Modification of Glycogen Phosphorylase *b* by Glutaraldehyde. Preparation and Isolation of Enzyme Derivatives with Enhanced Stability*

Jerry Hsueh-Ching Wang† and Jan-I Tu

ABSTRACT: Incubation of glycogen phosphorylase *b* with glutaraldehyde, a bifunctional protein reagent, resulted in inactivation of the enzyme. Approximately 60% inactivation could be observed after incubation of the enzyme with 0.05% reagent for 10 min. The partially inactivated enzyme showed multiple protein bands upon polyacrylamide gel electrophoresis. The various protein species were differentially susceptible to thermal denaturation. Heating at 50° for 5 hr resulted in the denaturation and precipitation of 90% of the modified enzyme. Gel electrophoretic analysis of the remaining protein showed only one major (80–90%) and one minor protein component. The minor protein component could be removed by gel filtration on a Sephadex G-200 column. This modified and heat-treated enzyme was considerably more resistant than native phosphorylase *b* toward heat, cold, or urea denaturation. Titration with trinitrobenzenesulfonic acid revealed that the stabilized enzyme contained 7–11 less free amino groups than native phosphorylase *b*. Amino acid analysis also indicated that the modified enzyme contained 10% less lysyl residues than native phosphorylase *b*. Titration of the enzyme with Ellman reagent suggested that sulfhydryl groups had not reacted in the modified enzyme. The specific activity of the modified and heat-treated phosphorylase *b* was approximately 65% that of the native enzyme. Although the affinities of this enzyme species toward glucose 1-phosphate and glycogen were the same as those of native phosphorylase *b*, the homotropic interaction of AMP which was observed with native enzyme could not be demonstrated with the stabilized phosphorylase *b*. Modification of phosphorylase *b* with monofunctional aldehyde was also investigated. Enzyme derivatives of these modifications were also more resistant to denaturation than native phosphorylase *b*.

The relative stability of 1% butyraldehyde-modified and glutaraldehyde-stabilized phosphorylase *b* depended upon denaturation conditions. While butyraldehyde-modified enzyme showed a higher resistance toward heat denaturation (50–51°) the glutaraldehyde derivative appeared to be more stable upon exposure to cold or urea solutions. Kinetic studies indicated that homotropic interactions of adenosine monophosphate was enhanced in the 1% butyraldehyde-modified phosphorylase *b*.

The regulatory significance of interconversion of glycogen phosphorylases *a* and *b* has long been recognized (Krebs and Fischer, 1962). In recent years, it has become increasingly clear that this enzyme may also be regulated through highly complexed allosteric interactions. Kinetic and structural studies have led many investigators to postulate that both phosphorylases *a* and *b* can undergo various conformational changes during allosteric transitions (Helmreich *et al.*, 1967; Lowry *et al.*, 1967; Engers and Madsen, 1968; Madsen and Shechosky, 1967; Wang and Black, 1968; Black and Wang, 1968; Kastenschmidt *et al.*, 1968; Buc and Buc, 1968). The nature of these conformational changes is far from clear.

Since protein conformations are determined by various intramolecular interactions, factors which effect such interactions may alter allosteric properties of the enzyme. Based on this view, the relationship between a specific type of interaction and certain allosteric behavior may be investigated. A recent study on the effect of polyamines on phosphorylase *b* has led us to postulate that a change in intramolecular electrostatic interactions may occur during AMP activation of this enzyme (Wang *et al.*, 1968). The desensitization of phosphorylase *b* by NaF or by controlled tryptic digestion has also been attributed to a change in electrostatic interaction of this enzyme (Sealock and Graves, 1967; Graves *et al.*, 1968).

In the present study, the modification of glycogen phosphorylase *b* by glutaraldehyde, a bifunctional reagent, was studied in an attempt to affect intramolecular interactions in this enzyme. A fraction of the modified enzyme was highly resistant to denaturation. This stabilized enzyme derivative was purified and characterized. In addition, modification of phosphorylase *b* by monoaldehydes, butyraldehyde and propionaldehyde, also resulted in stabilization of the enzyme. The allosteric properties of the modified enzymes were found to depend upon the reagent used for modification.

Materials and Methods

Crystalline phosphorylase *b* was isolated from rabbit muscle by the procedure of Fischer and Krebs (1958). Three- or four-times-crystallized enzyme which had been treated with Norit A to free the tightly bound AMP was used throughout this work. Cysteine hydrochloride, sodium glycerophos-

* From the Department of Biochemistry, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada. *Received June 23, 1969.* Work supported by Grant MA 2381 from the Medical Research Council of Canada.

† Medical Research Council Scholar.

phate, EDTA, DTNB,[1] TNBS, adenosine monophosphate, and shellfish glycogen were obtained from Sigma Chemical Co. The glycogen was purified according to the procedure of Sutherland and Wosilait (1956). Glutaraldehyde and butyraldehyde were obtained from Baker Chemical Co. Propionaldehyde and $NaBH_4$ were from Matheson Coleman & Bell. Chemicals for gel electrophoresis were from Canalco Ind. Co. with the exception of coomassie blue which was from Consolidated Laboratories.

Routine enzyme activity measurements were carried out in the direction of glycogen synthesis as described by Illingworth and Cori (1953). Enzyme concentration was determined spectrophotometrically at 280 mμ. An absorbancy index of 11.9 (Appleman *et al.*, 1963) for a 1% enzyme solution was used.

