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AN ENZYME THERMISTOR

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SUMMARY

A simple device comprising a heat sensor placed in close proximity to a matrix-bound enzyme preparation and connected to a temperature measuring unit has been developed to measure directly the temperature changes that occur in the environment of enzyme activities. The sensitivity of the system was established by pumping pulses of substrate past trypsin or apyrase immobilised to glass-beads. Injection of a pulse containing only one μmole trypsin substrate (benzoyl-L-arginine ethyl ester, BAEE) in Tris–HCl buffer gave a ΔT of 0.01 °C, corresponding to 10% full scale deflection, and a linear relationship was found within the range tested of upto 8 μmoles BAEE.

INTRODUCTION

The use of microcalorimetry for the study of biological systems, enzymatic reactions and particularly as a general analytical tool, has received considerable attention during the last few years [1]. Highly sensitive and precise microcalorimeters are now commercially available although wider application of these instruments may be hampered by their relatively high cost. In the present paper, we offer an alternative approach to the thermal analysis of biological systems using what we would like to call an enzyme thermistor. Here as in an enzyme electrode [2, 3], where the enzyme is arranged either as a liquid film around the electrode, or kept immobilised within a polymer film, the sensor is placed in close proximity to the site of the reaction. Thus, changes in heat caused by enzymic reactions can be readily measured since such changes are most pronounced in the "microenvironment" of the protein.

There are two possible ways of arranging the site of reaction and the sensor in close proximity; (a) immobilisation of the protein directly onto the thermistor by, for example, coating the latter with glutaraldehyde-crosslinked enzyme, by entrapping the enzyme within a dialysis bag around the thermistor or finally, by immersing the thermistor in the flow path of a bed containing glass- or polymer-bound enzyme; (b) encirclement of the thermistor by a coil of tubing packed with matrix-bound enzyme. Changes in heat could be detected with the alternatives given under (a), although a low yield of immobilised enzymatic activity or diffusional hinderance of substrate/product through the membrane caused problems. Immersion of the sensor

Abbreviation: BAEE, benzoyl-L-arginine ethyl ester.

directly in the flow path of a bed of immobilised enzyme has shown promising results Thus it was found that when the sensor was placed roughly in the centre of a micro-column (40 mm × 6 mm) containing about 1 ml of packed enzyme-bearing glass beads, comparable heat responses were obtained with the enzymes trypsin, apyrase and urease tested (Borgeryd, A. Danielsson, B. Scott, M. and Mosbach, K., unpublished observations). However, the simple device described below is the best tested device to data. As seen in Fig. 1, the device comprises a thermistor (Precision Thermistor (YSI No. 44106, Yellow Springs Instrument Co., Ohio, U.S.A. with a resistance of 10 kΩ at 25 °C) connected to an electronic temperature measuring instrument (Knauer, Wissenschaftlicher Geraetebau, W. Berlin). Changes in the resistance of the thermistor occasioned by temperature variations are measured with this electronic temperature measuring instrument, a modified Wheatstone bridge equipped with an ultrastable, high sensitivity amplifier. Temperature variations are recorded directly on a recorder (W+W recorder 3001 Kontron, Zürich, Switzerland). The equipment is capable of giving a dull scale deflectiin on the recorder (100 mV) for

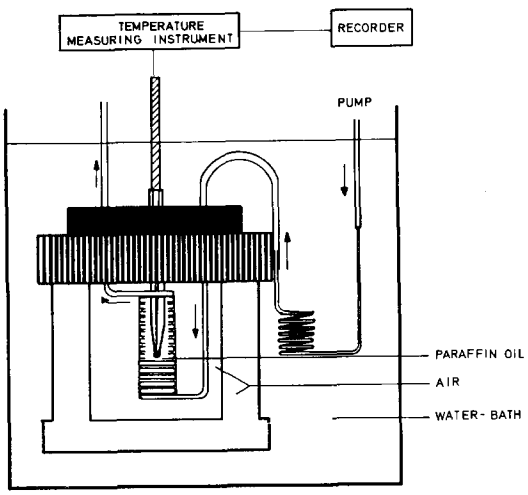

Fig. 1. The substrate solution is pumped through a stainless steel heat exchanger into the coil containing the glass-bound enzyme. The thermistor is placed inside the coil as seen in the schematic drawing. The entire measuring cell is immersed in a water bath. The temperature of the water bath was kept for all assays at 27.0 °C.

