The Sulfhydryl Groups of Rabbit Muscle Phosphoglucomutase*

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ABSTRACT: Titration of phosphoglucomutase (PGM) with *p*-hydroxymercuribenzoate (*p*-HMB) reveals the presence of six SH groups per mole. This agrees with the number of half-cystine residues per mole estimated by amino acid analysis. Consequently, the enzyme has no disulfide bonds. The over-all titration rate is much faster for the dephospho-PGM as compared with the phospho-PGM. This is primarily due to the greater accessibility of the third and the fourth sulfhydryl groups. Glucose 1-phosphate (G-1-P) accelerates the thiol titration rate of the enzyme only insofar as it converts the phospho to the dephospho form. Similarly, glucose 1,6-diphosphate (G-1,6-P) retards the rate only insofar as it converts the dephospho to the phospho form. Where the enzyme form is not altered by the substrate, as obtained in the reaction of G-1-P with the dephospho form, or G-1,6-P with the phospho form, no alteration in the rate of titration is observed. On this basis, the change in conformation of the enzyme is related to the phosphorylated state of the enzyme and not to an induced fit in an enzyme–substrate complex. Loss of enzymatic activity to a variable extent accompanies the blockage of sulfhydryl groups. Second-order rate constants of the titration reaction of individual thiol groups with *p*-HMB have been calculated.

The enzyme phosphoglucomutase (PGM)[1] exists in the phospho and dephospho forms according to the mechanism of action elucided previously (Najjar and Pullman, 1954).

$$\text{G-1-P} + \text{phosphoenzyme} \rightleftharpoons \text{G-1,6-P} + \text{dephosphoenzyme} \rightleftharpoons \text{G-6-P} + \text{phosphoenzyme}$$

Analysis of the amino acid composition of rabbit muscle phosphoglucomutase as reported by three different groups (Najjar, 1962; Yankeelov *et al.*, 1964; and Sloane *et al.*, 1964) indicates that the molecule has six to seven half-cystine residues. No effort was made to determine the nature of these residues. It is the purpose of this communication to show that the enzyme has a total of six cysteine residues per 74,000 molecular weight (Keller *et al.*, 1956) and, consequently, no disulfide bridges exist in the molecule. This was revealed by the present study of the kinetics of thiol titration with *p*-hydroxymercuribenzoate (*p*-HMB) (Boyer, 1954). Two residues were immediately accessible to the reagent, while two were very slow reacting. The remaining two residues were of intermediate character. It was the latter two residues that showed a substantial increase in the rate of reaction with the reagent in the dephospho form as compared to that of the phospho form. This was invariably the case whether substrate was present or not.

Recent observations on the ability of PGM to undergo manifold activation when preincubated under rigorous conditions of pH 3.0–10.5 (Bocchini *et al.*, 1964; Harshman *et al.*, 1964), urea, or guanidine hydrochloride (V. Bocchini, M. R. Alioto, and V. A. Najjar, 1966, manuscript in preparation) similar to that observed with Mg^{2+}-imidazole (Robinson and Najjar, 1960, 1961; Robinson *et al.*, 1965; Harshman *et al.*, 1965) strongly suggest that although regional areas of flexibility exist, a sufficiently rigid core must nevertheless be responsible for maintaining substantial integrity of the molecule. This, in addition to the fact that PGM is particularly stable to heat and its activity is destroyed by preincubation with cysteine (Najjar, 1948), would lead to the reasonable assumption that S–S bridges are present in the molecule (Boyer, 1959; Cecil, 1963). It has thus become important to explore the properties and the nature of the cysteine residues in this enzyme.

Experimental Procedure

Reagents. G-1-P (Schwartz Bioresearch Laboratories, N. Y.) substantially free of G-1,6-P was used for the preparation of dephosphoenzyme and for *p*-HMB titration experiments. For measurement of catalytic activity G-1-P (Nutritional Biochemical Corp., Cleveland, Ohio) was used as it contained sufficient quantity of the coenzyme to permit its use at saturation conditions. $MgCl_2$ (Mallinckrodt Chemical Works Co., St. Louis, Mo.) and imidazole (Aldrich Chemical Co., Inc., Milwaukee, Wis.) were recrystallized from

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[1] Abbreviations used: PGM, phosphoglucomutase; G-1-P, glucose 1-phosphate; G-6-P, glucose 6-phosphate; G-1,6-P, glucose 1,6-diphosphate; *p*-HMB, *p*-hydroxymercuribenzoate.

water. Urea (Sigma Chemical Co., St. Louis, Mo.) was recrystallized from 70% ethanol and fresh aqueous solutions were made just before use. No traces of cyanate were detectable (Werner, 1923) in a 10 M solution of the recrystallized urea. Other reagents, L-cysteine, β-glycerophosphate, and Tris (Sigma Chemical Co., St. Louis, Mo.), were used without further purification. G-1,6-P was prepared from yeast (Leloir and Paladini, 1957) except that acetone fractionation was substituted for column chromatography (Bartlett, 1959).

Solutions of *p*-HMB (Sigma Chemical Co., St. Louis, Mo.) were prepared by dissolving the reagent in a small volume of 0.05 N NaOH and then adjusting the volume with either 0.5 M Tris buffer, pH 7.4, or 0.1 M glycylglycine buffer, pH 7.4. The solutions were then standardized by spectrophotometric estimation of the *p*-HMB content at 232 mμ using a molar extinction coefficient equal to 1.69×10^4 (Boyer, 1954).

