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Selective Cysteine Modification in Glyceraldehyde-3-phosphate Dehydrogenase†

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

of the modified enzyme. The results of our investigations to be described in this communication reveal that a facile and specific modification can be effected on cysteine-153 leading to the formation of an active enzyme.

Materials and Methods

Materials. ENZYMES. Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was obtained commercially; its specific activity and purity were determined as previously reported (Moore and Fenselau, 1972). A description of the preparation of the apoenzyme and the values for the extinction coefficients also appear in this earlier publication.

ISOTOPICALLY LABELED COMPOUNDS. 1,3-Dibromo[2-^{14}C]-acetone (specific activity 0.55 Ci/mole) was the pure colorless crystals previously prepared (Moore and Fenselau, 1972).

N-Ethyl[1-^{14}C]maleimide was purchased from New England Nuclear (specific activity 5.95 Ci/mole). Following dilution of this material with recrystallized, unlabeled NEM[2] and determination of its purity by thin-layer chromatography, a specific activity (0.63 Ci/mole) was determined from the counts per minute in Bray's (1960) solution (efficiency 69–71%) containing a known amount of NEM (calculated from the absorbance at 305 nm, E = 620 OD M^{-1} cm^{-1}).

OTHER REAGENTS. Sodium iodosobenzoate and papain (twice crystallized) were purchased from Sigma. Trypsin (once recrystallized) was from Mann Chemical Company. Sodium tetrathionate was a product of K & K Laboratories. Sephadex G-25 and G-50 were obtained from Pharmacia Inc. All other reagents were of analytical or better grade.

Methods. ENZYMATIC ASSAYS AND COUNTING PROCEDURES. Assay and radioactive sample counting procedures used in this investigation are detailed elsewhere (Fenselau and Weigel, 1970; Fenselau, 1970).

PROTECTION OF CYSTEINE-149 FOLLOWED BY TREATMENT WITH ALKYLATING REAGENTS. Complete inactivation of holoenzyme (*ca.* 5 mg/ml, 35 μM) in 0.05 M Tris·HCl (pH 7.0, containing 1 mM EDTA) was accomplished using either iodoacetic acid (30 μl from a 10 mM methanol solution) in an 8:1 molar ratio of iodoacetic acid:enzyme for 35 min at 25° or sodium iodosobenzoate (20 μl from a 10 mM solution in 0.1 M NaOH) in a 6:1 ratio for 25 min at 25°. A 4:1 molar ratio of sodium tetrathionate:enzyme (15 μl of 10 mM in enzyme buffer) inhibited all dehydrogenase activity in 2 min at 0°. In all instances, the solutions were cooled to 0° before the addition of the alkylating reagent. Alkylation of these inactive, active site protected enzyme preparations was performed by adding either [^{14}C]dibromoacetone (58 mM in dry, diethyl ether so that the organic solvent remained less than 4% of the total volume) in a 10:1 molar ratio of [^{14}C]dibromoacetone to dehydrogenase or [^{14}C]NEM (56 mM in methanol) in a 21:1 molar ratio for 60 min at 0°. Termination of alkylation and removal of the protecting group (in the case of sodium tetrathionate and sodiuum iodosobenzoate) were accomplished by addition of mercaptoethanol (final concentration, 70 mM) and NAD$^+$ (2 mM) for 30 min at 0°. Prior to assaying, the enzyme (20 μl) was further incubated in a 0.18-ml solution of 0.05 M Tris·HCl (pH 7.0)–1 mM EDTA–2 mM NAD$^+$–10 mM mercaptoethanol for 30 min at 25°.

ACID HYDROLYSIS OF NEM-ALKYLATED ENZYME AND AMINO ACID ANALYSES. To quantitate the *S*-succinylcysteine formed from alkylation with NEM, acid hydrolysis was performed for 72 hr at 110° in order to completely hydrolyze the stable cysteine–NEM product. When these precautions were taken, the recovery of *S*-succinylcysteine approached 90%. The method of Smyth *et al.* (1964) was used to synthesize *S*-succinylcysteine. The amount of degradation of this product averaged 11% under the conditions employed here for amino acid analyses. Amino acid content of the hydrolyzed protein samples was determined as previously described (Moore and Fenselau, 1972).

