Pergamon

Tetrahedron 54 (1998) 8841–8846

TETRAHEDRON

Enzymatic Synthesis of 4-OH-Benzoic Acid from Phenol and CO_2: the First Example of a Biotechnological Application of a Carboxylase Enzyme.

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Received 30 April 1998; accepted 21 May 1998

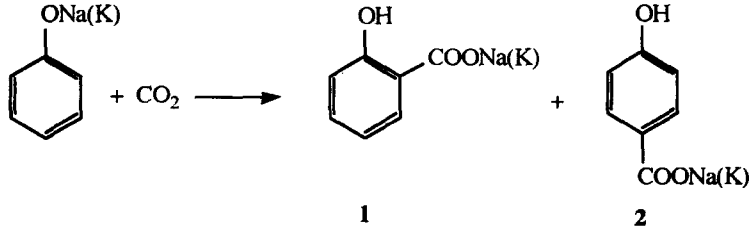
Abstract: 4-OH benzoic acid has been synthesized from phenol and CO_2 at room temperature and sub-atmospheric pressure of CO_2 with 100 % selectivity, using a Carboxylase enzyme. A cheap, fast and easy method for the phosphorylation of phenol has been also developed.
This represents the first biotechnological application of a Carboxylase enzyme.

INTRODUCTION

The chemical utilisation of carbon dioxide is a hot topic in these days.[1] In fact, it may be relevant to both the CO_2 mitigation[2] and the development of benign syntheses[3], avoiding toxic species and implementing direct instead of multistep processes.[4]

We have for a long time investigated both the coordination chemistry[5] of carbon dioxide and its use in the synthesis of organic products.[6] Recently, our interest has been focused on high-yield and selective processes avoiding toxic species,[7] using biomimetic catalysts and enzymes.[8]

In this paper we present an unprecedented utilisation of a supported Carboxylase enzyme for the net carboxylation of phenol to 4-OH-benzoic acid at ambient temperature and pressure. This product is currently synthesized using the Kolbe-Schmitt reaction[9] (Scheme 1).



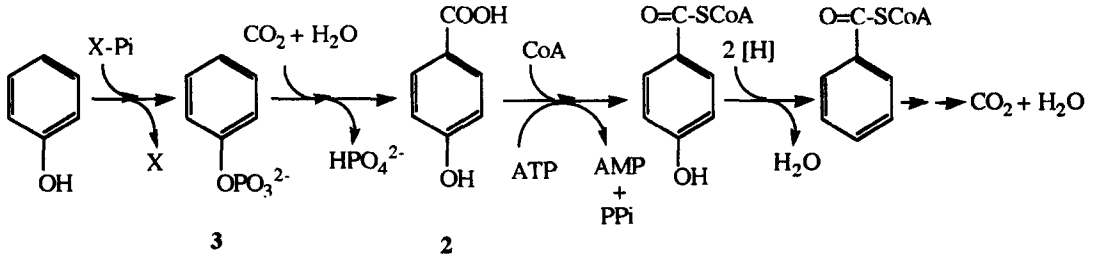
Scheme 1. The Kolbe-Schmitt reaction.

Na^+- or K^+-phenoxides are converted into a mixture of 2-OH- (**1**) and 4-OH-benzoic acid (**2**) salts. The yield (10-48 %) and selectivity (up to 90 %) depend on the reaction conditions (that are 373 - 430 K, 0.1 - 1.0 MPa CO_2) and the nature of the solvent. The selectivity of the reaction is driven by the Group 1 metal cation : Na^+

0040-4020/98/$19.00
PII: S0040-4020(98)00475-X

produces an excess of the *orto*, while with K^+ the *para* is the most abundant isomer.[9b] Complete conversion of phenol with high selectivity towards either of the two isomers is highly wished, for their industrial application: 2-OH-benzoic acid is used for the synthesis of aspirin, while 4-OH-benzoic acid finds an application for the synthesis of innovative materials.

We have recently characterized a Phenol carboxylase enzyme Mn^{2+}-dependent, extracted from Thauera aromatica bacteria which catalyzes with 100 % selectivity the phenyl-phosphate (**3**) carboxylation to 4-OH benzoic acid in mild conditions (300 K, P_{CO2}=0.1 MPa).[10] Thauera aromatica grows on phenol under stricly anaerobic conditions (N_2 or CO_2 atmosphere). If oxygen is present, Mn^{2+} is oxidized to Mn^{3+}. The phenol conversion, thus, implies the formation of catechol more than the carboxylation to 4-OH-benzoic acid.
The activity of various proteins involved at different levels in the phenol metabolism has been studied in detail.[11] The proposed mechanism of *in vivo* phenol degradation is reported in Scheme 2.



Scheme 2. Phenol metabolism of Thauera aromatica.