The time course of cold inactivation of phosphorylase *b* was determined according to the procedure of Graves *et al.* (1965). The enzyme (3–7 mg/ml) was diluted 50–150-fold into 0.04 M glycerophosphate–0.03 M cysteine at the desired pH and temperature to start the cold inactivation. For the kinetics of heat inactivation, the incubation at high temperature was started by dilution of the enzyme (3–7 mg/ml) 50–150-fold into buffer solutions at the desired temperature. The buffers contained 0.04 M glycerophosphate–0.03 M cysteine at pH 7.0. At various intervals of the incubation, aliquots (0.2 ml) were withdrawn and placed into ice-cold test tubes. The tubes were left on ice until all the aliquots were collected. The activity of the enzyme in these aliquots was measured at 30° for 5 min by addition of 0.2 ml of substrate containing 0.032 M glucose 1-phosphate, 2% glycogen, and 2 mM AMP.

Titration of amino groups by TNBS was according to the colorimetric method of Habeeb (1966). To determine the amount of amino group masked in the glutaraldehyde-modified phosphorylase *b*, the modified and the native enzymes were titrated concurrently at five or more different protein concentrations. The absorbance at 425 mμ was then plotted against protein concentrations using the least-squares fit. The ratio of slope for the modified enzyme to that for native phosphorylase *b* was taken as the per cent amino acid group remaining in the modified enzyme. Determination of sulfhydryl groups was performed in the presence of 1% sodium dodecyl sulfate using Ellman reagent, DTNB (Ellman, 1959). The procedure used by Battell *et al.* (1968) was closely followed.

Amino acid analysis was performed on a Spinco automatic amino acid analyzer. Enzyme samples were first dialyzed against two changes of 0.04 M glycerophosphate–0.03 M thioglycolate at pH 7.0, followed by two changes each of 0.02 M glycerophosphate–0.001 M EDTA and deionized water. The total time for this extensive dialysis was 3 days. Hydrolysis of the protein was carried out at 110° in 6 N HCl under vacuum for 48 hr.

Ultracentrifugal runs were performed on a Spinco Model E analytical ultracentrifuge at a rotor speed of 60,000 rpm. The temperature for all runs was maintained at 20 ± 1°. Sedimentation coefficients were determined with the aid of a Nikon Model 6C microcomparator.

Polyacrylamide gel electrophoresis was carried out at pH 8.3 and room temperature with the procedure of Ornstein (1964). Either Amido Black or coomassie blue was used for staining. The relative intensity of the protein staining was estimated by a densitometer.

[1] Abbreviation used that are not listed in *Biochemistry* 5, 1445 (1966), are: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNBS, 2,4,6-trinitrobenzenesulfonic acid.

Results

Preparation and Isolation of Glutaraldehyde-Stabilized Phosphorylase b. Incubation of glycogen phosphorylase *b* with glutaraldehyde, a bifunctional reagent, resulted in a loss in enzyme activity. Essentially complete inactivation of the enzyme (12 mg/ml) was obtained after incubation with 0.1% glutaraldehyde for 10 min at pH 7.5. At 0.05% reagent, approximately 60% inactivation was achieved. In addition to glutaraldehyde concentration, the enzyme concentration could also affect the extent of the inactivation. In three experiments with high phosphorylase *b* concentration (25–30 mg/ml), the enzyme was inactivated to greater than 90% with 0.05% reagent. The enzyme inactivation in these experiments was accompanied by heavy protein precipitation. Presumably, the bifunctional reagent favored intermolecular cross-links at high protein concentration.

Since glutaraldehyde has been shown to enhance the mechanical strength of protein crystals (Quiocho and Richards, 1964), the heat stability of 0.05% glutaraldehyde-modified phosphorylase *b* was compared with that of the native enzyme. In both samples, the enzyme was rapidly inactivated at 48°. The rate of inactivation for the modified enzyme, however, was appreciably reduced after approximately 70% of its original activity had been lost. Thus, although a loss of 70% of original activity could be demonstrated after 30 min, an additional 60-min incubation only resulted in an additional 10% loss of enzyme activity. These results suggested that modification of phosphorylase *b* by glutaraldehyde resulted in variously modified enzyme forms. Some of these modified enzymes might be more labile than the native enzyme whereas others, or a portion of others, were stabilized.

Disc gel electrophoresis on polyacrylamide gel showed that phosphorylase *b* after modification with 0.05% glutaraldehyde was indeed highly heterogeneous. Figure 1B showed that the partially inactivated enzyme could be separated into at least six protein bands. It is known that native phosphorylase *b* is homogeneous by this criterion (Huang and Madsen, 1966; Philips and Graves, 1968). The variously modified enzyme species were found to differ in their thermal stability. After heating of the modified enzyme at 51° for 5 hr, gel electrophoretic analysis of the remaining soluble protein showed only one major (80–90%) and one minor protein component (Figure 2A). These probably represented the protein species which were more resistant against heat denaturation.

