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KINETICS OF WATER-INSOLUBLE PHOSPHOGLYCERATE MUTASE

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SUMMARY

1. An insoluble form of phosphoglycerate mutase (2,3-diphospho-D-glycerate:2-phospho-D-glycerate phosphotransferase, EC 2.7.5.3) from rabbit muscle was prepared by mechanically immobilizing the crystalline enzyme within a water-insoluble carrier of highly cross-linked polyacrylamide. This procedure has previously been devised in our laboratory and is known to involve no covalent bonds between enzyme and carrier.

2. The kinetic behavior of the soluble and insoluble forms of this enzyme were identical in many respects, *i.e.* upon variation of the enzyme concentration, substrate concentration and temperature. Thus, their Michaelis constants, activation energies and susceptibilities to substrate inhibition were the same.

3. In contrast, the pH optimum of activity of the insoluble form of mutase was one whole unit lower than that of the water-soluble form, although the carrier of the insoluble enzyme was electrostatically neutral.

4. Insoluble mutase was also more sensitive to activation by 2,3-diphosphoglycerate than the soluble form.

5. A comparison between the insoluble forms of phosphoglycerate mutase and enolase, prepared by the same procedure, shows that differences between soluble and insoluble forms of these enzymes depend more on the nature of the enzyme than on that of the insoluble carrier.

INTRODUCTION

Water-insoluble forms of enzymes prepared from their soluble state have been the subject of markedly increased interest during recent years. Easy removal of insoluble enzymes from their reaction mixture without denaturing conditions provides great technical advantages. From a kinetic point of view, water-insoluble forms of many purified enzymes are more representative of their naturally occurring forms than the corresponding soluble products, because many enzymes actually fulfill their catalytic missions *in vivo* in a water-insoluble state while bound to insoluble cell material.

Soluble enzymes may be converted into insoluble forms by transforming them into insoluble derivatives involving substitution of certain chemical groups of the enzyme[1–3], or by attaching them to insoluble carriers by means of covalent bonds[4–9].

Insoluble enzymes may also be obtained by a considerably milder procedure[10-12] which does not require substitution of chemical groups of the native enzymes by covalent bonds; this method is, therefore, more generally applicable to practically all enzymes as well as to other macromolecular substances with biological activity, such as antigens[13,14].

The latter procedure consists in embedding the soluble enzymes in a highly cross-linked, insoluble synthetic polymer, *i.e.* polyacrylamide, whereby the enzymes become attached to the insoluble carrier through mechanical restriction rather than through covalent bonds. Insoluble forms of trypsin, chymotrypsin, papain, α- and β-amylases, ribonuclease and aldolase have been prepared by this method[10]. Work with ^{14}C-labeled, crystalline aldolase, rendered insoluble in the same manner, has suggested that only that portion of the embedded aldolase which is located at or near the surface of the carrier particle displays enzyme activity[11]. Once the enzyme has been embedded in the synthetic polymer, it can no longer be separated from its insoluble carrier, even after treatment in a powerful ultrasonic field. Insoluble enolase has been compared to the soluble form of the same enzyme, and the two forms of enolase have been shown to behave in most respects in an identical manner, with the exception of the temperature profile and of the inhibition by an excess of magnesium[12].

The present work deals with the kinetic behavior of crystalline phosphoglycerate mutase (2,3-diphospho-D-glycerate:2-phospho-D-glycerate phosphotransferase, EC 2.7.5.3) from rabbit muscle, rendered insoluble by the same procedure. The mutase was chosen for these studies because (1) it is derived from the same tissue as two of the enzymes previously investigated, namely aldolase and enolase; (2) it is metabolically related to these two enzymes and (3) it permits the study of the influence of a coenzyme, 2,3-diphosphoglycerate, on the activity of the insoluble form of the enzyme. The knowledge of the kinetic behavior of the individual enzymes will permit the study of water-insoluble multi-enzyme systems.

MATERIALS AND METHODS

Source of enzymes

Crystalline phosphoglycerate mutase from rabbit muscle was obtained commercially (Sigma Chemical Co. and Calbiochem.) as a suspension in concentrated $(NH_4)_2SO_4$ solution. For kinetic measurements of the soluble form of the enzyme, fresh dilutions from this suspension were made each day.

