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Kinetics of enzyme–substrate reactions in single living cells

The beam-splitter-supplemented CHANCE–LEGALLAIS microfluorimeter[1,2], which allows the recording of pyridine nucleotide fluorescence during microelectrophoretic addition of substrate (*e.g.*, Glc-6-*P* (glucose 6-phosphate)), is suitable for inspecting the kinetic properties of glycolytic enzymes in single living cells[3]. One aim of such a study is to determine to what extent these enzymes follow or deviate from first-order Michaelis kinetics[4,5,6] in the intact cell, since most regulatory enzymes are known to catalyze reactions that are kinetically of second order, or higher, with respect to substrates and regulating metabolites.

Provided the added Glc-6-*P* is metabolized along the Embden–Meyerhof pathway[7], the fluorescence pulse resulting from its rapid addition to ascites cells in culture (EL2 cells) represents the turnover of glycolytic substrate at the catalytic sites of enzymes in the above sequence, with DPN^+ reduction at the glyceraldehydephosphate dehydrogenase step acting as an indicator of the overall glycolytic flux[8,9]. In fact the fluorescence pulse closely resembles the kinetic curve of formation and disappearance of the enzyme–substrate complex, as described in transient-state relationships[4]. Such curves exhibit three significant portions corresponding to the rapid formation of the complex, a steady state and the slower disappearance of the complex as the substrate concentration is exhausted[4]. The reason the extramitochondrial fluorescence curve closely duplicates the kinetics of the enzyme–substrate complex is that the DPNH formed is readily oxidized by pyruvate and lactate dehydrogenase[10] rather than being accumulated. This fluorescence pulse can be analyzed[4] in terms of peak fluorescence response, $(p_1)_{max}$, whole area of the pulse, $\int_0^\infty p_1 \, dt$, half-time of fluorescence rise and decay, $t_{1/2\,off}$, *etc.* which are related to the actual amount of substrate added. The actual amount of substrate/μ^3 can be varied by regulating a microelectrophoretic current of constant duration (1 sec) or applying a uniform current to cells of varying sizes.

Using an EL2 cell, increasing doses of substrate can be added consecutively to the same cell (Fig. 1). When the microelectrophoretic current is increased from 1 × to

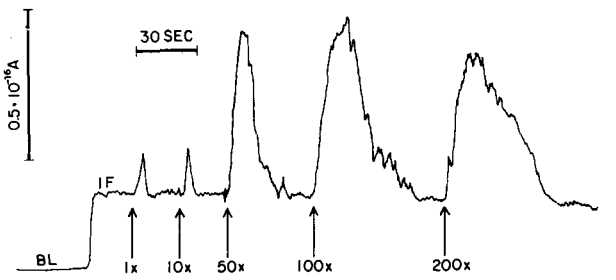

Fig. 1. Microfluorimetric recording of the fluorescence changes in the extramitochondrial (nuclear) region of an EL2 cell in response to repeated microelectrophoretic additions of Glc-6-*P*, in gradually increasing amounts (see arrows). 1 × corresponds to a microelectrophoretic current of $1 \cdot 10^{-10}$ A. The time scale proceeds from left to right. BL, baseline; IF, initial fluorescence. Expt. Expt. No. EK SBS68/4.

10 ×, there is practically no change in the fluorescence response; however, a very high and rapidly decaying pulse is obtained when the dose is increased to 50 ×. Further increases of the current to 100 × and 200 × result in broadening of the pulse (longer $t_{1/2\ off}$), but $(p_1)_{max}$ remains unchanged at first and then decreases.

There should be proportionality between the integrated fluorescence pulse ($\int_0^\infty p_1\,dt$) and the concentration, x_0, of added substrate, if first-order Michaelis kinetics are operative[4,5], according to the relation $k_3 = x_0/\int_0^\infty p_1\,dt$ (k_3 = velocity constant of the decomposition of the enzyme–substrate complex). However, in EL2 cells (Fig. 2), when a sufficiently wide range of substrate concentration is used, including values as low as can be accurately measured, a sigmoidal kinetic behavior is observed: the rate of increase in $\int_0^\infty p_1\,dt$ is maximum at intermediate concentrations and minimum at both extremes. $\int_0^\infty p_1\,dt$ changes very little when the microelectrophoretic current is increased from 1.10^{-10} to 1.10^{-9} A, but increases by 10-fold, *i.e.*, at a faster rate than the current, from $1 \cdot 10^{-9}$ to $5 \cdot 10^{-9}$ A. At higher substrate concentrations, there seems