Enzyme Preparation. Crystalline PGM was prepared (Najjar, 1948, 1962) from frozen rabbit muscle (Pel-Freez, Rogers, Ark.). All the enzyme preparations were homogeneous by paper electrophoresis in Veronal buffer at pH 8.6 or by sedimentation studies. The protein concentration was estimated by absorption at 278 mμ assuming $\epsilon^{1\%}$ 7.70 (Najjar, 1948).

The crystalline enzyme is exclusively of the phospho form. The dephospho form was prepared by repeated treatment of the enzyme with excess G-1-P according to the equation for the mechanism of action shown above. The excess substrate shifts the equilibrium concentration of the enzyme toward the dephospho form (Najjar and Pullman, 1954). Virtually complete conversion to the dephospho form was obtained as follows. The enzyme was treated with 10–20 times molar excess of G-1-P in 1×10^{-3} M $MgCl_2$–4×10^{-2} M imidazole buffer, pH 7.4. After 15 min at room temperature it was dialyzed for 3 hr at 5° against 400 volumes of 0.3 M Tris buffer, pH 7.1. The procedure was repeated two more times, the last treatment being followed by overnight dialysis against 0.3 M Tris buffer. The enzyme was then precipitated at 0° in 65% ammonium sulfate, redissolved in water, and similarly reprecipitated. This ensured the removal of any traces of contaminating sugar phosphates.

Thiol Titration. The generally accepted technique of thiol titration (Boyer, 1954) was used throughout. Titration was done in one of two ways: by stepwise addition of equivalent amounts of *p*-HMB to PGM and by a single addition of an excess of the reagent. The latter procedure was used when the kinetics of the reaction were studied. Mercaptide formation was measured by the increased absorbance at 250 or 255 mμ at pH 7.4 or 4.6, respectively. The increment per mercaptide formed on the enzyme ($\Delta\epsilon$) was obtained by adding an excess of the enzyme to the reagent and estimating the increase in optical density over that obtained by the reactants. An average $\Delta\epsilon$ value of 8×10^3 was found for PGM at 250 mμ as determined in 4×10^{-2} M imidazole and 1×10^{-3} M $MgCl_2$ at pH 7.4. This value was found to be the same for either phospho or dephospho form of the enzyme in the different buffers used. However, in agreement with earlier work (Swenson and Boyer, 1957) $\Delta\epsilon$ was found to alter with the urea concentration as well as with the pH. In 0.05 M phosphate buffer, pH 7.25, the $\Delta\epsilon$ for varying urea concentrations were as follows: 7×10^3 in 1.0 M, 6.15×10^3 in 2.0 M, and 5.25×10^3 in 5.0 M. In 0.1 M acetate buffer, pH 4.6, 6.0 M urea yielded a value of 6.60×10^3. These values were utilized to calculate the fraction of thiol groups titrated under these specified conditions of pH or urea concentration. Rate constants for the reaction between the individual thiol groups on the enzyme and *p*-HMB were calculated on the basis of second-order kinetics (Glasstone, 1946). The least-squares method was applied to calculate the slopes of the resulting straight lines. The latter were obtained by plotting the reaction time t against $\log (a - x)/(b - x)$, where x represents the amount of reagent consumed at time t, a and b the initial concentrations of the reactants, PGM SH groups and *p*-HMB, respectively. All titrations were performed at a working temperature of 23°, using a Model II Zeiss spectrophotometer.

Enzymatic Activity. This was measured in a reaction mixture containing G-1-P (5×10^{-3} M), imidazole (4×10^{-2} M), $MgCl_2$ (1×10^{-3} M), and Mg^{2+}–EDTA (2×10^{-3} M), pH 7.4. The assays were carried out in a temperature-controlled water bath at 30° (Najjar, 1948).

Results

The Number of SH Groups in Rabbit Muscle PGM. It has been shown (Boyer, 1954) that *p*-HMB is one of the more reliable reagents for measuring free thiol groups. The results of several experiments performed in a variety of buffers at two widely different pH values are presented in Table I. It appears that there are six titratable SH groups per mole of enzyme. This is based on a value of 5.69 representing the average of all values obtained under various conditions. The addition of urea, a potent denaturing agent for proteins, did not result in any significant deviation from this figure.

The Effect of the Substrate on the Rate of Titration of the Thiol Groups. It is quite apparent from the data presented in Table I that the addition of G-1-P to the enzyme did not alter the total number of SH groups that react with *p*-HMB. However, as will be noted below, the substrate does materially enhance the rate of this reaction. Thus in the presence of G-1-P (1×10^{-2} M) the time required to titrate the equivalent of three SH groups in a reaction mixture composed of 8.46 mμmoles of phospho-PGM, 91.0 mμmoles of *p*-HMB, in $MgCl_2$ (1×10^{-3} M) and imidazole (4×10^{-2} M) buffer at pH 7.4, was reduced from 8 to 4 min; similarly, the time required to titrate 4 SH equiv was reduced from 40 to 22 min.

