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Determination of the time course of an enzymatic reaction by ^{1}H NMR spectroscopy: hydroxynitrile lyase catalysed transhydrocyanation

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Abstract

The time course of the enzyme catalysed transhydrocyanation of benzaldehyde to give (*S*)-mandelonitrile was investigated using a hydroxynitrile lyase from *Hevea brasiliensis* as catalyst and acetone cyanohydrin as cyanide donor. Employing special techniques it was possible to apply ^{1}H NMR spectroscopy in aqueous medium to monitor the concentration changes of all substrates and products. By this technique strong evidence for inhibition of the enzyme at higher substrate concentrations was obtained.

Keywords: Aqueous system; Hydroxynitrile lyase; ^{1}H NMR spectroscopy; Transhydrocyanation

1. Introduction

In recent years the importance of the application of enantiomerically pure building blocks for the manufacture of many chiral products has been recognised. Enantiopure cyanohydrins are valuable structures for the chemical synthesis of compounds such as α-hydroxyketones, α-hydroxyacids, β-aminoalcohols and pyrethroid pesticides.

Several methods have been described for the enantiospecific addition of hydrogen cyanide to aldehydes or ketones. These methods can be divided into two main groups, those involving chiral chemical reagents [1] and those having enzymes as catalysts [2,3].

Enzymes used for the synthesis of enantiopure cyanohydrins are named either hydroxynitrile lyases or oxynitrilases. Their natural function is to catalyse the hydrolysis of cyanohydrins of cyanogenic glycosides into HCN and the corresponding aldehydes and ketones. Both (*R*)- and (*S*)-specific hydroxynitrile lyases have been isolated [4–8].

The hydroxynitrile lyase from the rubber tree

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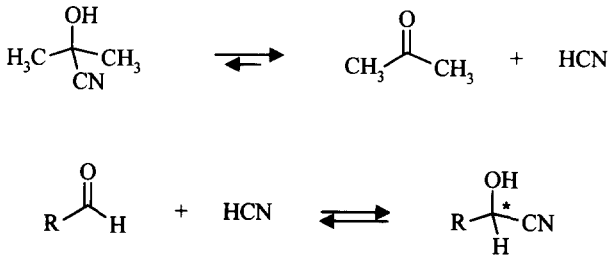

Fig. 1. Transhydrocyanation.

Hevea brasiliensis was used for the synthesis of various (*S*)-cyanohydrins with acetone cyanohydrin as cyanide donor (transhydrocyanation, Fig. 1 [9]. This reaction had previously been carried out using (*R*)-hydroxynitrile lyase from almond *Prunus amygdalus* [10,11].

The aim of this work was to follow the changes of concentrations of all substances during the course of the transhydrocyanation employing acetone cyanohydrin and benzaldehyde. GC and HPLC methods are well established for the detection of all substrates and products with the exception of HCN. However, sample preparation differs for every substance and includes several steps which contribute to considerable errors. Moreover, the reaction has to be carried out in a closed system with fixed substrate ratios.

To avoid these problems we have used ^{1}H NMR spectroscopy where all compounds can be detected in the reaction medium simultaneously. Usually the benefits and pitfalls of this method, when employed for quantitative analysis, are integration and overlapping peaks, respectively. However, as the signals in question did not overlap, this problem was avoided. Nearly all problems which occur in the quantitative determination of small amounts of substances in the presence of large quantities of other substrates can be avoided by careful experiments, so that enzymatic studies in aqueous solution are directly amenable to NMR analysis. The high concentration of water protons (about 110 M) causes dynamic range problems which have to be overcome by specific techniques such as presaturation [12,13], relaxation methods [14,15], binominal pulses [16,17], and gradient pulses [18,19], giving the best results.

