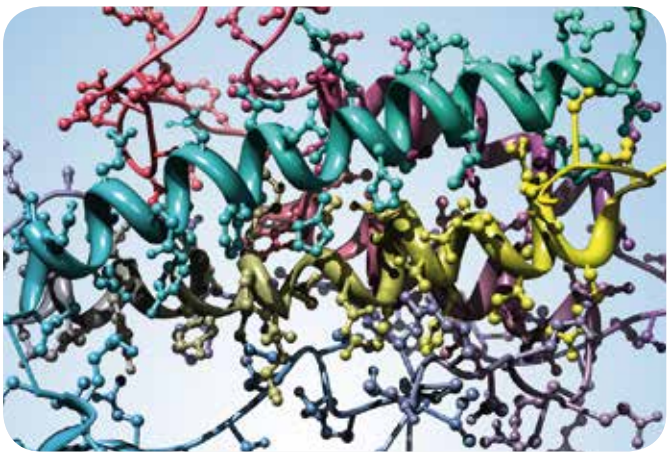

Application Note AN M74

Monitoring enzyme catalysis using the VERTEX 80 FTIR spectrometer in Rapid Scan mode

Introduction

α-chymotrypsin is a well characterised mammalian digestive enzyme that catalyses the hydrolytic cleavage of peptide bonds at the carboxyl side of aromatic residues. During the chymotrypsin-catalysed hydrolysis of N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPF-*p*NA; see figure 1), formation of the *p*-nitroaniline product can be followed spectroscopically at 410 nm while the peptide product can be monitored by FTIR spectroscopy due to formation of a new C-terminal carboxylate group. The VERTEX 80 FTIR spectrometer with the UltraScan™ linear air bearing scanner with True-Alignment™ technology is ideally suited for such kinetic studies, since at the fastest mirror velocity (320 kHz) more than 100 spectra at resolution 16 cm^{-1} can be collected per second.

Experimental

α-chymotrypsin was purchased from Sigma-Aldrich (St. Louis, MO; catalogue number 27270) and used without further purification. The concentration was determined by ε_{280} = 5104 M^{-1} cm^{-1}. We chose to use the substrate N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma catalogue number S7388) as it is a 'good' substrate with a tight K_m and relatively fast k_{cat} value (see reference 1). Additionally, aqueous solutions of the substrate are reasonably soluble to about 15 mM and cleavage can also be followed spectroscopically in the near-UV with ε_{315} (reactant) = 14000 M^{-1} cm^{-1} and ε_{410} (product) = 8800 M^{-1} cm^{-1}. The FTIR stopped-flow instrument consists of a drive unit, a thermostatted umbilical supply tube and an infrared cell with an integrated mixer (TgK Scientific, Bradford on Avon, UK) mounted in the sample compartment of a Bruker VERTEX 80 FTIR spectrometer. The stopped-flow unit and the mixing cell are both contained within an anaerobic Belle Technology glove box, which allows oxygen-sensitive reactions to be performed under a nitrogen environment containing <5 ppm oxygen. The infrared transmission cell has been described previously (see

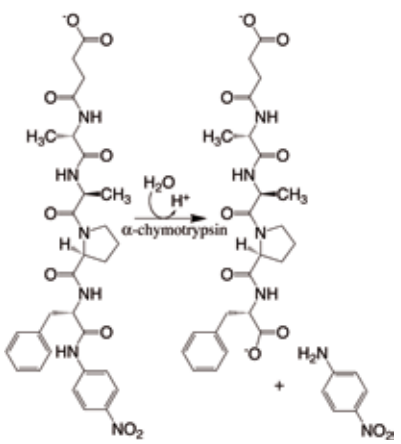

Figure 1: The α-chymotrypsin-catalysed cleavage of Suc-AAPF-*p*NA yielding Suc-AAPF-COO^- and *p*-nitroaniline.

reference 2). Briefly, the cell is a demountable stainless steel unit with an integrated T-mixer. It has 1.2 cm CaF_2 windows and the flow channels are 0.5 mm^2, together creating an 8 mm diameter observation chamber with a 100 µm path length. The stopped-flow mixing time is < 10 ms and the shot volume is variable, with a value of 200 µl used in this study. A photo of the apparatus is shown.

