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EFFICIENCY OF TWO ENZYMES IMMOBILIZED TO THE SAME SURFACE AND ACTING IN SEQUENCE

II. Relative Efficiencies of a Two-Enzyme Sequential Reaction in Either the Soluble or the Immobilized State

STANLEY Y. SHIMIZU[1] and HOWARD M. LENHOFF

Department of Developmental and Cell Biology and Molecular Biology and Biochemistry
University of California, Irvine, California 92717

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Phosphoglucomutase and glucose-6-phosphate dehydrogenase were immobilized to *s*-triazine trichloride activated cellulose to determine if perparations having two enzymes immobilized to the same surface could carry out the sequential reaction, glucose-1-P to glucose-6-P, glucose-6-P to 6-phosphogluconate more efficiently than preparations in which the enzymes were immobilized to separate particles or free in solution. The results of experiments examining the effect of dilution on the sequential rate indicate greater efficiency by enzymes immobilized to the same surface.

INTRODUCTION

The advantage of systems in which enzymes are close to one another rather than dispersed in solution lies in the potential for controlled flow of intermediates from one enzyme to the next. Examples of such systems include the sequential enzymes described in this paper, enzyme complexes (1–5), and those enzymes close to each other in natural membranes (6–9). One rationale for such efficient flows of substrates ascribes a possible role of the Nernst unstirred layer in which the intermediate may be trapped (10). Another involves concepts of substrate chaneling and compartmentalization (11,12).

Both views deal with the fixed proximity of enzymes acting in sequence to each other and the ability of the second enzyme in the sequence to utilize the intermediate (i.e., the product of the first enzyme) as fast as it is formed without allowing significant amounts to escape into the ambient solution.

[1] Present address: Tufts University, School of Medicine, Department of Biochemistry and Pharmacology, 136 Harrison Ave, Boston, Massachusetts 02111.

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We develop below our views on the various experimental factors that may allow the Nernst layer surrounding immobilized sequential enzymes to increase the efficiency of the overall rate of reaction of the two enzymes over that which would occur if the two enzymes were free in solution.

In this paper we describe an experimental system to test the influence of the Nernst layer in increasing the efficiency of the immobilized sequential enzyme system. This system is based on the premise that as preparations in which phosphoglucomutase and glucose-6-P dehydrogenase are not fixed in close proximity are diluted, that is, as the molecules of phosphoglucomutase and glucose-6-P dehydrognase move further and further apart, the overall rate of reaction (i.e., the rate of the two enzymes acting in sequence using glucose-1-P as the initial substrate) will no longer be the same percentage of the maximum rate or phosphoglucomutase rate (i.e., the rate of phosphoglucomutase measured in the presence of excess glucose-6-P dehydrogenase) as it was at higher concentrations of phosphoglucomutase and glucose-6-P dehydrogenase. Furthermore, we proposed that in preparations having both enzymes immobilized to the same surface and hence at a fixed distance apart, the overall rate will be a constant percent of the phosphoglucomutase rate regardless of the dilution used. The results presented herein conform to these views.

MATERIALS AND METHODS

All materials used in this section, as well as the methods for the preparation of the *s*-triazine trichloride cellulose, for binding of enzymes to the carrier, and for assaying the soluble and immobilized enzymes are described in the previous paper (13).

Basic Experiments for Determining Efficiency for Sequential Enzyme Reactions

We measured the rates of sequential reactions with the following four types of preparations: (I) both phosphoglucomutase and glucose-6-P dehydrogenase soluble, (II) immobilized phosphoglucomutase and soluble glucose-6-P dehydrogenase, (III) immobilized phosphoglucomutase and immobilized glucose-6-P dehydrogenase on separate particles, and (IV) immobilized phosphoglucomutase and glucose-6-P dehydrogenase on the same particles. The following types of experiments were performed. For each condition two or more concentrations of phosphoglucomutase ranging from 2 to 145×10^{-4} U/ml were selected. For each concentration of

