Reaction of Glyceraldehyde-3-phosphate Dehydrogenase with Dibromoacetone†

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ABSTRACT: Evidence for the proximal positioning of the catalytically essential cysteine residue and a histidine residue (or any residue bearing a nucleophilic side chain) was sought by means of chemical modification of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase using the bifunctional reagent, 1,3-dibromoacetone. At pH 7.0 amounts of the reagent stoichiometric with the concentration of active sites produced rapid, complete, and irreversible inactivation of the enzyme by alkylating only one cysteine residue per polypeptide chain. The presence of another reactive bromomethyl group within the active site following inactivation was clearly shown by its reaction with thiolnitrobenzoate. However, various methods for quantitating amino acid content revealed no further loss, by means of a cross-linking reaction, of a histidine, tyrosine, tryptophan, lysine, cysteine, or methionine residue. The evidence regarding a possible cross-link to a glutamic or aspartic acid residue is less conclusive. The same observations pertained for the reaction carried out under a variety of conditions: pH range from 5.6 to 8.8, protein concentrations from 1 to 10 mg/ml, temperatures from 0 to 15°, reaction times from 6 to 18 hr, and presence or absence of coenzyme and ammonium sulfate. Baeyer–Villager oxidation of the modified enzyme with performic acid yielded only two radiocarbon-containing species, *S*-carboxymethylcysteine sulfone and glycolic acid. The mass spectral data from the only radioisotopically labeled substance isolated from acid of the modified enzyme are also consistent with the interpretation that 1,3-dibromoacetone acts only as a monofunctional alkylating reagent for the catalytically essential cysteine residue. These results imply that the histidine residue modified by photooxidation is not within 5 Å of the sulfur atom of the essential cysteine residue and probably does not serve as a nucleophile in the *hydrolase* activity of the enzyme. Rather it would appear reasonable on the basis of these and other studies to postulate for the imidazole group, or any comparable group, a role as a nucleophile or general acid or base in the enzyme-catalyzed *phosphorolysis* reaction.

Information on the identity of functional groups involved in the various catalytic roles for glyceraldehyde-3-phosphate dehydrogenase is notably sparse in spite of the fact that the enzyme from both lobster tail muscle (Davidson *et al.*, 1967) and pig muscle (Harris and Perham, 1968) has been fully sequenced. Cysteine-149[1] has been shown to be essential for two of the enzyme activities, namely the physiologically important one of a substrate level oxidative phosphorylation, and the esterase activity. In addition lysine-183 is known to be within 5 Å of the essential sulfhydryl group in the rabbit muscle apoenzyme (Park *et al.*, 1966); however, except for a possible role in binding the coenzyme molecule NAD^+, no function in catalysis has been established for this residue. Photooxidation studies on the rabbit muscle dehydrogenase have been used to postulate the presence of an essential histidine residue in the active site (Friedrich *et al.*, 1964; Bond *et al.*, 1970). The validity of this proposal has been questioned on the basis of kinetic analyses of the esterase activity (Behme and Cordes, 1967). In an attempt to resolve these contradictory views and possibly to determine the presence of other residues near the essential cysteine residue we have employed 1,3-dibromoacetone in chemical modification studies directed toward cross-linking cysteine-149 with a proximal histidine or any other nucleophilic group. This bifunctional reagent has been used with great success by Lowe and his colleagues in establishing the proximity of a cysteine and a histidine residue in several plant sulfhydryl proteases (Husain and Lowe, 1968a,b, 1970).

Materials and Methods

General. Amino acid analyses were performed on a Spinco-120B amino acid analyzer using a modification of the system of Spackman *et al.* (1958). The most important change was the use of sodium citrate buffer (0.067 M) at pH 3.49 for the initial elution (at a flow rate of 90 ml/hr) of amino acids from the 52-cm column (packed with Beckman PA-28 resin); a buffer change to pH 4.40 was made at 58 min. Using these conditions the half-cystine peak was completely separated from the proline (or glycine) peak. A Gilford 222 spectrophotometer equipped with a thermostated cuvet holder (25°) and a Sargent SRL recorder were used for the measurement of absorbance changes in the ultraviolet and visible regions. Liquid scintillation counting was done on a Packard Tri-Carb spectrometer, and paper strips from chromatography and electrophoresis were monitored for radioactivity with a Packard Model 7200 radiochromatogram scanner. Mass spectral analyses were performed on a CEC mass spectrometer (Model 110C). Infrared spectra of samples in NaCl

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[1] Numerical assignments given to amino acids in the sequence of the rabbit muscle enzyme are those assigned by Harris and Perham (1968).

cells were obtained on Perkin-Elmer 137 spectrophotometer. Gas chromatography samples were analyzed on a Hewlett-Packard F and M Chromatograph (Model 700).

Materials. ENZYMES. Glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde 3-phosphate:NAD^+ oxidoreductase (phosphorylating), E.C. 1.2.1.12) from rabbit muscle was purchased from Boehringer Mannheim Corp. or Sigma Chemical Co. as an $(NH_4)_2SO_4$ suspension. Various lots of the enzyme revealed a specific activity for D-glyceraldehyde 3-phosphate of 50–65 μmoles of NADH/min per mg when assayed in 0.05 M pyrophosphate–1 mM EDTA (pH 8.5). Sodium dodecyl sulfate electrophoresis of the enzyme according to the procedure of Weber and Osborn (1969) showed no visible contaminants. Apoenzyme was prepared by batchwise charcoal treatment of the holoenzyme and generally had >94% of the activity of the untreated holoenzyme (with a ratio of absorbance at 280 nm to that at 260 nm of 1.15). Concentrations were determined using the extinction coefficients of Fox and Dandliker (1956) and a value for the molecular weight of 140,000. Lactate dehydrogenase was purchased from Sigma as an $(NH_4)_2SO_4$ suspension and used directly.

ISOTOPICALLY LABELED COMPOUNDS. Iodo[1-^{14}C]acetic acid (Volk; final specific activity, 5.1×10^5 dpm μmole^{-1}) was diluted with unlabeled iodoacetate obtained from Sigma (purified previous to use by twice extracting an aqueous solution with CCl_4 and subliming). Treatment of the iodo[1-^{14}C]-acetic acid (2 μmoles) with NaOH (0.5 ml, 0.8 M) for 18 hr at 25° afforded [1-^{14}C]glycolic acid. Thin-layer chromatography in chloroform–ethyl acetate (4:1) revealed a single spot with characteristics identical with those of the commercially available unlabeled glycolic acid. 1,3-Dibromo[2-^{14}C]-acetone was synthesized from sodium [1-^{14}C]acetate (Tracerlab) according to the published procedure (Husain and Lowe, 1968a). The product resembled the commercially available material (Dajac Lab.) with respect to its low melting point (*ca.* 26°) and its lachrymatory power. Thin-layer chromatography revealed the product moved as one spot in several solvents (heptane, acetone, and a 4:1 solution of chloroform–ethyl acetate) and in dry benzene (R_F 0.29, compared to the literature value of 0.27 (Husain and Lowe, 1968a). Gas chromatography confirmed its purity showing >98% 1,3-dibromoacetone and <1% methyl bromoacetate. The position of the characteristic carbonyl absorption band at 1720 cm^{-1} was identical with the literature value. Mass spectral analysis indicated the correct molecular weight (216) and a fragmentation pattern essentially identical with that of the commercially available reagent. The specific activity (0.55 Ci/mole) was determined from counts per minute in Bray's (1960) solution containing a known amount of dibromoacetone (calculated from its reaction with the thiophenolate anion of DTNB).[2]

OTHER REAGENTS. NAD^+ and glyceraldehyde 3-phosphate (as the diethyl acetal barium salt) were purchased from Sigma. Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid), *N*-acetyltyrosine, and *N*-acetylcysteine were from Calbiochem; 5-amino-1*H*-tetrazole, from Eastman. Fox Chemical Co. supplied α-*N*-acetyllysine and α-*N*-acetylhistidine. From the appropriate N-acetylated amino acids were synthesized the carboxymethyl derivatives according to the published procedures (Crestfield *et al.*, 1953).