These results with phospho-PGM are essentially in full agreement with the data obtained by over-all iodoacetamide titration, both in the accessibility of

TABLE I: Titration of PGM with *p*-HMB.[a]

Expt Performed	Buffer (M)	Additions: G-1-P (M)	Additions: Urea (M)	Thiol Residues/ Mole of Enzyme
14	$MgCl_2$ (1×10^{-3})–imidazole (4×10^{-2}), pH 7.4	—	—	5.55
5	$MgCl_2$ (1×10^{-3})–imidazole (4×10^{-2}), pH 7.4	1×10^{-2}	—	5.46
1	$MgCl_2$ (1×10^{-3})–imidazole (4×10^{-2}), pH 7.4	1×10^{-3}	—	6.10
2	$MgCl_2$ (1×10^{-3})–imidazole (4×10^{-2}), pH 7.4	1×10^{-4}	—	5.74
1	$MgCl_2$ (1×10^{-3})–imidazole (4×10^{-2}), pH 7.4	1×10^{-2} (β-glycero-P)	—	5.48
5	Phosphate (5×10^{-2}), pH 7.4	—	—	5.75
2	Phosphate (5×10^{-2}), pH 7.4	—	1.0	5.25
2	Phosphate (5×10^{-2}), pH 7.4	—	2.0	5.60
2	Phosphate (5×10^{-2}), pH 7.4	—	3.0	5.60
2	Tris (1×10^{-1}), pH 7.4	—	—	5.54
3	Acetate (1×10^{-1}), pH 4.6	—	6.0	5.70
Total 39			Av value	5.69

[a] The crystalline enzyme was collected by centrifugation from an ammonium sulfate suspension and dissolved in an appropriate volume of buffer. Titration was done in one of two ways: (a) by stepwise addition of 10-mμmole aliquots of *p*-HMB to 10–40 mμmoles of enzyme and (b) by a single addition of the reagent in 10–15 molar excess to 5–20 mμmoles of enzyme. In this case the number of thiol groups titrated per mole was calculated from $\Delta\epsilon$ values obtained under the specified set of conditions (see Experimental Section). Where more than one experiment was done under a particular set of conditions, about one-half of these was done according to a; the rest according to b. In all experiments performed in the presence of urea, the reaction was started by the addition of the enzyme. This minimized the increase in spontaneous oxidation of the enzyme caused by standing in urea. Titration was terminated when no further change in optical density was discernible. This was attained after the lapse of several hours.

the thiol groups and in the inhibition of catalytic activity. These two parameters were correspondingly greater in the presence of substrate than in its absence (Yankeelov and Koshland, 1965). However, similar results were obtained by these authors when these experiments were repeated with dephospho-PGM. It is here that we find the opposite to be the case. The results of SH titration, detailed below, clearly show that in the absence of substrate, the reactivity of the dephosphoenzyme is greater than the phosphoenzyme. Furthermore, the reactivity of the dephosphoenzyme is unaffected by substrate whereas that of the phosphoenzyme is increased to the level of the dephosphoenzyme. This important discrepancy between the two laboratories cannot be readily explained. It is possible that the different thiol reagents might be responsible. This is unlikely since both reagents affect the rate of inhibition of enzyme activity in a similar manner. It is important, at this point, to indicate that phosphoenzyme in the presence of substrate exists in an equilibrium state between the phosphorylated and dephosphorylated forms. However, the substrate concentration in the system under investigation is in considerable excess, with a molar ratio (substrate: enzyme) of 2.8×10^3. Based on the equilibrium constants of the two steps in the mechanism of the reaction (see below), the amount of phosphorylated form would be negligible. In the thiol titration experiments, whether performed with iodoacetamide or *p*-HMB, the contribution of the phospho form would be correspondingly small. Furthermore, since both forms react with the thiol reagent, the change in the position of the equilibrium, resulting from the unequal rate of mercaptide formation in the two forms, would not be detectable.

The increased reactivity of the thiol groups, when substrate is added to phospho-PGM, is indeed indicative of an increase in the accessibility of the sulfhydryl groups of the enzyme. This lends itself to two possible interpretations. (a) It is possible that some of the cysteine residues are close to the active site such that the removal of the transferrable phosphate group from the serine residue of the enzyme allows ready approach of the reagent. This would otherwise be hampered by charge repulsion of the *p*-HMB by the similarly charged phosphate or simply by steric hindrance of the bulky phosphate group. (b) The other possibility is that the presence of substrate in some

