A Mid-Infrared Flow-Through Sensor for Label-Free Monitoring of Enzyme Inhibition

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Label-free monitoring of acetylcholinesterase (AChE) activity was achieved with a mid-infrared flow-through sensor. The flow-through sensor comprised agarose beads, carrying covalently immobilized AChE, which were placed in a temperature-controlled (37 °C) CaF_2 flow cell with an optical path of 60 μm. The sensor was incorporated into a computer-controlled sequential injection (SI) system for automated liquid handling. Different mixtures of enzyme substrate acetylcholine (ACh) and inhibitor (tacrine) were prepared and fed into the flow-through sensor. The flow was stopped as soon as the prepared mixtures reached the sensor. Enzymatic hydrolysis of ACh by AChE was directly monitored as it took place in the flow-through sensor. The inhibition effect of tacrine was calculated from the reaction-induced spectral changes, revealing an important decrease in the activity of AChE, approaching zero when the inhibitor concentration is high enough. The developed mid-infrared flow-through sensor is flexible and can be used to study the inhibitor activity of different target molecules as well as different enzymes.

Index Headings: **Flow-through sensor; Fourier transform infrared spectroscopy; FT-IR spectroscopy; Sequential injection analysis; SIA; Enzyme; Inhibition kinetics; Acetylcholinesterase.**

INTRODUCTION

The development of new sensors for the fast, sensitive, and label-free detection of biologically active compounds is considered to be one of the principal trends of modern analytical chemistry.[1] Flow-through optical sensors are based on the "solid-phase spectrophotometry" (SPS) technique, which was originally reported by Yoshimura et al. in 1976.[2] The main idea of SPS involves the use of a suitable solid support, packed into the flow-cell. In such systems, samples are injected in a carrier stream and driven towards the detector where the analyte or a suitable reaction product is retained and detected on the solid support.[3] The main advantages of the use of solid supports retained in the flow cell are related to (1) fast conversion of the analyte due to the mass transfer, which is more efficient in the solid–liquid interface, while the concentration of the reagent is maximum at the interface; and (2) a minimization of reagent consumption, since reagents do not flow continuously towards the detector and only the required amount reacts on passage of the analyte.[4] Thus, flow-through optical sensors combine the advantages of SPS (enhanced selectivity and sensitivity) and flow methods (automation, increased sampling rate, and cost-effectiveness).

Flow-through sensors based on Fourier transform infrared (FT-IR) spectroscopy are based on the same idea, with the main advantage being that FT-IR sensors provide direct molecular-specific information. In this sense, mid-infrared flow-through sensors based on transmission spectroscopy have been successfully developed to determine acetic and malic acid,[5] tannins in wine samples,[6] carbohydrates in beer,[7] and water hardness based on an indirect method.[8]

Fourier transform infrared spectrometry can also be used for determination of enzyme activities in solution. This has been demonstrated for the alkaline phosphatase[9] and α-amylase[10] in human serum and amyloglucosidase[11] and β-fructo-furanosidase in aqueous solutions.[12,13] Also, due to the high information content of FT-IR spectra, simultaneous determination of enzyme activities catalyzing similar reactions is possible. This has been shown by the simultaneous determination of α-amylase and amyolglucosidase,[14] as well β-fructo-furanosidase and amyolglucosidase,[15] respectively.

This paper describes immobilization of acetylcholinesterase (AChE) on agarose polymer beads contained in a temperature-controlled flow cell. This mid-infrared flow-through sensor was used to monitor the enzymatic hydrolysis of acetylcholine (ACh) *in situ*. Label-free measurement of the course of enzymatic reactions in the presence of the inhibitor (tacrine) at different concentrations revealed its inhibitory effect on acetylcholinesterase.