Methods. ENZYMATIC ASSAYS. Assay procedures used in this investigation are detailed elsewhere (Fenselau and Weigel, 1970).

AMINO ACID HYDROLYSIS. After exhaustive dialysis against water, the samples of protein (*ca.* 5–12 mg/ml or 35–85 nmoles/ml) were frozen, lyophilized, and placed into a clean hydrolysis tube. Hydrochloric acid (6 N, 0.5–1.0 ml) previously deaerated with N_2 was added (to give about 2 mg of protein/ml), and the suspension was frozen, evacuated to *ca.* 0.1 mm, and sealed. The tubes were opened after 24 hr at 110°, and the contents were taken to dryness under a stream of N_2.

ENZYME OXIDATION AND REDUCTION. Performic acid oxidation was done by dissolving 10 mg of lyophilized protein in 0.1 ml of 90% HCO_2H and adding at 0° 0.1 ml of HCO_3H (made by allowing a 9:1 solution of HCO_2H–H_2O_2 (30%) to sit for 2 hr at 25°). After 4 hr at 0° the contents were transferred to 30 ml of cold water, frozen, and lyophilized.

Borohydride reduction of the protein was accomplished by dissolving lyophilized protein (10 mg) in 6 M guanidinium chloride (previously deaerated with N_2). Fresh $NaBH_4$ (25 mg/ml, 0.1 ml) was added and N_2 was bubbled through the solution for 60 min at 40°. After lowering the pH to 6.0, the solution was dialyzed against water before hydrolysis.

DETERMINATION OF CONTENT OF MISCELLANEOUS AMINO ACIDS. Diazoniumtetrazole was prepared from 5-amino-1*H*-tetrazole according to the published procedure (Sokolovsky and Vallee, 1967). Following titration of the enzyme solutions (2.1–3.3 mg/ml) the absorbances at 550, 480, and 600 nm were employed in determining histidine and tyrosine content.

Tryptophan content was determined by the Edelhoch procedure (1967) using solutions containing 0.32 and 0.67 mg of protein per ml. Little deviation from linearity was noted. Titration of the enzyme with DTNB (Ellman, 1959) provided a value for the extinction coefficient of the anion of thionitrobenzoate of 12,900 OD at 412 nm per M·cm (lit. value; 13,600) when the number of sulfhydryl groups per enzyme subunit was assumed to be 4.0.

MASS SPECTROMETRY. Approximately 20 mg of the radioactive amino acid derivative (previously reduced with $NaBH_4$) was isolated from the long column of the amino acid analyzer by pooling the collections of several runs. Desalting (to remove amino acid column buffer salts) was done using a Dowex 50X column (0.6 × 35 cm) with H_2O as a wash and 1 M NH_4OH as the eluting agent. The complete removal of salt was detected by the absence of AgCl formation upon adding 1 drop of 1 M $AgNO_3$ to 1–2 ml of the wash. The dried sample was esterified in a capillary tube using methanol–HCl (0.1 ml), dried, and acylated with an identical volume of a 1:1 acetic acid–acetic anhydride solution. After a final drying to remove all solvents (under reduced pressure, 0.1 mm), the sample was subjected to mass spectral analysis.

INACTIVATION OF GLYCERALDEHYDE-PHOSPHATE DEHYDROGENASE WITH [^{14}C]DIBROMOACETONE AND COUNTING PROCEDURES. 1,3-Dibromo[2-^{14}C]acetone in dry diethyl ether (58 mM) was added to the enzyme solution (5–12 mg/ml) so that the organic solvent remained <4% of the total volume. Samples for amino acid hydrolysis were prepared by incubating at 0° for 4 hr and 15° for 2 hr before termination of the reaction by the addition of mercaptoethanol (10 mM).

Incorporation of radiocarbon into the protein with [^{14}C]-dibromoacetone or iodo[1-^{14}C]acetic acid was followed by precipitating the protein into a glass fiber disk (Whatman GF/C) (Fenselau, 1970). The dried disk was placed into a vial with 5 ml of scintillation fluid (500 ml of toluene–250 ml of Triton X-100–4.15 g of 2,5-diphenyloxazole–94 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] benzene and counted with an efficiency of 62–64%. All samples were counted for at least five 10-min periods and averaged.

[2] Abbreviation used is: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GAP, glyceraldehyde phosphate.

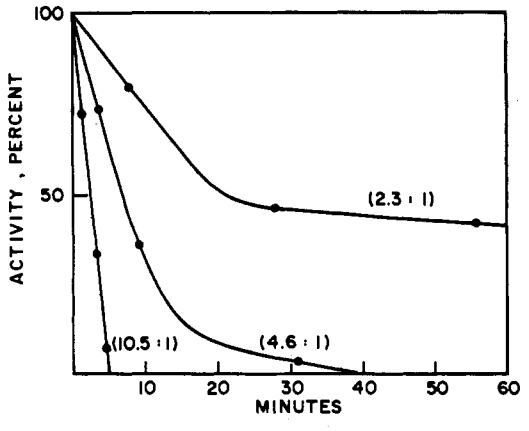


FIGURE 1: Inactivation of glyceraldehyde-3-phosphate dehydrogenase by dibromoacetone. Three separate tubes containing the enzyme (OD_{280}:OD_{260} = 1.14; 76 nmoles in 5.0 ml at 0.5 M Tris·HCl containing 1 mM EDTA at pH 7.0) were incubated at 0° with [^{14}C]dibromoacetone at various ratios of concentrations of inhibitor to enzyme: A, 2.3:1 (174 nmoles of reagent); B, 4.6:1 (348 nmoles of reagent); C, 10.5:1 (812 nmoles of reagent). At the designated times, 0.2 ml of the reaction mixture was removed, mercaptoethanol (2 nmoles) in excess of the alkylating reagent was added to it, and the solution was incubated for 30 min at 0° before assaying.

The radioactive eluates from the amino acid analyzer columns were monitored by collecting 2-min fractions (2.0 ml) from the columns and placing a 1.0-ml aliquot from each tube into 10 ml of Bray's solution and counted.

Results

Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (holoenzyme) is inactivated rapidly at pH 7.0 by 1,3-dibromo-[2-^{14}C]acetone as shown in Figure 1. At a low molar ratio of reagent:enzyme (4.6:1), all activity is abolished in 30 min. Since this protein is composed of four identically reactive subunits, this ratio represents slightly more than a 1:1 ratio per active site. Complete inactivation is seen in 5 min at higher ratios (10.5:1), and a corresponding limited inactivation is noted at less than stoichiometric amounts of reagent to the active site (2.3:1). As the pH is increased from 5.6 to 8.5 the rate of incorporation of the radioisotopic label initially increases, but levels out when about 4 moles of reagent (originally at a ratio of 4.1:1 to the enzyme) have been incorporated in a mole of enzyme (Figure 2A). The loss in dehydrogenase activity under the same conditions closely parallels the data in Figure 2A (Figure 2B).