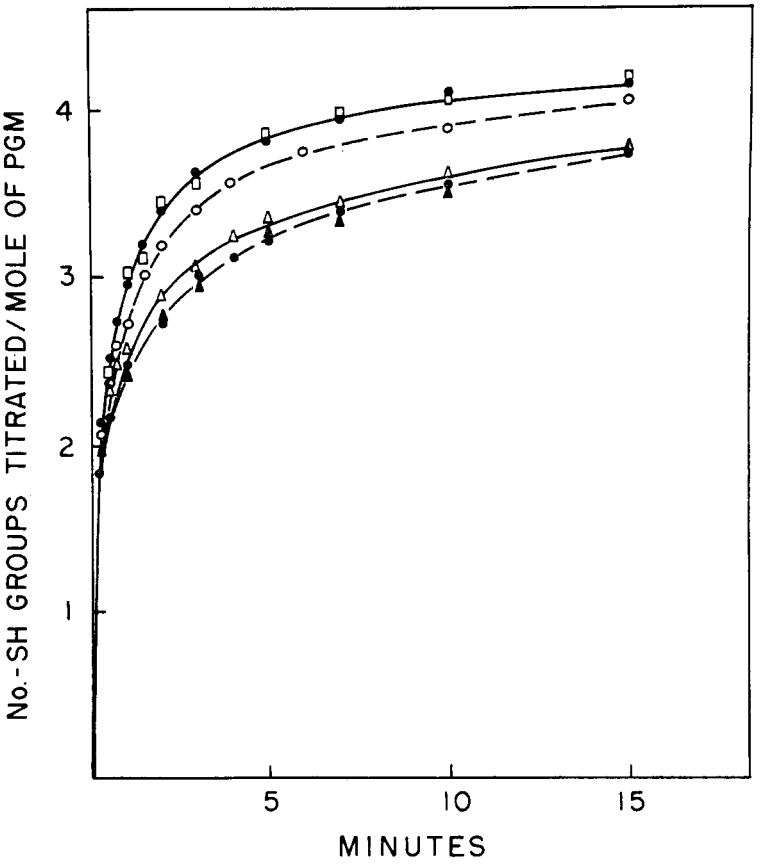

FIGURE 1: Titration of SH groups in phospho- and dephospho-PGM in the presence and absence of the substrates. Enzyme (7.0 mμmoles) was titrated with 90.0 mμmoles of *p*-HMB in 4×10^{-2} M imidazole, 1×10^{-3} M $MgCl_2$, and 3×10^{-1} M Tris, pH 7.4, and the indicated modifications in a final volume of 1.0 ml: ●——●, dephospho-PGM plus β-glycerophosphate (1×10^{-2} M); □——□, dephospho-PGM plus G-1-P (1×10^{-2} M); △——△, dephospho-PGM plus G-1,6-P (1.2×10^{-5} M); ●——●, phospho-PGM plus β-glycerophosphate (1×10^{-2} M); ○——○, phospho-PGM plus G-1-P (1×10^{-2} M); and ▲——▲, phospho-PGM plus G-1,6-P (6×10^{-6} M). In all cases the titration was started 15 min after the addition of the substrate to the enzyme.

manner alters the conformation of the enzyme. This may be due to the formation of an enzyme–substrate complex as such or due to the dephosphorylation of phospho-PGM by substrate. This possibility was suggested some time ago (Robinson and Najjar, 1961; Najjar, 1962) when it was shown that the addition of substrate to PGM (phospho form) effected a change in $[\alpha]_D^{22}$ of +3.2°. The change was found to be due not to the presence of substrate as such or as a possible enzyme–substrate complex, but to the consequent formation of dephosphoenzyme since the latter showed a specific negative rotation lower than that of the phospho form by the same magnitude. Parallel observations of the effect of substrate on PGM have since been made using spectral differences in absorbancy, fluorescence, and thiol titration (Yankeelov and Koshland, 1965). These observations have been interpreted in such a manner as to favor the hypothesis of induced fit (Koshland *et al.*, 1962). However, one important point has consistently been ignored, namely that in the presence of excess substrate the bulk of the enzyme is by far in the dephospho form irrespective of the formation of enzyme–substrate complex. This can be clearly deduced from the equilibria of the two steps of the reaction mechanism (see above). The equilibrium constant for the second step for the muscle enzyme is 3.76 (Sidbury and Najjar, 1957). Similar values of 3.82 and 4.09 for shark and flounder PGM, respectively, were found by Hashimoto and Handler (1966). In the absence of added glucose diphosphate and the presence of a large excess of monophosphate, little if any detectable phospho form exists. This being the case, no support could be harnessed to favor the induced fit theory if these changes caused by substrate are due to the dephospho form, as was shown in the case of optical rotation (Robinson and Najjar, 1961). A clear decision on this point can be made by comparison of the rates of reaction of *p*-HMB with (a) phospho-PGM, (b) phospho-PGM plus G-1-P, and (c) dephospho-PGM. In the event that the rate of SH titration in the presence of G-1-P is characteristically that of a reaction with the dephospho

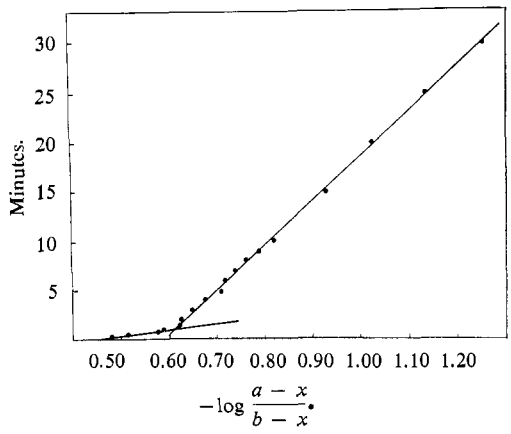

FIGURE 2: Second-order reaction rates of the second and the third SH groups of PGM with *p*-HMB. The titration of the first and fastest thiol group was complete in 15 sec and was not recorded. The PGM preparation used for titration gave a total of 5.87 titratable SH groups/mole. Accordingly, the amount of enzyme used in these experiments, 15.37 mμmoles, contained 90.22 mμequiv of SH. The slight excess of *p*-HMB added, 48.11 mμmoles, was sufficient to form 3.13 mμmoles of mercaptide/mole. The reaction was carried out in $MgCl_2$ (1×10^{-3} M), imidazole (4×10^{-2} M), and G-1-P (1×10^{-4} M) at pH 7.4.

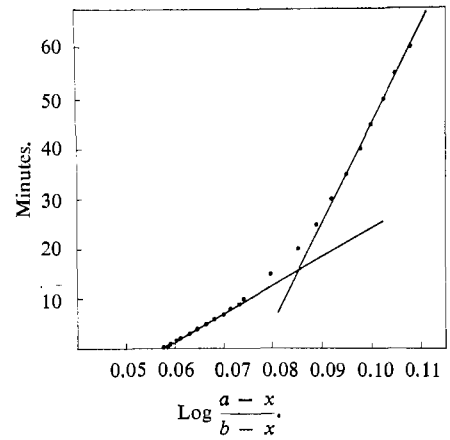

FIGURE 3: Second-order reaction rates of the fourth and fifth SH groups in PGM with *p*-HMB. The graph represents the continuation of the experiment reported in Figure 2. After the first three SH groups were titrated, a second aliquot of 48.11 mμmoles of *p*-HMB was added. The reaction was not followed to completion and at the 60th min only 4.5 SH groups/mole had been titrated. Details as in Figure 2.

form, the following should then be obtained. (i) The rate of mercaptide formation with dephospho-PGM should be faster than that with the phospho form, and (ii) should approach or equal that obtained with the phosphoenzyme in the presence of G-1-P. Similarly, the titration rate of dephospho-PGM in the presence of G-1,6-P should (iii) approach or equal that with the phospho form. In addition, (iv) the presence of G-1-P should not influence the rate of SH titration in the dephospho form, nor (v) should the presence of G-1,6-P affect the SH titration rate of the phospho form. The results of experiments designed to verify these points are shown in Figure 1.