AChE is one of the most crucial enzymes for nerve response and function, catalyzing the hydrolysis of acylcholinesters with a relative specificity for acetylcholine.[16] This enzyme has been frequently used for the construction of chemical sensors for pesticides[17] and drug activity[18] where the inhibitory effect of the analytes was exploited for their quantification. AChE is also in the focus of pharmaceutical research, due to its importance in the treatments of different illnesses as well as aiding the recovery of victims of organophosphate-type insecticide and nerve agent intoxications. The inhibition of the AChE activity is the basis of many types of pharmaceuticals, especially in the treatment of Alzheimer's disease.[19]

EXPERIMENTAL

Reagents. All reagents were of analytical grade. Acetylcholinesterase (AChE) (E.C.3.1.1.7, type VI-S, from electric eel, 200–1000 U mg^{-1}); acetylcholine (ACh) chloride; *N*-ethyl-*N*A-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC); 9-amino-1,2,3,4-tetrahydroacridine hydrochloride (tacrine hydrochloride); pyridine-2-aldoxime methochloride; and choline solution (50% v/v in water) were supplied by Sigma (Schnelldorf, Germany) and TRIS (2-amino-2-(hydroxymethyl)-1,3-propanediol) was supplied by AppliChem GmbH (Darmstadt, Germany). Sepharose 4B polymer beads (4% agarose beads, particle size 40–165 mm, spacer arm 10-carbon with 7–11 mmol amino groups per mL drained gel) were also purchased from Sigma.

Enzyme Immobilization Procedure. The immobilization procedure was adapted from K. Ragnitz, M. Pietzsch, and C. Syldatk.[20] Approximately 2 mL of the bead suspension was washed with 150 mL 0.5 M sodium chloride solution and 150 mL water. Afterwards the beads were suspended in 25 mL 0.1

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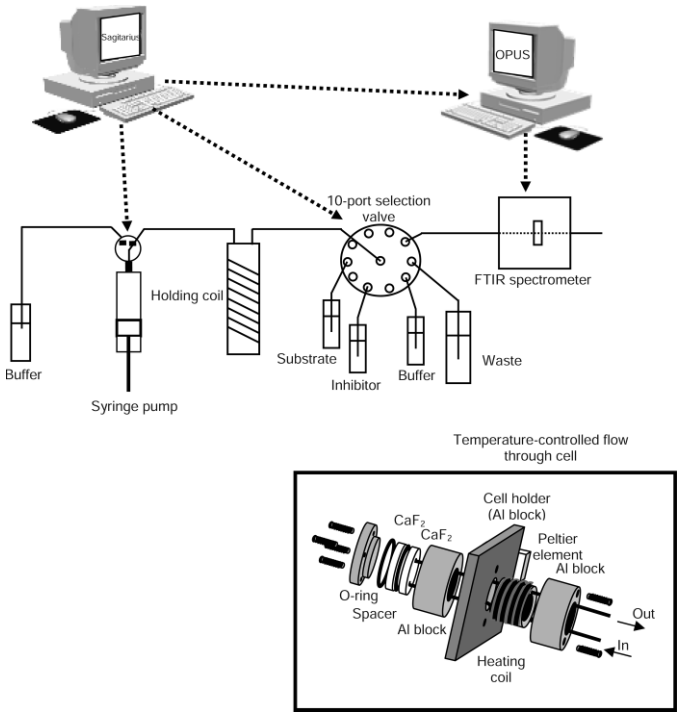

FIG. 1. Scheme of the experimental setup consisting of an automated flow system and a temperature-controlled mid-infrared flow-through sensor containing AChE immobilized on agarose beads.

M EDC solution containing 1 mg AChE and mixed with an excentric rotator for 18 h. Subsequently, the beads were washed alternately with 0.1 M sodium acetate (pH 4) and 0.1 M TRIS (pH 8.3), both containing 0.5 M sodium chloride, and stored in the refrigerator in 0.1 M phosphate buffer solution at pH 7.0.

Temperature-Controlled Flow-Through Sensor Cell. An important parameter in biochemical reactions is the influence of temperature. The major effect of temperature is often not only on rates of biochemical reaction, but also on the possible conformation changes before and after the chemical step.[21] So, in this context, control of the temperature in the sensor cell is highly desirable.

