mg of $BaCl_2 \cdot 2H_2O$ and the mixture lyophilised. Direct precipitation with ethanol as described in the literature[3] was not successful in this case. A proton decoupled ^{31}P NMR spectrum of the product was obtained by dissolving 120 mg of the barium salt in 2 ml of D_2O and treating the solution with 70 mg of Na_2SO_4 in 1 ml of D_2O. The precipitate was separated by centrifugation and the solution adjusted at pH 7. The spectrum showed two major signals at δ 3.1 and 4.2 (reference external phosphoric acid)|| in agreement with the spectra described in the literature.[3,24,25] Minor signals (8%) presumably due to phosphorylated impurities were also present in the crude PLC preparate thus suggesting that no other phosphorus containing compound is formed during the enzymatic hydrolysis.

The present procedure appears to be suitable for the preparation of other monophosphates of natural origin. Experiments on this line are in progress.

Received, 7th August 1995; Com. 5/05283A

Footnotes

† PC (soy beans) fatty acid chains natural composition: palmitic 11.6, stearic 3.4, oleic 4.6, linoleic 66.4, linolenic 8.7%.

‡ The preparation of dipalmitoyl PDHA and its possible use as precursor for DHAP has recently been reported (ref. 19).

§ This observation is confirmed in the PLC catalysed hydrolysis of PC: the presence of 20% PA completely stops the reaction in conditions in which it should otherwise be complete in 20 min.

¶ IPV is The Istituto di Patologia Vegetale, Facoltà di Agraria, Università di Milano, Via Celoria Milano, Italy.

|| The two signal are due to the keto and hydrated forms of DHAP. See ref. 24.

References

1 A. E. Walts, D. G. Schena, E. M. Fox, J. T. Davis and M. R. Mischke, in *Chirality in Industry*, ed. A. N. Collins, G. N. Sheldrake and J. Crosby, Wiley, Chichester, 1992, p. 223.
2 S.-H. Jung, J.-H. Jeong, P. Miller and C.-H. Wong, *J. Org. Chem.*, 1994, **59**, 7182 and references therein.
3 D. C. Crans and G. M. Whitesides, *J. Am. Chem. Soc.*, 1985, 107, 7019
4 A. Pradines, A. Klaébé, J. Périé, F. Paul and P. Monsan, *Tetrahedron*, 1988, **44**, 6373.
5 *Phospholipids Handbook*, ed. G. Cevc, Marcel Dekker, New York, 1993.
6 M. Heller, *Adv. Lipid Res.*, 1978, **16**, 267
7 M. Kates and P. S. Sastry, *Methods Enzymol.*, 1978, **14**, 197.
8 E. A. Dennis, *Methods Enzymol.*, 1991, **197**.
9 S. Shuto, S. Ueda, S. Imamura, K. Fukukawa, K. Matsuda and T. Ueda, *Tetrahedron Lett.*, 1987, **28**, 199.
10 L. R. Juneja, N. Hibi, T. Yamane and S. Shimizu, *Enzyme Microb. Technol.*, 1987, **9**, 350.
11 L. R. Juneja, T. Kazuoka, T. Yamane and S. Shimizu, *Biochim. Biophys. Acta*, 1988, **960**, 334.
12 L. R. Juneja, T. Kazuoka, T. Yamane and S. Shimizu, *Biochim. Biophys. Acta*, 1989, **1003**, 277.
13 P. Wang, M. Schuster, Y.-F. Wang and C.-H. Wong, *J. Am. Chem. Soc*, 1993, **115**, 10487.
14 P. D'Arrigo, L. de Ferra, V. Piergianni, A. Ricci, D. Scarcelli and S. Servi, *J. Chem. Soc., Chem. Commun.*, 1994, 1709.
15 M. Takami and Y. Suzuki, *Biosci. Biotech. Biochem.*, 1994, **58**, 1897.
16 M. Takami, N. Hidaka, S. Miki and Y. Suzuki, *Biosci. Biotech. Biochem.*, 1994, **58**, 1716.
17 M. Takami, N. Hidaka and Y. Suzuki, *Biosci. Biotech. Biochem.*, 1994, **58**, 2140.
18 J. Nakajima, T. Nakashima, Y. Shima, H. Fukuda and T. Yamane, *Biotech. Bioeng.*, 1994, **44**, 1193.
19 M. Takami and Y. Suzuki, *Biosci. Biotech. Biochem.*, 1994, **58**, 2136.
20 J. Brotherus and O. Renkonen, *Chem. Phys. Lipids*, 1974, **13**, 11.
21 G. Carrea, P. D'Arrigo, V. Piergianni, S. Roncaglio, S. Secundo and S. Servi, *Biochim. Biophys. Acta*, 1995, **1255**, 273.
22 G. Michal and H. Ottobeutler, in *Methods of Enzymatic Analysis*, 2nd edn., ed. H. U. Bergmeyer, Verlag Chemie, Berlin, 1974, vol. III, p. 1314.
23 C. Little, *Methods Enzymol.*, 1981, **71**, 725.
24 G. R. Gray, *Biochemistry*, 1971, **10**, 4705.
25 S. C. Blacklow, R. T. Raines, W. A. Lim, P. D. Zamore and J. R. Knowles, *Biochemistry*, 1988, **27**, 1158.