phosphoglucomutase held constant, the amount of glucose-6-P dehydrogenase was varied. Each preparation was assayed (*a*) for its overall rate (hereafter called the *sequential rate*), (*b*) for the amount of glucose-6-P dehydrogenase activity, and (*c*) for the amount of phosphoglucomutase activity alone. This latter value was obtained by adding sufficient soluble glucose-6-P dehydrogenase to the preparation to be assayed so that the phosphoglucomutase would be operating at its maximal possible rate. Using the values obtained from (*a*) and (*c*), a number, which we might have designated the percent $V_{\text{max PGM}}$ was determined. The percent $V_{\text{max PGM}}$ of each preparation was calculated by dividing the sequential rate by the maximum rate of the phosphoglucomutase and multiplying by 100. This value is an expression of the percent V_{max} of the preparation, because it compares the actual rate of the sequential enzyme preparations to that which is experimentally possible in an excess of the second enzyme (glucose-6-P dehydrogenase), that is, if the phosphoglucomutase is allowed to react at its maximum rate. Although the procedures for measuring the rates of preparations I, II, III, and IV were similar, the procedures for determining the amount of each type of preparation were somewhat different. These are described next.

Determining the Amount of Glucose-6-P Dehydrogenase Needed to Follow a Fixed Level of Immobilized Phosphoglucomutase Acting at its Maximum Rate

A fixed amount of immobilized phosphoglucomutase was placed in a fritted glass disk crucible and suspended in 10 ml of assay solution. The maximum rate of the reaction of the phosphoglucomutase was measured by adding 20 μl containing approximately 0.2 U of soluble glucose-6-P dehydrogenase to the 10 ml of assay solution. The assay solution was discarded and the particles were washed with distilled water to remove any soluble glucose-6-P dehydrogenase that may have adsorbed to them. To the crucible was added 11 ml of assay solution and soluble glucose-6-P dehydrogenase; 1 ml was then removed to assay the activity of the soluble glucose-6-P dehydrogenase. The sequential rate and the rate of the glucose-6-P dehydrogenase for the preparation of soluble glucose-6-P dehydrogenase and immobilized phosphoglucomutase was then measured as described elsewhere (13). After the assays were completed, the assay solution was removed, more soluble glucose-6-P dehydrogenase was added, and the sequential rate and the rate of the glucose-6-P dehydrogenase were measured again. This procedure was repeated with increasing amounts of glucose-6-P dehydrogenase until the sequential rate equaled the maximum rate of the phosphoglucomutase as determined with soluble glucose-6-P

dehydrogenase. This entire procedure was repeated for several different concentrations of immobilized phosphoglucomutase.

Determining the Amount of Immobilized Glucose-6-P Dehydrogenase Needed To Follow a Fixed Level of Immobilized Phosphoglucomutase

These measurements were made as described above except for the following steps: (*a*) immobilized glucose-6-P dehydrogenase was used rather than soluble glucose-6-P dehydrogenase and (*b*) the volume of the suspension of immobilized phosphoglucomutase in assay solution placed in the fritted glass crucible was 10 ml rather than 11 ml. The 10-ml suspension was then treated exactly as described in the above paragraph.

Determining the Amount of Glucose-6-P Dehydrogenase Needed to Follow a Fixed Level of Phosphoglucomutase Acting at its Maximum Rate When Both Enzymes are Immobilized to the Same Particles

Preparations of glucose-6-P dehydrogenase and phosphoglucomutase immobilized to the same particles were made using attachment solutions in which varying amounts of soluble glucose-6-P dehydrogenase were added to fixed amounts of soluble phosphoglucomutase. This procedure resulted in preparations having different amounts of phosphoglucomutase and glucose-6-P dehydrogenase activities. Because this ratio of the bound enzymes to each other did not always correlate directly with the ratio of the enzymes in the attachment solutions, it was necessary to make a wide range of enzyme mixtures in order to obtain a few preparations of particles having ratios of bound enzymes that could be used in the experiments described below in Fig. 4. The actual assays were carried out in the same manner as described above in the case of glucose-6-P dehydrogenase and phosphoglucomutase immobilized to separate particles.