The graphs in this figure represent two sets of conditions. One set depicts conditions in which the dephosphorylated species of the enzyme is exclusively or predominantly present: (a) dephosphoenzyme plus β-glycerophosphate, (b) dephosphoenzyme plus G-1-P, and (c) phosphoenzyme plus G-1-P. In the other set, conditions are such that the phosphorylated species is the exclusive form or greatly predominates. These conditions are: (a) phosphoenzyme plus β-glycerophosphate and (b) dephosphoenzyme plus G-1,6-P. All ester phosphates are used in considerable molar excess relative to the enzyme. The β-glycerophosphate is used as control and provides an ester phosphate similar in charge to that of G-1-P. The results clearly show that where dephosphoenzyme is present, whether prepared as such or formed by reaction of the phospho form with the added G-1-P, the rates of mercaptide formation were similar and within reproducible limits of the experimental technique. Similarly, in all instances where phosphoenzyme was present, whether prepared as such or formed in the reaction of the dephospho-form with G-1,6-P, the rates are also comparable. In all cases, the rate of titration is faster with the dephosphorylated species. It appears, therefore, that the presence of substrate as such has no relevance to the reaction rate except insofar as it converts one species of the enzyme to the other. This is further reinforced by the observation that the addition of G-1-P to dephospho-PGM or G-1,6-P to the phosphoenzyme does not alter the respective reaction rates of these forms of enzyme with *p*-HMB. In both instances there is no transfer of phosphate between the added glucose phosphates and the enzyme, thereby leaving the dephospho and the phospho forms unaltered (Najjar and Pullman, 1954). It can be concluded, therefore, that if a conformational change in the protein molecule is involved in the transition from one form to the other, as suggested by the change in optical rotation (Robinson and Najjar, 1961), such a change can obtain only when the proper substrate actually reacts with the proper form of the enzyme.

Reaction Rates for Individual Sulfhydryl Groups. Although the over-all titration rate of SH groups has been reported to follow second-order kinetics for a number of proteins (Boyer, 1954; Madsen and Cori, 1956), some instances have been found where this does not appear to be the case (Madsen and Cori, 1956; Fujioka and Snell, 1965). In the latter case, the results may well relate to the different yet overlapping degrees of accessibility of the various SH groups to the titrating reagent. The simple titration of phosphoglucomutase by the successive addition each of 1 equiv of *p*-HMB/mole of enzyme yielded

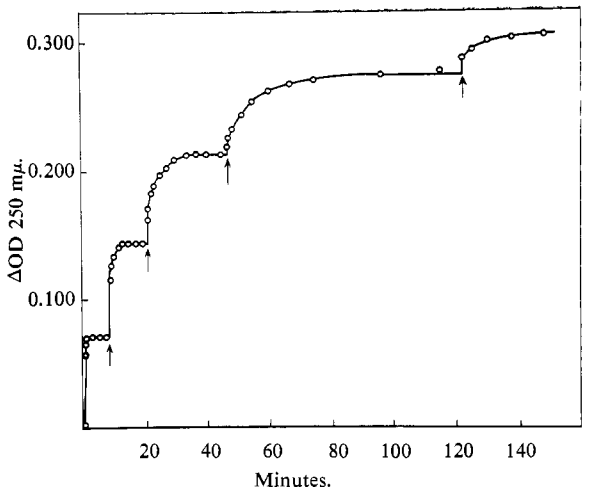

FIGURE 4: Titration of single SH groups of PGM by stepwise addition of 1 equiv of *p*-HMB. The titration was carried out in imidazole (4×10^{-2} M), $MgCl_2$ (1×10^{-3} M), Tris (3×10^{-1} M), and β-glycerophosphate (1×10^{-2} M), pH 7.4, final volume 1.0 ml. To 10 mμmoles of enzyme 10 mμmoles of *p*-HMB was added at the points indicated by the arrows. The titration of the fifth SH group was terminated before completion.

rates sufficiently different so as to indicate minimal overlapping (Figure 4). Furthermore, an analysis of the over-all titration rate with excess reagent, sufficient to react with all the thiol groups, showed complex second-order kinetics. A plot of the rate revealed a number of straight lines with differing slopes. This result suggested that PGM represents a case in which the SH groups react on the whole in a slightly overlapping sequential series. This being the case, each line should represent an individual SH group with the slope approximately reflecting the rate of that reaction. Moreover, the lines representing the various thiol groups would intersect at a point on the time axis corresponding to the time required to complete the titration of the faster group. This indeed proved to be the case, as shown by the following experiment. The phospho form of the enzyme was treated with *p*-HMB in amounts sufficient to titrate only three SH groups per mole of enzyme. When the reaction was completed, another addition of 3 equiv of the reagent was made and the rate of mercaptide formation was recorded at frequent intervals until 2 equiv was titrated. The data obtained for each of the two *p*-HMB additions were calculated and plotted according to second-order kinetics. Two straight lines with different slopes were obtained for the first titration involving the three thiol groups (Figure 2). The titration of the fastest thiol equivalent was complete within 15 sec and was not recorded. The two straight lines on the time axis, shown in the figure, represent the rate of mercaptide formation involving only the second and third thiol groups. These intercept at about 1 min, which compares favorably with the experimentally determined value for complete titration of the two fast SH groups. A kinetic analysis of the data obtained for the reaction following the second addition of 3 equiv of *p*-HMB yielded two lines also with different slopes (Figure 3). The intercept of both lines on the time axis at 18 min, which represents the end point for the titration of the fourth thiol group, corresponds reasonably well with the value of 17 min obtained experimentally. It is thus possible to obtain adequate rate constants under these conditions for the single thiol groups involved.