2. Experimental

2.1. Nuclear magnetic resonance measurements

The NMR measurements were performed in an aqueous buffer system with only a small amount of D_2O (10%) to establish the spectrometer lock on Bruker AM 360 MHz and MSL 300 MHz spectrometers. Due to lack of gradient coils in both NMR systems, in addition to the jump and return method [16], the binominal $\bar{1}$ 3 $\bar{3}$ 1 Redfield sequence [17] has been used to minimise the water signal which we found leads to the best suppression. The delay τ between the pulses, which allows choice of the proper excitation area, was adjusted to cover the signals of interest with a similar amplitude (i.e. that the Redfield pulse is in the area of interest roughly a 90° pulse). Acquisition time was 2 s and relaxation delay was 3 s. The signals used for the kinetics were for benzaldehyde and mandelonitrile, the benzylic protons at 10.00 ppm and 5.82 ppm; and the methyl groups of acetone (2.27 ppm) and acetone cyanohydrin (1.67 ppm, see Fig. 2).

2.2. Enzyme

The enzyme used was a recombinant (*S*)-hydroxynitrile lyase from *Hevea brasiliensis*. The specific activity was 40 IU mg^{-1} [20].

2.3. Reaction conditions

Reactions were carried out in 50 mM sodium phosphate buffer pH 3.75. The low pH value was necessary to suppress the non-enzymatic addition of HCN to benzaldehyde which would decrease the enantiomeric excess of the product.

The total volume of the sample was 0.5 ml and the reaction temperature 25°C. To prevent HCN from entering the vapour phase a tight fitting flexible plastic stick was inserted into the NMR tube to the liquid miniscus.

Acetone cyanohydrin and benzaldehyde were always used in the ratio of 2.5:1. Former experiments indicated that higher concentrations of acetone cyanohydrin were not appropriate for the determination by ^{1}H NMR spectroscopy and with lower concentrations the enzymatic reaction becomes very slow.

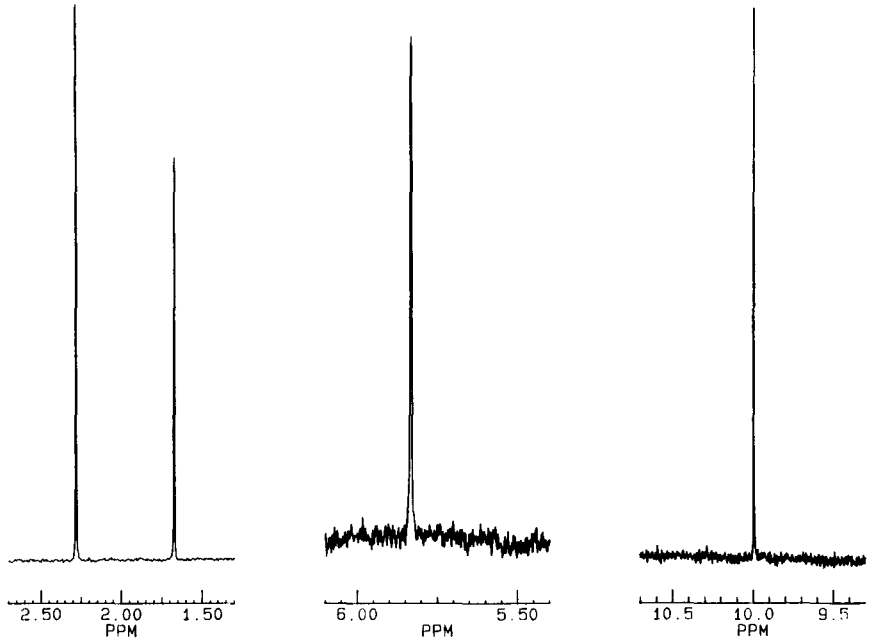

Fig. 2. Spectra of all measured substances (mandelonitrile, benzylic proton at 5.82 ppm; benzaldehyde, benzylic proton at 10.00 ppm; acetone, the methyl-groups at 2.27 ppm and acetone cyanohydrin, the methyl-groups at 1.67 ppm).

3. Results and discussion

Because the transition probability for all protons is equivalent, NMR is, despite its low sensitivity, suited for all types of kinetic studies. A number of publications illustrate the value of NMR for kinetic studies; however, to our knowledge, only a single paper describes an enzymatic study which was followed in aqueous solution. In this study [14] a T_2 reagent was added to remove the large water signal.

The determination of acetone, acetone cyanohydrin and benzaldehyde was easily realised as either the isolated methyl groups or the benzylic proton were measured. Some problems arose with the detection of mandelonitrile as the peak is too close to the water signal and therefore also suppressed by the binominal Redford sequence. This gave a small signal compared with other peaks and only detection of higher concentrations was possible.