The inset in Fig. 1 shows a schematic diagram of the temperature-controlled flow cell. The cell is constructed completely of aluminum, due to its light weight and thermal conductivity. The Peltier element, connected to the back side of the cell holder, controls the temperature of the flow cell and the heating coil, providing an operating temperature range from 2 to 37 °C. The 75 mm heating coil, placed before the flow-through cell, provides the same temperature for the liquid stream to that of the flow-through cell.

Beads carrying covalently bound AChE were placed between the CaF_2 windows (thickness 2 mm) of the flow cell, which were separated by a polyethylene spacer of 60 μm. It is important to ensure a homogenous layer that covers the whole width of the spacer channel, providing an adequate reaction rate without a noticeable increase of the pressure in the system.

Sequential Injection System and Fourier Transform Infrared Instrument. The sequential injection (SI) manifold (Fig. 1) was set up with a Cavro (Sunnyvale, CA) XP 3000 syringe pump (syringe size 5000 μL) and a Valco (Houston, TX) 10-port selection valve. Poly(tetrafluoroethylene) (PTFE) tubing (inner diameter: 0.5 mm, length: 400 mm from valve to flow-through sensor) and fittings were obtained from Global FIA (Gig Harbour, WA).

All experiments were carried out on a Bruker (Karlsruhe, Germany) Equinox 55 FT-IR spectrometer equipped with a narrow band mercury cadmium telluride (MCT) detector. Spectra were recorded by co-adding 128 scans at a resolution of 8 cm^{-1} and a scanner velocity of 100 kHz HeNe frequency. The whole setup was automatically controlled by coupling the Sagittarius 3.0.25 software, an in-house-written software program based on MS Visual Basic 6.0 (Microsoft), of the SI manifold, with the OPUS 6.1 software (Bruker, Karlsruhe, Germany) of the spectrometer.

Measurement Procedure. For the measurements of the calibration set (0.8–6.4 g L^{-1}) an ACh stock solution of 10.0 g L^{-1} in 0.1 M phosphate buffer adjusted to pH 7.0 was prepared. The appropriate dilutions were prepared automatically in the SI system and injected into the carrier stream. The flow was stopped as soon as the sample had reached the flow-through sensor cell. After stopping the flow, spectra were recorded for fifteen minutes to monitor the course of the reaction taking place in the flow-through sensor cell.

A standard solution of tacrine hydrochloride (inhibitor) of 1 g L^{-1} was mixed at different ratios with the standard solution of 10.0 g L^{-1} ACh, diluted to the appropriate concentration with phosphate buffer (pH 7.0), and injected and analyzed as described above.

RESULTS AND DISCUSSION

Batch Immobilization. It is well established that the kinetic behavior of AChE is consistent with the role of a recycling catalyst.[21] A single molecule of AChE (enzyme) combines reversibly with a single molecule of ACh (substrate) to form an enzyme–substrate complex, which is transformed irreversibly to free enzyme and the products of reaction, choline, and acetate. This is a monomolecular chemical reaction in which the enzyme plays the role of a recycling catalyst.

In order to verify the success of the immobilization process and also the kinetic mechanism of the AChE covalently bound to the beads, a standard solution of 4 g L^{-1} ACh was mixed in a batch system with the beads carrying the enzyme. After one hour of reaction time the solution was decanted and an infrared spectrum of the clear solution was recorded. This spectrum was compared to those of the substrate (ACh) and the products. As can be seen from Fig. 2, the disappearance of the ACh band at 1736 cm^{-1}, corresponding to the carbonyl of the ester moiety, and the appearance of the symmetric stretch vibration of the carboxylate of acetate at 1417 cm^{-1} suggest complete hydrolysis of acetylcholine into choline and acetic acid, which is neutralized to acetate in the phosphate buffer media. Therefore, it can be concluded that the immobilization procedure carried out in batch was successful.

