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Rabbit Muscle Triosephosphate Isomerase: Activity and Inactivation by Sulfhydryl Reagents as Affected by Enzyme Concentration[1] (38599)

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The amino acid sequence of rabbit muscle triosephosphate isomerase, EC 5.3.1.1, (TIM) has been established by Corran and Waley (1). The enzyme is a dimer with a molecular weight of 53,257. Each sub-unit contains five cysteine residues. There have been conflicting reports as to the effects of sulfhydryl reagents on the activity of this enzyme. Burton and Waley (2) found the sulfhydryl groups of the enzyme to react slowly with iodoacetate and to be unreactive toward 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) except in the presence of guanidine. They inferred that the sulfhydryl groups are buried in the interior of the molecule. Burton and Waley reported that the enzyme after treatment with iodoacetate lost activity only after a latent period of several hours. During this latent period most of the sulfhydryl groups of the enzyme had been alkylated. Their conclusion was that the sulfhydryl groups are inessential for enzymatic activity. On the other hand, Krietsch *et al.* (3) reported that the TIM from rabbit muscle and liver but not that from yeast was inactivated by *p*-mercuribenzoate (*p*-MB), Ellman's reagent, iodoacetate, and 2,4-dinitrofluorobenzene. Poole *et al.* (4) observed inhibition of rabbit muscle TIM by low concentrations of 2,2′-dithiodipyridine and 6,6′-dithiodinicotinic acid, an effect presumed to involve reaction of these compounds with the sulfhydryl groups of the enzyme. Davis *et al.* (5) reported that rabbit muscle TIM was inactivated by maleimides, mercurials, tetrathionate, and Ellman's reagent with increase of the Michaelis constant but without change of the maximum velocity. Silver and mercuric ions decreased the maximum velocity.

In this report we shall show that the concentration of the enzyme in solution has important effects on the inhibition of rabbit muscle TIM by sulfhydryl reagents. This suggests that concentration affects configuration of the protein so as to alter the reactivity of its sulfhydryl groups. These effects of enzyme concentration may underlie some of the discrepancies in the literature.

Materials and Methods. TIM was the crystalline rabbit muscle enzyme supplied by Sigma Chemical Co. as a suspension of 10 mg protein/ml in 2.7 M $(NH_4)_2SO_4$ and 0.5 m*M* EDTA. Glycerolphosphate dehydrogenase (GDH) was the crystalline rabbit muscle enzyme supplied by Sigma as a suspension of 10 mg protein/ml in 2.0 *M* $(NH_4)_2SO_4$ and 0.3 m*M* EDTA. DL-Glyceraldehyde-3-phosphate (GAP) was prepared from the diethylacetal barium salt (Sigma). Disodium NADH and sodium iodoacetate were from Sigma and sodium *p*-MB from Calbiochem.

All solutions used in incubations and assays were in a buffer containing 0.4 mole triethanolamine hydrochloride and 0.144 mole NaOH per liter (pH 7.6).

Incubation of TIM with inhibitors. The suspension of crystalline enzyme was diluted with buffer to the desired concentration. The solutions of enzyme of 1 mg/ml and 0.1 mg/ml used in the experiments of Table II were dialyzed against 100 vol of buffer before incubation with *p*-MB. Incubations were at 37°.

Assay of TIM activity. The rate of isomerization of GAP was measured in the system described by Meyer-Arendt *et al.* (6). At the end of an incubation period, the solution was diluted with buffer so as to give a final

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concentration of the isomerase of 2–10 ng/ml. Solutions of NADH, GDH, and DL-GAP were then added rapidly. Except in the experiments of Fig. 2, in which different concentrations of GAP were used, the final concentrations were: GDH, 2.2 μg protein/ml; NADH, 5×10^{-5} *M*; DL-GAP, 2×10^{-3} *M*. Immediately after the final additions, decreasing absorbancy at 340 nm was followed in a recording spectrophotometer, the cuvette being at room temperature (about 25°).