According to the knowledge we have of the enzymatic phenol metabolism, it is first phosphorylated to phenyl-phosphate. The Phenyl phosphate carboxylase catalyzes then the carboxylation of the activated phenol to 4-OH-benzoate (**2**), which is subsequently converted into 4-OH benzoyl-CoA and dehydroxylated to benzoyl-CoA.
The phenol metabolism of Thauera aromatica represents one of the few known examples of a degradative pathway implying, as a preliminary step, the substrate carboxylation whith an increment, thus, of the molecular complexity of the metabolite before its degradation.[12] However, the modification of the molecular structure of a substrate for making possible its utilisation, is found to be a common feature to other bacterial degradation pathways.

We became interested in investigating the possibility of using the enzymatic pool, or the semi-purified enzyme, for synthetic purposes, i.e. for driving the net carboxylation of phenol to 4-OH-benzoic acid.

RESULTS AND DISCUSSION.

Enzyme extraction and immobilisation on low melting agar.

If Phenylphosphate carboxylase has to be used for the synthesis of 4-OH benzoic acid from phenol, the use of the whole cell is precluded as the target product is further used by the bacteria as a source of carbon. However, the cells were broken using a French-press and either the crude extract, obtained after ultracentrifugation, or the partially purified enzyme, obtained after gel filtration on Sepharose CL-6B, was used.

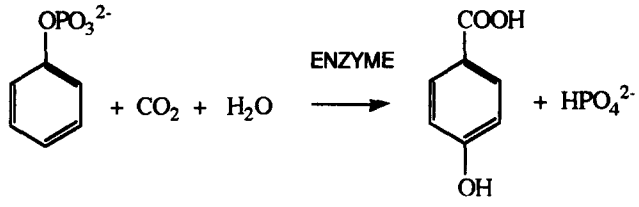
The purification of the enzyme was carried out under dinitrogen or CO_2, with the absolute exclusion of air. This procedure allows to eliminate most of the proteins and other cell components that are not implied into the carboxylation reaction.

The cell extract obtained as reported in the literature[10] or the carboxylase containing fractions obtained after protein separation on Sepharose CL-6B (30 % pure enzyme) were supported on sterile low melting agar, that results to be the best support among those investigated (zeolites, pumice, polyacrylamide).

The supported enzyme mantains its activity for more than one week, while in solution the enzyme looses its activity after a few hours.

Phenylphosphate synthesis.

As shown in Scheme 3, the phenol O-phosphoryl ester more than phenol is the species carboxylated by the enzyme. Interestingly, while *in vivo* the complete process described in Scheme 2 is operative, *in vitro* the cellular extract of Thauera aromatica or the purified enzyme is unable to phosphorylate phenol, as the kinase protein responsible for the phosphorylation reaction is lost with the membrane fragments. In order to get the phenyl-phosphate, required for carboxylation to 4-OH benzoate a simple *in situ* phosphorylation of phenol was developed.

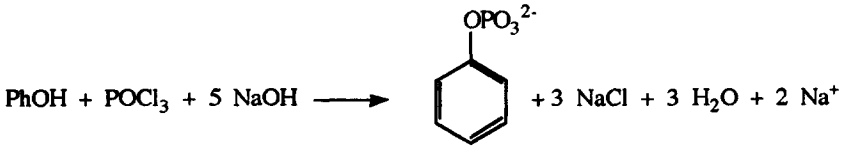


Scheme 3. Phenylphosphate carboxylation reaction.

The phosphorylation of alkoxo-, and aryloxo- compounds has been reported to occur using expensive products such as sodium cyclo-triphosphate,[13] dibenzyl phosphite,[14] or through the activation of trialkyl phosphites with molecular iodine.[15]

These products are too expensive for a practical application. Moreover, the time required for the phosphorylation reaction to be complete ranges from 1 h to more than 24 h for the different reagents.

However, although phenylphosphate is a commercial product, for laboratory purposes (monitoring of the overall reaction using ^{31}P nmr) we have used $POCl_3$, a cheap and easily available reagent, and NaOH (Scheme 4) under controlled conditions to produce $PhO\text{-}PO_3^{2-}$ with good yield and selectivity.



Scheme 4. Phenol phosphorylation reaction.

The formation of $PhO\text{-}PO_3^{2-}$ has been also monitored by HPLC.

Synthesis of 4-OH-benzoic acid.

Once the reaction is completed, after pH correction (to pH 7) the water solution of the phosphorylated phenol is incubated with the agar-supported enzyme under N_2 or CO_2 atmosphere. Phenylphosphate is converted into 4-OH-benzoate that accumulates in water. HPLC analysis of the water phase allows to follow the kinetics of the conversion. After a few hours, a 70 % conversion is observed. The water phase can be withdrawn and more phenylphosphate added. The extraction procedure can be repeated over a few days. The overall yield of 4-OH benzoic acid is more than 90% with respect to the added phenyl phosphate.

No other products are formed. Considering the purity of the enzyme and its molecular mass (280 kDa) a TOF of ca. 600 has been calculated.