A procedure was developed for the preparation and isolation of the stabilized phosphorylase *b*. Phosphorylase *b* (~15 mg/ml) was incubated at 0° with 0.05% glutaraldehyde in buffer containing 0.04 M glycerophosphate–0.001 M EDTA (pH 7.5). After incubation for 10 min, an equal volume of 1 mg/ml of $NaBH_4$ in 0.3 M glycerophosphate–0.001 M EDTA (pH 7.0) was added to the enzyme solution. The reaction mixture was allowed to stand on ice for 30 min. The addition of $NaBH_4$ was then repeated twice at 30-min intervals. The enzyme was then precipitated with addition of solid $(NH_4)_2SO_4$ to the final concentration of 35%. The

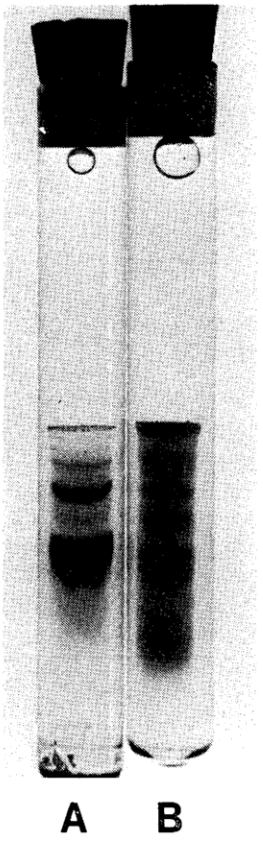

FIGURE 1: Polyacrylamide gel electrophoresis of glutaraldehyde-modified phosphorylase *b*. (A) Phosphorylase *b* modified with 0.05% glutaraldehyde and then heated at 51° for 5 hr. (B) Sample as in part A prior to heating at 51°. Electrophoresis was carried out at 2.5 mA/tube for 2 hr at room temperature.

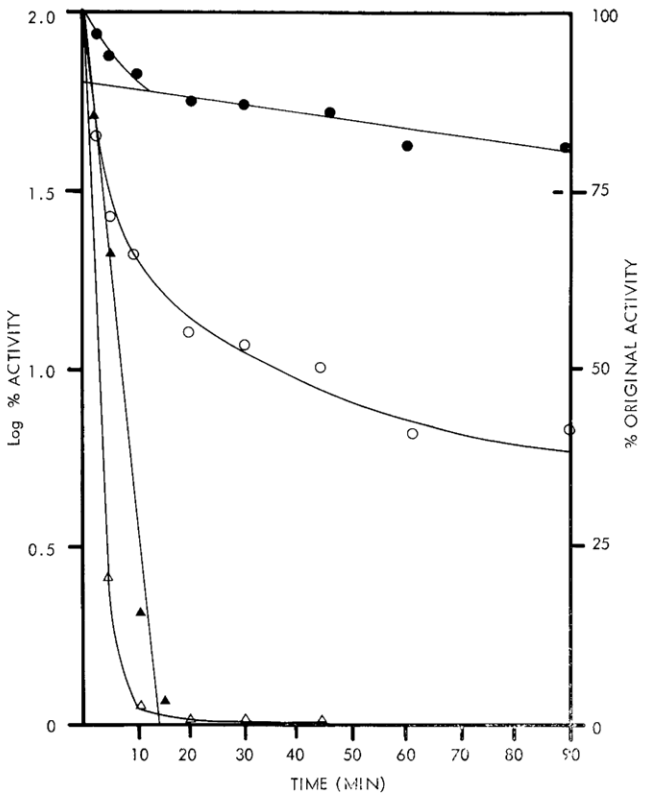

FIGURE 2: Comparison of kinetics of heat inactivation of native and glutaraldehyde-stabilized phosphorylase *b*. Activity of phosphorylase *b* was plotted as per cent original activity (△), or log per cent original activity (▲) as a function of incubation time at 51°; stabilized enzyme activity expressed as per cent (○) and log per cent original activity (●).

precipitate collected from centrifugation was then suspended in 0.04 M glycerophosphate–0.001 M EDTA buffer at 7.0 and dialyzed against 0.04 M glycerophosphate–0.03 M cysteine (pH 7.0). The glutaraldehyde-modified enzyme had a low specific activity. When it was heated at 50–51° for 0.5 hr, a large amount of protein precipitate developed and was removed by centrifugation. The enzyme remaining in solution was then precipitated by addition of solid $(NH_4)_2SO_4$ to 35%. The precipitate was dissolved in a small volume of 0.04 M glycerophosphate–0.03 M cysteine buffer at pH 7.0 and dialyzed against the same buffer. This enzyme was then subjected to incubation at 50–51° for 4.5 hr and the precipitate was removed. In Table I the yield and specific activity for a typical preparation are presented. The specific activity of the enzyme after the second heat treatment was usually 30–40% lower than that of native phosphorylase *b*.

The stability of the isolated enzyme was routinely examined as a test for the quality of the preparation. The kinetics of the heat inactivation shown in Figure 2 indicated that the stabilized enzyme was more resistant toward heat inactivation than native phosphorylase *b*. While incubation of native phosphorylase *b* at 51° for 10 min resulted in a greater than 95% loss of enzymic activity, the stabilized enzyme still retained 70% of its original activity. In Figure 2, the kinetics of heat inactivation were also plotted according to the first-order plot. Although inactivation of the native enzyme could be described as a first-order reaction, the stabilized enzyme showed apparent deviation from linearity. These data suggest that the sample represented a mixture of enzyme derivatives with different thermal stability. The later stage of the inactivations, however, could be approximated by a linear first-order plot. Presumably, this linear region represented the kinetics of heat inactivation of the most stable enzyme species in the original mixture. Based on this assumption, the amount of the most stable species in the enzyme preparation might be estimated by extrapolating the slope of the linear region to the initial incubation time. Results in Figure 2 gave an extrapolated value of 65%. In addition to heat inactivation, the kinetics of cold inactivation of the stabilized enzyme were examined and compared with that of native phosphorylase *b*. Figure 3 showed that the rate of cold inactivation of the stabilized phosphorylase *b* was much slower than that of native phosphorylase *b*. The first-order kinetic plot of the cold inactivation of the stabilized enzyme was also found to show apparent deviation from linearity. A linear region could be obtained at the later stage of this cold inactivation. When the extrapolation procedure was applied to the estimation of the amount of the most stable enzyme species, a value of 64% was obtained. This value compared favorably with that obtained from the kinetic data of heat inactivation of the sample (Figure 2).