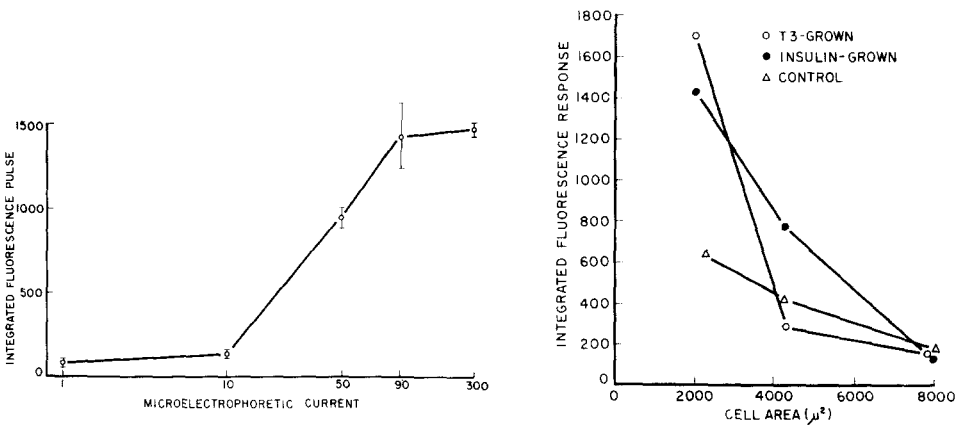

Fig. 2. The relationship between the integrated fluorescence pulse (whole area of the pulse = $\int_0^\infty p_1 dt$) and the microelectrophoretic current (as a measure of the added Glc-6-*P*) in EL2 cells. $P_1 dt$ is expressed in arbitrary units. A microelectrophoretic current of 1 corresponds to $1 \cdot 10^{-10}$ A. The current is plotted on a logarithmic scale. The standard error of the mean was calculated according to S.E. = $\sqrt{\Sigma d^2/n(n-1)}$.

Fig. 3. The change in the integrated fluorescence response in radiation-produced giant cells (EL2G) maintained under various conditions when the microelectrophoretic current is kept constant and the size of the cell varied. The integrated fluorescence response, ($\int_0^\infty p_1 dt$) is expressed in arbitrary units. For each cell the product of the major and minor axes is taken as an expression of the cell area. Since the cells investigated were of approximately the same thickness, the differences in cell area were roughly proportional to the differences in cell volume.

to be a first-order relationship between $\int_0^\infty p_1\,dt$ and x_0, since the former is twice as large when the latter is increased from $5 \cdot 10^{-9}$ to $1 \cdot 10^{-8}$ A. There is practically no further change over $1 \cdot 10^{-8}$ A.

Similar properties are observed in radiation-produced giant cells, EL2G cells[3]. Upon microelectrophoretic addition of Glc-6-*P* to these cells, the substrate concentration throughout the cell equilibrates quite evenly from the first second of addition (as confirmed by visual observation of the fluorescence), while the fluorescence pulse has a half-time of about 20 sec. Therefore, if the microelectrophoretic current is kept

constant, the amount of substrate/μ^3 is largest in the smallest cells (Fig. 3). The area of the whole pulse, $\int_0^\infty p_1 \, dt$, does not change linearly with substrate concentration especially in triiodothyronine (T_3)-grown EL2G, but rather increases more rapidly, than concentration in the smallest cells.

The sigmoidal kinetics observed are consistent with the properties of allosteric enzymes[6,11] (although there could be other interpretations, *e.g.* relaxation effects[6,12] in enzyme transitions from active to inactive conformation). Allosteric enzymes can act as biological amplifiers[11] by accelerating the reaction velocity faster than the rate of increase in substrate concentration. These enzymes are relatively inefficient catalysts at very low substrate concentrations, as more than one molecule of substrate must be bound before the enzyme becomes catalytically active; however, at higher concentrations of substrate their activity increases as a power function of the concentration/dissociation constant of the enzyme–substrate complex[10], depending upon the number of subunits[6,11].

The allosteric properties of an enzyme are usually more labile than its catalytic properties[10]. Thus, enzymes which could exhibit simple first-order Michaelis kinetics in purified preparations may have allosteric properties in the intact cell[10]. The ultimate aim of the above experiments should be to determine the activity and possibly, from the kinetic properties, the configuration[6,9] (polymerization, number of subunits, *etc.*) of enzymes in the various compartments of single living cells. While the turnover of glycolytic substrate can be estimated from the fluorescence pulse, the kinetics of formation of the enzyme–substrate complex before steady-state level is reached can be studied only from the very rapidly increasing part of the ascending branch of the pulse[4]. Thus, in the future, a microfluorimeter with a shorter response time[1,2] will be essential.

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