Substance concentrations were measured by the height of the signals similarly to kinetic studies, only the differences are important. Moreover, signals without coupling are of Lorentz-type in shape which implies that height strongly correlates with volume.

Acetone cyanohydrin and acetone give the same signal height at the same concentrations, so that initial concentrations were easily calculated.

For benzaldehyde and mandelonitrile a factor was determined to relate the heights of the peaks to their concentrations.

All substances were measured except HCN which was calculated as the difference of the concentrations of acetone cyanohydrin and mandelonitrile. Curves in Figs. 3 and 4 are fitted and not simulated. The signal-to-noise ratio is poor due to the proximity of the signal at 5.82 ppm (benzylic proton of mandelonitrile) to the water signal. Therefore, only two measured values are shown in Fig. 3. Lower concentrations could not be measured at all (Fig. 4). After 25 min HCN seems to have reached a nearly stationary concentration under conditions shown in Fig. 3. The equilibrium of the reaction shown in Fig. 1 ap pears to be established after about 60 min. When both substrates were provided at half concentrations the reaction was complete within 3 min. Thus the reaction could not be followed by ^1H

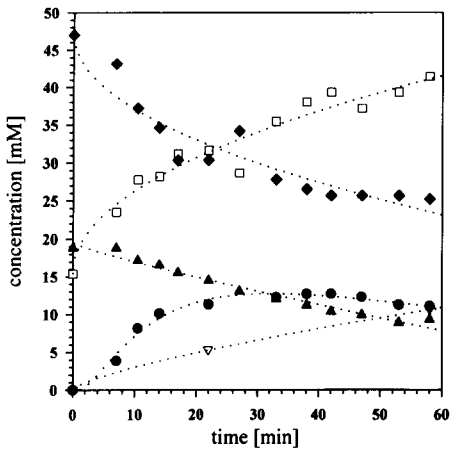

Fig. 3. Changes of substrate concentrations during the course of the transhydrocyanation over a period of 60 min. Initial concentrations: acetone cyanohydrin, 47 mM; benzaldehyde, 18.8 mM; enzyme, 3.4×10^{-3} mM (acetone cyanohydrin (◆), acetone (□), mandelonitrile (▽), benzaldehyde (▲) and HCN (●), calculated).

NMR spectroscopy. After reduction of the enzyme concentration from 3.4×10^{-3} mM to 1.7×10^{-4} mM the time course of Fig. 4 was obtained. Despite this, the reaction proceeded faster, a result which can be probably explained best by inhibition at higher substrate concentrations.

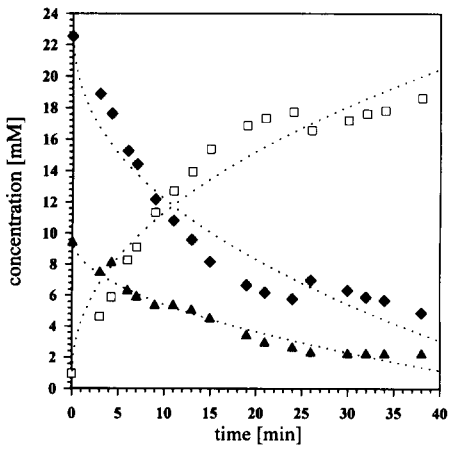

Fig. 4. Changes of substrate concentrations during the course of the transhydrocyanation over a period of 40 min. Initial concentrations: acetone cyanohydrin, 23.5 mM; benzaldehyde, 9.4 mM; enzyme, 1.7×10^{-4} mM (acetone cyanohydrin (◆), acetone (□) and benzaldehyde (▲)).

4. Summary

The enzyme catalysed transhydrocyanation has been studed by ^{1}H NMR spectroscopy in aqueous solution. The Redfield $\bar{1}$ 3 $\bar{3}$ 1 sequence was an excellent method to eliminate the water signal. Because the reaction had to be carried out in a closed system with fixed substrate ratios it was an immense advantage that sample taking was not necessary because of the possibility of the simultaneous measurement of all substrates.

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