RESULTS AND DISCUSSION

Comparison of the Percent Efficiency of the Four Preparations

With both enzymes soluble (Fig. 1) it can be seen that as the amount of phosphoglucomutase in the preparations is lowered, the relative amount of glucose-6-P dehydrogenase needed to maintain maximal efficiency is higher. This effect did not occur when the phosphoglucomutase levels were 9.75×10^{-4} U/ml or higher. With phosphoglucomutase immobilized and

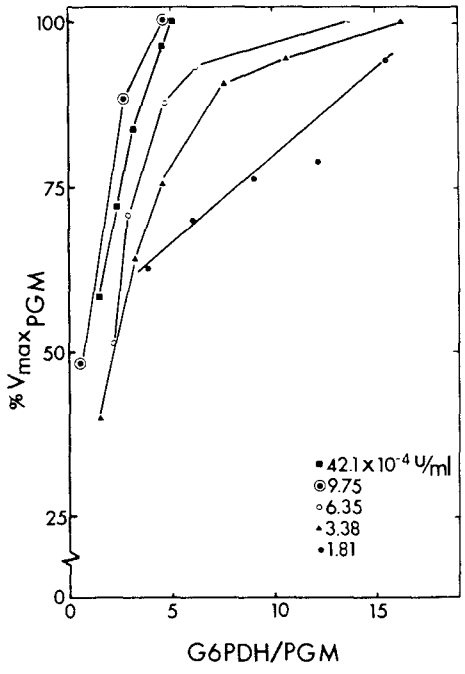

FIG. 1. The percent $V_{max\,PGM}$ for different ratios of glucose-6-P dehydrogenase activity to phosphoglucomutase activity for preparations of soluble phosphoglucomutase and soluble glucose-6-P dehydrogenase. These measurements were done for various levels of soluble phosphoglucomutase. The preparation and description of the assays used is given in the text under Materials and Methods.

glucose-6-P dehydrogenase soluble (Fig. 2), and with both enzymes immobilized to separate particles (Fig. 3), the same general trend was observed.

On the other hand, when both enzymes were immobilized to the same carrier (Fig. 4), the amount of glucose-6-P dehydrogenase to give maximal efficiency was the same regardless of the amount of phosphoglucomutase in each preparation. Hence, it will be noticed in Fig. 4 that there is no family of curves, but rather all the experimental points appear to fit a common line. Examination of Figs. 1–4 shows that at levels of phosphoglucomutase greater than 42.1×10^{-4} U/ml, the slope of the line describing the percent $V_{max\,PGM}$ (Table 1) was similar (19.8, 20.67, 22.0, and 15.6, respectively). With preparations I, II, and III, however, the slope became smaller as the level of the phosphoglucomutase decreased.

For preparations of phosphoglucomutase and glucose-6-P dehydrogenase immobilized to the same particles, the slopes were 15.6

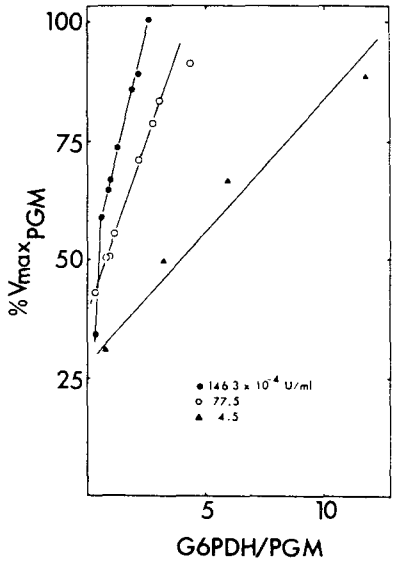

FIG. 2. The percent $V_{max\,PGM}$ for different ratios of glucose-6-P dehydrogenase activity to phosphoglucomutase activity for preparations of immobilized phosphoglucomutase and soluble glucose-6-P dehydrogenase. Various levels of immobilized phosphoglucomutase were used.

regardless of the level of phosphoglucomutase used. Note, for example, that the solid circles refer to preparations having 1.99 to 10.0×10^{-4} U/ml, the open circles 10 to 43.7×10^{-4} U/ml, and the solid triangles 55.0 to 175.0×10^{-4} U/ml. Because the preparations having both enzymes immobilized to the same carrier always gave a slope of about 15.6, and because the maximum slope attained with any of the preparations (Table 1) was about 20, we assumed that a slope of 15 to 20 indicated high efficiency of the rate of reaction of the sequential enzyme preparations. Hence, preparations giving slopes significantly below this range can be considered less efficient.