It is of considerable interest to compare [^{14}C]dibromoacetone alkylation of the enzyme in the presence and absence of NAD^+ since the structural changes accompanying NAD^+ binding have been well documented (Listowsky *et al.*, 1965; deVijlder and Slater, 1967). Incorporation of [^{14}C]dibromoacetone (when the reagent:enzyme is 4:1) occurs at a somewhat faster rate in the absence of NAD^+ which is consistent with earlier observations on the effects of NAD^+ on the reaction rate of cysteine-149 with uncharged alkylating reagents (Fenselau, 1970). Removal of ammonium sulfate by dialysis led to a very slight increase in rate of alkylation but had no effect on the extent of alkylation.

Protection from [^{14}C]dibromoacetone alkylation occurs when the thiolester enzyme intermediate of glyceraldehyde 3-phosphate is prepared by the method of Krimsky and Racker (1963). No incorporation of either [^{14}C]dibromoacetone or iodo[1-^{14}C]acetic acid, a relatively specific alkylating agent for the essential cysteine of this enzyme, occurs when the enzyme is acylated in this manner with its natural substrate (Figure 3). This complete absence of incorporation of [^{14}C]dibromoacetone into the *S*-acyl-enzyme provides more concrete evidence for the specificity of the reagent for the active-site region, and most likely, the essential cysteine-149. Further confirmation for this observation was obtained by first inactivating the enzyme with dibromoacetone and then adding iodo[1-^{14}C]acetic acid; no [^{14}C]carboxymethyl groups were incorporated into the enzyme. The identity of cysteine as the amino acid residue being modified by the bifunctional reagent was further demonstrated by means of titration with DTNB (Table I). In total, this evidence supports the proposition that dibromoacetone is a highly selective reagent for modifying at least the active-site cysteine residue.

Information was then sought on the presence of the second reactive bormomethyl group at the active site. The reaction of the bromomethyl ketone with the highly colored thiophenolate anion derived from DTNB was used to monitor the stability of the reagent under various conditions. At 0° the times required to hydrolyze half of the reactive bromomethyl groups were found to be much greater than 5 hr at pH 5.6 and 7.0,

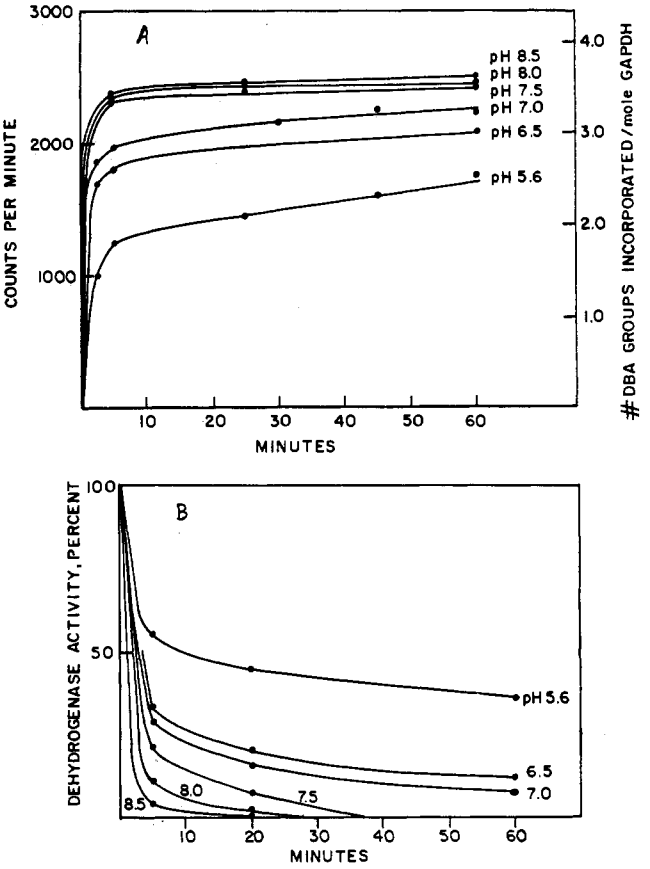


FIGURE 2: pH dependence of enzyme inactivation and rate of incorporation of dibromoacetone (DBA) into glyceraldehyde phosphate dehydrogenase (GAPDH). GAPDH (42 nmoles in 0.7 ml) was placed in buffers at pH 6.5–8.5 using 0.05 M Tris·HCl with 1 mM EDTA and at pH 5.6 using 0.05 M sodium acetate with 1 mM EDTA. [^{14}C]Dibromoacetone (58 mM, 174 nmoles) was added to each enzyme solution. Aliquots were removed at the designated times for counting (15 μl) (Figure 2A) and for assaying (20 μl) (Figure 2B). Incubation before assaying was done for 30 min at 0° in 0.18 ml of 0.05 M Tris·HCl containing 20 mM mercaptoethanol–1 mM NAD^+–1 mM EDTA (pH 8.5).

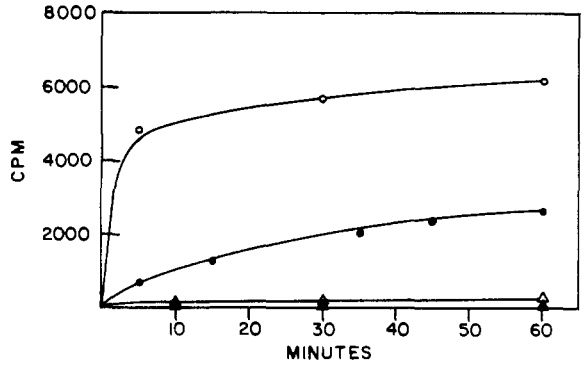


FIGURE 3: Protection from alkylation with iodo[1-^{14}C]acetic acid or [^{14}C]dibromoacetone by presence of phosphoglyceroyl group at the active site of glyceraldehyde-3-phosphate dehydrogenase. The thiolester enzyme was prepared by allowing 0.6 ml of holoenzyme (6.0 mg, 42 nmoles in 0.09 M Tris·HCl and 2 mM EDTA at pH 7.0), 0.30 ml of NAD^+ (0.023 M, pH 7.0), 10 μl of sodium pyruvate (0.1 M, pH 7.0), 0.45 ml of GAP (17.5 mM, pH 6.3), and lactate dehydrogenase (0.01 ml, 50 μg) to incubate 90 min at 0° (final pH 7.1). The control (unacylated enzyme) was prepared in an identical fashion except the GAP was omitted. Iodo[1-^{14}C]acetic acid (50 mM, 3 μl) and [^{14}C]dibromoacetone (58 mM, 1 μl) were added to 0.25-ml aliquots of the acylated and control enzymes. At the designated times, aliquots (40 μl) were removed and counted as described in Methds. Dehydrogenase + iodoacetic acid (●) or + dibromoacetone (○); phosphoglyceroyl-enzyme + iodoacetic acid (▲) or + dibromoacetone (△).