temperature change (ΔT) of 0.02 °C. The thermistor is placed inside a coil of polyvinylchloride tubing (internal diameter 1 mm) that has been glued together with epoxy resin to form a cup with an inner diameter of 4 mm. The thermistor is surrounded by paraffin oil, a fluid known to have low heat capacity giving good thermocontact. The coil assembly is placed in an air jacket whence the whole measuring cell is immersed in a water bath (Hetotherm, 05 PG 623 UO, Heto Birkeroed, Denmark). No reference thermistor was found to be required. The coil (total volume 180 μl) contains approx. 100 mg (wet wt) of enzyme bearing glass beads and the substrate solution is pumped through the system with a peristaltic pump (LKB-Beckman, Perpex) at a flow rate of 10 ml/h. Matrix-bound enzyme preparations are easily pumped into the tubing and prevented from escape by a plug of glass fibre. Buffer and substrate solution were alternatively pumped through the cell through a three-way tap.

The two enzymes tested, trypsin (Sigma, twice crystallised 36 units/mg) and apyrase (Sigma, potato, Grade 1, 1000 units (5′-ATPase activity) per 0.417 g solid; 5′-ATPase 2.4 units/mg, 5′-ADPase 2.9 units/mg, 5′-AMPase 0.05 units/mg) were coupled separately to alkylamino glass (diameter 70–150 μm, pore diameter 700 Å) following activation by 2.5 % glutaraldehyde as developed by Weetall and described in the literature [4]. Trypsin (15 mg) and apyrase (50 mg) were coupled to 0.5 g of dry glass. The coils carried a total enzymatic activity of approx. 3 units (trypsin) or 1.5 units (apyrase) based on separate spectrophotometric determinations. Fig. 2a exemplifies the response obtained after injection of a pulse of 1 ml of 6 mM benzoyl-L-arginine ethyl ester (BAEE) in 0.1 M Tris–HCl buffer (pH 8.0) into the system containing glass-bound trypsin. For this and all subsequent measurements, a sensitivity

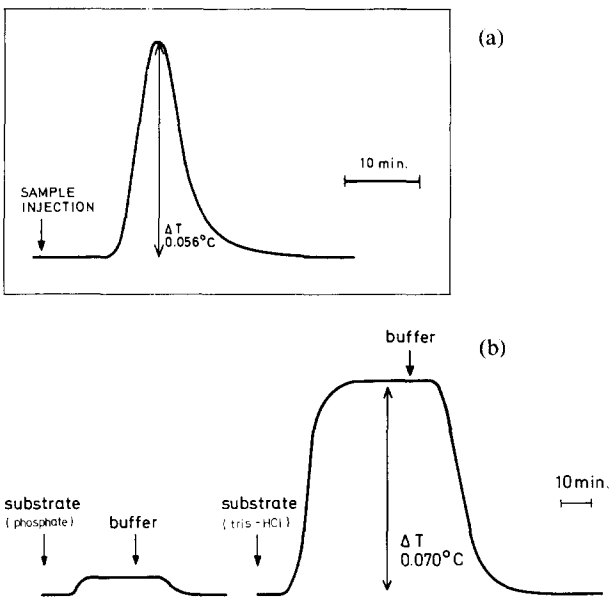

Fig. 2. (a) Experimental curve obtained after injection of a pulse of 1 ml of 6 mM BAEE solution into the Tris–HCl buffer flow. (b) Experimental curve obtained by continuously flowing substrate, 6 mM BAEE, through the enzyme thermistor, in phosphate buffer and in Tris–HCl buffer.

region was chosen that full scale deflection of the recorder corresponded to a ΔT of 0.1 °C. In Fig. 2b the response to the same concentration continuously flowing through the system is depicted. The fact that the recorded ΔT is somewhat higher in the flow-through system is because the amount of substrate present in the pulse is not sufficient to give heat equilibrium and is in agreement with previous findings using conventional flow-microcalorimeters [5]. A lower response in ΔT was found when the reaction was carried out in 0.1 M phosphate buffer (pH 8.0) instead of Tris buffer. It should be emphasised that ΔH values for ester hydrolysis reactions are usually close to zero and that the main contribution to the total recorded heat is caused by proton transfer from the acid formed to the buffer. In line with this finding are the literature data on the heats of protonisation of the corresponding Tris and phosphate buffer systems, $\Delta H = -47.48$ kJ/mole [6] and -4.74 kJ/mole [7], respectively.