Effect of the Level of Phosphoglucomutase on the Ratio of Glucose-6-P Dehydrogenase to Phosphoglucomutase to Give 50% $V_{max\,PGM}$

Another way to evaluate the effectiveness of a phosphoglucomutase–glucose-6-P dehydrogenase preparation is to compare the ratio of the two enzymes giving 50% of the V_{max} of the phosphoglucomutase (50% $V_{max\,PGM}$). These values were determined for preparations I, III, and IV at a number of different concentrations at phosphoglucomutase and are sum-

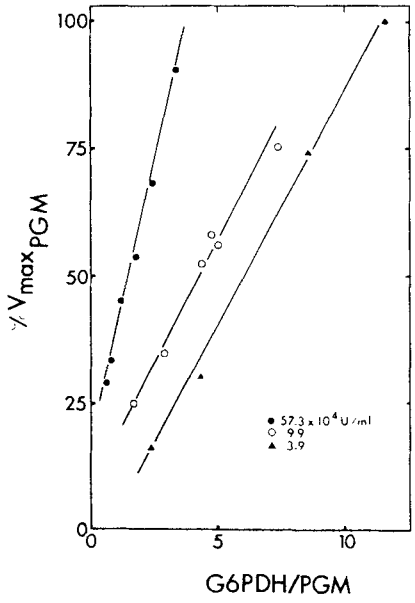

FIG. 3. The percent $V_{max\,PGM}$ for different ratios of glucose-6-P dehydrogenase activity to phosphoglucomutase activity for preparations of immobilized phosphoglucomutase and immobilized glucose-6-P dehydrogenase. Various levels of immobilized phosphoglucomutase were used.

marized in Fig. 5. At phosphoglucomutase concentrations greater than 15×10^{-4} U/ml, all preparations gave 50% $V_{max\,PGM}$ for glucose-6-P dehydrogenase to phosphoglucomutase ratios of around 1.2 to 1. As the level of phosphoglucomutase was decreased in preparations I and III, the ratio of glucose-6-P dehydrogenase to phosphoglucomutase required for 50% $V_{max\,PGM}$ increased. At the lowest amount of phosphoglucomutase examined, 4×10^{-4} U/ml, the ratio of glucose-6-P dehydrogenase to phosphoglucomutase increased to 2.5 and 6.5 for preparations I and III, respectively. Preparation IV, however, remained relatively unchanged throughout this range of concentrations. Hence, we can conclude that the efficiency of the preparations in which both enzymes are immobilized to the same carrier remains maximum and unchanged regardless of the concentration of the phosphoglucomutase used.

These results indicate that the sequential reaction of preparations having both enzymes bound to the same surface are unaffected by the lowering of the number of particles per ml in solution. Similar results have been seen upon dilution of multienzyme complexes (14,15). At low levels of

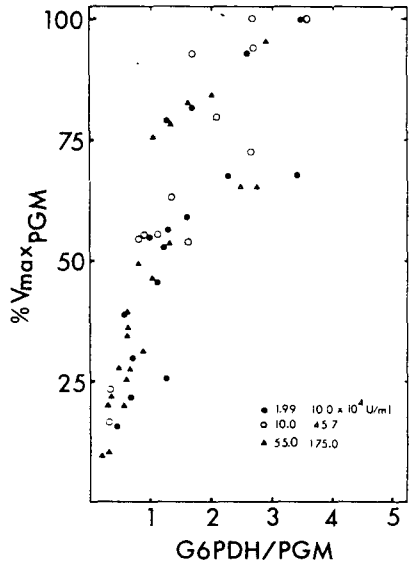

FIG. 4. The percent $V_{max\,PGM}$ for different ratios of glucose-6-P dehydrogenase activity to phosphoglucomutase activity for preparations of phosphoglucomutase and glucose-6-P dehydrogenase immobilized the same surface. Various levels of immobilized phosphoglucomutase were used. Description of the preparation of the immobilized enzymes is given in the text under Materials and Methods.

the first enzyme, the placing of the two enzymes on the same surface allows the overall reaction to proceed in a more efficient manner than if the same two enzymes were free in solution.