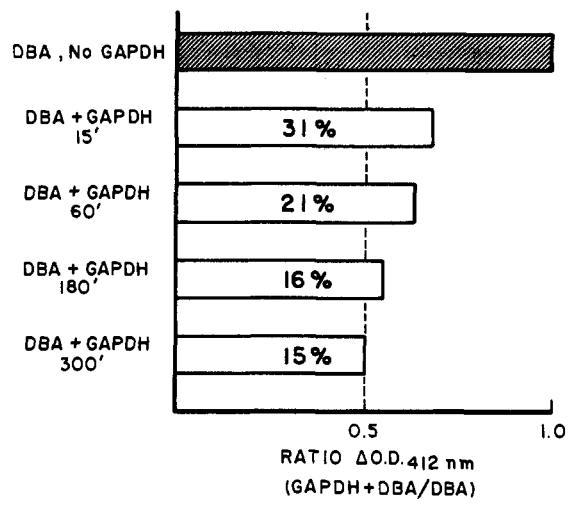


FIGURE 4: Determination of the presence of a reactive bromomethyl group within the active site of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Holoenzyme (2.8 mg, 9.7 nmoles) in 0.50 ml of Tris·HCl (0.05 M, 1 mM EDTA, pH 7.0) was allowed to react with dibromoacetone (DBA) (35 nmoles, 30 μl) for various lengths of time at 0°. At the end of the designated time, 0.10-ml portion was removed and added to 2.90 ml of a stock solution of thiolnitrobenzoic acid (prepared by reducing DTNB in methanol with $NaBH_4$, initial OD_{412} = 0.51). Absorbance measurements were made after 10 min at 25°. At the same time 2 μl of the enzyme solution was added to a cuvet containing the standard assay mixture plus mercaptoethanol (10 mM), and the activity was determined after 3 min. Corrections were made by measuring OD_{412} for enzyme alone (no dibromoacetone added) and dibromoacetone alone (no GAPDH added). Results are expressed in terms of the ratio of the change (decrease) in absorbance for enzyme and dibromoacetone to only dibromoacetone. (The measured dehydrogenase activities are shown in the bar plot.) The dotted line corresponds to a theoretical result, assuming complete enzyme inactivation and the availability of the second bromomethyl group for subsequent reaction (with other enzyme residues or thiolnitrobenzoate).

whereas at pH 8.5 the time observed was 3.5 hr. When the reagent was added at pH 7.0 to the enzyme (in slight excess), the results shown in Figure 4 were obtained. Quite clearly inactivation of the enzyme does not lead to rapid loss in the ability of the remaining bromomethyl group to react with the thiolnitrobenzoic acid.

Having established that under one set of conditions dibromoacetone was actually first reacting with cysteine-149 at the active site and that the second bromomethyl group was still available for further reaction, it was necessary to ascertain if any other amino acid residues were being alkylated. Furthermore, the scope of our studies would have to be extended to determine the generality of our results. Thus studies were carried out on the enzyme (>1 mg/ml) in the presence and absence of NAD^+ and at different pH values from 5.6 to 8.8. Reaction with dibromoacetone was allowed to proceed with the same consequences either for 4 hr at 0° and 1 hr at 15° or overnight at 4°. For comparison the studies of Husain and Lowe (1968a,b) were conducted with papain (1.4 mg/ml) at pH 5.6 and 0°, resulting in the alkylation of one residue of both cysteine and histidine by dibromoacetone in 10 min. Over this pH range the dehydrogenase has been shown to undergo no drastic structural change, according to studies involving spectrophotometric titrations (Libor *et al.*, 1965) and circular dichroism (Shibata and Kronman, 1967). However, various ionizable groups on the protein that might react with the bromomethyl group by a nucleophilic displacement mechanism should show dramatic changes in reactivity depending on the state of protonation. Characteristic pK_a values for different groups are 6.0–7.0 for histidine, 8.0–8.5 for cysteine, 10.0–10.5 for lysine, and 9.5–10.5 for tyrosine. Finally both the apo- and holoenzyme were studied over this pH range since it is known that the binding of NAD^+ to the

TABLE 1: DTNB Titrations of Glyceraldehyde Phosphate Dehydrogenase.[a]

Enzyme Sample	OD_{412}	No. of SH Groups/ Subunit
Glyceraldehyde-phosphate dehydrogenase	0.490	4.0
Glyceraldehyde-phosphate dehydrogenase + iodoacetic acid	0.382	3.1
Glyceraldehyde-phosphate dehydrogenase + dibromoacetone	0.361	3.0

[a] Glyceraldehyde-phosphate dehydrogenase (10.0 mg/ml, 71.4 nmoles in 3.0 ml of 0.05 M Tris·HCl–1 mM EDTA (pH 7.0)) was divided into three 1.0-ml portions. To one was added iodo[1-^{14}C]acetic acid (50 mM, 0.01 ml, 500 nmoles); to another, 10 μl of methanol (as a control). A portion (0.8 ml) of the last 1.0-ml sample was inactivated with [^{14}C]dibromoacetone (58 mM, 4 μl, 232 nmoles). After 60 min at 0°, all samples were dialyzed against 500 ml of the same buffer. An aliquot (0.10 ml) was used for each DTNB titration. Each value represents the average of three determinations.

TABLE II: Comparison of Cysteine Content as Determined by Several Different Methods.

Enzyme Treatment	Amino Acid Analyses[a]			DTNB Titrations No. SH Groups/ Subunit	Radiocarbon Content No. of Groups Introduced/ Subunit (^{14}C-Containing Reagent)
	Half-Cystine	Cysteic Acid	CM-Cysteine		
(1) Untreated	3.7	Tr[b]	0	4.0	
(2) HCO_3H treated	0	3.5	0		
(3) Urea + iodoacetic acid	0	Tr	3.6	0.2	3.9 (iodoacetic acid)
(4) +NAD + iodoacetic acid	2.8	Tr	0.95	3.1	0.9 (iodoacetic acid)
(5) +NAD + iodoacetic acid; HCO_3H treated	0	3.6[c]	0		
(6) +Dibromoacetone (1.2:1)[d]	2.9	Tr	0	2.9	1.0 (dibromoacetate)
(7) +Dibromoacetone (1.2:1); HCO_3H treated	0	3.8[c]	0		
(8) +Dibromoacetone (1.2:1); urea + iodoacetic acid					3.1 (iodoacetic acid)

[a] Calculations are based on the content of phenylalanine = 14.0 per subunit. All values are stated per subunit. [b] Abbreviations used are: Tr, trace; +NAD + iodoacetic acid, iodoacetate added to native enzyme in the presence of coenzyme; urea + iodoacetic acid, enzyme unfolded in 8 M urea and 1 mM β-mercaptoethanol, then treated with excess iodoacetate and dialyzed. [c] Carboxymethylcysteine sulfone (from carboxymethylcysteine or dibromoacetone–cysteine adduct) elutes at the same position as cysteic acid (from cysteine and dibromoacetone–cysteine adduct). [d] Ratio of concentration of reagent to that of enzyme subunit.

enzyme causes marked structural changes (Listowsky *et al.*, 1965; deVijlder and Slater 1967). The apoenzyme is more susceptible to proteolysis and its structure is not as compact as the holoenzyme indicating a more flexible structure (Krimsky and Racker, 1963). Therefore, the bifunctional reagent within the active site of the apoenzyme might see a different environment than it sees when NAD^+ is bound. For example, a lysine residue is acetylated by acetyl phosphate in the apoenzyme but not in the holoenzyme (Mathew *et al.*, 1967).