TRYPTIC DIGESTION OF ALKYLATED GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE. Holoenzyme (40 mg, 286 nmoles) in 4.0 ml of a solution containing 0.05 M Tris·HCl (pH 7.0)–1 mM EDTA was allowed to react directly with [^{14}C]dibromoacetone in the molar ratio (reagent:enzyme) of either 4:1 (with or without sodium tetrathionate protection) or 10:1 (no sodium tetrathionate protection). The modified enzyme in 6 M guanidinium chloride was treated with $NaBH_4$ as described previously (Moore and Fenselau, 1972). After extensive dialysis against H_2O, each enzyme solution was frozen, lyophilized, and dissolved in 5.0 ml of deaerated 8.0 M urea (pH 8.5). Following the addition of mercaptoethanol (10 μl, 10 μmoles), the solution was kept under N_2 for 60 min at 25° before the addition of iodoacetate (30 μl, 30 μmoles). The pH was then adjusted to 8.5, and the solution was allowed to stir under N_2 for 160 min at 25°. Another 40 μmoles of mercaptoethanol was added, and the solution was allowed to stir under N_2 for 60 min at 25° before the final addition of 60 μmoles of iodoacetate. The pH was again adjusted to 8.5, and the solution was stirred for 180 min under N_2 at 25° before dialyzing it against 0.1 M NH_4HCO_3 (*ca.* 700 ml, pH 8.0). The enzyme recovered from lyophilization was resuspended in 0.1 M NH_4HCO_3 (3.0 ml, pH 8.0) and digested with trypsin (50 μl, 0.5 mg) for 4 hr at 37°. Tosyl fluoride (0.50 ml of a 10 mM methanol solution) was added to inhibit the protease, and the soluble portion was frozen and lyophilized. A small amount of insoluble material generally resulted, but was found to contain less than 2% of the total radioactivity. The identical procedure was employed when inactivation was accomplished using [^{14}C]NEM (21:1 molar ratio of NEM:enzyme in presence of sodium tetrathionate and 5:1 molar ratio in its absence).

In a separate experiment, 60 mg of dehydrogenase (0.43 μmole) in 5.0 ml of the same Tris buffer used above was treated with [^{14}C]dibromoacetone as above in the presence and absence of sodium tetrathionate. After 60 min at 0° and the addition of mercaptoethanol, the solution was dialyzed and treated as before. The enzyme was digested with trypsin at 37° as in the previous paragraph. At the end of 24 hr papain (0.7 mg) was added and allowed to digest the tryptic peptides for 5 hr at 37° before the addition of tosyl fluoride and iodoacetate. This digest was treated identically with the tryptic digest as above.

COLUMN CHROMATOGRAPHY OF THE TRYPTIC PEPTIDES. The Sephadex columns (60 × 0.9 cm) were packed with the previously swollen G-50 gel (0.1 M NH_4HCO_3, pH 8.0). The columns were routinely equilibrated by overnight elution with with the same buffer. The lyophilized protein digest was dissolved in 2.5 ml of the NH_4HCO_3 buffer and 0.2 ml (*ca.* 16 × 10^3 cpm), mixed with 2 drops of glycerin, was added to the top of the column and allowed to penetrate before filling the column with buffer. Flow rate was adjusted to 0.4 ml/min (1.2 ml/tube). Peptides were monitored for 280-nm absorption and radiocarbon content by counting a 0.10-ml aliquot in Bray's solution.

[2] Abbreviations used are: NEM, *N*-ethylmaleimide; DTNB, 5,5-dithiobis(2-nitrobenzoic acid).

