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A STOPPED-FLOW CALORIMETER WITH A THERMOCOUPLE ARRAY SENSOR FOR STUDIES OF ENERGETIC ASPECTS OF ENZYMATIC REACTIONS

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SUMMARY

A stopped-flow calorimeter has been constructed using a newly developed thermocouple array sensor. It can measure temperature changes of 1 mK with an accuracy of 0.2 mK, the dead time of 13 ms and time resolution better than 5 ms. The instrument has proved useful in studies of kinetics and thermodynamics of the pre-steady state of myosin-catalyzed hydrolysis of ATP.

INTRODUCTION

Stopped-flow calorimetry provides kinetic and thermodynamic information of a chemical reaction simultaneously. Although a number of instruments have been built (refs. 1-3), they have had inadequate sensitivity and hence limited biochemical applications.

There are three major problems in designing a stopped-flow device to be adopted for calorimetry: (a) to construct an observation tube with a rapid heat flow from the reaction system to the temperature sensor; (b) to minimize a temperature difference between solution in the observation tube and reactant solutions in the flow-path ready for next run; and (c) to make the flow rate of reactant solutions reproducible as much as possible. In addition, the observation tube should be thermally insulated from the immediate environment. Here we give an outline of a stopped-flow calorimeter which is an improvement in these respects over earlier designs.

DESCRIPTION AND CONSTRUCTION OF THE INSTRUMENT

Temperature sensor

The sensor is a nickel/copper thermocouple array which is still under development so that its details will be described elsewhere. In short, by using essentially the same technique as for making semiconductor devices, nickel and copper patterns are made and sandwiched by layers of silicon nitride (thickness, 50 μm) on a surface of a square-shaped zirconia ceramic plate (5 × 7 × 0.05 mm). The thermocouple density is 10 mm^{-1} and the total thermal sensitivity of 1.0

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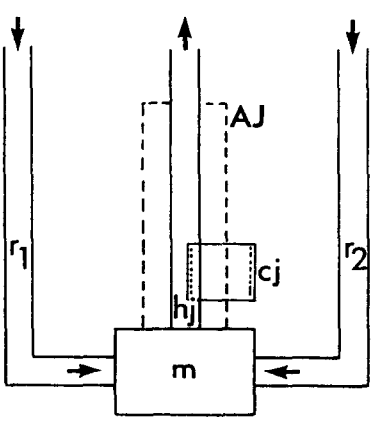
mV/K at room temperatures. The impedance is 1.1 kΩ.

Observation tube and mixer

The sensor is inserted into the observation tube (polystyrene, i.d. 2mm/o.d. 3mm) so that all "hot" junctions are aligned along the middle of the flow-path of solution (Fig. 1). The observation tube is jacketed with a Perspex cylinder (i.d. 8 mm/o.d. 10 mm). The "cold" junctions, which are covered by a epoxy-resin block, are on the outer surface of the cylinder. The mixer is a double 2-jet type, made of a 18-K gold block and a Teflon insert. The distance between the mixing center and the sensor is 20 mm. At a flow rate of 1.5 m/s, therefore, theoretical dead time of the instrument is 13 ms.

Solution delivery

The mixer is connected through 18-K gold tubings (40-cm long; i.d. 2 mm/o.d. 2.1 mm) and Teflon tubings (20-cm long; i.d. 2 mm /o.d. 3 mm), via solenoid-operated 3-way valves, to gas-tight syringes (Hamilton 1002TEFLL, 2.5-ml volume) placed outside the air bath. Syringe plungers are pushed by a pneumatic/hydraulic linear feeder (type ZYZ-63-80, Fest, Esslingen, West Germany), delivering 0.2 ml each of two reactant solutions through the mixer and observation tube into the water bath. The fluctuation of the push rate (monitored by a linear potentiometer) is within 0.5%.



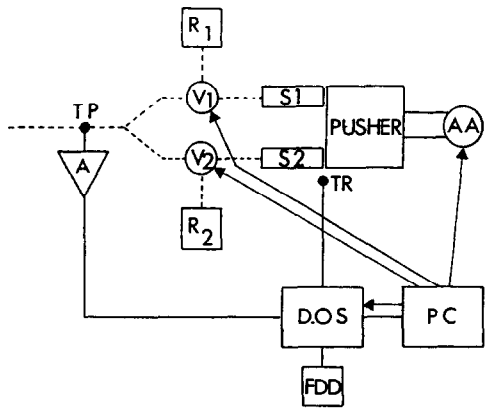


Fig. 1. Observation tube/mixer assembly. OB, observation tube; AJ, air-jacket; hj, hot-junction alignment; cj, cold-junction alignment; m, mixer; r_1/r_2, reactant solution tubings. Arrows indicate flow directions of solution.