GENERAL DISCUSSION AND THEORY

The same factors, such as enzyme purity, binding methods, and nature of carrier, that affect the activity of a single enzyme immobilized to a carrier (16) take on even greater importance with two enzymes acting in sequence and immobilized to the same carrier. Crucial in these-sequential enzyme reactions is the presence of the Nernst layer (17–23). Recall that the Nernst layer, also called the unstirred diffusion layer, is that liquid layer which surrounds surfaces (Fig. 6). In this layer there is relatively less fluid movement and, hence, less mixing of substrates, coenzymes, and intermediates than in the ambient bulk solution. A model of a two-enzyme sequential reaction covalently bound to an uncharged nonporous matrix is presented in Fig. 6. The Nernst layer is the region between the surface of the particle

TABLE 1. **Slopes of the Linear Portion for the Curves Given in Fig. 1–4**[a]

System	Phosphoglucomutase (U/ml × 10^4)	Slope: % V_{maxPGM} (Units G6PDH/Units PGM)
I	42.1	16.4
	9.75	19.84
	3.38	10.6
	1.81	2.64
II	146.26	20.67
	79.47	14.8
	4.51	7.09
III	57.26	22.0
	9.87	9.67
	3.95	9.18
IV	1.99–10.0	15.5
	10.0–45.7	15.6
	55.0–175.0	15.6

[a]The slopes for the line for the linear portion of the curve given by plotting the percent $V_{max\,PGM}$ for varying ratios of glucose-6-P dehydrogenase activity to phosphoglucomutase activity for fixed levels of phosphoglucomutase. System I refers to the data from preparations of soluble phosphoglucomutase and soluble glucose-6-P dehydrogenase; System II, immobilized phosphoglucomutase and soluble glucose-6-P dehydrogenase; System III, phosphoglucomutase and glucose-6-P dehydrogenase immobilized to separate particles; and System IV, phosphoglucomutase and glucose-6-P dehydrogenase immobilized to the same particle.

(where $x = 0$) and extends a distance, $x = L$, into the surrounding solution. In the region between $x = 0$ and $x = L$ movement of substrate and product is diffusion controlled. In the space beyond $x = L$, solute is homogeneously distributed and is known as the ambient bulk solution.

The Nernst layer takes on particular importance when dealing with coupled enzymes immobilized on a surface, because the product of the first enzyme may become entrapped in this zone, allowing the concentration of the intermediate to be much higher in the vicinity of the second enzyme than in the ambient solution (24). As a consequence, the rate of reaction of the immobilized enzyme will be faster than that of an enzyme in the ambient solution. One would expect, therefore, that immobilization of the two enzymes, acting in sequence to the same carrier, would lead to a more efficient reaction that would occur if they were both immobilized to separate carriers (24–37). To take advantage of the Nernst layer, the distance between the two active enzyme species should not be greater than the thickness of that layer, since the likelihood of the intermediate escaping into the ambient solution becomes greater as the distance between the enzymes increases. At best the six factors (enzyme purity and specific activity, binding

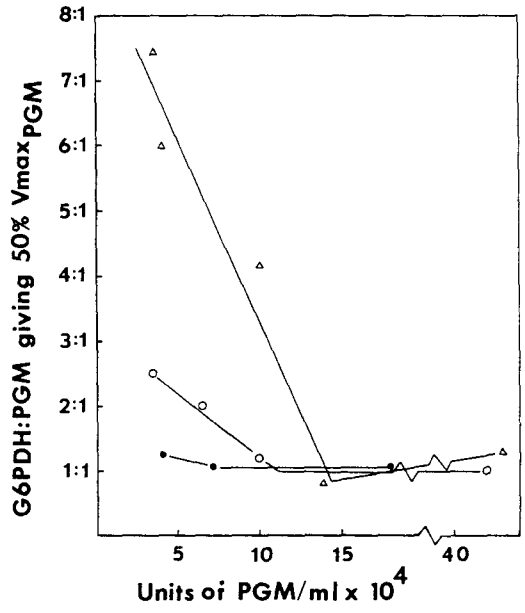

FIG. 5. The ratio of glucose-6-P dehydrogenase activity to phosphoglucomutase activity required to give 50% $V_{max\,PGM}$ for various levels of phosphoglucomutase using preparations I, III, and IV.