Characterization of the Flow-Through Sensor. In order to monitor the enzyme catalyzed reaction, the beads carrying the AChE were placed between the CaF_2 windows and the temperature of the flow cell heating coil was fixed to a constant value of 37 °C. The ACh solution was injected into the sensing cell and the flow was stopped. Sample spectra were recorded, using the cell filled with phosphate buffer as background, at equal time intervals of 30 seconds yielding difference spectra containing the spectral fingerprint of the reaction under investigation. As can be seen from Fig. 3, the

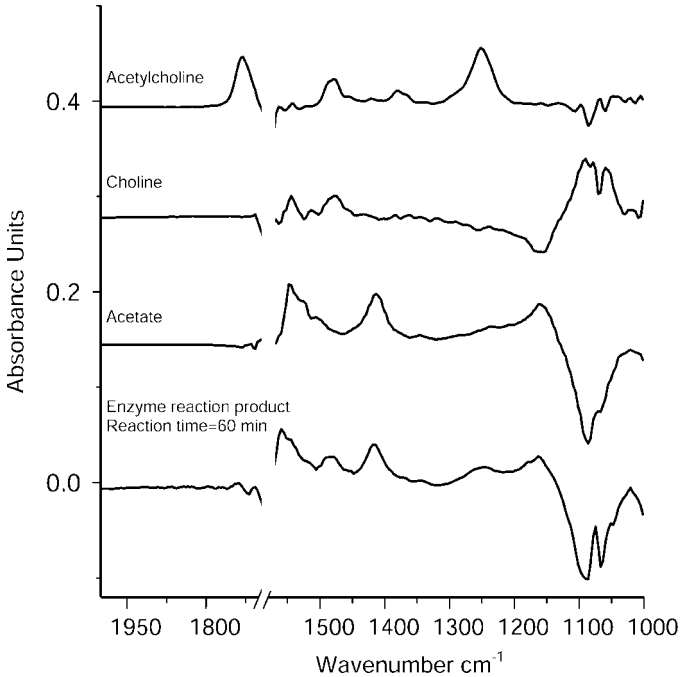

FIG. 2. Absorbance spectra of (***top to bottom***) 4 g L^{-1} acetylcholine standard, 2 g L^{-1} choline standard, 2 g L^{-1} acetic acid (acetate), and enzyme reaction products after a batch reaction time of 1 hour. Spectra were obtained using 128 co-added scans, 8 cm^{-1} nominal resolution, and 0.1 M phosphate buffer as background. Note: Spectra were shifted on the *y*-axis for clarity.

peaks at 1736 cm^{-1} (C=O stretch) and 1382 cm^{-1} (symmetric CH_3 deformation vibration in acetylcholine) decrease with time, while the band at 1417 cm^{-1} (symmetric carboxylate stretching due to acetate)[22] increases, indicating the hydrolysis of ACh in the flow cell.

The progress of the enzymatic reaction was monitored by following the decrease in intensity at 1736 cm^{-1} in the difference spectra corresponding to 120 seconds of reaction time. This value was used to calculate the concentration-dependent reaction rate. The graph of initial reaction rate plotted against the concentration of substrate is a section of a right rectangular hyperbola. When the concentration of enzyme is much lower than the concentration of substrate, the progress of enzymatic reaction gradually decreases due to depletion of substrate and the initial rate of reaction responds nonlinearly to

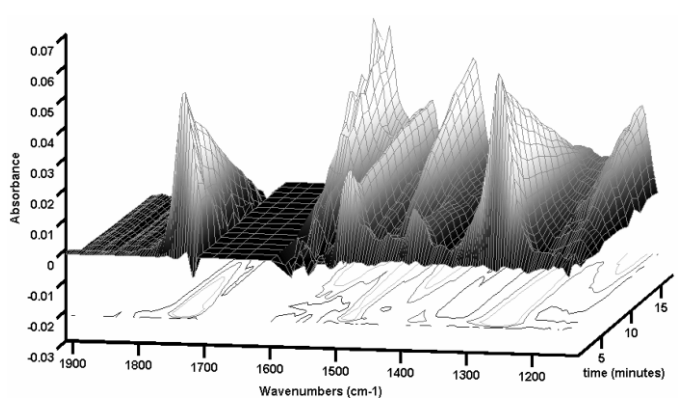

FIG. 3. Three-dimensional plot of the enzyme-catalyzed reaction, using the sensor cell filled with phosphate buffer as background and starting the measurements immediately after stopping the flow.