Amino acid analyses were performed in order to identify and determine the numbers of any residues in addition to cysteine being modified by dibromoacetone under all the different conditions. The cysteic acid content (shown in Table II) could not be used to determine the amount of unmodified cysteine, due to the coelution of the two products from the performic acid oxidation and hydrolysis of the dibromoacetone–cysteine adduct, namely cysteic acid and *S*-carboxymethylcysteine sufone. Rather than carry out additional treatment of the modified enzyme, such as with urea and iodoacetate, we decided to employ half-cystine content following only acid hydrolysis of the enzyme derivative as a means for confirming cysteine modification. In Table II can be seen the correlations between half-cystine content and cysteic acid or *S*-carboxymethylcysteine (as measured by the analyzer), sulfhydryl group content (as measured by Ellman's procedure), or radiocarbon content introduced by a cysteine-modifying reagent. Since no discrepancies were noted among these methods, half-cystine content was subsequently employed as a measure of cysteine content. The results for both apo- and holoenzyme in Table III show no loss in any residue except cysteine. The content of histidine, tyrosine, and methionine can be reliably measured by amino acid analysis; however, the value of lysine, although virtually unchanged throughout this pH range and for the apo- and holoenzymes, cannot be accepted as being truly reliable since it is present to the extent of 26 residues/monomer. However, the results from performic acid treatment of the dibromoacetone-inactivated enzyme (see below) also indicate no change in lysine content. In addition any ester formed from dibromoacetone and the carboxyl group of aspartic and glutamic acids would not survive the conditions of acid hydrolysis and consequently reduce the content of these residues.

Since the existence of an essential histidine has been postulated in rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, the determination of histidine content was accomplished by a second, independent method to confirm the results of amino acid analyses by acid hydrolysis. The reagent, diazonium-1*H*-tetrazole, has been employed in the quantitation of both histidine and tyrosine residues (Horinishi *et al.*, 1964). The spectrophotometrically measured histidine and tyrosine contents of the untreated enzyme (8.9 and 7.0 residues per subunit, respectively) were found to be the same as the dibromoacetone-treated enzyme (8.8 and 7.1). However, the histidine and tyrosine content measured by this method is about two residues less (in both cases) than the number measured by the amino acid analyses. This is due to the fact that only the bisazo derivatives contribute to the spectral measurements; the unmodified residues and monoazo derivatives can not be detected. When the enzyme preparations (untreated or treated with dibromoacetone) following reaction with diazoniumtetrazole were hydrolyzed completely and the amino acid content was determined, an additional 1.30 tyrosine residues and 0.75 histidine residue were found for both preparations. Because of the complete identity of the results for both untreated and dibromoacetone-treated enzyme, we must conclude that no histidine or tyrosine residue has reacted with dibromoacetone.

Tryptophan has potential as an important residue since all three residues in the lobster and pig muscle enzyme are found in homologous positions (Harris and Perham, 1968; Davidson *et al.*, 1967). One hypothesis for its role in the functioning of the enzyme is that it participates in a charge-transfer complex

TABLE III: Amino Acid Analysis of Dibromoacetone-Treated Glyceraldehyde-3-phosphate Dehydrogenase.[a]

Amino Acid (Elution Vol, ml)	pH 5.6	6.5	7.0	7.5	8.0	8.8	Control
			Holoenzyme				
Lysine (55)	26.5	26.9	26.3	25.7	25.9	26.0	25.8
Histidine (69)	10.9	11.0	10.8	10.9	11.2	11.1	11.1
Arginine (101)	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Aspartic acid (49)	42.1	41.9	39.8	40.5	38.7	39.2	38.5
Threonine (63)	22.1	22.4	21.8	21.9	22.3	21.5	21.8
Serine (67)	18.1	17.8	17.7	17.5	17.1	17.7	18.1
Glutamic acid (71)	19.7	19.5	19.1	20.5	20.6	19.3	19.2
Proline (81)	12.1	12.3	11.8	11.7	12.1	11.9	11.8
Half-cystine (88)	*3.1*	*2.8*	*3.1*	*3.2*	*2.9*	*2.9*	3.9
Glycine (95)	34.3	34.1	33.8	34.5	33.4	33.6	33.8
Alanine (100)	33.2	33.4	33.0	33.4	32.8	32.5	32.9
Valine (124)	29.2	32.1	31.1	30.8	29.8	30.1	31.1
Methionine (157)	8.8	8.9	9.0	8.7	8.9	8.9	8.9
Isoleucine (168)	16.4	16.5	17.6	16.8	16.3	16.8	17.2
Leucine (175)	18.9	18.1	19.7	18.5	18.4	19.2	18.8
Tyrosine (210)	8.9	9.0	8.8	8.9	9.1	9.0	8.9
Phenylalanine (217)	14.0	14.0	14.0	14.0	14.0	14.0	14.0
			Apoenzyme				
Lysine	25.5	26.1	25.9	26.3	25.8	25.4	25.8
Histidine	11.0	10.7	10.9	10.8	11.1	11.0	11.1
Arginine	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Aspartic acid	40.6	39.7	38.9	39.7	40.1	38.2	38.5
Threonine	22.5	20.3	20.4	21.3	22.0	21.5	21.8
Serine	18.6	17.5	17.9	18.4	18.0	18.1	18.1
Glutamic acid	19.1	19.7	20.4	19.9	19.1	19.5	19.2
Proline	11.5	11.7	12.5	12.4	11.9	12.1	11.8
Half-cystine	*3.0*	*2.9*	*3.0*	*3.0*	*2.9*	*2.9*	3.9
Glycine	34.5	33.1	33.4	34.7	34.3	34.7	33.8
Alanine	32.7	32.9	33.0	32.8	31.7	31.8	32.9
Valine	30.4	32.3	32.0	31.9	30.7	31.5	31.1
Methionine	9.1	9.1	9.0	9.0	8.7	8.8	8.9
Isoleucine	16.6	17.5	17.1	16.5	16.1	16.0	17.2
Leucine	18.3	18.5	19.3	18.7	19.0	18.8	18.8
Tyrosine	8.8	8.9	9.0	8.9	9.0	9.1	8.9
Phenylalanine	14.0	14.0	14.0	14.0	14.0	14.0	14.0

[a] The inactivation of holo- and apoenzymes [5–12 mg/ml in 0.05 M Tris·HCl with 1 mM EDTA (pH 6.5–8.8) and 0.05 M sodium acetate with 1 mM EDTA (pH 5.6)] by [^{14}C]dibromoacetone was carried out as described in Methods. After extensive dialysis against H_2O, the samples were frozen, lyophilized, and hydrolyzed in a sealed evacuated tube for 24 hr at 110° as in Methods. The numbers in the table are based on residues per monomer and the presence of 10.0 arginine and 14.0 phenylalanine residues per monomer. Each value in the table represents the average of two or more determinations. Any differences from the untreated enzyme (control) are italicized.

with NAD^+ yielding the broad absorption band at 360 nm (Cilento and Giusti, 1959). Tryptophan is a reactive amino acid toward alkylating agents (Barman and Koshland, 1967), but direct quantitation by routine acid hydrolysis is impossible for this acid-labile residue. The spectrophotometric method of Edelhoch (1967) was used to determine tryptophan content in the enzyme inactivated with dibromoacetone. The tryptophan content of the untreated enzyme (2.9 residues per subunit) was the same in the dibromoacetone-inactivated enzyme (2.7), which in both instances equals the literature value of three residues per monomer (Harris and Perham, 1968).