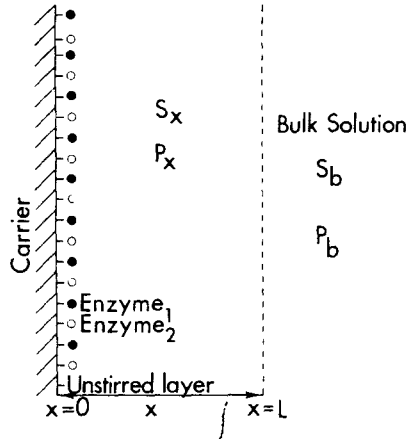

FIG. 6. Model of two enzymes covalently bound to a nonporous matrix. The unstirred or Nernst layer is of thickness L. S_x and P_x are the concentrations of the substrate and product at some point, x, in the unstirred layer. S_b and P_b are the concentrations of the substrate and product in the bulk solution.

methods, K_m, carrier, spatial arrangements, and reactor design) that we have discussed elsewhere have to be considered (38).

In addition to the six factors, an additional set of criteria have to be kept in mind in order to evaluate the overall efficiency of the sequential enzymes bound to a single carrier as compared to the efficiencies of the same two enzymes in the soluble state, bound to separate carriers, or a mixture having one enzyme not bound and the other bound to a carrier. We have devised four criteria, one described herein, and three described in the following paper in this series (39).

Here we consider the optimal ratio of the second enzyme (G6PDH) to the first (phosphoglucomutase) that will allow the rate of reaction of the sequential system to equal the rate of the phosphoglucomutase acting alone. For sequential enzymes to act efficiently, it is essential that the second enzyme follow first-order kinetics so that the intermediate is utilized as fast as it is formed and stays at relatively low steady-state levels. Thus, it would appear that larger quantities of the second enzyme would be needed to surround the first one in order to utilize the intermediate as soon as it is released. It is important to use the minimum amount of the second enzyme to give the maximal rate in order that each covalent binding site for enzyme on the carrier be occupied by enzyme functioning at its maximal capacity.

It is not simply the ratio alone of the glucose-6-P dehydrogenase to phosphoglucomutase that will determine the efficiency of the sequential reaction because the number of units of phosphoglucomutase per unit volume takes on different meanings depending upon the type of system used. For example, in any situation in which phosphoglucomutase and glucose-6-P dehydrogenase are not bound to the same particle, one would expect the ratio of glucose-6-P dehydrogenase to phosphoglucomutase giving maximal efficiency to increase when phosphoglucomutase is of sufficiently low concentration. That is, with small amounts of phosphoglucomutase, more glucose-6-P dehydrogenase would be needed to maintain the density of glucose-6-P dehydrogenase surrounding phosphoglucomutase to give the highest efficiency. On the other hand, in a system in which phosphoglucomutase and glucose-6-P dehydrogenase are immobilized to the same carrier, an efficient preset density of glucose-6-P dehydrogenase surrounding phosphoglucomutase will always be the same regardless of the amount of phosphoglucomutase used in the reaction vessel. Thus, the efficiency given by that combination would always be the same. This line of reasoning is supported by the data described in Figs. 1–5 and already discussed above under Results and Discussion.

In the following paper in this series (39), we discuss and show how (*a*) the changes in lag time, (*b*) the concentrations of the intermediate, glucose-6-P, and (*c*) the more efficient use of glucose-1-P by phosphoglucomutase

immobilized with glucose-6-P dehydrogenase on the same surface, all reflect the efficiency of the sequential enzyme system.

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