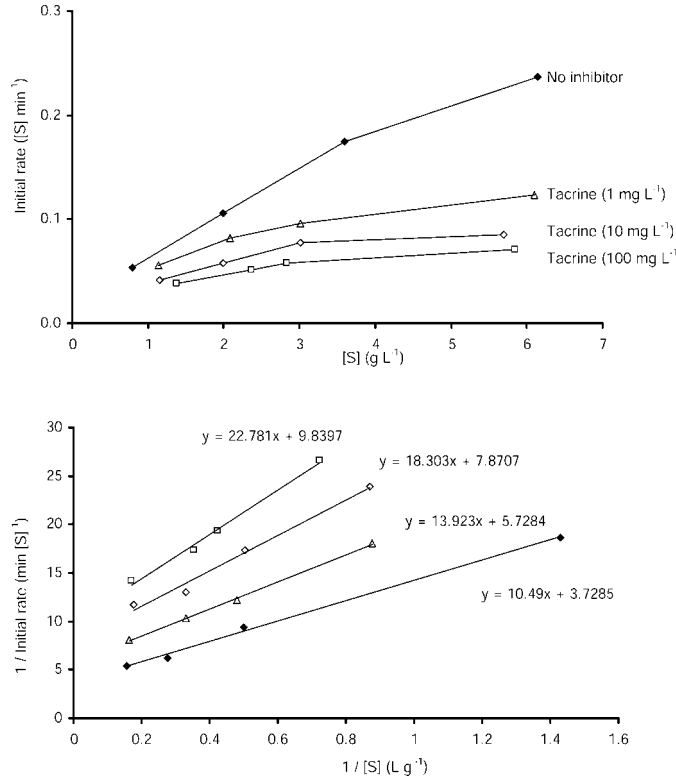

FIG. 4. (**Top**) Inhibition of the hydrolysis of ACh by tacrine at different concentrations. (**Bottom**) Lineweaver–Bulk plot of initial reaction rates against AChE concentration in the presence (at different concentrations) and absence of tacrine.

increasing concentrations of substrate. The Michaelis–Menten constant (K_M) of an enzyme is, therefore, the substrate concentration at which the reaction occurs at half of the maximum rate. K_M can be determined in conditions of zero-order kinetics with respect to the substrate and it can be assumed at the beginning of the reaction. A K_M value of 19.23 mmol L^{-1} (2.81 g L^{-1}) was determined for the immobilized enzyme and ACh by using a Lineweaver–Burk plot.

Inhibition of AChE by Tacrine. Tacrine hydrochloride is a reversible inhibitor that forms a noncovalent complex with AChE and thus reduces the amount of enzyme available for participation in the reaction with acetylcholine. Computational studies suggested that tacrine binds to the AChE near to the active site, occupying part of it.[23] There is a lack of agreement in the literature on the type of inhibition caused by tacrine; however, the recommendation of the International Union of Biochemistry is to consider a mixed competitive-uncompetitive inhibition.[24]

Here the inhibitor activity of tacrine on the hydrolysis of ACh catalyzed by AChE at different inhibitor concentrations

TABLE I. Kinetic constants obtained for the ACh hydrolysis catalyzed by immobilized AChE in the presence and absence of tacrine.