TABLE II: Second-Order Rate Constants of the Stepwise Titration Reaction of the First Four SH Groups in Phospho- and Dephospho-PGM with *p*-HMB.[a]

Reaction Mixture Titrated with *p*-HMB (M)		Approximate Constants (l. mole^{-1} sec^{-1})			
		k_1	k_2	k_3	k_4
1. Phospho-PGM	(1×10^{-5})	151.3	49.74	12.10	—
Tris	(3×10^{-1})				
2. Phospho-PGM	(1×10^{-5})	171.1	46.86	13.41	8.46
Tris	(3×10^{-1})				
Imidazole	(4×10^{-2})				
$MgCl_2$	(1×10^{-3})				
β-Glycero-P	(1×10^{-2})				
3. Phospho-PGM	(1×10^{-5})	184.6	56.06	20.00	13.13
Tris	(3×10^{-1})				
Imidazole	(4×10^{-2})				
$MgCl_2$	(1×10^{-3})				
G-1-P	(1×10^{-2})				

[a] To 10 mμmoles of enzyme 10 mμmoles of *p*-HMB was added and the reaction followed to completion, at which point another 10 mμmoles was again added, etc. Other conditions as in Figure 4. For details see the text.

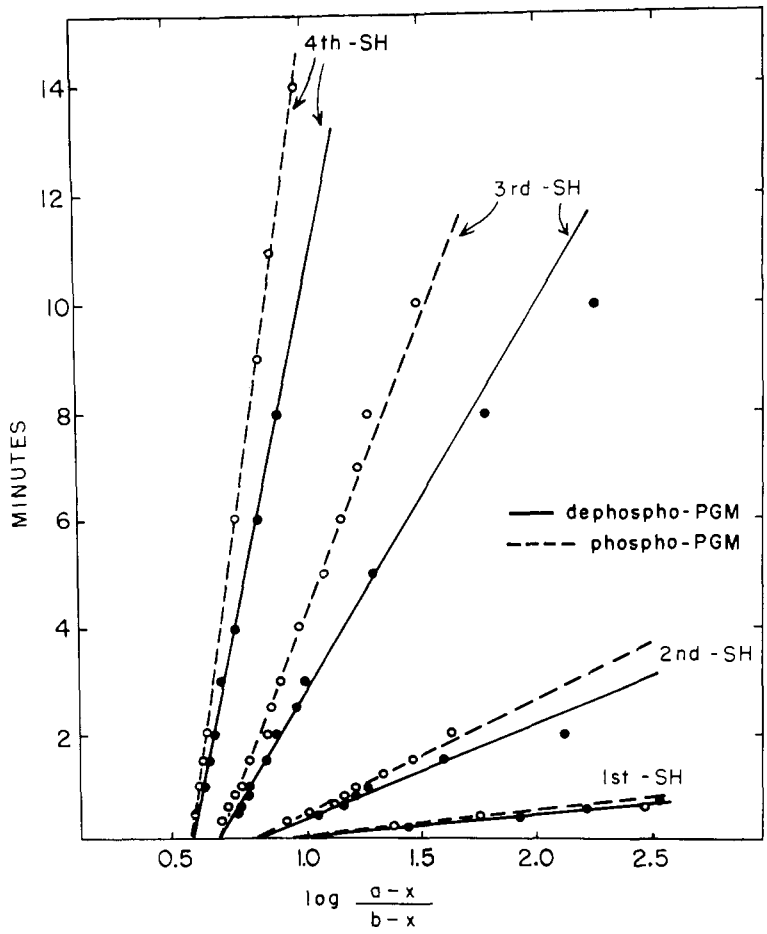

FIGURE 5: Second-order reaction rates of *p*-HMB with the first four SH groups of PGM in the presence and absence of G-1-P. Experimental conditions were as indicated under Figure 4. O- - -O in the presence of β-glycerophosphate (1×10^{-2} M); ●——● in the presence of G-1-P (1×10^{-2} M). The enzyme contained 5.5 titratable SH groups/mole.

Since it has been suggested that the two forms of the enzyme differ in conformation (Robinson and Najjar, 1961), an evaluation of the kinetics of the reaction for each individual SH group should indicate whether dephosphorylation of the enzyme results in a generalized increase in availability of all the SH groups or in the specific exposure of selected SH groups. To that end, 10.0 of mμmoles both forms of PGM was titrated by the successive addition of 10.0-mμmole aliquots of *p*-HMB. After each addition the reaction was allowed to proceed to completion until no further change in absorbance at 250 mμ could be observed. The next aliquot of the reagent was then added, thus making it possible to titrate one SH group at a time. A graphic representation of such a titration is given in Figure 4. From these multiple curves it was possible to calculate the second-order rate constant for each addition of *p*-HMB and therefore for each SH group. The results of such an analysis for the first four additions of *p*-HMB are presented in Figure 5. The straight lines indicate that each independent addition of *p*-HMB does follow simple second-order kinetics, thus indicating well-defined different reactivities for each sulfhydryl group. The technique of individual titration made it possible to delineate a clear difference between the first and the second SH. The approximate rate constants calculated from the slopes are presented in Table II. Analysis of the data permits two conclusions. (a) The first and the second sulfhydryl groups in both forms of the enzyme react much faster than do the third and fourth; (b) the major difference in the over-all rate of titration observed between phospho- and dephospho-PGM can be accounted for largely by the difference in the rates observed for the third and fourth SH groups. These results indicate that with the formation of the dephospho form of the enzyme, there is an alteration in the conformation of the molecule in such a manner as to make the third and the fourth thiol groups more available for reaction with *p*-HMB.