Enolase, needed in rather large quantities for the enzyme assay, was purchased (Sigma Chemical Co.) as a lyophilized powder prepared from yeast. This source, rather than the crystalline enzyme from rabbit muscle, was chosen for economical reasons. The enolase preparation used was found to be free from phosphoglycerate mutase, since incubation of 3-phosphoglycerate with enolase in concentrations and under conditions employed in the mutase assay (see below), but without added mutase, yielded no change in optical density, as was the case in the absence of enolase.

Source of substrate

The barium salt of 3-phosphoglyceric acid (Sigma Chemical Co.) was transformed into the corresponding sodium salt by the addition of sodium sulfate in an excess of 5% over the theoretical amount and removal of the $BaSO_4$ formed by centrifugation.

A fresh solution of the sodium salt was obtained each day. This substrate preparation was found to be free from its isomer, 2-phosphoglycerate, for the same reasons that the enolase preparation was found to be free from phosphoglycerate mutase (see above).

Source of coenzyme

The coenzyme of phosphoglycerate mutase, 2,3-diphosphoglycerate, was purchased (Sigma Chem. Co.) and used as its pentacyclohexylammonium salt.

Preparation of the insoluble mutase

The original method called Procedure II for enzymes (Procedure I is best suited for antigens) was used[10]. It is identical with that employed for the study of the kinetics of water-insoluble enolase[12]. Twenty batches of insoluble mutase, each starting with 2 mg of crystalline enzyme (0.2-ml commercial mutase suspension) were prepared simultaneously, and the insoluble polymers from all batches were combined, washed 4 to 6 times as described earlier[11,12] and filled up with water to a total volume ranging from 50 to 75 ml.

Enzyme assay

The "coupled assay method" described by GRISOLIA[15] was used. Conditions of the assay were exactly the same for both the soluble and insoluble forms of the enzyme. Incubation with both forms of the mutase was carried out under mechanical stirring of the reaction mixture.

The concentration of substrate (3-phosphoglycerate, sodium salt, 16.7 mM), Na_2SO_4 (the excess remaining after the transformation of the barium salt of substrate into the sodium salt, 0.8 mM), enolase (50 μg/ml), $MgCl_2$ (3.33 mM) in the digestion mixture, as well as the pH (6.7, 0.05 M imidazole buffer) and the temperature (26.8°) were the same throughout, except where specifically stated otherwise. The concentration of mutase was 1 μg of the soluble enzyme per ml of digest or that quantity of insoluble polymer per ml of digest which had been formed in the presence of 50–80 μg of soluble mutase, usually 1 ml of the standard suspension of insoluble mutase in 10 ml of digest.

The incubation time ranged from 150 sec (for the soluble enzyme under optimum conditions) to 50 min for the study of the insoluble enzyme. The duration of incubation was selected in such a manner that the increase in absorbance due to the enzyme reaction did not exceed 0.5 but was at least 0.05, whenever possible. Six 3-ml aliquot samples were withdrawn at regular time intervals during this period (including one at zero time) to measure the progress of the reaction. Enzyme action was arrested by the addition of an equal volume of 10% perchloric acid to the aliquot sample of the digestion mixture. After about 10 min, the sample was neutralized by the addition of 3 ml of a solution which contained NaOH (5.45%, w/v) and Tris (1 M); the resulting pH was 8.4. Finally, 350 mg activated carbon (Norit A, Fisher Scientific) were added, and the mixture was shaken to remove trace amounts of low molecular weight, soluble polyacrylamide which exhibits some light absorption in the ultraviolet range. The mixture was filtered through Whatman No. 2 filter paper, and the absorbance at 240 mμ was measured in a quartz cell of 10-mm light path.

Reaction rates were determined from the six values obtained for each digestion mixture by the method of least squares, and were expressed in terms of increase of

absorbance $\times 10^3$ at 240 mμ in a 10-mm light path, per min of incubation time. Specific enzyme activity was computed per unit quantity of enzyme contained in 3 ml of the digest. This unit quantity of enzyme was 1 μg of the soluble form of the mutase or that amount of insoluble polymer which had been formed in the presence of 100 μg soluble, crystalline phosphoglycerate mutase.