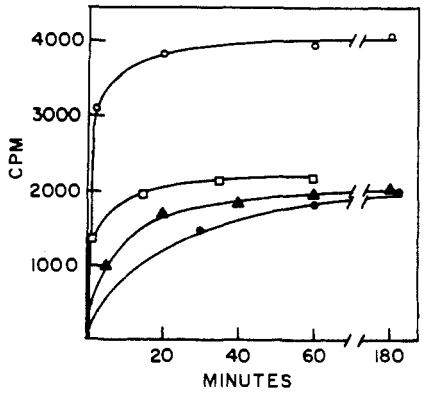

FIGURE 1: Alkylation with dibromoacetone of glyceraldehyde-3-phosphate dehydrogenase previously inactivated with sodium iodosobenzoate, sodium tetrathionate, or iodoacetate. To a 0.48-ml solution of the dehydrogenase (7.3 mg/ml, 25.0 nmoles) in 0.05 M Tris·HCl (pH 7.0) and 1 mM EDTA was added either iodoacetate (20 μl, 200 nmole) (●), sodium iodosobenzoate (15μl, 150 nmoles) (▲), or sodium tetrathionate (10 μl, 100 nmoles) (■). Complete inhibition was checked by assaying for dehydrogenase activity. [^{14}C]Dibromoacetone (4 μl, 232 nmoles) was added to 0.30 ml of each solution of inactivated enzyme as well as to a 0.30-ml solution of active, unmodified enzyme (○). An aliquot (15 μl) was removed and placed on a glass fiber disk and counted as described in Methods.

ELECTROPHORESIS AND CHROMATOGRAPHY. Electrophoresis was carried out on a flat plate system using either pH 6.4 buffer (pyridine–HOAc–H_2O, 25:1:349) or pH 1.9 (formic acid–acetic acid–H_2O, 1:4:45). Cooling was accomplished with a Haake constant temperature circulator set to maintain plate temperature at 20°. Electrophoresis was done at 3000 V (*ca.* 30 V/cm) for 90 min. The paper was air-dried, and ninhydrin-positive regions were detected by spraying with the ninhydrin–collidine reagent (0.1% ninhydrin in absolute ethanol, 5% collidine). Descending paper chromatography was performed with the following buffer systems: (1) 1-butanol–HOAc–H_2O, 4:1:5; (2) 1-butanol–pyridine–HOAc–H_2O, 15:10:3:12. Chromatography was performed until the solvent front reached three-fourths of the length of the paper.

TABLE I: Alkylation of Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) by Dibromoacetone at Various Ratios of Reagent to Enzyme.[a]

Ratio [Dibromoacetone]:[GAPDH]	No. of Dibromoacetone Groups Incd/GAPDH Molecule
2.5	2.1
5.0	3.4
15.1	3.5
30.2	3.5
60.4	3.6

[a] Five separate tubes containing GAPDH (7.1 mg/ml, 0.46 ml, 23.2 nmoles) in 0.05 M Tris·HCl (pH 7.0) and 1 mM EDTA were incubated at 0° with [^{14}C]dibromoacetone at various ratios of concentrations of inhibitor to enzyme after complete inactivation with sodium tetrathionate (as in Figure 1). An aliquot (20 μl) from each solution was placed on the glass fiber disk after 1 hr and counted as in Figure 1.

TABLE II: DTNB Titrations of Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH).[a]

Enzyme Sample	OD_{412}	No. of SH Groups/ GAPDH Subunit
GAPDH, no additions	0.824	4.0
GAPDH, sodium tetrathionate protected	0.811	3.9
GAPDH, S-carboxymethylated	0.639	3.1
GAPDH, sodium tetrathionate protected + dibromoacetone	0.593	2.9

[a] GAPDH (3.5 ml of a solution containing 8.7 mg/ml or 60 μM in 0.05 M Tris·HCl (pH 7.0) and 1 mM EDTA) was divided into four 0.80-ml portions. To one was added [^{14}C]-iodoacetate (10 μl, 500 nmoles) and to another, methanol as a control. Into each of the remaining two aliquots was added sodium tetrathionate (20 μl, 200 nmoles). After 2 min at 0° (0% activity), [^{14}C]dibromoacetone (10 μl, 580 nmoles) was added to one of these samples. After 60 min at 0° (25° for the iodoacetic acid treated sample), mercaptoethanol was added (10 mM) to reduce the sulfenyl thiosulfate groups, and the solutions were dialyzed against cold buffer (500 ml) to remove excess alkylating reagent and reducing agents. An aliquot (0.20 ml) was used for each DTNB titration.