Fig. 2. Block diagram of stopped-flow calorimeter showing a net work of system operation and control. TP, thermopile; A, amplifier; TR, trigger pulse generator; DOS, digital storage oscilloscope; FDD, floppy-disk drive; PC, personal computer (Epson 386X); R_1/R_2, reactant solution reservoirs; V_1/V_2, solenoid-operated 3-way valves; S_1/S_2, driving syringes; PUSHER, pneumatic/hydraulic linear feeder; AA, solenoid-operated air-actuator of the feeder. Components with arrows directed from the computer are under sequence control.

Thermostatting

The observation tube/mixer assembly and a part of the solution tubing of each side are housed in between two aluminum blocks (120 × 120 × 20 mm), which is in turn submerged in a water bath (10-liter volume) placed in a precision air bath (temperature fluctuation < ± 0.01 K). Thus, the essential part of the calorimeter is surrounded by a small amount of water which is effectively occluded from bulk bath water by aluminum blocks of a large heat capacity.

Operation and control

The output of the sensor is fed to a differential amplifier (OP27 × 2 + OP07, PMI) and recorded with a digital oscilloscope (Nicolet 310C). The push/pull movement of the feeder, opening/closing of valves in the flow path and data acquisition/storage are all under sequence control by a microcomputer (Fig. 2). Thus, the whole system could be left in automatic operation and heat records could be obtained at regular intervals.

PERFORMANCE

Heat production on mixing

On mixing water alone (both sides of the flow path were filled with distilled water), the calorimeter output began to rise rapidly when the plungers were started off and reached a maximum value when they stopped. The output then underwent a slow decay. The rise of the output is probably due to energy

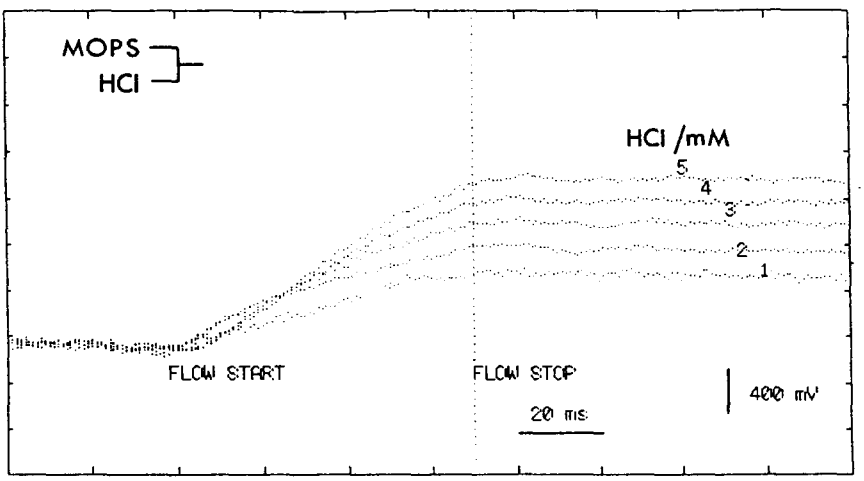


Fig. 3. Heat records for mixing of MOPS with HCl. The concentrations of HCl given are those after mixing. The calorimeter output was digitized at 1 ms intervals. Five or more mixings were made, and heat records were averaged for each concentration of HCl and processed by a digital filtration method (smoothing).

dissipation as heat on a rapid fluid flow through the mixer. The maximum output was in fact proportional to square of the calculated flow rate. This heat production was very reproducible at a given flow rate. This is of fundamental importance in measuring the magnitude of a small, rapid heat change accompanying a very fast reactions which would be complete within the dead time of the instrument, for example, the substrate binding step in an enzymatic reaction. The decay is caused by heat loss from the solution around the "hot" junctions.