The activity of commercial preparations of the soluble mutase varied considerably from one batch to another, and the soluble enzyme frequently lost some activity upon storage, even when kept under refrigeration and as a crystalline suspension in concentrated $(NH_4)_2SO_4$ solution. For these reasons, all measurements involving one given variable, such as for instance the temperature, were carried out with the same batch (or with the same lot of batches) of commercial mutase for both the soluble and the insoluble forms of the enzyme, and were performed approximately at the same time. The same precaution was taken for each of the other variable parameters investigated, *i.e.* pH, substrate concentration, coenzyme concentration, *etc.*

RESULTS

There was a linear relationship between the concentration of insoluble mutase and the enzyme activity observed (Fig. 1). This linearity prevailed as long as the enzyme concentration and the incubation time were selected in such a manner as to prevent the absorbance of an aliquot of the digestion mixture, after addition of perchloric acid and neutralization, to exceed the value of 0.5, *i.e.* as long as the substrate turnover was kept below 6%. In this respect, the soluble and insoluble forms of the mutase behaved in an identical fashion.

When the concentration of the substrate (3-phosphoglycerate) was varied, there was a continuous rise in specific enzyme activity with increasing substrate concentrations for both the soluble and insoluble forms of the mutase (Fig. 2). Maximum specific activity was reached at about the same substrate concentration for both forms of the enzyme, *i.e.* at about 16.7 μ-moles/ml, and pronounced substrate inhibition started to become noticeable at higher substrate concentrations for both forms of the

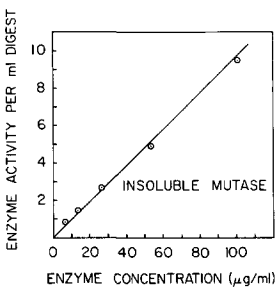


Fig. 1. Influence of enzyme concentration on the activity of insoluble rabbit muscle phosphoglycerate mutase at 26.8° and pH 6.7 (0.05 M imidazole buffer) in the presence of 16.7 mM substrate (3-phosphoglycerate), 3.33 mM $MgCl_2$ and an excess of enolase (50 μg/ml).

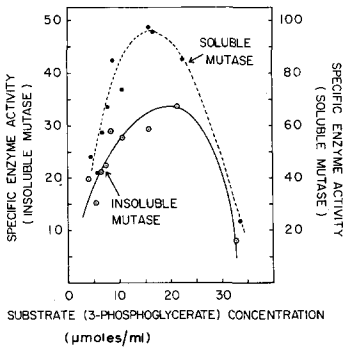


Fig. 2. Influence of substrate (3-phosphoglycerate) concentration on the specific activity of the soluble and insoluble forms of rabbit muscle phosphoglycerate mutase, at 26.8° and pH 6.7 (0.05 M imidazole buffer), in the presence of 3.33 mM $MgCl_2$ and an excess of enolase (50 μg/ml).

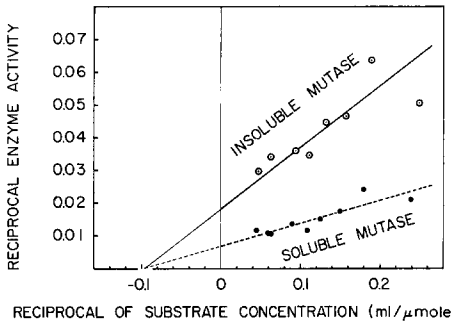
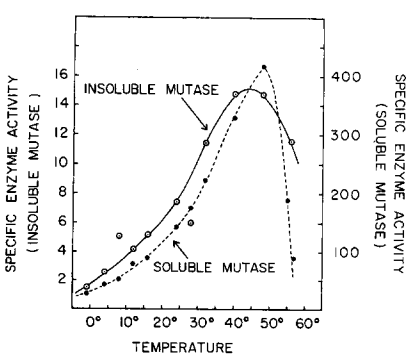


Fig. 3. Lineweaver–Burk plots of the relationship between substrate concentration and specific enzyme activity for the soluble and insoluble forms of rabbit muscle phosphoglycerate mutase.

mutase. At a concentration of $33 \cdot 10^{-3}$ μmoles 3-phosphoglycerate per ml, this inhibition reached about 75% in both instances.