As the supported catalyst remains active for more than one week a TON of more than 10^4 moles of phenylphosphate carboxylated per mole of catalyst has been observed. Although $POCl_3$ is not the best reagent for practical purposes, it was quite useful in the reaction study at the laboratory scale as it is soluble in organic solvents and reacts very fast with phenol affording only the phenyl-phosphate and phosphate clearly shown by ^{31}P n.m.r. For practical purposes other phosphorylating agents can be used, as P_2O_5.

This paper represents the first report on a supported Phenyphosphate carboxylase protein and the unique demonstration of the use of a Carboxylase enzyme in synthetic application. The specificity for CO_2 has been clearly stated. Work is in progress in order to ameliorate the TON for a better definition of the potential application of the synthetic methodology.

Aknowledgements.

We aknowledge the financial support by MURST and CNR-Rome Contribute n° 96.2599.

Experimental.

General : All manipulation were conducted under dinitrogen or CO_2 atmosphere by using vacuum-line techniques. All used solutions were made anaerobic and sterile by boiling at 120 °C in autoclave and degassing under N_2 flow.

Bacterial cells were grown and harvested as reported in the literature.[10]

NMR spectra were obtained at 81 MHz with a Varian XL-200 instrument. ^{31}P chemical shifts are referred to 85% H_3PO_4 using the high-frequency-positive convention.

All GC-MS analyses were carried out with a HP 5890 gas chromatograph linked to a HP 5970 mass detector (SE-30 capillary column: 30 m x 0.00032 m, 0.25 μm film thickness).

Procedure for the immobilisation of the protein in low melting agar: a) 0.16 g of low melting agar were suspended, under dinitrogen atmosphere, in 10 mL of imidazole/HCl buffer solution (10^{-1} M, pH 7) and heated at the temperature of 353 K, to allow complete solubilization of agarose, then cooled to 303 K and added with 10 mL of crude extract (22.88 mg of proteins mL^{-1}), 2 mL of a $MnCl_2$ solution ($4 \cdot 10^{-2}$ M) and 4 mL of a $NaHCO_3$ solution ($5 \cdot 10^{-1}$ M).

b) A parallel experiment has been carried out by substituting the crude cell extract by the semi-purified enzyme obtained after separation of the crude extract proteins on a Sepharose CL-6B column. Part (30 mL) of the

solution fractions eluted from the column which contained the Phenylphosphate carboxylase enzyme (test for the phenylphosphate carboxylase activity performed according to the procedure described in the literature[11]) were used. The concentration of all other cosubstrates was kept constant.

Synthesis of Phenylphosphate : a) $POCl_3$ (0.1 mL, 1.1 mmol) was dissolved in 1 mL of THF and PhONa (0.13 g, 1.1 mmol dissoved in 5 mL of THF) was added dropwise.
The ^{31}P NMR spectrum of the solution at pH 13 showed two peaks of high intensity due to Pi [δ 5.73 ppm (s)], $PhOPO_3^{2-}$ [δ 0.73 ppm (s)] and a signal of quite low intensity due to $(PhO)_2PO_2^-$ at around -5 ppm. These were the only products of the reaction.
The conversion yield of $POCl_3$ was 100 % and the selectivity towards $PhOPO_3^{2-}$ (extimated by HPLC) was in the range 55-70%.
b) $POCl_3$ (1.8 mL, 20 mmol) and PhOH (1.8 g, 20 mmol) were reacted in 20 mL of distilled water with NaOH (4 g, 100 mmol) at room temperature for 1 hour. The resulting solution was analyzed by HPLC (elution mixture 20% CH_3CN, 80% phosphate buffer 1.5 10^{-1} M, pH 3.5, Nucleosil C18 column) and found to contain $PhOPO_3^{2-}$ and some free phosphate. $(PhO)_3PO$, that separates as a white solid from the reaction mixture, was the other phosphate-functionalisation product. The $POCl_3$ conversion was 100 %, and the selectivity as $PhOPO_3^{2-}$ was 50-75 %. Phenol eventually not reacted separated from the reaction mixture as a low density liquid phase and was easily recovered by washing with diethyl-ether.

Carboxylation of Phenylphosphate : The agar-protein gel system obtained as described above was put in contact with 10 mL of the solution containing $PhOPO_3^{2-}$ (4 10^{-2} M), $MnCl_2$ (4 10^{-3} M) and $NaHCO_3$ (10^{-1} M) and stored at 300 K under N_2 or CO_2. At fixed intervals of time a sample of the solution was withdrawn and monitored by HPLC. After 24 h the solution was removed, acidified to pH 2 with concentrated hydrochloric acid and the solid product formed filtered off. The yield of the crude 4-OH-benzoic acid was 90 %. Recrystallization from water gave 4-OH-benzoic acid in 70 % yield. The chromatographic analysis of the water solution of phenylphosphate in contact with the supported enzyme showed formation of 4-OH-benzoic acid during 8 days. A TON of 16000 was estimated.

Characterization of 4-OH benzoic acid: 4-OH benzoic acid : Mp 215 °C;
4-OH benzoic acid methyl ester (esterification with BF_3/CH_3OH): MS *m/z* 152 (M^+).

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