Calibration of sensitivity

Figure 3 shows the calorimetric records for the reaction between half-neutralized MOPS (3-(*N*-morpholino)propanesulfonic acid, protonation heat, 21.0 kJ/mol, ref. 4) in excess (0.2 M, pH 7.2) and HCl of a series of different concentrations (2 - 10 mM). On mixing of the two solutions, there was a rapid temperature rise which reached a maximum value when the flow stopped and was then followed by a decay. The maximum output was found to be proportional to the concentration of HCl and, therefore, to heat production in the calorimeter. A similar experiment was repeated with phosphate (heat of protonation for the second dissociation, $H_2PO_4^{-1} \rightleftharpoons H^+ + HPO_4^{-2}$, 5.2 kJ/mol, ref. 5), the result of which is shown in Fig. 4, indicating that the instrument has the thermal resolution of 1 mK with an accuracy of 0.2 mK.

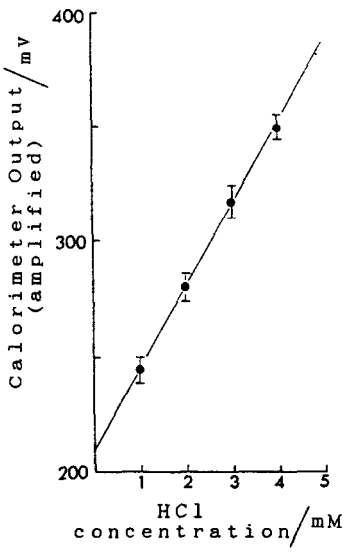


Fig. 4. Relationship between the maximum deflection of calorimeter output and concentration of HCl which was mixed with potassium phosphate (0.2 M, pH 6.8). The maximum deflection was calculated by subtracting the averaged output for 200 ms before mixing from the averaged output between 100 and 200 ms after the flow stop. Each point is the average of 5 or more calculated deflections for each concentration of HCl. Error bars indicate ± 1 S.D.

APPLICATION

The instrument has been in use for studying the ATP hydrolysis by myosin subfragment-1(SF-1, preparation retaining the enzyme activity of myosin obtained by chymotryptic digestion). A preliminary result is shown in Fig. 5, which indicates that during the pre-steady state of the reaction, at least three phases are seen in sequence, which are distinct from each other in kinetics and thermodynamics. Thus, on mixing SF-1 with ATP, there was large heat burst which was completed before the flow stop and was followed by a rapid heat absorption. The rate of this heat absorbing phase was approximately 100 s^{-1}, which is essentially the same as for the rate of ATP cleavage step determined by other kinetic methods under comparable conditions (ref. 6). Thus, the preceding rapid heat burst probably corresponds to ATP binding to myosin subfragment-1 (the rate constant > 1000 s^{-1}, ref. 6), whereas the subsequent slow heat production would correspond to the release of inorganic phosphate from SF-1. It should be noted that the calorimeter used in earlier studies (ref. 8) had response time of 1s, which was too slow to make thermal separation of ATP binding and cleavage, and hence only a small heat burst (binding and cleavage together) and subsequent slow heat production could be observed.

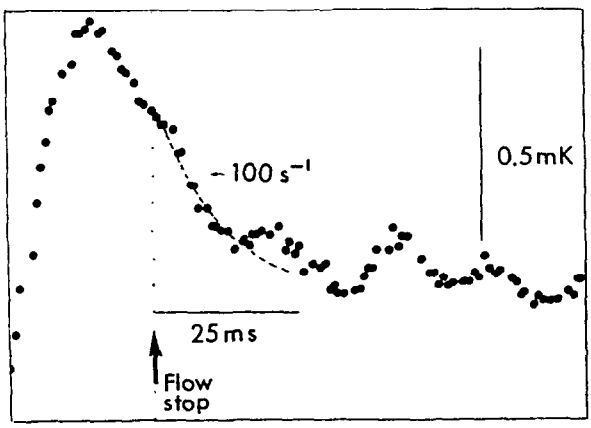


Fig. 5. Heat record of interaction of myosin subfragment-1 with ATP. A preparation of SF-1 was dialyzed for 24 hr against 100 volumes of buffer containing 100 mM KCl, 20 mM MOPS (pH7.2), and 10 mM $MgCl_2$ as previously described (ref. 7). The SF-1 (0.2 mM) was first mixed with the buffer used for dialysis. The mixing was repeated five times at 10 min intervals. The heat records thus obtained were averaged and processed as described in Fig. 3 (dilution control). The SF-1 was then mixed with ATP solution made up in the same buffer supplemented with 1 mM MgATP and the averaged result was obtained in the same way as for dilution control. The heat record shown here was obtained by subtracting the heat record of dilution control from the averaged heat record for SF-1/ATP mixing.

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