Since the stable enzyme isolated by the above procedure represented less than 10% of the original glutaraldehyde-

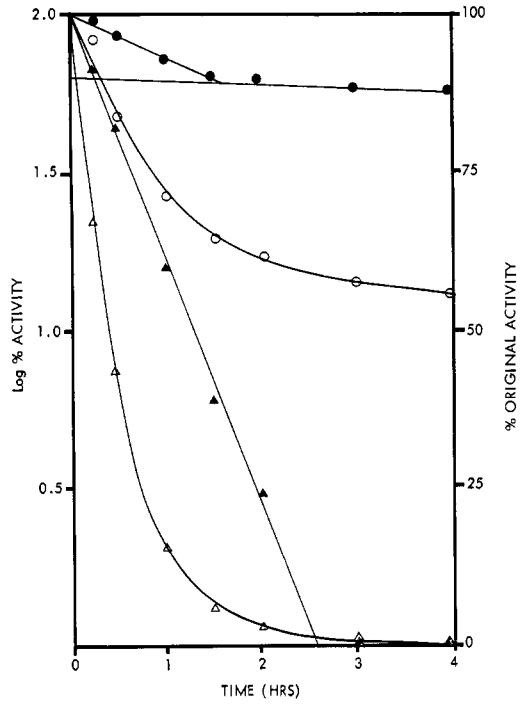

FIGURE 3: Comparison of kinetics of cold inactivation of native and glutaraldehyde-stabilized phosphorylase *b*. Activity of phosphorylase *b* expressed as per cent (△) and log per cent (▲) original activity as a function of incubation time at 0° in 0.04 M glycerophosphate–0.03 M cysteine (pH 5.9); activity of stabilized enzyme as per cent original activity (○) and log per cent original activity (●).

modified enzyme (Table I), the possibility that a small fraction of stable phosphorylase *b* could also be isolated from the native enzyme was tested. In one experiment, phosphorylase *b* (13 mg/ml) was heated at 51° for 3.5 hr. The remaining soluble enzyme was concentrated by $(NH_4)_2SO_4$ precipitation and then dialyzed against 0.04 M glycerophosphate–0.03 M cysteine (pH 7.0) buffer. It was found that the kinetics of heat as well as cold inactivation of this enzyme were identical with those of the untreated phosphorylase *b*.

Phosphorylase *b* in its free state exists as a dimeric species with an $s_{20,w}$ value of 8.2 S. Ultracentrifugal analysis of the stabilized enzyme showed that it also existed mainly as a dimeric form. A small amount of fast sedimenting component with an $s_{20,w}$ of 12.8 S was, however, evident (Figure 4, inset). This sedimentation constant is similar to that of the tetrameric form of this enzyme. Molecular sieving chromatography on Sephadex G-200 also revealed the existence of a small amount

TABLE I: Preparation of Glutaraldehyde-Stabilized Phosphorylase *b*.

Fraction	Vol (ml)	Sp Act. (units/mg)	Protein Yield (%)
Original enzyme	25.0	1205	100
Glutaraldehyde modified	25.0	510	87
First heat	6.5	495	20
Second heat	6.0	740	6.5

TABLE II: Effect of Monoaldehydes on Phosphorylase *b* Activity.[a]

Reagent	Enzyme Act. (%)[b]
No	100
Propionaldehyde (0.5)[c]	100
Propionaldehyde (1)	92
Propionaldehyde (3)	62
Butyraldehyde (0.5)	97
Butyraldehyde (1)	88
Butyraldehyde (3)	56

[a] Phosphorylase *b* (17 mg/ml) in 0.04 M glycerophosphate (pH 7.5) was incubated with the aldehyde at 0°. After incubation for 10 min, an equal volume of 1 mg/ml of $NaBH_4$ in 0.3 M glycerophosphate (pH 7.0) was added. The $NaBH_4$ addition was repeated once after 30 min. The reaction mixture was then dialyzed against 0.04 M glycerophosphate–0.03 M cysteine (pH 7.0). [b] Enzyme activity is expressed as per cent of native enzyme activity. [c] Numbers in parentheses represent reagent concentration in per cent.

of aggregated enzyme which appeared as a leading shoulder in the elution profile (Figure 4). The elution fractions under the major peak were pooled and concentrated. Gel electrophoresis of this enzyme sample showed essentially one protein component corresponding to the major component in Figure 1A. The minor gel electrophoretic protein component observed in Figure 1A, therefore, was probably the tetrameric form of the glutaraldehyde-modified enzyme. As was described above, first-order kinetic plots of the heat or cold inactivation of the stabilized enzyme might be used to estimate the amount of the most stable molecular species in an enzyme preparation. When this procedure was applied to an enzyme preparation prior to and after Sephadex G-200 chromatography, the amount of the most stable enzyme derivative was found to be 68 and 85%, respectively. It appeared, therefore, that molecular sieving chromatography on Sephadex G-200 could be a useful step in purification of the most stable phosphorylase *b* derivative.