Lineweaver–Burk plots of these data (deleting all values where substrate inhibition had been noted, *i.e.* those obtained at substrate concentrations higher than 16.7 mM) yielded straight lines for the two forms of the mutase (Fig. 3). These lines, when extrapolated beyond the ordinate, intersect at one and the same point with the negative branch of the abscissa. Hence, within the limits of error of the measurements, the Michaelis constants characterizing the dissociation constants of the enzyme–substrate complexes, are the same for the soluble and insoluble forms of the mutase; *i.e.* 10 mM at 26.8° and pH 6.7 in 0.05 M imidazole buffer. Experiments carried out with each of the two forms of the enzyme to which 2,3-diphosphoglycerate (1.67 mM) had been added, or with the soluble mutase after dialysis for 36 h against 0.05 M imidazole buffer, did not show evidence for any changes in the Michaelis constants under these conditions, although the addition of coenzyme increased the rates of reaction (see below), especially with the insoluble form of the mutase, whereas dialysis of the soluble form decreased the reaction rate.



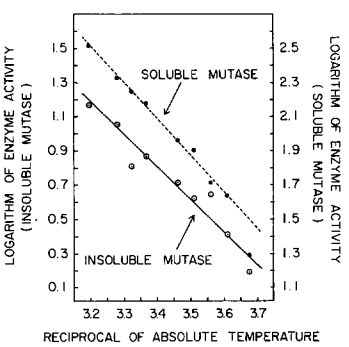


Fig. 4. Influence of temperature on the specific activity of the soluble and insoluble forms of rabbit muscle phosphoglycerate mutase, at pH 6.7 (0.05 M imidazole buffer), in the presence of 16.7 mM substrate (3-phosphoglycerate), 3.33 mM $MgCl_2$ and an excess of enolase (50 μg/ml).

Fig. 5. Arrhenius plots of the temperature-specific activity relationship for the soluble and insoluble forms of rabbit muscle phosphoglycerate mutase: logarithm of specific activity *versus* reciprocal absolute temperature × 10^3.

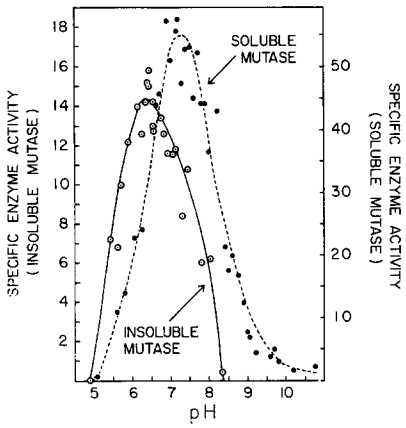


Fig. 6. Influence of pH on the specific activity of the soluble and insoluble forms of rabbit muscle phosphoglycerate mutase, at 26.8°, in the presence of 16.7 mM substrate (3-phosphoglycerate), 3.33 mM $MgCl_2$ and an excess of enolase (50 μg/ml). Buffers used were: 0.05 M imidazole (pH 4.88–7.36); 0.1 M Tris–malonate (pH 5.10–6.72); 0.1 M Tris–HCl (pH 6.74–8.62); 0.1 M glycine (pH 8.06–10.83).

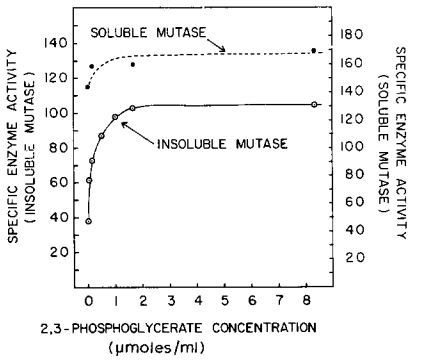


Fig. 7. Influence of the concentration of added coenzyme (2,3-diphosphoglycerate) on the specific activity of the soluble and insoluble forms of rabbit muscle phosphoglycerate mutase, at 26.8° and pH 6.7 (0.05 M imidazole buffer), in the presence of 16.7 mM substrate (3-phosphoglycerate), 3.33 mM $MgCl_2$ and an excess of enolase (50 μg/ml).