The scan for radioactivity was done on the air-dried paper (unsprayed with any reagents) by cutting a 2-in. wide strip (1 in. on either side of the origin of application) and placing it into the Packard chromatogram scanner.

Sample application for both of these methods consisted of concentrating a larger sample (0.2–0.3 ml, (16–24) × 10^3 cpm) to 0.1 ml and applying it in 15- to 20 μl increments with drying (hair dryer) after each application. Diffuse spots were noted after the electrophoretic or chromatographic runs if the point of application of the sample was allowed to become too large. The application of *ca.* 15 × 10^3 cpm was an ideal amount for detection on the scanner (efficiency 10–15%).

Results

In the course of our studies on the reaction of dibromoacetone with rabbit muscle glyceraldehyde-3-phosphate dehydrogenase we observed that at higher ratios of reagent to enzyme (75:1) more than one residue per monomer was being alkylated. By using dibromoacetone in a 15 to 1 ratio with either holoenzyme or enzyme preparations in which the active site cysteine is blocked irreversibly with iodoacetate or reversibly with sodium tetrathionate (Pihl and Lange, 1962) or sodium iodosobenzoate (Rafter, 1957), we were able to demonstrate that only one more residue per monomer was being alkylated by the reagent (Figure 1). In fact, protection of the enzyme with sodium tetrathionate followed by reaction with varying amounts of dibromoacetone revealed that at ratios greater than 4:1 (reagent:enzyme) incorporation always leveled out at the same value (3.5–3.6 groups incorporated per protein molecule) and a corresponding lower extent of incorporation

TABLE III: Amino Acid Analysis of Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH).[a]

Amino Acid	GAPDH	GAPDH + TET[b]	GAPDH + TET + DBA[c]	GAPDH + IAc[d]	GAPDH + IAc + DBA
Lysine	26.3	26.8	26.5	26.1	25.8
Histidine	10.9	11.1	11.0	11.0	10.9
Arginine	10.0	10.0	10.0	10.0	10.0
Aspartic acid	39.8	39.4	40.7	41.5	38.7
Threonine	21.7	20.8	22.1	22.3	21.5
Serine	18.1	18.5	18.4	18.2	17.9
Glutamic acid	20.4	21.5	19.8	20.3	20.7
Proline	12.3	11.8	11.9	12.1	11.8
Half-cystine	3.5	3.7	*2.8*	*3.1*	*1.9*
Glycine	34.2	33.9	34.1	34.1	34.0
Alanine	33.1	33.2	32.8	33.0	32.9
Valine	31.7	32.3	32.1	32.5	32.1
Methionine	9.1	9.0	9.0	8.9	8.9
Isoleucine	17.3	17.8	17.3	17.1	17.0
Leucine	18.9	18.1	98.5	19.2	18.8
Tyrosine	8.9	9.0	8.9	8.9	9.0
Phenylalanine	14.0	14.0	14.0	14.0	14.0
S-Carboxymethylcysteine				*0.8*	*0.8*

