Biochimica et Biophysica Acta, 452 (1976) 285—291

BBA 67972

EXPERIMENTS AND CALCULATIONS CONCERNING A THERMAL ENZYME PROBE

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(Received May 19th, 1976)

Summary

A simple device capable of measuring almost any reactant in an enzyme-catalyzed reaction is created when an enzyme is immobilized onto one thermal sensor of a differential thermometer. Experiments are described in which two thermistors, one bare and one coated with immobilized enzyme, are immersed in a well-stirred solution. The response of this device to increases in glucose-ATP concentration was observed using hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1), and to increases in glucose concentration using glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4). A simple model is presented whose predictions are in reasonable agreement with the experimental results.

Introduction

The application of enzymes to chemical analysis has had considerable impact on the development of measurement systems featuring high chemical specificity. However, the need to interface the enzyme-catalyzed reaction with a sensor, such as a spectrophotometer, fluorimeter, polarigraphic electrode or ion-specific electrode, has placed a major limitation on the widespread use of enzymes for chemical analysis. In cases in which the substrate or product of an enzyme-catalyzed reaction cannot be detected by one of the above sensors, multienzyme sequences that culminate in a detectable product have been used. However, the difficulties of multienzyme systems increase with the number of steps involved.

The development of systems based on immobilized enzymes has further increased the interest in using enzymes for chemical analysis, primarily because

immobilization of enzymes increases the number of measurements that can be performed per unit of enzyme, and facilitates the development of continuous and automated analysis systems. However, the problems of interfacing the reaction(s) with an appropriate sensor remain.

We have explored a system that incorporates the advantages of immobilized enzymes and, in principle, can be used with any enzyme. Specifically, we employ calorimetry to exploit the enthalpy change, which is universal to all chemical reactions, while enzymes provide specificity. Although the application of calorimetry to biochemical analysis is a well-established technique [1—3], most enzyme calorimeters are difficult to use for routine chemical analysis. With this as motivation, we have performed some experiments and calculations on a thermal enzyme probe that in more advanced form should be applicable to either discrete or continuous sampling [4,5]. The Thermal Enzyme Probe consists of two thermal sensors (e.g. thermistors); active enzyme(s) is immobilized onto one of them (Fig. 1). This is in contrast to other devices described as "enzyme-thermistors" in which a thermistor is either located within or downstream from a column of immobilized enzyme [6—9] and in which a laminor flow of substrate solution is passed through the column.

In our experiments, the two thermistors comprise half of a high-precision Wheatstone bridge, whose electrical output is proportional to the temperature difference (ΔT) between the thermistors if ΔT is small. When the Thermal Enzyme Probe is in a solution containing the enzyme substrate, the enzyme-catalyzed reaction(s) occurs only near the thermistor bearing the enzyme, and a ΔT develops between the thermistors. As calculation shows, the magnitude of the steady-state ΔT, and therefore the electrical output of the bridge, is expected to be proportional to the steady-state reaction rate. Thus, the steady state ΔT exploited by this technique is in principle applicable to the analysis of any chemical for which is an enzyme that can be immobilized next to one of the thermal sensors.

Methods and Results

The apparatus shown in Fig. 1 was used for a number of experiments in which the substrate concentration of the well-stirred inner bath was increased sequentially by the addition of small amounts of concentrated substrate. In one set of experiments, a Thermal Enzyme Probe using hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) was exposed to a series of increased glucose-ATP concentrations, and the ΔT response was observed (Fig. 2a). Subsequently, the enzyme-coated thermistor was separately assayed by spectrophotometric analysis using soluble glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$ 1-oxidoreductase, EC 1.1.1.49) to produce NADPH. The spectrophotometric assay allowed us to: (1) compare the steady-state thermal and chemical response of the thermistor pair, and (2) measure independently the apparent K_m for the immobilized enzyme. The apparent K_m value determined from the thermal response is 2.0 mM, while the value of 2.5 mM was obtained by chemical analysis. Thus, the response of the Thermal Enzyme Probe to glucose-ATP is nearly the same in both cases. Furthermore, the apparent K_m value is about 10 times the value found for the soluble enzyme, and is