Under the conditions of this assay, any possible effect of the inhibitors on GDH could not have made the reduction of dihydroxyacetone phosphate the rate-limiting reaction. This was shown in preliminary experiments in which the unreacted inhibitors were removed in large part from the isomerase solution by dialysis before assay. The degree of inhibition shown by the assay was unaffected by dialysis.

Increase of activity with time after dilution. In 18 of 19 experiments in which the crystalline suspension of triosephosphate isomerase was diluted with buffer to give final concentrations of protein between 1 mg/ml and 5 ng/ml, progressive increase of enzymatic activity with time was observed. Activity sometimes increased to levels greater than twice the value observed immediately after dilution. In some experiments, activity was still increasing after six hours; whereas in others, no further increase occurred after one hour. Examples are shown in Table I.

Microscopic examination showed that the enzyme crystals dissolve rapidly on addition of buffer. The slow increase of activity on dilution cannot be accounted for by delay in solution of the crystals.

Effect of the concentration of TIM on the inhibitory concentration of p-MB. Table II shows the concentration of the mercurial required to reduce the activity of the isomerase to about half of the uninhibited value after 30 min incubation. The higher the concentration of enzyme, the higher the concentration of mercurial required to inhibit. However the ratio of the inhibitory concentration of the mercurial to the concentration of enzyme increases with decreasing enzyme concentration. If the crystalline enzyme is assumed to be pure, the molar ratio of mercurial to enzyme increases from 5:1 at the highest enzyme concentration to 1000:1 at the lowest.

TABLE I. Examples of Change of Enzymatic Activity of Rabbit Muscle TIM Occurring After Dilution of the Crystalline Suspension.

Concentration of TIM[a]	Enzymatic activity at designated time after dilution[b]					
	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr
5 ng/ml	104	128	140	136	132	136
10 ng/ml	138	169	166	181	197	219
1 mg/ml	103	118	132	159	232	224

[a] Concentration at which the enzyme was incubated in the 0.4 *M* triethanolamine buffer, pH 7.6.

[b] Activity expressed as percentage of that found immediately after dilution of the crystalline suspension with the buffer. Times shown are those intervening between initial dilution of the crystalline suspension and assay. During this time the enzyme was incubated at 37°.

TABLE II. Inhibition of Rabbit Muscle TIM by *p*-MB[a].

Concentration of TIM	Inhibitory concentration of *p*-MB[b]
mg/ml	*M*
1	1×10^{-4}
10^{-1}	8×10^{-6}
10^{-2}	2×10^{-6}
10^{-3}	2×10^{-6}
10^{-4}	8×10^{-7}
10^{-5}	2×10^{-7}

[a] The enzyme was incubated with the inhibitor for 30 min at 37° before assay.

[b] The concentration reducing the rate of enzymatic conversion of glyceraldehyde-3-phosphate to dihydroxyacetone phosphate to approximately 50% of the uninhibited value. Concentrations are expressed to the nearest power of two times a power of 10.

Course of inactivation of TIM by p-MB. The inactivation of the enzyme in a concentration of 8 ng/ml by several concentrations of the mercurial is shown as a function of time in Fig. 1. With the lowest concentration of mercurial there was a period of 4 hr during which there was no detectable inactivation. Activity then began to be lost. With the next higher concentration (twice the first), the lag period was 0.5 hr. With higher concentra-