Modification of Phosphorylase b by Propionaldehyde and Butyraldehyde. In contrast to glutaraldehyde, monoaldehydes such as butyraldehyde and propionaldehyde did not inactivate phosphorylase *b* at reagent concentrations below 0.5% (Table II). At 1–3% of propionaldehyde or butyraldehyde, approximately 10–40% inactivation of the enzyme could be demonstrated. If the incubation of the enzyme with aldehydes was not followed by additions of $NaBH_4$, no inactivation of the enzyme could be observed. Thus Schiff base probably was the primary product of the reaction between enzyme and monoaldehydes.

These monoaldehyde-modified enzymes also exhibited enhanced resistance toward heat denaturation. Figure 5 showed that all the enzyme derivatives of monoaldehyde modification were more stable than native phosphorylase *b* at 50°. Among them, the 1% butyraldehyde-modified phosphorylase *b* was most stable.

In order to test whether the mechanisms of stabilization

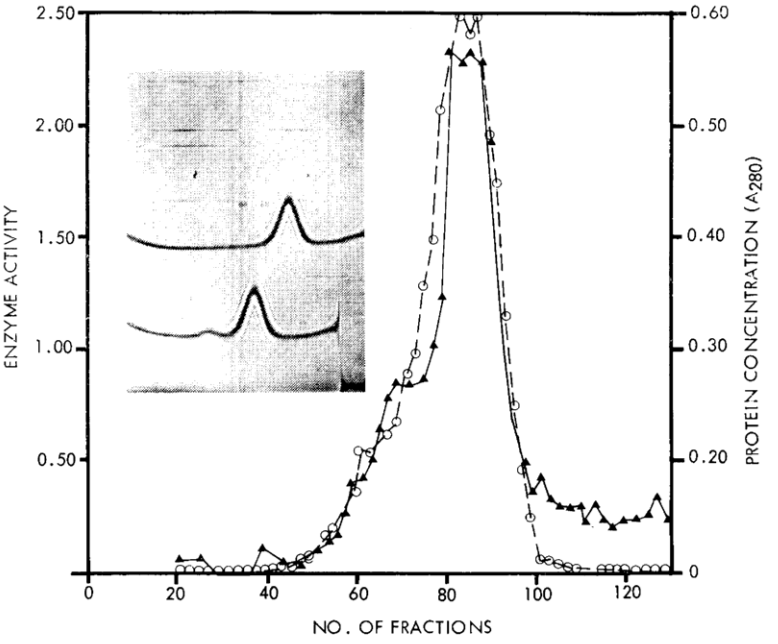

FIGURE 4: Molecular size heterogeneity of glutaraldehyde-stabilized phosphorylase *b*. Sephadex G-200 molecular sieving chromatography of the stabilized phosphorylase *b* (14 mg/2 ml). The column (2.5 × 45) was equilibrated with 0.02 M glycerophosphate–0.015 M β-mercaptoethanol (pH 7.0). Chromatography was carried out at room temperature. Fractions containing 1.1 ml of eluent were collected and analyzed for both enzyme concentration (▲) and activity (○). Inset: sedimentation patterns of native (upper curve) and stabilized (lower curve) phosphorylase *b*.

by glutaraldehyde and by monoaldehyde modifications were the same, the relative stability of the glutaraldehyde- and 1% butyraldehyde-stabilized enzymes was examined. For these studies the 1% butyraldehyde-modified enzyme was first heated at 50° for 5 hr to free it from less stable enzyme derivatives. In one experiment, the concentration of the modified enzyme prior and after the heat treatment was 17 and 8.8 mg per ml, respectively. Thus, less than 50% of the modified enzyme was precipitated during this prolonged heating. The treated enzyme was homogeneous as judged by gel electrophoresis. The specific activity of this enzyme was 1350 units, similar to that of native phosphorylase *b*. This preparation was used throughout this work. Figure 6A showed that this 1% butyraldehyde-modified phosphorylase *b* was as stable as glutaraldehyde-stabilized phosphorylase *b* upon exposure to high temperature (51°). It appeared, however, less stable than the glutaraldehyde-stabilized enzyme when they were subjected to cold inactivation (Figure 6B). After incubation at 0° and pH 5.9 for 31 hr, while glutaraldehyde derivative still retained 50% original activity, the butyraldehyde derivative only possessed approximately 10% of its original activity. These results suggested that the relative stability of these two enzyme derivatives depended upon the conditions used to affect their inactivation. This suggestion is further substantiated by a comparison of the stability of these enzymes in urea solution (Figure 7). At low concentrations (<1.5 M) of this denaturant, the monofunctional aldehyde-modified phosphorylase *b* appeared to be more stable than glutaraldehyde-modified enzyme; the opposite was observed at higher urea concentrations (>1.5 M). Indeed, at 2 M or higher urea concentration, the monoaldehyde-modified enzyme could be inactivated as readily as native phosphorylase *b*.

Preliminary Chemical and Kinetic Characterization of the Glutaraldehyde-Stabilized Phosphorylase b. To determine the amount of amino groups masked in the stable enzyme derivatives, the glutaraldehyde enzyme derivatives, along with a native enzyme sample, were titrated with TNBS. In three experiments using three different preparations, the enzyme derivatives were found to contain 7, 10, and 11% less amino groups than the control sample. Similar results were obtained when the amino acid compositions of native and the modified phosphorylase *b* were determined and