The Effect of Blocking of Thiol Groups on Enzymatic Activity. When the extent of inactivation of PGM was examined in relation to the number of sulfhydryl groups titrated, it became clear that these two parameters were not directly linked. In fact, the first two sulfhydryl groups could be titrated with only mini-

TABLE III: The Effect of SH Group Titration on the Catalytic Activity of PGM.[a]

Expt	Buffer (M), pH 7.2	Initial Reactants (moles of *p*-HMB/ mole of PGM)	Sulfhydryl Groups Blocked (moles/mole of PGM)		
			2 Act. (%)	3 Act. (%)	4 Act. (%)
1	Phosphate (0.1)	17.31	—	80.4	64.6
2	Glycylglycine (0.1)	10.35	90.0	74.0	53.0
3	Glycylglycine (0.1)	6.56	85.0	78.2	64.6
4	Tris (0.1)	10.65	—	78.4	44.0
5	Tris (0.1)	10.52	—	79.2	38.4
6	Tris (0.1)	6.27	—	77.0	51.0
7	Tris (0.3)	2.01	90.0	—	—
8	Tris (0.3)	3.07	86.6	80.0	—
9	Tris (0.3)	4.07	—	79.0	60.0
10	Tris (0.3)	5.07	—	72.0	40.0
11	Tris (0.3)	10.14	—	63.0	9.0

[a] PGM was treated with *p*-HMB under conditions specified in the table. The rate of mercaptide formation was followed spectrophotometrically. At the proper time intervals, when 2–4 equiv of thiol groups was titrated, a small aliquot was taken directly from the reaction cuvet and immediately assayed for enzyme activity. Samples of enzyme not treated with *p*-HMB were run simultaneously under otherwise identical conditions and served as control. Activity was expressed as per cent of control.

mal but reproducible effect on the enzymatic activity. Further blocking uniformly suppressed the activity by about 25% for the third group and 40–60% for the fourth (Table III). The results were obtained with molar ratios of *p*-HMB:PGM ranging from 2 to 17 and with three different buffers: phosphate (0.1 M), glycylglycine (0.1 M), and Tris (0.1–0.3 M), pH 7.4. The observed lack of a direct correlation between SH titration and the suppression of enzymatic activity indicates that SH groups *per se* are not critical for catalytic activity. This finding is consistent with those reported by Milstein (1961). Furthermore, the data also suggest that the suppression of activity might be the result of conformational changes induced by the covalent binding of *p*-HMB.

In view of the observation that the rate of mercaptide formation with the dephosphoenzyme is greater than that with the phospho form, a determination of the rate of inactivation of the enzyme under these conditions was made. PGM (10.3 mμmoles) was mixed with a large excess of G-1-P (10.0 μmoles) and treated with 100 mμmoles of *p*-HMB in $MgCl_2$ (1×10^{-3} M) and imidazole (4×10^{-2} M), pH 7.4 (Figure 6). The rate of inhibition of the enzyme activity in the presence of G-1-P was faster and paralleled the increased rate of mercaptide formation with dephosphoenzyme as compared to the phospho form. These results provide further evidence that the mere presence of substrate *per se* does not exert any measurable protective effect on the titration of the SH groups and further indicate that thiol groups are not directly involved at the substrate binding site of the enzyme. The inhibition of enyzme activity following blockage of four thiol groups was not reversed by the addition of 2 moles of L-cysteine/mole of mercaptide. These results are also consistent with the view that *p*-HMB blockage of the SH groups (particularly the third and fourth) induces a conformational change in the enzyme that is not readily reversed and results in a diminution of its catalytic capacity.

Discussion

The data recorded in Table I indicate that PGM possesses six SH groups per mole. This accounts for the six half-cystine residues per mole of enzyme found by amino acid analysis from different laboratories (Najjar, 1962; Yankeelov *et al.*, 1964; Sloane *et al.*, 1964). The lower value of about 4 in 3.0 M urea (Ray and Koshland, 1962) is significantly lower than that obtained subsequently by the same laboratory (Yankeelov *et al.*, 1964), a discrepancy that was not explained by these authors.

The relative stability of this enzyme to thermal denaturation and to moderate concentrations of urea and guanidine (V. Bocchini, M. R. Alioto, and V. A. Najjar, 1966, manuscript in preparation) must reside in stabilizing bonds other than the disulfide. The possibility that the enzyme might be a large circular peptide would be in harmony with this property and explain the failure to detect an amino- or a carboxy-terminal residue (Harshman and Najjar, 1965). The

absence of disulfide bonds would add another enzyme to a number already known to contain only reduced half-cystine residues (Boyer, 1959).