In an attempt to obtain positive identification of the modified amino acid, the effluent from the amino acid analyzer columns was monitored for radioactive components. Husain and Lowe (1968b) have demonstrated that the cross-link after $NaBH_4$ reduction and acid hydrolysis eluted just before NH_4^+ on an amino acid analyzer short column, and that HCO_3H oxidation yielded *N*-1-carboxymethylhistidine and *S*-carboxymethylcysteine sulfone on the long column. Glyceraldehyde-3-phosphate dehydrogenase, after complete inactivation with [^{14}C]dibromoacetone, was reduced with $NaBH_4$ and hydrolyzed with 6 N HCl. All of the radioactive

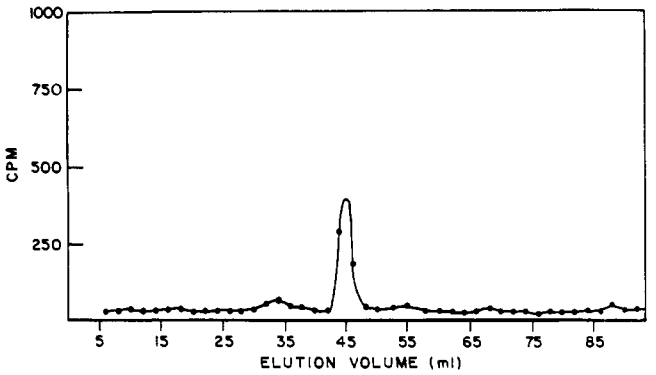


FIGURE 5: Elution patterns of radioactive components of dibromoacetone-treated glyceraldehyde-3-phosphate dehydrogenase after reduction with $NaBH_4$. The enzyme following inactivation with [^{14}C]dibromoacetone was reduced with $NaBH_4$ and hydrolyzed as described in Methods. Approximately 1200 cpm of the hydrolysate was placed on the long column of the amino acid analyzer and the radioactive sample was collected as described in Methods.

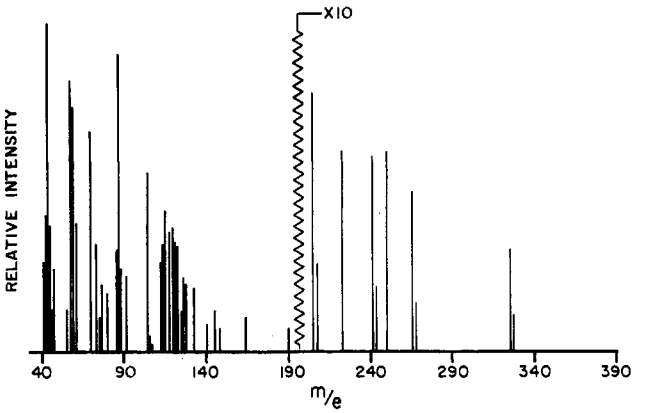
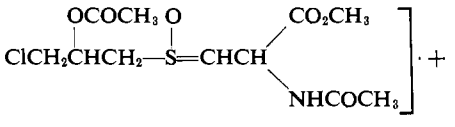


FIGURE 6: Mass spectrum of dibromoacetone-cysteine adduct from glyceraldehyde-phosphate dehydrogenase following reduction with $NaBH_4$ and acid hydrolysis. The procedures were identical with those described in Figure 3. Approximately 20 mg (60 nmoles) of the dibromoacetone–cysteine adduct was collected by pooling material collected from 10 runs (each containing 1.5 nmoles of enzyme) as described in Methods. The entire amount was converted to the volatile derivative (see Methods) and subjected to mass spectral analysis at a probe temperature of 180°.

material was found to elute from the short column where the acidic and neutral amino acids emerge, as determined by carrying out a normal elution of the sample on the same column and detecting its presence by the ninhydrin reaction. Although the buffer system used here differs from that of Husain and Lowe (1968a,b), the reduced cross-link of cysteine and histidine still elutes at the same relative position, *i.e.*, just before NH_4^+ (P. Farmer, 1971, personal communication). Since no radiocarbon component is retarded on the short column, this same hydrolysate was placed on the long column (Figure 5). All of the radioactive material placed on this column is accounted for in the single, relatively neutral species which elutes close to serine and threonine, suggesting that the radioactive derivative is a small, hydrophilic species.

Since a cross-link between cysteine and some other residue yields a high molecular weight compound (>400, assuming that it cross-links to the smallest reactive amino acid residue mentioned earlier, cysteine), the identification of this molecule might be readily determined by mass spectrometry from its molecular ion and fragmentation pattern. The material from the peak in Figure 5 was collected and converted into a volatile *N*-acetyl methyl ester. The mass spectrum of this derivative (Figure 6) provides no useful information at *m/e* values below 200 due to the large amount of contaminating derivatives of serine and threonine which elute at about the same position as the radiocarbon species. The complete absence of peaks higher than *m/e* 350 strongly suggests that no cross-linking to another residue has occurred. More positive evidence that dibromoacetone has not behaved as a bifunctional reagent is seen from the peaks between *m/e* 206 and 328. The presence of a chlorine atom (detected by its characteristic ratio of ^{37}Cl:^{35}Cl of 1:3) (Biemann, 1962) is noted in peaks at *m/e* 206 and 208, 266 and 268, and 326 and 328. The tentative structures assigned to these fragment ions are in agreement with a chloromethyl derivative of cysteine sulfone. The chlorine-containing ion of masses 326 and 328 is assigned the following structures



Those peaks at *m/e* 266 and 268 and 206 and 208 correspond to the ions formed by the loss of one and two neutral $C_2H_4O_2$ fragments, respectively, from the ion whose structure is shown. Although these structures are not the only ones which can be assigned to these ions, this interpretation is consistent with a chlorinated derivative of cysteine sulfone. The chlorine substituent most probably was introduced into either the bromomethyl or hydroxymethyl derivative during the period of acid hydrolysis in 6 N HCl. Therefore, mass spectral analysis of the dibromoacetone–cysteine adduct eliminates the possibility that the alkylating reagent has cross-linked to another residue, with the exceptions of glutamic and aspartic acids whose ester linkages to the reagent would have undergone hydrolysis during acid treatment.

