ABSTRACT

A COMPUTER INTERFACED RAPID SCANNING ABSORBANCE AND FLUORESCENCE STOPPED-FLOW SYSTEM AND ITS APPLICATION TO THE KINETICS OF BIOLOGICAL REACTIONS

BY

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A computer interfaced, double-beam, thermostated stopped-flow system for fixed wavelength and rapid scanning of absorbance or light emission(fluorescence, luminescence and reflectance) spectra has been constructed. The entire system was constructed such that the solutions contact only Pyrex, Teflon, Kel-F, polypropylene and quartz. A specially designed all quartz, double 4-jet mixing and observation cell(2 mm ID) with two optical path lengths has been made. The syringes were made out of heavy-walled Pyrex precision bore tubing(0.396" ID). Steel plungers with easily adjustable Teflon cap seals were made to fit them. Two metal flags mounted on the stopping plunger provide timing pulses by interrupting two light beams which are detected by separate phototransistors. The thermostat bath, which was made of Plexiglas to provide good visibility, surrounds the flow system and allows for temperature-dependent studies and for establishing thermal equilibrium between the solutions and the system. A rapid scanning monochromator is used to disperse either the excitation or the emission beam. In the

absorbance mode, light beams are transmitted via quartz fiber optics to both the sample cell and the reference cell, and then to a pair of photomultipliers. The emitted light(or reflected light), which is observed at 90° to the excitation beam at the short path length, is conducted with a separate quartz fiber bundle from the sample cell to the monochromator and then to a photomultiplier. Sample and reference photocurrents are converted to absorbance by use of a log ratio operational amplifier, or into partially corrected fluorescence by means of a ratio operational amplifier. The absorbance or the fluorescence analog signal is sampled and digitized at a nominal rate of 20.4 KHz, which is synchronized to the rotation of the monochromator mirror with the aid of a phase-locked-loop circuit. The signal is transmitted to a remote PDP-8/I computer for data acquisition and processing. The entire system was tested extensively by studying several well-characterized fast reactions. Its performance characteristics are excellent. The dead volume was estimated to be 60 μl for the short path length and 146 μl for the long path length, which resulted in dead times of 2.5 msec. and 6.5 msec., respectively, under a pushing pressure of ~50 psig. This instrument is especially suited to kinetics studies when only small volumes of the reagents are available. At least 6 good pushes can be obtained from only 5 ml of each reagent.

Scanning absorbance experiments on the redox reaction of chromic acid and hydrogen peroxide in acidic solutions

were carried out at 22°C. The observed first-order rate constants and the the calculated third-order rate constants were computed and were found to be in good agreement with published values.

The computer simulation of complex enzyme-catalyzed reactions has been explored. Five hysteretic enzyme models were investigated by numerically simulating the full time course of the reaction progress curve. The Bates and Frieden Model(D. B. Bates and C. J. Frieden, J. Biol. Chem., 248, 7878 (1973)) gives kinetically hysteretic behavior but does not show allosteric behavior in the saturation of enzyme activity. The Fit-Induced Model and the Monod-Wyman-Changeux (MWC) Model(with n=2) both show kinetically hysteretic response as well as allosteric saturation in the enzyme activity. Slow conformational changes give hysteresis in the time response of the reaction, while substrate binding at a second site causes the allostery in the enzyme activity. The cases with " zero cooperativity " and with " negative cooperativity " for the MWC Model(with n=2) were also simulated. The concept that an enzyme may have primitive Michaelis-Menten activity together with conformational-change-regulated Michaelis-Menten activity has been developed and the dependence of the full time course of the reaction on the initial substrate concentration and on the total enzyme concentration was also numerically simulated.

The new instrument was first used to scan the kinetics of firefly luciferase catalyzed reactions. Both the decay of

luciferin and the growth of enzyme-bound oxyluciferin occur in at least two steps, each of which is first-order. A transient intermediate absorption band was observed between ~420 nm and ~440 nm. A pseudo-isosbestic point was observed at ~353 nm.

The binding reactions of 1-anilino-8-naphthalene sulfonate and bovine serum albumin were scanned by measuring the fluorescence changes. At least two steps are present in the mechanism: an unobservably fast association process(the quenching of BSA fluorescence) and a slower first-order process(the enhancement of ANS fluorescence). Both processes increase the fluorescence of ANS. The first-order process shows dependence on the ratio of the equivalent concentrations, $(ANS)_o/5(BSA)_o$, with a decrease in the first order rate constant as the equivalent concentration ratio is increased.

The first scanning fluorescence experiments with an enzyme were done with a reaction catalyzed by lactate dehydrogenase. The reaction was followed in both directions by observing the changes in the NADH fluorescence.

The final scanning experiments were done with AMP aminohydrolase catalyzed reactions by scanning the absorbance from ~257 nm to ~300 nm. The full time course of the reactions for various concentrations of 5'-AMP at a fixed AMP aminohydrolase concentration of 0.209 mg/ml and for various total AMP aminohydrolase concentration at a fixed 5'-AMP concentration were determined. The dependence of the

full time course on various initial substrate and enzyme concentrations shows characteristics similar to the hysteretic enzyme model - MWC Model(with n=2). A set of the kinetics and thermodynamic parameter values, which approximately fit the experimental data, were found and the simulated full time course behavior was compared with the corresponding experimental data.

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To

Shiu-Ying and My Parents

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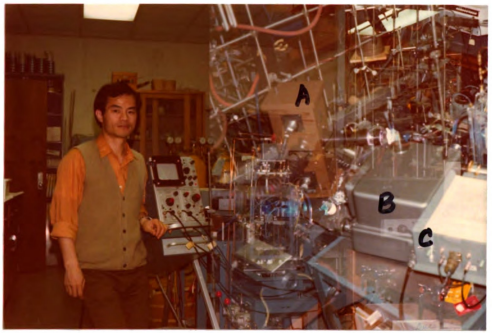

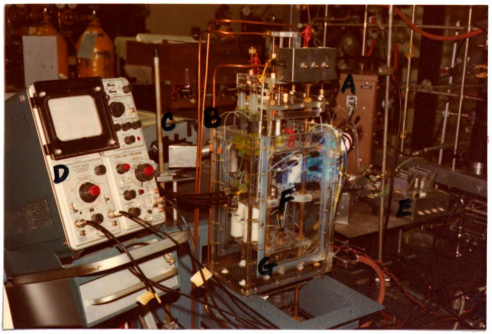

Author and the Rapid Scanning Absorbance and Fluorescence Stopped-Flow System. A - 1 kw Xenon lamp light source, B - rapid scanning monochromator, C - photomultiplier tube, D - storage oscilloscope, E - log ratio amplifier, F - mixing and reference cells, G - thermostat.

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