The finding that both dephospho-PGM in the presence or absence of G-1-P, and phosphoenzyme in the presence of G-1-P, exhibited similar rates of reaction with *p*-HMB which were considerably faster than those obtained with phospho-PGM alone, indicates that the effect of the substrate on the *p*-HMB reaction rate is a result of the conversion of the phosphorylated to the dephosphorylated form. Our conclusion is further supported by the fact that the addition of G-1,6-P to dephospho-PGM, a standard procedure for regenerating the phosphorylated form of the enzyme (Najjar and Pullman, 1954), causes a shift to a slower rate of mercaptide formation similar to that observed with isolated phospho-PGM.

Similarly, the rate of enzymatic inactivation by *p*-HMB observed for dephospho-PGM was correspondingly faster than that observed with the phosphorylated enzyme. It appears, therefore, that no protective effect on the enzyme is displayed by the substrate. Our own observations can readily be related to the difference in conformation between the phospho- and dephospho-PGM rather than to substrate-induced conformational changes (Koshland *et al.*, 1962; Yankeelov and Koshland, 1965). No linear correlation was obtained between the rate of inhibition of enzyme activity and the rate of thiol titration. The inhibition occurred at a faster rate than mercaptide formation. It is reasonable to assume that the loss of enzymatic activity is due to a concomitant disorientation of the catalytic site rather than to the blockage of an SH group at or near the active site. Furthermore, had there been no distortion of the tertiary structure, cysteine treatment should have restored full activity. Structural changes in other enzymes induced by *p*-HMB (Li *et al.*, 1962; Elödi, 1960) and lack of restoration of enzymatic activity by cysteine have been reported (Wolfe and Nielands, 1956; Mahler and Elowe, 1954; Dogson *et al.*, 1955; Sher and Mallette, 1954). Milstein (1961) also reported loss of enzymatic activity following treatment of PGM with a number of sulfhydryl reagents. There was an apparent restoration of activity of the *p*-HMB-inhibited enzyme by cysteine treatment. However, it is not clear whether the activity observed was a true restoration or resulted from cysteine activation as occurs with the normal enzyme.

The finding that the sulfhydryl groups in a protein molecule may have different reactivities toward a sulfhydryl reagent is not an unusual one (Boyer, 1954; Benesch *et al.*, 1955; Swenson and Boyer, 1957; Pihl and Lange, 1962; Di Sabato and Kaplan, 1963; Fujioka and Snell, 1965; Thoma *et al.*, 1965; Misaka and Nakanishi, 1965; Pontremoli *et al.*, 1965). In some cases, the extent of inhibition of enzymatic activity has been used as a criterion for assigning different reactivities to the SH groups. This has not proved to be uniformly valid. Thus the extent of inactivation of liver alcohol dehydrogenase appears to depend

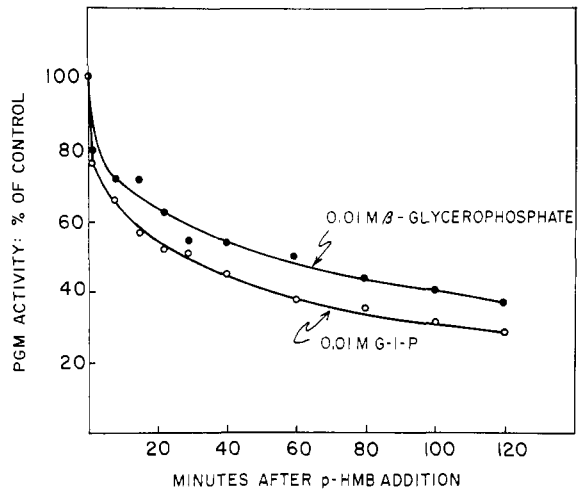

FIGURE 6: The effect of G-1-P on the inhibition of the catalytic activity of PGM by *p*-HMB. Enzyme, 10.33 mμmoles, was treated with 100 mμmoles of *p*-HMB in imidazole (4×10^{-2} M), $MgCl_2$ (1×10^{-3} M), and Tris (3×10^{-1} M), pH 7.4, in presence of 1×10^{-2} M β-glycerophosphate or G-1-P in a final volume of 1.0 ml. Controls, lacking the reagent, were prepared and assayed under otherwise identical conditions. At the indicated time intervals, aliquots of the enzyme were removed and assayed for enzymatic activity.

on the nature of the reagent used (Li and Vallee, 1965). Furthermore, in the case of phosphoglucomutase, blockage of the third and fourth SH groups gave about similar increments of inhibition of catalytic activity, yet the rate constants are markedly different (Table II).

The rate constants for each of the four thiol groups (Table II) show definite differences. This is equally true for the phospho and the dephospho forms of PGM. In the phosphoenzyme, the first sulfhydryl group has a rate constant, k, of 171.1 l. mole^{-1} sec^{-1}, which is about four times more reactive than the second, 13 times more reactive than the third, and 20 times that of the fourth SH group. A definite and characteristic difference also exists in the second-order rate constants between dephospho and phospho forms of PGM isolated as such or generated in the reaction with the proper phospho sugars G-1-P and G-1,6-P, respectively. The rate constants in Table II and the corresponding graphs in Figure 5 clearly show that the third and fourth sulfhydryl groups are considerably more reactive in dephospho-PGM as compared to phospho-PGM.

A comparison of the rate constants of PGM with those found for other proteins shows a record high rate for the first (fastest) SH group under comparable conditions. In ovalbumin and β-lactoglobulin values of 20.9 and 7.5 l. mole^{-1} sec^{-1}, respectively, were obtained at 23° in 0.05 M phosphate buffer, pH 7.0 (Boyer, 1954). Similar studies on muscle phosphorylase

yielded a value of 51 l. mole^{-1} sec^{-1} at 21° (Madsen and Cori, 1956).

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