Further evidence supporting this conclusion arises from the results of HCO_3H oxidation of the apo-or holoenzyme treated with [^{14}C]dibromoacetone at pH 5.6–8.8. A Baeyer–Villager oxidation by the peracid converts the ketone to an ester which is hydrolyzed in 6 N HCl to the corresponding acid and alcohol. The alcohol spontaneously deformylates leaving the free amino acid while the acid is the stable carboxymethyl derivative. Since this type of oxidation can occur randomly on either side of the ketone carbonyl group, approximately equal amounts of carboxymethyl derivatives from both amino acids involved in the cross-link are expected (Husain and Lowe, 1968b). The results of a HCO_3H oxidation are shown in Figure 7. As anticipated two radioactive peaks are detectable following oxidative cleavage. The identification of the peak eluting first as *S*-carboxymethylcysteine sulfone was made by comparing the results of an identically treated iodoacetate-inactivated enzyme sample. This peak also contained unlabeled cysteic acid, formed by the alternative pathway in the oxidative cleavage of the ketone. The other labeled product was identified as glycolic acid because it does not react with ninhydrin and its position on the amino acid analyzer (determined by means of radioisotopic label) is identical with that found for glycolic acid synthesized from iodo[1-^{14}C]acetic acid. This identification was simplified because no ninhydrin-positive peak was detected in this position in the normal amino acid analyses. If dibromoacetone were covalently attaching two different amino acids, two carboxymethyl

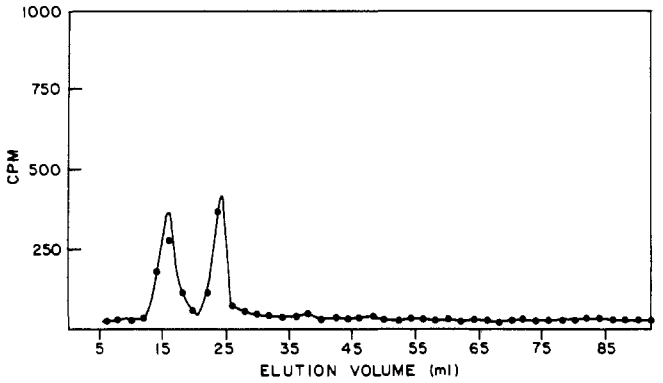


FIGURE 7: Elution pattern on long column of radioactive components of dibromoacetone-treated glyceraldehyde-3-phosphate dehydrogenase after oxidation with HCO_3H. The enzyme following inactivation with [^{14}C]dibromoacetone was oxidized with performic acid and hydrolyzed with 6 N HCl (see Methods). Approximately 2000 cpm of the hydrolysate was placed on the long column of the amino acid analyzer and radioactive material was collected as described in Methods.

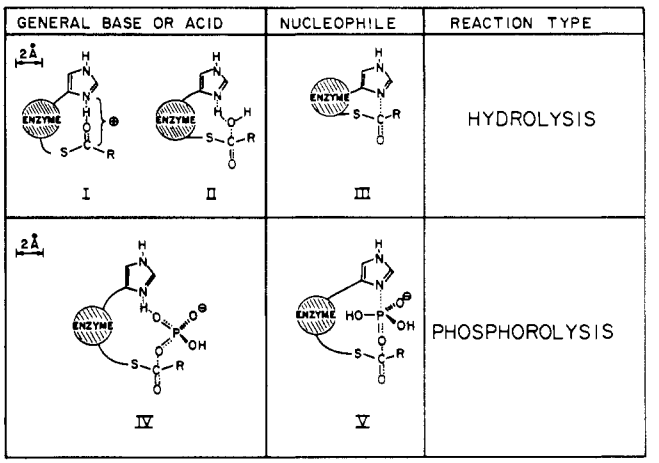


FIGURE 8: Possible catalytic roles for a nucleophilic group in the hydrolysis and phosphorolysis of a thiolester enzyme intermediate. These structures are drawn from scale (see insert) using space-filling models. All atoms are placed at appropriate bonding distances from one another. Dotted lines indicate positions of bond making and breaking.

derivatives would result after HCO_3H oxidation. The position of other carboxymethyl derivatives of several amino acids, including *S*-carboxymethylcysteine sulfone, 1,3-dicarboxymethylhistidine, *S*-carboxymethylcysteine, 1- and 3-carboxymethylhistidine, *O*-carboxymethyltyrosine, and ϵ-*N*-carboxymethyllysine, were determined by model studies and, except for the cysteine derivatives, none of these acidic derivatives is observed after oxidation with HCO_3H. The problems mentioned earlier concerning the quantitation of lysine are resolved using the peracid treatment, since ϵ-carboxymethyllysine elutes at a position well removed from other amino acids under these oxidative conditions. The presence of this acidic derivative of lysine cannot be detected in either the radiocarbon elution profiles or in the course of routine amino acid analyses. The inability of dibromoacetone to react with the ϵ-amino group of the lysine residue known to be in close proximity to cysteine-149 in the apoenzyme may be attributed to the fact that dibromoacetone hydrolyzes rapidly in the pH range where the amino group is unprotonated and, hence, acts as a better nucleophile. These findings confirm that cross-linking of the dibromoacetone molecule from cysteine-149 to the other residues mentioned (lysine, tyrosine, cysteine, histidine, methionine, and tryptophan) has not occurred.

Discussion

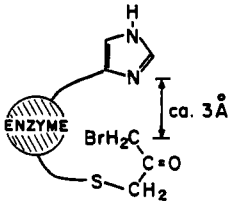
In the case of papain and several other plant sulfhydryl proteases a molecule of 1,3-dibromoacetone has been shown to cross-link rapidly and irreversibly an essential cysteine to a neighboring histidine residue (Husain and Lowe, 1968a,b, 1970). The role postulated for these residues in the enzymatic hydrolysis of polypeptides and low molecular weight substrates can be visualized in terms of the structures I, II, and III in Figure 8. In these the nucleophilic imidazole group may function as a general acid (I), general base (II), or nucleophile (III). The mechanisms of enzyme action porposed for papain on the basis of crystallographic, chemical modification, and kinetic evidence favor a role for histidine as either a general acid (I) or base (II) (Drenth *et al.*, 1970; Lowe, 1970). Using space-filling models and assuming that the positions of the sulfhydryl group and imidazole ring are relatively fixed, the distance separating the two in structures I, II, and III can be estimated to range from about 3 to 4.5 Å. Consequently for these proteases, the facility of the cross-linking reaction with dibromoacetone, which can span distances of 3–5 Å, is not surprising.

A histidine residue, identified as histidine-38 in rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, has been postulated as serving as a nucleophile in the esterase and transferase activities of this sulfhydryl enzyme (Olson and Park, 1964; Mathew *et al.*, 1967). This function would require a spatial relationship between the histidine and essential cysteine residues very similar to that depicted by III (in Figure 8) and would indicate a high probability on steric grounds for observing a facile cross-linking reaction with dibromoacetone. The results discussed in this paper clearly demonstrate that such a facile reaction with dibromoacetone most certainly does not occur between cysteine-149 and a histidine or, for that matter, a tyrosine, a second cysteine, a methionine, or a tryptophan. No modification of a lysine residue was observed; however, at the higher pH value where alkylation of the ϵ-amino group of lysine would be expected to occur, the reagent was found to be quite unstable. Although the evidence regarding a possible cross-link to a glutamic or aspartic acid is less conclusive, preliminary studies on the tryptic peptides of the dibromoacetone-modified enzyme indicate there are no major differences from the peptides of the unmodified enzyme. These negative data have been accumulated by studying the effects on the dibromoacetone reaction and the resulting products of a number of variables, including reaction time, temperature, pH, enzyme concentration (>1 mg/ml), presence or absence of NAD^+ and other substrates. Thus, 1,3-dibromoacetone does not appear to behave as a bifunctional reagent with rabbit muscle glyceraldehyde-3-phosphate dehydrogenase.