[a] GAPDH (4–7 mg/ml in 0.05 M Tris·HCl, pH 7.0 with 1 mM EDTA) was inactivated with sodium tetrathionate (a 4:1 molar ratio of sodium tetrathionate:GAPDH) for 2 min at 0°. [^{14}C]Dibromoacetone (in molar ratios of 5 to 60:1 of reagent to GAPDH) was added to an aliquot of this sodium tetrathionate inactivated sample and allowed to stand for 60 min at 0°. The remaining sodium tetrathionate treated sample served as the control. After 60 min at 0°, mercaptoethanol (10 mM) was added to the control and dibromoacetone-modified samples, which were then subjected to amino acid analysis. Iodoacetate (an 8:1 molar ratio of reagent to GAPDH was used for inhibiting the enzyme) was similarly employed, except alkylation with dibromoacetone was allowed to proceed for 150 min at 0° before the addition of mercaptoethanol (10 mM). The values shown in this table represent residues per monomer and are based on 10.0 arginine and 14.0 phenylalanine residues per monomer. Since no variation in residues was seen in those samples treated with 5 to 60:1 molar ratios of dibromoacetone to enzyme, all of these samples were averaged to give a single column. The controls (no dibromoacetone) represent the average of three determinations. Any differences from the untreated GAPDH are italicized. [b] TET, sodium tetrathionate. [c] DBA, dibromoacetone. [d] IAc, iodoacetic acid.

at lower ratios (Table I). The effects of substrates on this reaction were then investigated. When sodium tetrathionate inactivated apoenzyme was treated with the reagent (11:1), the rate of incorporation was faster in the presence of NAD^+ than in its absence (Figure 2). These results stand in contradistinction to those noted in the alkylation of the essential sulfhydryl group by dibromoacetone (Moore and Fenselau, 1972). However, as was reported in the studies on the reaction of dibromoacetone with cysteine-149, when the phosphoglyceroyl enzyme was prepared, no reaction with dibromoacetone was detected. These two observations will be discussed later.

All of the studies so far described were conducted at pH 7.0, so therefore the pH dependency of this reaction was determined. As had been previously observed in the modification of the essential cysteine residue by dibromoacetone, maximal activation of the reactive amino acid side chain occurs at a pH value greater than 7.5. Also the extent of incorporation at the different pH values remains the same as at pH 7.0. Since these results implicate a second cysteine residue as the one reacting with dibromoacetone, DTNB titrations were performed. Treatment of the enzyme with first sodium tetrathionate and then dibromoacetone, followed by addition of mercaptoethanol and an overnight dialysis, produced a protein derivative whose sulfhydryl content (determined by the Ellman (1959) procedure) was about one group (on a subunit basis) less than an appropriate control (Table II). Further confirmation of the reaction of a second cysteine residue with dibromoacetone was obtained by amino acid analyses (Table III), which also show the loss of only one residue, a cysteine residue (measured as half-cystine), per monomer upon treatment in the manner first described. As discussed previously (Moore and Fenselau, 1972), the use of half-cystine content as a measure of cysteine content was justified on the basis of corroborative data from the determination of half-cystine and *S*-succinylcysteine content (by the analyzer) and of sulfhydryl group and radiocarbon content. The sodium tetrathionate or iodoacetic acid inactivated enzyme modified with [^{14}C]dibromoacetone was also reduced with $NaBH_4$ or oxidized with HCO_3H prior to complete acid hydrolysis. The radiocarbon elution patterns from the amino acid analyzer were determined as previously described in our studies on the alkylation of cysteine-149 and were found to be identical with those reported previously. That is to say, *S*-carboxymethylcysteine sulfone and the product identified as glycolic acid appear after performic acid oxidation; the same neutral species is also present after $NaBH_4$ reduction. Therefore, dibromoacetone appears to be able to alkylate another reactive cysteine in rabbit muscle glyceraldehyde-3-phosphate dehydrogenase and does not further react in a cross-linking alkylation.

N-[^{14}C]Ethylmaleimide was used in order to demonstrate the generality of this reaction with a second cysteine residue.