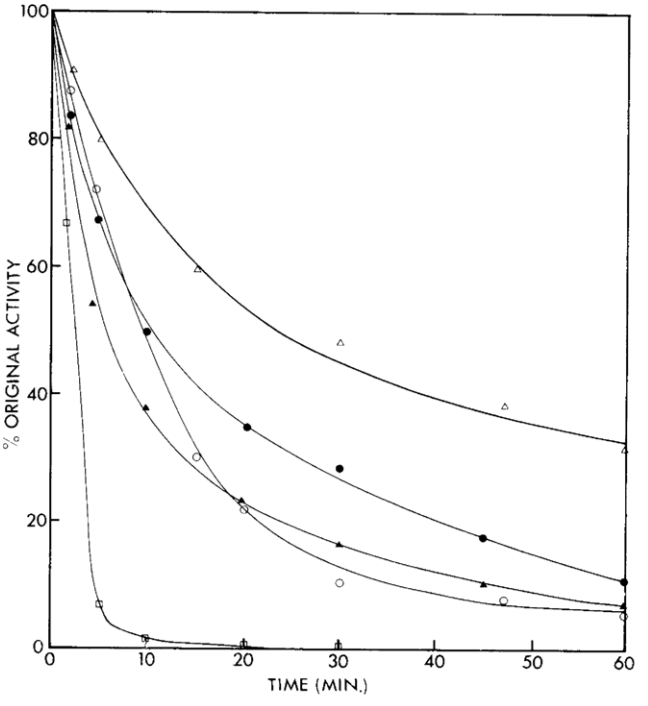

FIGURE 5: Heat inactivation of monoaldehyde-modified phosphorylase *b*. Time course of enzyme inactivation at 51° of native (□), 1% propionaldehyde-modified (○), 3% propionaldehyde-modified (●), 1% butyraldehyde-modified (△), or 3% butraldehyde-modified phosphorylase *b* (▲).

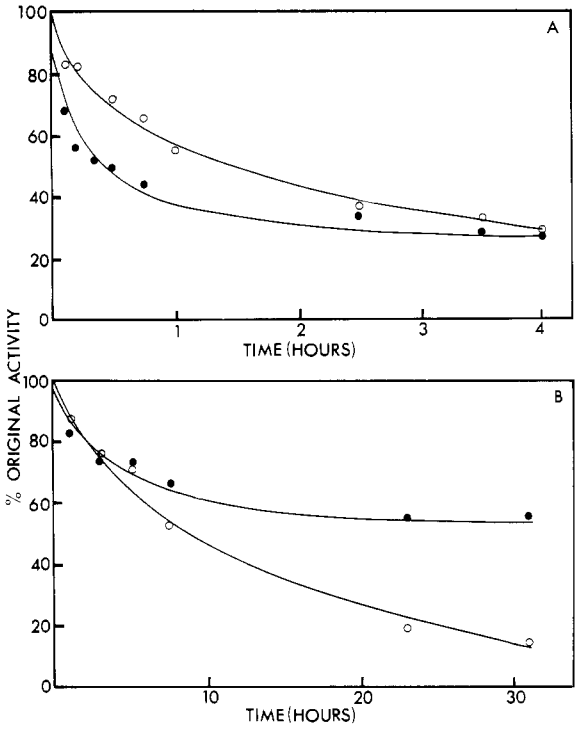

FIGURE 6: Comparison of heat and cold stability of glutaraldehyde- and 1% butyraldehyde-stabilized phosphorylase *b*. (A) Time course of heat inactivation at 51° for the glutaraldehyde- (●) and 1% butyraldehyde-stabilized phosphorylase *b* (○). (B) Time course of cold inactivation of the glutaraldehyde- (●) and 1% butyraldehyde-stabilized phosphorylase *b* (○).

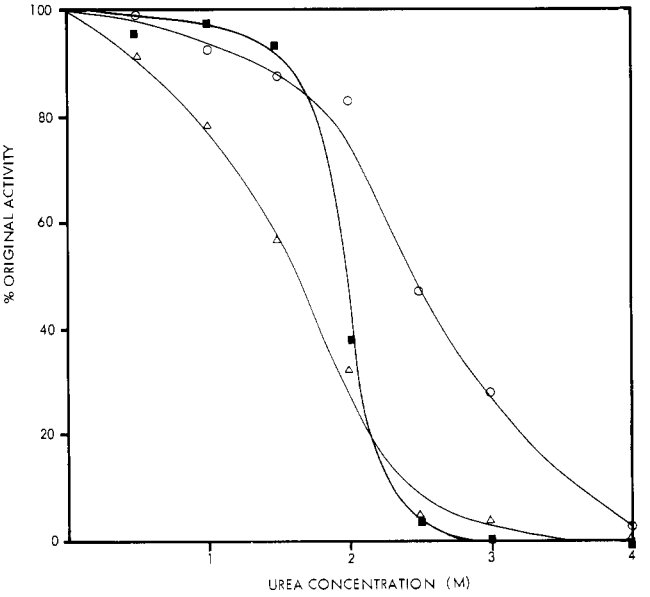

FIGURE 7: Urea inactivation of phosphorylase *b*. Native (△), glutaraldehyde-stabilized (○), and 1% butyraldehyde-stabilized phosphorylase *b* (■) were incubated at 30° in 0.02 M glycerophosphate–0.015 M cysteine (pH 6.9) with various concentrations of urea. After 30 min the samples were diluted 50-fold in 0.04 M glycerophosphate–0.03 M cysteine (pH 6.9) and enzyme activities were measured. Samples incubated in the absence of urea were taken as 100% activity.

compared. The modified enzyme contained 10% less lysyl residues. None of the other amino acids in the modified and native phosphorylase *b* differed by more than 5%, with the exception of serine and methionine. The difference of these two amino acids between the two enzyme samples was not considered significant. Serine is known to be partially destroyed during acid hydrolysis whereas methionine content in both analyses was too low to be accurately measured (<0.05 μM). For the amino group content in 1% butyraldehyde-modified enzyme, only TNBS titration was carried out. In comparison with native phosphorylase *b*, the modified enzyme contained 9.7% less amino groups.