Variation of the temperature did not reveal any significant differences in behavior between the two forms of the mutase (Fig. 4). Maximum activity was achieved between 40 and 45° for both forms, and the activity increment with rising temperatures was practically the same for soluble and insoluble mutase. The Arrhenius plots of the same data (with the exclusion of those obtained at the higher temperatures where the activity declines) yielded straight lines of almost the same slope (Fig. 5). Accordingly, the activation energies, calculated from these data, are approximately the same for the two forms of phosphoglycerate mutase, *i.e.* 9000 cal/mole for the soluble enzyme and 9100 cal/mole for the insoluble form.

In contrast to the findings presented so far, variations of the pH resulted in a marked difference between the behavior of the two forms of mutase (Fig. 6). The insoluble form showed an optimum of activity at pH 6.3, whereas the soluble mutase was most active at pH 7.3. Addition of 2,3-diphosphoglycerate to either form did not influence their pH optima. At an intermediate pH of 6.7, at which all other determinations were carried out with the two forms of mutase, the insoluble enzyme exhibited from 86–94% of its maximum activity; the corresponding figure for the soluble enzyme was 74–78%.

A further distinction was observed between the two forms of phosphoglycerate mutase in their behavior toward the coenzyme, *i.e.* 2,3-diphosphoglycerate (Fig. 7). Whereas the activity of the soluble form was only slightly enhanced by the addition of the cofactor, the insoluble enzyme exhibited a 2.5-fold increase in specific activity under the same conditions. In either case, full activation was achieved by a coenzyme concentration of 1.67 μmoles/ml of digest (in the case of the soluble enzyme, one-tenth of that concentration also had the same effect) and additional amounts of coenzyme

TABLE I

EFFECT OF 2,3-DIPHOSPHOGLYCERATE (COENZYME) ON THE ACTIVITY OF THE TWO FORMS OF PHOSPHOGLYCERATE MUTASE

Form of enzyme	*Specific enzyme activity**	
	Without added coenzyme	*With added coenzyme (0.5 mM)*
Soluble mutase	80	93
Soluble mutase after dialysis**	6.0	11.2
Insoluble mutase	38	88

* At 16.7 mM substrate concentration (pH 6.7) (imidazole buffer) and 26.8°.
** 36 h against 0.05 M imidazole buffer (pH 6.7).

produced no further changes in the activities. The behavior of the insoluble form of mutase toward added coenzyme is somewhat similar to that of the soluble form after dialysis, in that both forms are activated to approximately the same extent (Table I). However, dialysis of the soluble enzyme against buffer solutions results in considerable losses of enzyme activity which cannot be recovered by the addition of coenzyme, but the insoluble form may be washed with distilled water without incurring irreversible losses of activity.

The apparent amount of mutase activity, which had become associated with the insoluble polymer, varied by a factor of more than 10 for different preparations of the insoluble form of phosphoglycerate mutase (Table II). There was no manifest correlation between the apparent amount of enzyme activity associated with the insoluble carrier (Table II, last column) and the specific activity of the batch of soluble mutase from which the insoluble form had been prepared (fourth column).

TABLE II

APPARENT AMOUNT OF MUTASE ACTIVITY ASSOCIATED WITH THE INSOLUBLE POLYMER AS INFLUENCED BY THE PARAMETER STUDIED

Parameter studied	*See Fig. No.*	*Condition of comparison*	*Specific activity of*		*Apparent amount of enzyme activity associated with the insoluble carrier***
			Soluble mutase	*Insoluble mutase**	
Substrate concn.	2	16.7 mM	97	33	0.34
Temp.	4	26.8°	163	8.8	0.055
pH	6	pH 6.7	42	13.5	0.32
Cofactor	7	No cofactor added	144	38	0.26
Cofactor	7	1.67 mM	165	105	0.64

* Each parameter studied represents a different batch of insoluble enzyme, but for each parameter, the same lot of commercial soluble mutase served for both the kinetic study of the soluble form and the preparation and kinetic study of the insoluble form.
** Specific activity of insoluble form, divided by specific activity of soluble form.