Even though the absence of positive evidence cannot be used to rigorously establish or eliminate a hypothesis, we believe the results of this study, when combined with other published evidence, produce significant limitations on any proposed mechanisms for the dehydrogenase action. The evidence that bears on this matter may be reviewed briefly. In the photooxidation studies that lead to the identification of histidine-

38 as a possible catalytic residue, the hydrolase and dehydrogenase activities were inhibited only 50–60%. One possible explanation for this incomplete inactivation is that the modified histidine is not absolutely essential in these catalytic functions or its role may be more passive and its location may not be necessarily proximal to cysteine-149, *e.g.*, histidine-38 might only be required for maintaining a more highly active conformation about the essential cysteine. The kinetic studies on the acylation of the enzyme with *p*-nitrophenyl acetate indicate that only the cysteinethiol anion can be implicated in the attack on the ester. However, no definitive mechanism for the deacylation of the resulting thiolester could be suggested from the kinetic studies. Another objection arises from the relative unimportance of histidine-38 in the primary sequence of the enzyme; in the lobster tail muscle enzyme this residue is replaced by a glutamic acid residue. Counterarguments to these objections have been put forward by Bond *et al.* (1970).

There exists additional information on this matter that has not been considered by Bond *et al.* (1970). Other relatively nonspecific cross-linking reagents, 1,5-difluorodinitrobenzene (Shaltiel and Tauber-Finkelstein, 1971) and *p,p'*-difluoro-*m,m'*-dinitrophenyl sulfone (Givol, 1969), have so far failed to covalently attach to a histidine residue, although cysteine-149 has been linked by these reagents to lysine-183 and possibly to another cysteine residue in the apoenzyme. Finally inadequate attention has been paid to the role of this imidazole group in the physiologic reaction for which the enzyme is specifically designed, namely the *phosphorolysis*, and not the *hydrolysis*, of the intermediate thiolester. Due to the extraordinary reactivity of histidine-38 in the photooxidation reaction and the subsequent effects on enzyme catalysis, this residue obviously still merits serious consideration in explaining enzyme functioning. Possible roles for the imidazole group, or any other comparable nucleophilic group, such as a carboxyl group (for example, consider ribonuclease T_1 (Takahashi *et al.*, 1967)), are depicted by structures IV and V in Figure 8. If this group serves to increase the reactivity of inorganic phosphate by acting as a general acid in removing completely a second proton or as a nucleophile attacking the electron-deficient phosphorus atom, then a minimum distance from N-1 in the imidazole ring to the sulfur atom would measure approximately 6 Å. By assuming that this distance between the two atoms cannot be further reduced, *e.g.*, by ligand-induced protein conformational changes, the inability of dibromoacetone to cross-link can be explained graphically by structure VI. The distance between the reactive



VI

bromomethyl group and nucelophilic group, shown as an imidazole ring nitrogen, exceeds the normal C–N bond length by over an ångström unit. Since inorganic phosphate (with a pK_{a_2} of 7.2) at physiological pH values exists as equimolar amounts of the mono- and dianionic species, the role played by histidine as a general base (IV) need not be absolutely essential and loss of this histidine residue need not result in complete inactivation of the enzyme. Indeed on the basis of this discussion it seems clear that the phosphorolysis reaction requires careful investigation at various pH values with the native and photoxidized enzymes; the resulting data can then be best interpreted with the aid of comparable model studies on the phosphorolysis of α-thiovalerolactone or other thiolesters (Bruice *et al.*, 1963).

In summary, the results of our studies do not exclude the possiblity that a histidine residue contributes to the proper functioning of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. What they do suggest is that the histidine residue does not serve as a nucleophile in the hydrolase activity of the enzyme and, furthermore, that the role of this histidine or a comparable nucleophilic residue might be better ascertained from studies on the phosphorolysis reaction.

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Selective Cysteine Modification in Glyceraldehyde-3-phosphate Dehydrogenase†

John Moore, Jr.,‡ and Allan Fenselau*

ABSTRACT: A structural feature common to the glyceraldehyde phosphate dehydrogenases from a wide variety of sources is the amino acid sequence about the catalytically essential cysteine residue (Cys*): -Cys*-Thr-Thr-Asn-Cys-. The second cysteine can be modified selectively in rabbit muscle glyceraldehyde-3-phosphate dehydrogenase by first blocking, irreversibly or reversibly, the essential cysteine residue and by then treating the inactive enzyme with a SH reagent. Specific protecting of the essential SH group was afforded by iodoacetate (irreversible) and by sodium iodosobenzoate and sodium tetrathionate (reversible). Employing either *N*-ethylmaleimide or 1,3-dibromoacetone (which did not behave as a cross-linking reagent) as alkylating reagents lead to the modification of 0.90–0.95 cysteine residue/polypeptide chain in the protected enzyme and 1.9–2.0 cysteine residues in the unprotected, native enzyme. From the tetrathionate-treated enzyme 80–90% of the original dehydrogenase activity could be recovered by the addition of thiol reducing agents (such as mercaptoethanol or dithioerythritol) to the enzyme alkylated by either reagent. The location of the second reactive cysteine residue was determined by the following indirect methods: (a) complete absence of incorporation of modifying reagent into the phosphoglyceroyl enzyme, (b) comparative peptide studies following trypsin and papain treatment, and (c) protection from irreversible inactivation caused by intrachain disulfide bond formation.

A problem of paramount importance facing the protein chemist is the ability to study the dynamic functioning of the protein. One approach to solving this problem requires the incorporation at some particular target site in the macromolecule of a spectroscopic structural probe. Testimony to the fruitfulness of this approach may be found in many diverse studies, most notably in the elegant work of McConnell and coworkers on spin-labeled hemoglobin (McConnell, 1971) and of Vallee and his colleagues on carboxypeptidase containing an azo probe (Johansen and Vallee, 1971).

The enzyme glyceraldehyde-3-phosphate dehydrogenase appeared to us to be amenable to this approach of selective chemical modification on the basis of several reported observations (Fenselau, 1968; Vas and Boross, 1970; Wassarman and Major, 1969). In brief, these studies on the dehydrogenase from several sources indicated that a second cysteine residue, presumably cysteine-153[1] that is four residues removed from the catalytically essential cysteine-149, shows a reactivity with certain reagents comparable to that of the essential cysteine and much greater than that of the remaining cysteine residues. Chemical modification of this second cysteine residue, to the exclusion of cysteine-149, would possibly provide us with an active enzyme that could be employed in studies analogous to those just mentioned. Furthermore, cysteine-153, if this is the modified residue, is present in all of the "active-site" peptides that have so far been studied (Allison, 1968). This means that not only could these studies on the dynamics of enzymic function be extended to cover the dehydrogenase from different cells displaying a wide range of metabolic (and specifically, glycolytic) activities but also that the function, if any, of this highly conserved cysteine residue might be assessed.

The format of our study was to modify selectively and *reversibly* the essential sulfhydryl group, to attach covalently and *irreversibly* a different moiety on the second reactive sulfhydryl group in the polypeptide chain, and then to deprotect the essential cysteine residue and study the properties

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‡ Supported by National Institutes of Health Training Grant GM 00184-13. This work forms part of the dissertation submitted to the Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Present address: Department of Genetics, Stanford University School of Medicine.

[1] Numerical assignments given to amino acids in the sequence of the rabbit muscle enzyme are those assigned by Harris and Perham (1968).