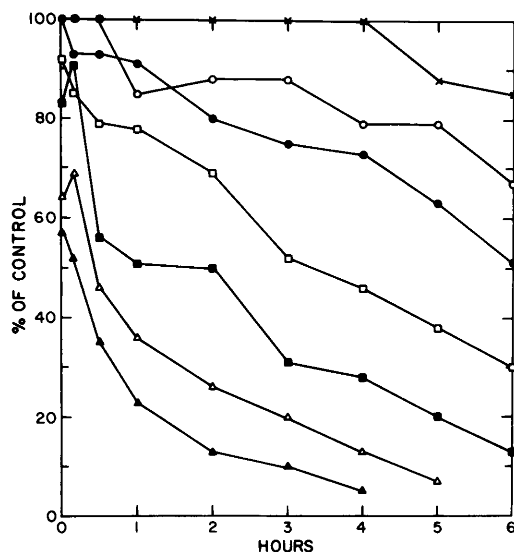

FIG. 1. Inactivation of TIM by *p*-MB as a function of time. The time shown is that between the time of addition of the inhibitor to the enzyme solution and the time of dilution of the sample for assay. The values shown are enzymatic activities in samples treated with inhibitor as percentages of those in control samples identical except for absence of inhibitor and incubated for the same length of time. Concentrations of *p*-MB: X, 1.25×10^{-8} *M*; ○, 2.5×10^{-8} *M*; ●, 5×10^{-8} *M*; □; 10^{-7} *M*; ■, 2×10^{-7} *M*; △, 4×10^{-7} *M*; ▲, 8×10^{-7} *M*. These concentrations are those in the initial incubation. The concentration of TIM at this stage was 8 ng protein/ml. Incubations were at 37°.

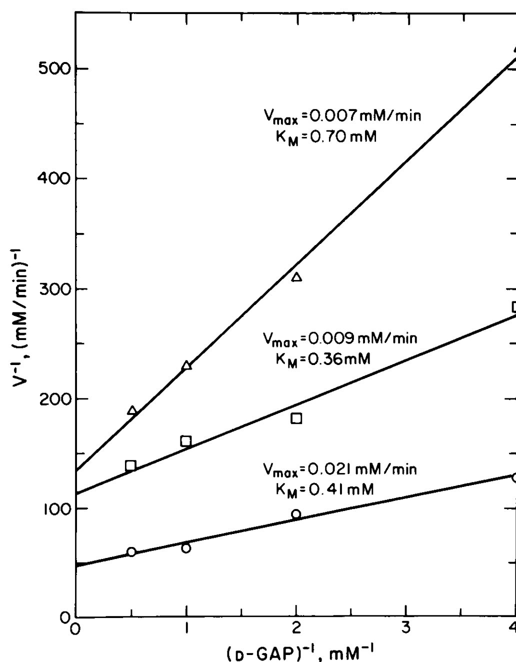

FIG. 2. Lineweaver-Burk plots of the isomerization of GAP by TIM as affected by *p*-MB. The concentration of the isomerase during the incubation was 32 ng protein/ml and in the assay 9.3 ng/ml. Concentrations of *p*-MB in the initial incubation: ○, none; □, 2×10^{-7} *M*; △, 4×10^{-7} *M*. The enzyme was incubated with the mercurial for 30 min. at 37° before assay.

tions, no lag period was seen. With the four highest concentrations of mercurial studied, a considerable degree of inactivation had already occurred by the time the first sample was taken at 1 min. Thereafter, activity continued to disappear but at rates not comparable to those of the first minute. The extent of the initial rapid inactivation was greater the greater the concentration of drug.

Effect of p-MB on the relationship of substrate concentration to isomerization rate. Lineweaver–Burk plots relating concentration of GAP as substrate to the rate of isomerization are shown in Fig. 2. The effects of two concentrations of mercurial acting on the isomerase in a concentration of 32 ng/ml were studied. The lower concentration of mercurial reduced maximum velocity to less than half the control value without significantly affecting the Michaelis constant. The higher concentration of mercurial caused some further reduction in maximum velocity and a large increase in the Michaelis constant.