However, in contrast to amino groups, the amount of sulfhydryl groups in the glutaraldehyde enzyme derivatives was essentially identical with that in native phosphorylase *b*. The sulfhydryl groups were determined by using Ellman reagent in the presence of 1% sodium dodecyl sulfate. In two experiments, the amount of sulfhydryl groups per modified phosphorylase *b* was found to be 11 and 12, whereas the respective control samples were found to contain 11 and 13 sulfhydryl groups per enzyme.

The low specific activity observed with the glutaraldehyde-stabilized phosphorylase *b* (Table I) appeared to have arisen from a low maximum velocity of this enzyme. Figure 8 showed that the K_m's of the glutaraldehyde-stabilized enzyme for its substrates glycogen and glucose 1-phosphate were essentially identical with those of native phosphorylase *b*. These results suggested that the enzyme affinities toward substrates were not affected by this modification and the subsequent heat treatment. The maximum velocity of this enzyme with respect to either of the substrates was found to be 65% that of native phosphorylase *b*. Values ranging from 62 to 78% were obtained. The kinetics of the stabilized and native phosphorylase *b* with respect to AMP were also examined. Figure 9 shows that AMP activation of the glutaraldehyde-stabilized enzyme did not exhibit homotropic interaction, whereas the expected homotropic interaction of the nucleotide with native phosphorylase *b* (Helmreich and Cori, 1964; Sealock and Graves, 1967; Madsen and Shechosky, 1967) could be demonstrated. The K_a of AMP for the stabilized enzyme was 6 to 8 $\times 10^{-5}$ M, whereas the nucleotide concentration required for half-maximum activation of native phosphorylase *b* ranged from 4 to 7 $\times 10^{-5}$ M in thee experiments. Although the maximum velocity of the stabilized enzyme in this experiment was approximately 75% that of native phosphorylase *b*, the activity of this enzyme derivative at low concentrations of AMP (<1.5 $\times 10^{-5}$ M) was higher than that of the native enzyme. This probably resulted from the elimination of the homotropic interaction. The kinetics of AMP activation of the 1% butyraldehyde-modified phosphorylase *b*, in contrast, showed a more pronounced homotropic interaction than the native enzyme. In addition, the monoaldehyde enzyme derivative exhibited a lower affinity toward AMP. The AMP concentration required for half-maximum activation of this enzyme was 1.4 $\times 10^{-4}$ M, approximately two to three times that required for native phosphorylase *b*.

Discussion

Glutaraldehyde has been used successfully in preparing cross-linked protein crystals which possess greatly enhanced

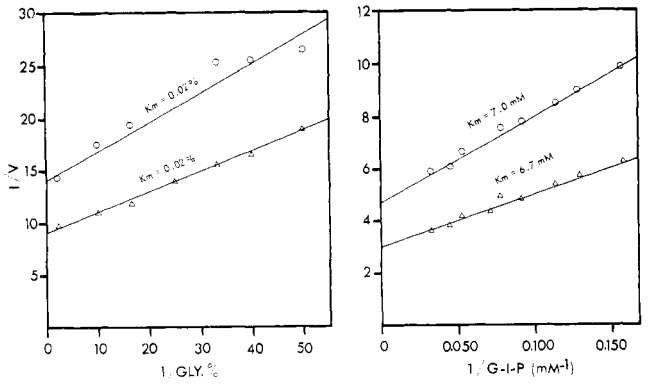

FIGURE 8: Reciprocal plot for native and glutaraldehyde-stabilized phosphorylase *b* with respect to glucose 1-phosphate and glycogen. (A) Assay mixture contained 0.016 M glucose 1-phosphate and 0.001 M AMP. (B) Assay mixture contained 0.001 M AMP–1% glycogen. Initial velocity was expressed as phosphate production (absorbancy at 660 mμ) per 5 min per mg of enzyme. (△) and (○) reresents initial velocities for native and stabilized phosphorylase *b*, respectively.

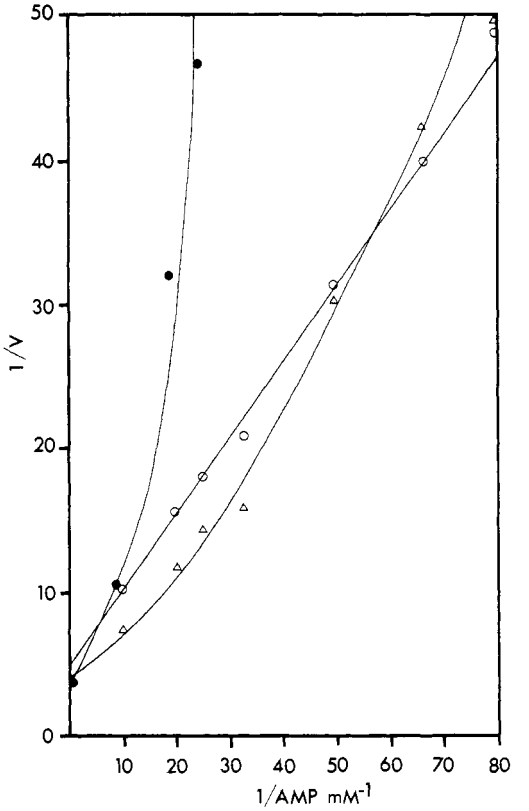

FIGURE 9: Reciprocal plot for native and stabilized phosphorylase *b* with respect to AMP. Assay mixture contained 1% glycogen–0.016 M glucose 1-phosphate with varying concentrations of AMP. Other conditions as in Figure 9. (△), (○), and (●) for native, glutaraldehyde, and butyraldehyde-stabilized phosphorylase *b*, respectively.