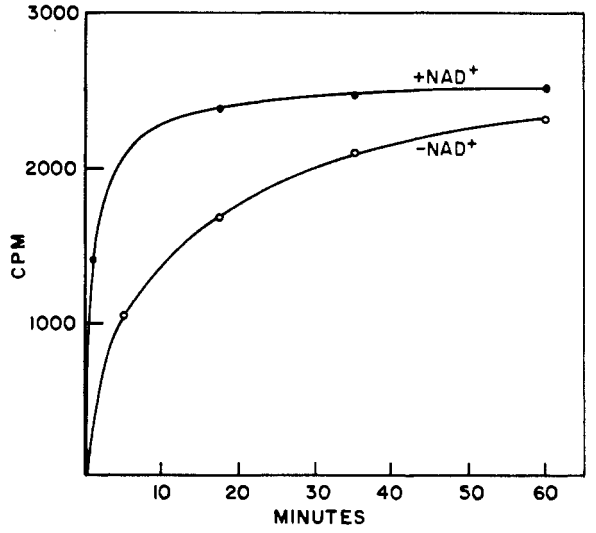

FIGURE 2: Effect of NAD^+ on dibromoacetone alkylation of sodium tetrathionate inactivated enzyme. Two 0.5 ml solutions of charcoal-treated dehydrogenase (A_{280}:A_{260} = 1.84, 6.4 mg/ml, 22.9 nmoles) in 0.05 M Tris·HCl (pH 7.0) and 1 mM EDTA were treated as follows. To one was added NAD^+ (10 μl, 185 nmoles, pH 7) (●); to the other, 10 μl of buffer (○). To each solution was then added sodium tetrathionate (10 μl, 100 nmoles), and after 2 min at 0° neither solution contained any enzyme activity. [^{14}C]Dibromoacetone (5 μl; 290 nmoles) was added to both samples; aliquots (20 μl) from each were placed on disks at the designated times and counted as in Figure 1.

This reagent has been shown to react specifically with cysteine at neutral pH and low reagent concentration (Smyth *et al.*, 1964) and produces the easily identified product, *S*-succinylcysteine, upon 72-hr acid hydrolysis of the modified protein. The position of this derivative on the amino acid analyzer is separated from the other regularly occurring amino acids and, by using an extinction coefficient of 1.08 times that for aspartic acid, its content can be easily determined. The results found in Figure 3 indicate that [^{14}C]NEM is also capable of alkyating rabbit muscle dehydrogenase when the active site is blocked with either iodoacetic acid or sodium tetrathionate. The extent of incorporation reaches, per mole of enzyme, 7.9 groups for unprotected and 3.8 groups for protected enzyme. The rate of alkylation is slower and the product more unstable than that alkylated with dibromoacetone. At room temperature, the enzyme quickly precipitates (20 min) when [^{14}C]-NEM is used to alkylate this second cysteine. The iodoacetic acid inhibited enzyme shows a much slower rate of alkylation with [^{14}C]NEM than does the sodium tetrathionate inhibited enzyme. When a molar ratio of 21:1 of NEM:enzyme is used for the sodium tetrathionate inacticated enzyme, the reaction goes to completion within 60 min at 0°. The sodium tetrathionate inhibited dehydrogenase begins to lose activity irreversibly after this time unless mercaptoethanol (or some other thiol reducing agent) is added. Table IV contains the results of amino acid analyses performed on these samples. Assuming 10–12% of the original amount of *S*-succinylcysteine is degraded during acid hydrolysis, a corrected amount of 3.8 *S*-succinylcysteine residues per mole of enzyme appears after acid hydrolysis of a NEM-modified enzyme—in excellent agreement with the radioisotope incorporation studies. These two independent methods of quantitating changes produced by NEM alkylation, along with changes in half-cystine content, strongly argue that one cysteine per monomer is being modified when the active site is blocked.