Course of inactivation of TIM by iodoacetate. In Fig. 3 are shown the effects of iodoacetate on the activity of the enzyme as a function of time. Two concentrations of the inhibitor were studied with two concentrations of enzyme. Activities are expressed as percentages of those in identical control samples lacking iodoacetate. Activities in iodoacetate-treated samples have consistently been found to be higher than in corresponding controls immediately after addition of the reagent. This suggests that iodoacetate initially accelerates the process of increase of activity occurring on dilution, as described above. In the solutions of enzyme containing 1 mg/ml, activity in samples treated with iodoacetate did not begin to fall below control levels until after 2 hr, even with the

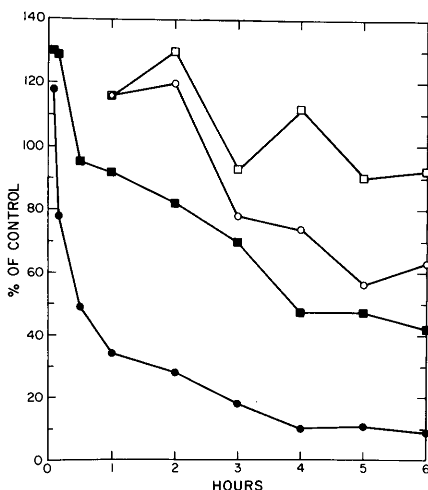

FIG. 3. Effect of iodoacetate on activity of TIM. The time shown is that between the time of addition of the inhibitor to the enzyme solution and the time of the dilution of the sample for assay. Incubations were at 37°C. The values shown are enzymatic activities in samples treated with inhibitor as percentages of those in control samples identical except for absence of inhibitor and incubated for the same length of time. Concentrations of the isomerase in the initial incubations: ○ and □, 1 mg protein/ml: ● and ■, 8 ng protein/ml. Concentrations of iodoacetate in the initial incubations: □ and ■, 10^{-2} *M*; and ○ and ●, 2×10^{-2} *M*.

higher concentration of inhibitor. In the 8 ng/ml solutions of enzyme, iodoacetate caused progressive loss of activity without a lag period of more than a few minutes.

Discussion. The conclusion of Burton and Waley (2) that the sulfhydryl groups of rabbit muscle TIM are relatively unreactive and that their integrity is inessential for enzymatic activity has not been corroborated by the work of Krietsch *et al.* (3) and Davis *et al.* (5) and by unpublished work in our laboratory showing the enzyme to be inactivated by a number of compounds representative of several chemical classes reactive toward sulfhydryl groups. A sulfhydryl group would not necessarily play a direct role in the catalytic process. All of the cysteine residues of the rabbit muscle enzyme are sequentially remote from the active site as identified by affinity labeling of a glutamyl residue with haloacetolphosphates or glycidolphosphate (1, 7, 8). Yeast TIM has the same sequence of amino acids around this glutamyl residue as has the rabbit muscle enzyme (9) but is insensitive to sulfhydryl reagents (3, 5). Reaction of sulfhydryl groups in the rabbit muscle enzyme could result in conformational changes of the whole molecule that could in some way destroy its catalytic activity.

Consideration of the discrepancies among the reports in the literature and between them and the present report suggests that these discrepancies may have arisen from the use of different concentrations of the enzyme. There has been inadequate appreciation of the possibility that the concentration of a protein in solution might affect the reactivity of its sulfhydryl groups.

Burton and Waley (2) did not specify the concentration of rabbit muscle TIM used in the experiments in which iodoacetate caused inactivation only after a long latent period and in which the enzyme was unreactive toward Ellman's reagent. It appears likely that these concentrations were relatively high. In our experiment of Fig. 3 in which the concentration of enzyme was 1 mg/ml, inhibition by iodoacetate appeared only after a long period of incubation. When the concentration of enzyme was 8 ng/ml, inhibition began promptly on addition of iodoacetate and proceeded in a manner to be expected if it were reflecting the progress of a chemical reaction. Since iodoacetate was in very large molar excess even in the more concentrated enzyme solution, it seems unlikely that the difference can arise from different molar ratios of reagent to protein. A more plausible hypothesis is that conformational differences in different concentrations of the enzyme are associated with different reactivities of the sulfhydryl groups.