In Fig. 3, the heights of the peaks (ΔT) are plotted as a function of the amount of substrate (1 ml of solution, 1–8 mM) added to immobilised trypsin. An almost linear relationship exists between the recorded ΔT and the amount of substrate added. The response obtained after addition of 1 μmole of substrate gives a ΔT of 0.01 °C corresponding to 10% full scale deflection of the recorder chart and is easily and reproducibly detected. Equivalent relationships are obtained when the integrated areas beneath the peaks are plotted against the amount of substrate added or when the gradient at the point of inflection of the front face of the curve is plotted. It could be shown by enzymatic spectrophotometric analysis of the effluents, that passage through

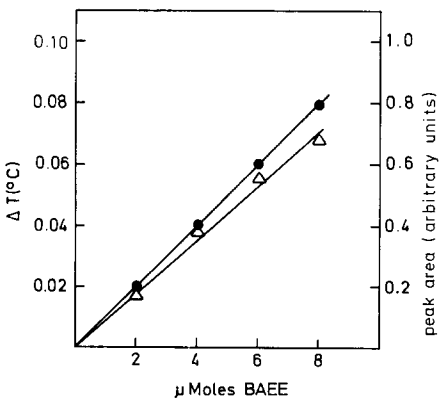

Fig. 3. Measured peak heights (ΔT) in °C (△—△) and the corresponding integrated peak area (●—●) as a function of the amount of BAEE substrate injected to glass-bound trypsin. A ΔT of 0.1 °C of the recorder represents full scale deflection.

the coil containing the glass-bound trypsin totally hydrolysed the added ester at all concentrations applied.

The ΔT obtained on enzymic hydrolysis of ATP with glass-bound apyrase was followed as a function of substrate concentration. The enzyme preparation contained 5′-ATPase, 5′-ADPase and 5′-AMPase (about 1% of the total activity) and thus essentially the reaction generates AMP from ATP. Fig. 4 shows that almost a direct linear relationship was obtained between the amounts (1 ml of solution, 1–8 mM in 0.1 M veronal–HCl buffer (pH 6.7), 1 mM $CaCl_2$) of ATP added and the ΔT recorded. Furthermore, as in the case of trypsin, linear relations were found between the amount of added substrate and the integrated surface area of the responses and the gradients of the inflection points of the curves. In a control experiment, the effluent, after addition of a pulse of 1 ml containing 8 μmoles of ATP, was assayed for inorganic phosphate [8]. At this concentration of ATP, the amount of inorganic phosphate found corresponds to total hydrolysis of all the added ATP to AMP in the passage through the coil.

A pulse of 1 ml of 10 mM BAEE solution was added to this coil to assure in an indirect fashion that the heat evolved is the result of a specific enzymatic reaction and not heat effects due to, for instance, substrate dilution. No change in temperature was recorded. Alternatively, on addition of a pulse of 1 ml of 10 mM ATP solution to

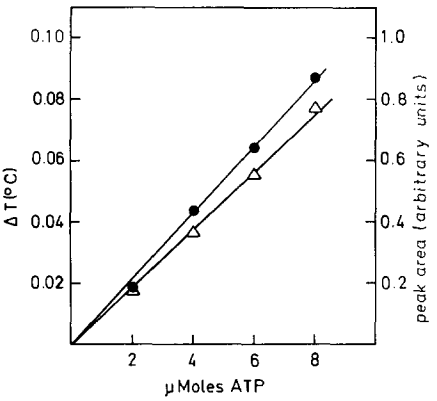

Fig. 4. Measured peak heights (ΔT) in °C (△—△) and the corresponding integrated areas (●—●) as a function of the amount of ATP injected to glass-bound apyrase.

the coil containing trypsin, only a negligible thermistor response was recorded (<5% of the total heat with apyrase).

Summarising, the responses measured for both enzymic reactions were sufficiently large to permit safe determination of amounts of substrate down to 1 μmole. The following calculation can be made based on the reported heat of protonisation in Tris buffer. Since at a flow (10 ml/h) of 6 mM BAEE 800 μW are formed ($0.0167 \cdot 10^{-6}$ moles/s × 47.48 × 10^3 J) then the found ΔT of 0.07 °C (Fig. 2b) implies that a deflection of 0.01 °C corresponds to about 101 μW. This compares favourably with the sensitivity in response obtained w th advanced commercially available flow microcalorimeters equipped with modified flow cells [7]. It is this principle of placing sensors in close proximity to the site of the reaction that eliminates the need for special shields or thermophiles required in conventional microcalorimeters.

The enzyme thermistor described has a number of advantages. One is that the matrix-bound enzyme preparations, which represent a part of the system, are often stable and can be re-used. In our studies with glass-bound trypsin no diminution of the heat response over the long periods of assays during 10 days was found. When the enzyme becomes denatured, the coil can be repacked easily. The inherent potential of enzyme thermistors as an analytical tool is obvious but their use may be extended to incorporate other areas for example as model systems for affinity chromatography.

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