DISCUSSION

The soluble form of muscle phosphoglycerate mutase, used in the present experiments, behaved in all respects like enzyme preparations from the same source described in the recent literature. This refers in particular to the pH optimum of activity, reported to be 7.0 by COWGILL AND PIZER[16], to the Michaelis constant of 5 mM (ref. 16), to an activation energy of 10 500 cal/mole, reported by GRISOLIA[15], and to the behavior toward diphosphoglycerate[17]. The same batch of soluble enzyme was used to prepare the insoluble form and to study the kinetics of the soluble form under conditions identical to those employed with the insoluble form.

Like the water-insoluble form of enolase, insoluble phosphoglycerate mutase possesses essentially the same kinetic characteristics as its soluble form. This is true with regard to the effect on the enzyme activity caused by variations of enzyme concentration, substrate concentration, including inhibition by excess of substrate and temperature. Consequently, the Michaelis constants of the two forms of the mutase are the same, within the limits of error of the assay method, as are their activation energies.

However, the pH optimum of activity of the insoluble mutase is about one pH unit lower (more acid) than that of the soluble form, and this difference appears to be highly significant. Shifts of the pH optimum, when enzymes are rendered water-insoluble, have been reported in the literature, but they have always been attributed to a polyelectrolyte nature of the insoluble enzyme carrier. Thus, water-insoluble trypsin, bound to a polyanionic maleic acid–ethylene copolymer, proved to have a more alkaline pH optimum than the water-soluble form of trypsin[18], and aminoacylase, rendered insoluble by complexing it with a polycationic carrier, namely DEAE-cellulose, exhibited a more acid pH optimum than water-soluble aminoacylase[19].

Such shifts in the pH optima of two different enzymes attached to polyelectrolytic carriers would seem to indicate that polyacrylamide, used as the carrier of the insoluble form of phosphoglycerate mutase in our own experiments, was of a polycationic nature. There are no indications, however, to substantiate such a hypothesis because (1) on theoretical grounds, amides are well known to be electrostatically neutral and hydrolysis of the carrier, a phenomenon which is most unlikely to occur in the present case, would yield acid rather than basic groups and (2) from experimental observations it follows that the polymer does not stain with acidic dyes, *e.g.* eosin and that enolase, rendered insoluble by embedding it in the same synthetic polyacrylamide carrier used for the mutase, did not exhibit any change in its pH optimum of activity[12]. It, therefore, appears unlikely that the shift of the pH optimum of the insoluble mutase is caused by a polyelectrolyte nature of its carrier. No reason for this pH shift is forthcoming from our experiments.

An additional distinction between the soluble and insoluble forms of the mutase is that the latter proved to be much more susceptible to activation by diphosphoglycerate than the former. Since the insoluble form of the enzyme had been washed extensively with water, it can be assumed that a large amount of the coenzyme had been removed, and washing of the insoluble form has actually been found to have an effect on the enzyme similar to that of dialysis on the soluble form. The variations in specific activity between different batches of insoluble mutase (see Table II) are obviously the result of more or less complete removal of the coenzyme during washing

of the insoluble form. In contrast to the soluble form of mutase which can only be dialyzed against dilute salt solutions, the insoluble form of the enzyme can safely be washed with distilled water, and activity can be restored, as long as coenzyme is added. GRISOLIA AND CLELAND[20] have demonstrated in their interesting studies on the "Ping Pong" mechanism of the enzyme reaction that the coenzyme of phosphoglycerate mutase is much more firmly bound to the enzyme protein at low rather than at high salt concentrations. Nonetheless, a marked degree of dissociation can be achieved in the practical absence of any electrolyte, as is shown by the present findings on the removal of the cofactor from the insoluble form of mutase by washing with distilled water.

An examination of the similarities and differences between the soluble and insoluble forms of the two enzymes studied so far, *i.e.* enolase[12] and phosphoglycerate mutase, shows that the two forms of enolase have an entirely different temperature profile, but exhibit the same pH dependency, whereas the situation is exactly reversed in the case of phosphoglycerate mutase, *i.e.* there is identity in the temperature relationship but difference in pH optima. Thus it appears that any differences between soluble and insoluble forms of an enzyme depend on the nature of each individual enzyme rather than on the insoluble carrier.

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