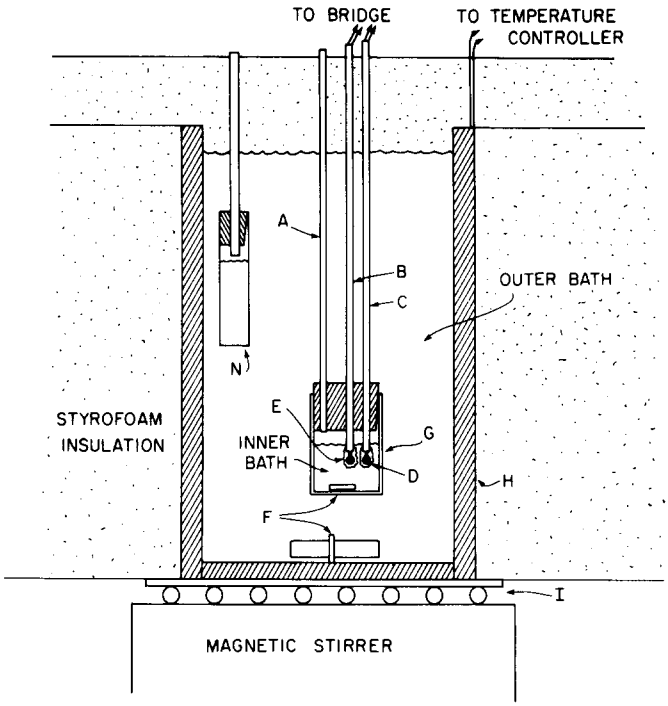

Fig. 1. The Thermal Enzyme Probe stirred-bath configuration used for thermal response measurements. (A) Sample injection tube, (B,C) Stainless steel thermistor tubes, (D,E) thermistors, (F) Magnetic stirring bars, (G) Glass inner bath wall, (H) Brass outer bath wall, (I) Cooling coil, (N) Temporary storage reservoir for concentrated substrate prior to injection into the inner bath.

consistent with a reaction limited by diffusion across a thin aqueous layer. The response of the Thermal Enzyme Probe is linear up to 3.0—4.0 mM gluclose-ATP.

In another set of experiments we used a pair of thermistors with glucose oxidase (β-D-Glucose:oxygen 1-oxidoreductase, EC 1.1.3.4). The ΔT response is given in Fig. 2b, and is linear up to 1.5 mM glucose. The apparent K_m value is $7 \cdot 10^{-4}$ M, which is about 3.5 times the K_m value for dissolved O_2. Saturation with respect to O_2, not glucose, is believed to be the limiting factor.

The increased Michaelis-Menten constant for the immobilized enzymes is typical of immobilized enzymes and consistent with diffusional limitations on the reaction rate. A desirable aspect of the diffusional limitation is that it extends upward the useful linear response with respect to C_s, the substrate concentration.

The major problems of the Thermal Enzyme Probe involve the physics of differential thermometry, as both the predicted and observed ΔT values are small. In agreement with Bowers and Carr [10], some of our other experiments [5] suggest that the present limitation is hydrothermal noise associated with the thermistors when used in a "well-stirred" bath. Also in agreement with Bowers and Carr [10], we find that if a very small inner bath is used without stirring, then (after any flow has damped out) the noise of a bare thermistor pair is significantly lower, and is equal to the noise measured with a pair of

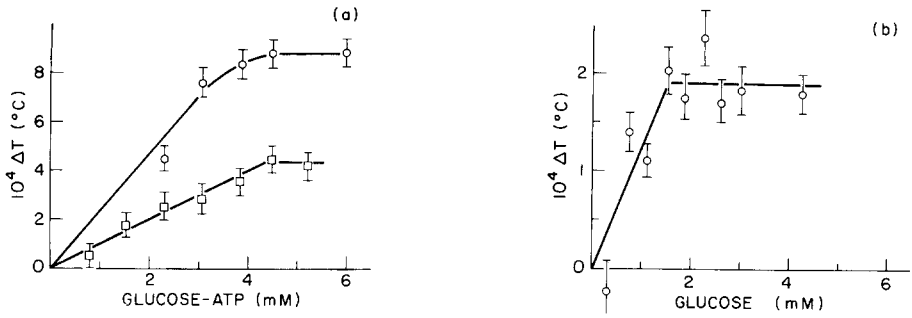

Fig. 2. a: ΔT vs. glucose-ATP concentration curve for the hexokinase Thermal Enzyme Probe. The response is corrected for a slight blank response which occurs only after several 50-μl additions of liquid have been made, and which appears to be associated with increased bath level of the inner bath. ○ initial run, □ 24 h later (probe stored in bath at 35°C; the reason for the decay is unknown). The form of this curve is consistent with Michaelis-Menten kinetics; the V (ΔT_{max}) decreases with time due to an unknown mechanism of enzyme decay, and the constant K_m value of $2.0 \cdot 10^{-3}$ M is in agreement with the spectrophotometrically determined value of $2.4 \cdot 10^{-3}$ M. b: ΔT vs. glucose concentration for a glucose-oxidase Thermal Enzyme Probe. A diffusional limitation with respect to oxygen, not glucose, appears to restrict the response.