2 mM α-chymotrypsin was mixed with an equal volume of 15 mM Suc-AAPF-*p*NA in 50 mM potassium phosphate/D_2O, pD 8.4 at room temperature within the stopped-flow FTIR apparatus. A narrow band MCT detector was used for speed and sensitivity. In addition, a long wave pass optical filter <1828 cm^{-1} is required. The interferogram acquisition mode of "double-sided forward-backward" gave spectra every ~ 68 ms for ~35 s with a spectral resolution of 4 cm^{-1} (see figure 2).

Further post measurement processing by splitting the interferograms by software enables a four fold increase in time resolution down to ~17 ms.

Results

The C-terminal carboxylate moiety of the product is monitored as an increase in both C=O and C-O stretches at 1604 and 1322 cm^{-1} respectively (see difference spectra in figure 3). The disappearance of the peaks at 1521 and 1344 cm^{-1} is assigned to the NO_2 group. Representative reaction traces are shown in figure 4 with their colour referring to the peaks marked in the difference spectra. The difference spectra show clear isosbestic points suggestive of a single chemical reaction and reaction traces show that the reaction is completed after ~10 s.

Acknowledgements

These experiments were performed in the Manchester Interdisciplinary Biocentre at the University of Manchester, England.

References

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2. Thumanu, K., Cha, J., Fisher, J.F., Perrins, R., Mobashery, S., Wharton, C. (2006) Proc. Natl. Acad. Sci. USA, 103, 10630

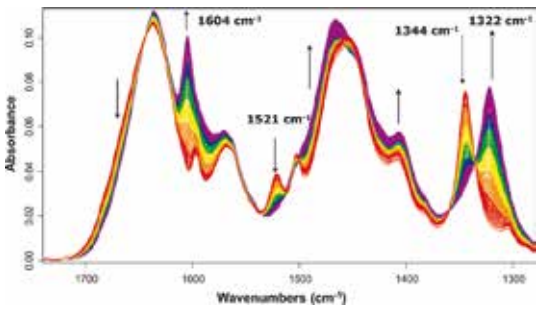

Figure 2: Progressive FTIR spectra recorded over the time course of the reaction

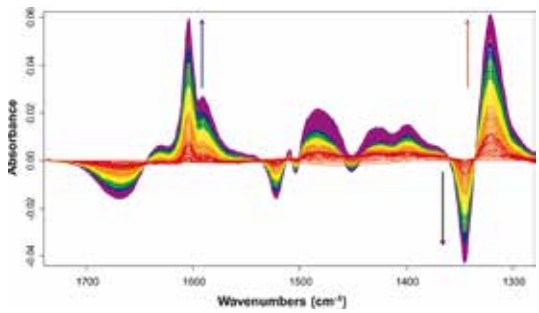

Figure 3: Difference FTIR spectra after subtraction of the first spectrum which highlight the intensity changes

Photo showing stopped flow unit in foreground and VERTEX 80 with IR cell in background

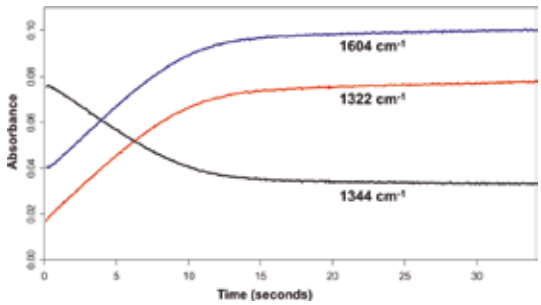

Figure 4: Peak heights of selected bands of the FTIR spectra versus time which monitor the reaction rate.

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