mechanic stability (Quiocho and Richards, 1964). In the present study, modification of glycogen phosphorylase *b* in an aqueous solution also produced more stable enzyme derivatives. It is not clear, however, whether such stabilization is directly related to bifunctional modification of this enzyme. Modification of glycogen phosphorylase *b* by monoaldehyde also strengthens the enzyme against denaturation. Since the relative stability of the glutaraldehyde- and butyraldehyde-modified phosphorylase *b* depends upon the denaturation conditions, the mechanisms of stabilization by these two types of modification are probably different.

In recent years, evidence has accumulated to support the view that conformational changes in enzymes may play important roles in catalysis (Koshland and Neet, 1968). This view has been most useful in the formulation of the current concept of allosteric interactions (Monod *et al.*, 1963). In the case of glycogen phosphorylase *b*, recent studies have suggested that allosteric transitions of this enzyme may involve various conformational changes. Since protein denaturation represents changes from an organized to a largely disorganized structure, the aldehyde derivatives of phosphorylase *b*, which possess enhanced resistence toward denaturation, may be expected to exhibit altered allosteric properties. In the preliminary kinetic characterizations reported in this study, both glutaraldehyde- and butyraldehyde-modified phosphorylase *b* have allosteric properties different from those of the native enzyme. The observation that the allosteric properties of these two types of modified enzyme are different is in agreement with the suggestions that these enzyme derivatives are stabilized by different interactions.

Since no homotropic cooperativity of AMP can be observed with the glutaraldehyde-stabilized phosphorylase *b*, this enzyme derivative may be considered as "desensitized." An essential step in the preparation and isolation of the glutaraldehyde-stabilized enzyme is a heat treatment at 51°. However, prolonged heating of the native enzyme at this temperature does not result in desensitization of homotropic cooperativity. Thus heat treatment does not appear to be the primary cause of desensitization of the glutaraldehyde-stabilized enzyme, in spite of the classic examples of desensitization of allosteric enzymes by high temperature (Gerhart and Pardee, 1962; Changeux, 1962).

Although glutaraldehyde has been widely used as a cross-linking reagent, the chemical nature of its reaction is still obscure. Amino groups and possibly sulfhydryl groups are the sites of attack in proteins by glutaraldehyde;[2] however, positive identification of the amino acid derivatives has not yet been successful (Wold, 1967). Recently Richards and Knowles (1968) have shown that glutaraldehyde may exist in various polymerized forms. This property of the reagent has, on the one hand, made it a good cross-linking reagent, and, on the other hand, contributed to the difficulty in chemical identification of the reaction products. In the present study, only the loss of amino acid residues and/or functional groups have been determined with the modified enzymes.

Although desensitization of homotropic cooperativity of AMP in phosphorylase *b* upon modification of a small number of sulfhydryl groups has been demonstrated (Gold, 1968; Kastenschmidt *et al.*, 1968), no significant difference in sulfhydryl content between the glutaraldehyde-modified and native phosphorylase *b* has been observed in the present study. Thus, the mechanism of desensitization of homotropic inter-

[2] Although other amino acid side chains have also been implicated in the reaction of glutaraldehyde and proteins (Habeeb and Hiramoto, 1968), they require high concentrations of the reagent.

actions in the stabilized enzyme differs from those involving sulfhydryl groups. Furthermore, the enzyme derivatives of sulfhydryl reagents exhibit very low affinity for AMP (Gold, 1968; Kastenschmidt *et al.*, 1968; Damjanovich *et al.*, 1967) whereas the K_a of the glutaraldehyde-stabilized enzyme for this nucleotide is comparable to that of native phosphorylase *b*. In contrast to sulfhydryl groups, approximately 10% amino groups has reacted in the glutaldehyde-stabilized phosphorylase *b*. It is difficult to attribute the desensitization of homotropic interaction for AMP in the glutaraldehyde-enzyme derivative to the masking of these amino groups. Butyraldehyde-modified phosphorylase *b*, which also contains 10% less amino groups, exhibits strong homotropic cooperativity for AMP. Indeed, the strength of the interaction in the monoaldehyde-modified enzyme is even greater than that in native phosphorylase *b*. The possibility that the two reagents have modified different amino groups thus causing different effects cannot be completely ruled out.

The effect of chemical modification of lysine groups on the structure and activity of glycogen phosphorylase has been investigated by two groups of workers. Huang and Madsen (1966) have shown that inactivation of phosphorylase *b* by KCNO involves the modification of 20 amino groups. The inactive enzyme derivatives show an ultracentrifugal pattern indicative of partial dissociation of the enzyme. Philips and Graves (1968), on the other hand, achieved essentially complete inactivation of phosphorylase *b* upon dinitrophenylation of 4–5 lysyl residues. In the present study, modification of 7–11 amino groups in phosphorylase *b* with glutaraldehyde results in only 30–40% inactivation of the enzyme. When similar amounts of amino groups had reacted with butyraldehyde, essentially no inactivation of the enzyme was observed. The ultracentrifugal pattern of the modified phosphorylase *b* is essentially identical with that of the native enzyme. Thus, these modified amino groups appear to represent an additional class which is neither obligatory for enzyme activity nor essential for subunit association.

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