Tacrine conc. (mg L^{-1})	K_M (g L^{-1})	V_{Max} (g min^{-1})
0	2.81	0.28
1	2.43	0.17
10	2.32	0.13
100	2.31	0.10

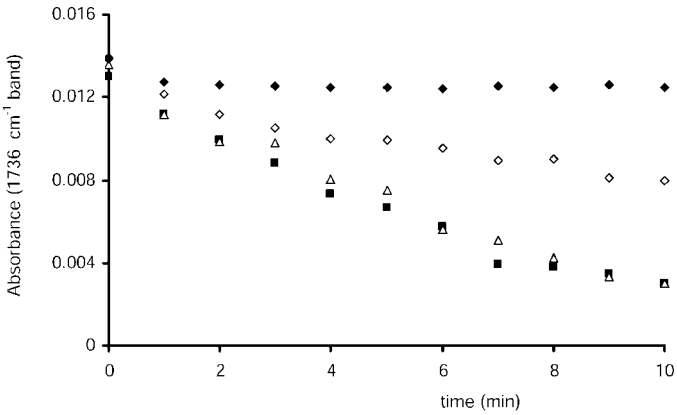

FIG. 5. Time course of AChE activity monitored by the decrease in absorbance at a characteristic wavenumber (1736 cm^{-1}) of the substrate. Traces recorded before and after inhibition with 4 g L^{-1} tacrine as well as traces reflecting reactivation by dilution with phosphate buffer and by treatment with oxime are shown. (■) AChE hydrolysis of 4 g L^{-1} ACh; (♦) inactivation of the activity by addition of 4 g L^{-1} tacrine for 15 min; (◇) reactivation by 10 min washout with 0.1 M phosphate buffer; and (△) reactivation with 4 g L^{-1} pyridine-2-aldoxime methochloride ($t = 10$ min).

was studied. An important decrease of the activity of the AChE in the presence of increasing concentrations (from 1 to 100 mg L^{-1}) of the inhibitor can be observed in the top panel of Fig. 4. From the Lineweaver–Bulk plot depicted in the bottom panel of Fig. 4, the apparent Michaelis–Menten constants can be determined in conditions of zero-order kinetics with respect to the substrate at the beginning of the reaction. A decrease from 19.23 mmol L^{-1} (2.81 g L^{-1}) is observed in absence of inhibitor to 15.8 mmol L^{-1} (2.31 g L^{-1}) in the presence of 100 mg L^{-1} tacrine (see Table I).

Additionally, complete inhibition of AChE by tacrine is observed, as the reaction rate approaches zero when the inhibitor concentration is high enough.

Enzyme Reactivation. AChE can be inactivated after prolonged contact with high concentrations of tacrine. The reactivation methods can vary from simple dilution to more complicated dialysis or treatment with oxime-based compounds. The reactivation treatment of AChE after addition of tacrine (4 g L^{-1}) is presented in Fig. 5.

After measuring the hydrolysis rate of 2 g L^{-1} ACh, the enzyme was treated with a solution containing 2 g L^{-1} ACh and 4 g L^{-1} tacrine. As a consequence, virtually all activity was lost, as evidenced by the constant absorbance level at 1736 cm^{-1} in the difference spectra. From Fig. 5, it can be noted that inhibition of AChE by tacrine was partially reversed by dilution. However, treatment with pyridine-2-aldoxime methochloride was necessary to achieve an AChE reactivation of nearly 100% within 10 minutes.

CONCLUSION

The inhibition of the catalytic activity of AChE has been successfully studied by means of a mid-infrared flow-through sensor. The enzyme immobilized on agarose beads placed inside a 60 μm transmission cell provided excellent catalytic activity, presenting an appropriate way to measure the inhibition kinetics of a target molecule. The combination of the flow-through mid-infrared sensor with a SI system allowed automated control of the whole analysis procedure. The sensor has demonstrated excellent robustness, as it is possible to reactivate the enzymatic activity of the AChE covalently bound to the beads after inhibition with high doses of tacrine using an oxime based treatment.

Therefore, it can be concluded that the flow-through mid-infrared system developed can be exploited to analyze the inhibitor activity of different molecules on AChE. It may also be of use for different biochemical systems, especially in cases where quantification of the course of reaction is difficult to achieve by conventional analysis techniques, as may be the case if indicator reactions or separations are required prior to detection.

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