The kinetics of inactivation with *p*-MB appear more complex than with iodoacetate. From the experiments of Fig. 1, in which the concentration of the enzyme was 8 ng/ml, it would seem that sulfhydryl groups essential for enzymatic activity are accessible to rapid reaction only when the concentration of mercurial is above a certain threshold level. At lower concentrations, reaction with nonessential sulfhydryl groups may in time bring about conformational changes that expose essential groups to reaction. The concept of the progressive reaction of sulfhydryl groups

resulting in conformational changes not necessarily inactivating the enzyme but increasing the accessibility of other groups to reaction might account for the irregular course of enzymatic decay seen with some concentrations of mercurial.

The discrepancies between our work with *p*-MB and that of Davis *et al.* (5) with that and other mercurials can probably be accounted for by differences in enzyme concentrations. In the experiments of Davis *et al.* (5) several different concentrations of enzyme were used and some of the incubations contained EDTA, a compound that we have found to increase greatly the concentration of mercurial required for inhibition. In one of the kinetic studies of Davis *et al.* (5) the concentration of rabbit muscle enzyme was 2.5 mg/ml. At this concentration of enzyme we have been unable to obtain inhibition with *p*-MB except at concentrations that cause precipitation. As shown in Fig. 2, when the concentration of enzyme is 32 ng/ml, a small concentration of mercurial causes a decrease in maximum velocity with little charge in the Michaelis constant. This result, which might be expected with a non-competitive inhibitor, is contrary to those reported by Davis *et al.*

The spontaneous increase of enzymatic activity that occurs after dilution of the suspension of crystalline rabbit muscle TIM is also in accord with the concept that conformation of the enzyme is dependent on its concentration in solution. The change in conformation following dilution is not instantaneous but proceeds rather slowly. It would appear that in more dilute solutions the enzyme assumes a conformation in which it is catalytically more active as well as having its sulfhydryl groups more accessible to chemical attack.

Chatterjee and Noltmann (10) in a study of rabbit muscle phosphoglucose isomerase and its inactivation by mercurials found effects of enzyme concentration suggestive of concentration-dependent conformational changes of the same nature that we are postulating for TIM. With the mercurial at a constant concentration in large excess, the inactivation rate increased with decreasing enzyme concentration until that concentration reached about 10^{-6} *M*. As an explanation, Chatterjee and Noltmann suggested that the enzyme undergoes dissociation on dilution, with an accompanying increase in accessibility of its sulfhydryl groups.

Chemical titration of protein sulfhydryl groups as followed by spectrophotometric methods must of necessity use relatively high concentrations of protein. It should be kept in mind that the results of such titrations may not necessarily give a correct picture of the reactivity of the sulfhydryl groups when the protein is in more dilute solution.

Summary. Progressive increase of enzymatic activity with time occurs after dilution of a crystalline suspension of rabbit muscle TIM.

The concentration of *p*-MB required to inhibit the enzyme is higher the greater the concentration of enzyme. With the lowest concentrations of mercurial that are ultimately inhibitory to the enzyme in dilute solution, there is a lag period before inactivation begins. With higher concentrations of mercurial, some degree of inactivation occurs almost immediately, and enzymatic activity thereafter decreases at a slower rate. With an enzyme concentration of 32 ng/ml, a low concentration of *p*-MB decreases V_{max} without change of K_M. A higher concentration of mercurial also increases K_M.

With an enzyme concentration of 1 mg/ml, iodoacetate begins to inactivate it only after a lag period. In enzyme solutions containing 8 ng/ml, inactivation by iodoacetate progresses without significant initial lag.

Discrepancies between the present work and that of other authors can probably be accounted for by differences in the concentrations of enzyme used. It is suggested that the conformation of rabbit muscle TIM in solution is dependent on its concentration. In more dilute solutions the enzyme assumes a conformation in which it is catalytically more active and in which its sulfhydryl groups are more accessible to chemical attack.

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