wire-wound resistors of the same resistance. An additional contribution to ΔT noise arises from the current rather poor common mode rejection ratio ($CMRR_T \simeq 10$; the $CMRR_T$ is defined as 1/(apparent ΔT) which is measured when the common temperature of both thermistors is raised by 1°C) of the present thermistors, which results in fluctuations in the average bath temperature appearing as fluctuations in ΔT of one-tenth the magnitude. Selection of better sensors and more carefully controlled-flow conditions (e.g. laminar flow in a flowthrough cell) should lead to much better performance, e.g. to about 10^{-6} °C [10,11]. For typical enzyme systems [12] with $\Delta H \simeq -10$ kcal $\cdot$ mol^{-1}, this implies a minimum detectable substrate concentration of the order 10^{-5} M. At this level, the thermal enzyme probe would have the potential for widespread use as a simple, convenient device in many areas of biochemical analysis and research.

Experimental Details

Hexokinase and glucose oxidase were immobilized by first attaching hexokinase or glucose oxidase Enzygel (Boehringer Mannheim GmbH) to the surfaces of both thermistors by means of a special glue [13], which was UHU Kontakt 2000 (UHU-Werk, H.u.M. Fischer GmbH, West Germany) in our experiments. Specifically, 1 ml of glue was first diluted with 2 ml of CH_2Cl_2. A thermistor was carefully cleaned with ethanol and was then dipped twice into the glue solution. After 5—10 s, the tip was carefully rolled in finely powdered Enzygel. After two hours the coated thermistor was ready for use. Two thermistors were activated in this way, with one subsequently deactivated by immersion in a 25% aqueous glutaraldehyde solution for 10 min.

Thermal response experiments were conducted in the apparatus of Fig. 1 using triethanolamine buffer, 0.1 M, pH 7.0 at 35°C. The thermistors were encased in Teflon (model No. 44108 of Yellow Springs Instrument Co., Yellow

Springs, Ohio), with a nominal $3 \cdot 10^4$ Ω resistance, and temperature coefficient $\alpha \simeq 4 \cdot 10^{-2}\,^{\circ}K^{-1}$. The d.c. excitation voltage across each thermistor was about 0.5 V; under our experimental conditions of turbulent, well-stirred flow a short-term noise (rms) of 1 μV was obtained, which corresponds to a short-term ΔT noise of about $5 \cdot 10^{-5}\,^{\circ}C$. The outer bath (Fig. 1) was controlled to $1 \cdot 10^{-3}\,^{\circ}C$ by precision circuitry designed and constructed by J. Williams [14].

The hexokinase activity on a thermistor was measured by dipping it into a 3.5-ml cuvette containing 1.75 ml of 0.1 M triethanolamine buffer, pH 7, 0.1 ml of 1 M glucose, 0.01 ml of glucose-6-phosphate dehydrogenase (Sigma Chemical Co., 210 units/ml), 0.10 ml NADP (40 mg/ml), and 0.50 ml of ATP (8 mg/ml). The cuvette was continuously stirred with a magnetic stirring bar and absorbance increase measured at 340 nm.

Theory

A simple mathematical model (Fig. 3) can be used to estimate the expected steady state ΔT as a function of substrate concentration, and to identify parameters for interpretation of our experimental results. A number of assumptions are made for simplicity. The steady state substrate flux density, J_S, from the bulk solution to a layer of immobilized enzyme is given by:

$$J_S = D_S(C_S - C_S')/x \tag{1}$$

where D_S is the diffusion constant of the substrate, C_s and C_S' are the concentrations in the bulk solution and at the enzyme, respectively, and x is a simple, one-dimensional boundary-layer thickness. The boundary layer is treated as a stationary, unstirred aqueous layer including any trapped water or porous gel surrounding the active enzyme. The heat flux density, J_Q, which is conducted away from the probe, is simply:

$$J_Q = K\Delta T/x \tag{2}$$

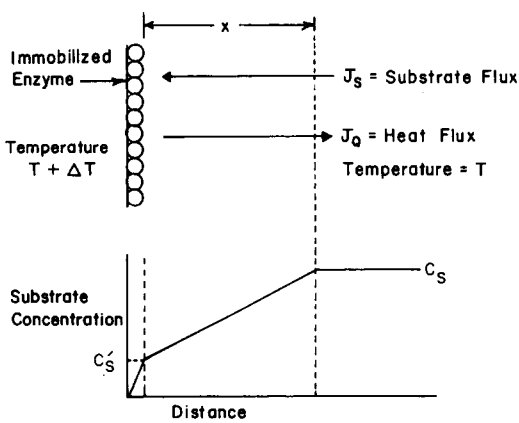

Fig. 3. Simple configuration upon which the mathematical model is based. The bulk substrate concentration is C_S, while the substrate concentration at the immobilized enzyme layer is C_S'. T is the bulk solution temperature, and the enzyme-coated thermal sensor is ΔT above T. A one-dimensional unstirred layer of thickness x (typically 10^{-2} cm) is assumed.

where K is the thermal conductivity of the unstirred layer, and ΔT is the temperature difference between the probe and the bulk solution. The rate of reaction per unit area, V, at the probe surface is assumed to be given by the Michaelis-Menten model:

$$V = V_m C_S'/(K_m + C_S') \tag{3}$$

where in this case V_m is the maximum reaction rate per unit area, and K_m is the concentration of substrate at one-half of V_m. For $C_S << K_m$, Eqn. 3 may be simplified to:

$$V \simeq (V_m/K_m)C_S' \tag{4}$$

Because $J_s = V$, and at steady state $V\Delta H = J_Q$, where ΔH is the sum of enthalpy changes for all the reactions localized near the enzyme, we find:

$$\Delta T \simeq \left\{\left(\frac{(D_S/x)(V_m/K_m)}{(D_S/x) + (V_m/K_m)}\right)\left(\frac{x\Delta H}{K}\right)\right\} C_S \tag{5}$$

Often the reaction is diffusion-limited because $V_m/K_m >> D_s/x$, and in this case

$$\Delta T \simeq \left\{\frac{D_S \Delta H}{K}\right\} C_S \tag{6}$$

and ΔT is proportional to the concentration of the substrate in the bulk solution. This form shows explicitly that in the linear region where $C_S << K_m$, apparent, we can model the enzyme layer as a perfect sink for substrate which is connected in series with an effective permeability, D_S/x. Significantly, this simple model shows that when its assumptions are realized, the reaction in the linear region is diffusion-limited, and neither the enzyme concentration at the surface nor the boundary layer thickness affects the steady state ΔT. When C_S is greater than K_m, however, the saturation response is dependent on the amount of enzyme per unit area.

This model also predicts the time response caused by substrate diffusion to be of order $\tau_D \simeq x^2/4D_S \simeq 5$ s for a well-stirred system in which $x \simeq 10^{-2}$ cm. Because our thermistors (2-mm diameter) immersed in water typically have thermal time constants of 1—2 s, it can be seen that diffusional limitations control the fundamental response time observed previously [4]. However, in our present apparatus (Fig. 1) the time constant for heat exchange between the inner and outer bath is approximately 40 s, which dominated the Thermal Enzyme Probe transient response because of the common mode response (with respect to ambient temperature) of the thermistor pair when we added a sample or blank at a slightly different temperature [5]. Thermal sensors with a significantly better common mode rejection ratio could be used to minimize this effect, so that the time response will be fundamentally limited by τ_D. By substituting typical values into Eqn 6, we can estimate the expected ΔT for a given value of C_S. For example, if the sample were glucose in blood, a ten-fold diluted sample might be $1.1 \cdot 10^{-3}$ M; the diffusion constant of glucose in water at 25°C is $D_S = 6.7 \cdot 10^{-6}$ cm$^2 \cdot$ s^{-1}, the thermal conductivity of water at 25°C

is $1.3 \cdot 10^{-3}$ cal $\cdot$ s^{-1} $\cdot$ cm^{-1} $\cdot$ °C^{-1}, and the enthalpy of the reaction catalyzed by hexokinase in the presence of adenosine triphosphate is −6.6 kcal $\cdot$ mol^{-1} [9] (the heat of protonization in triethanolamine buffer is about 8.2 kcal $\cdot$ mol^{-1}), so that ΔH = −14.8 kcal $\cdot$ mol^{-1}. Thus, the calculated steady state ΔT is $8.4 \cdot 10^{-5}$°C. Although small, a ΔT of this size is measurable by using available thermistors in a sensitive bridge circuit [5,10,11].

Acknowledgements

This work received support from Boehringer Mannheim, GmbH, Tutzing, West Germany and the MIT Undergraduate Research Opportunities Program. We gratefully acknowledge the technical assistance of Rita Ouellet.

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