That [^{14}C]dibromoacetone or [^{14}C]NEM compete for the same site is shown in Figure 4. The full incorporation of either

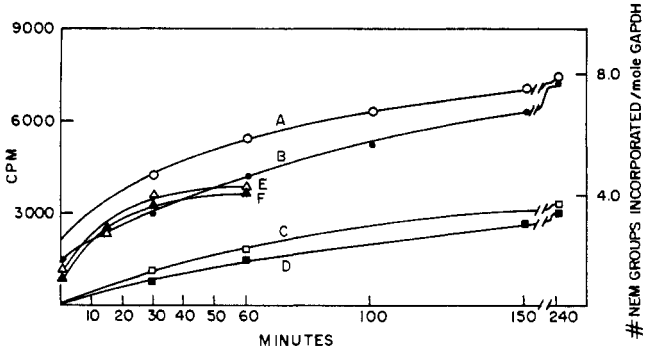

FIGURE 3: Alkylation with NEM of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) previously inactivated with sodium tetrathionate or iodoacetate. A solution of GAPDH (2.89 mg/ml) in 0.05 M Tris·HCl (pH 7.0) and 1 mM EDTA was divided into six 0.60-ml aliquots (1.73 mg. 12.4 nmoles) (A–F). To one (A) was added 5 μl (615 nmoles) of a stock [^{14}C]NEM solution (○) and to another (B), 2 μl (246 nmoles) (●). Two aliquots were inactivated by the addition of iodoacetate (10 μl, 100 nmoles) for 30 min at 25° and cooled to 0°. A 5-μl (615 nmoles) portion of the stock [^{14}C]NEM solution was added to one (C) (□); 2 μl (246) nmoles) to the other (D) (■). To each of the remaining two solutions of only GAPDH was added sodium tetrathionate (5 μl, 50 nmoles); after 2 min at 0°, 516 nmoles of [^{14}C]NEM was added to one (E) (△), and 246 nmoles, to the other (F) (▲). Aliquots (50 μl) were removed at the designated times and counted as described in Figure 1.

TABLE IV: Amino Acid Analysis of Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) Alkylated with NEM in the Presence and Absence of TET and IAc.[a]

Amino Acid	GAPDH	GAPDH + TET + NEM	GAPDH + IAc + NEM
Lysine	26.3	26.1	26.5
Histidine	10.9	10.8	11.0
Arginine	10.0	10.0	10.0
Half-cystine	3.7	*2.9*	*1.8*
Tyrosine	8.9	9.0	9.0
Phenylalanine	14.0	14.0	14.0
S-Succinylcysteine		*0.95*	*0.90*
S-Carboxymethylcysteine			*0.90*

[a] GAPDH (5–7 mg/ml) was treated as previously described for the modification of the active site cysteine (by sodium tetrathionate or iodoacetic acid) and subsequent alkylation by NEM of the enzyme. Mercaptoethanol was added at the end of 60 min for the sodium tetrathionate treated enzyme (150 min for the iodoacetic acid treated enzyme), and the enzyme solutions were dialyzed against water before being prepared for acid hydrolysis. The numbers in the table represent values for residues per monomer and are based on 10.0 arginine and 14.0 phenylalanine residues per monomer. The *S*-succinylcysteine values have been corrected for loss during acid hydrolysis (11%). Any differences from the untreated GAPDH are italicized. TET, sodium tetrathionate; IAc, iodoacetic acid.

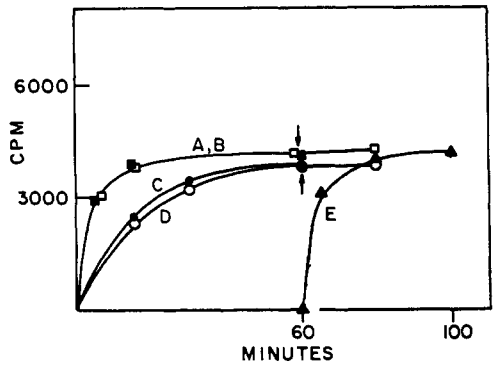

FIGURE 4: Competition between NEM and dibromoacetone for the second reactive cysteine. Five 0.7-ml portions of the dehydrogenase (2.89 mg, 20.6 nmoles) (A–E) in 0.05 M Tris·HCl (pH 7.0) and 1 mM EDTA were placed in separate tubes, to each of which was added sodium tetrathionate (8 μl, 80 nmoles) at 0°. After 5 min, one of these (A) was allowed to react with only [^{14}C]dibromoacetone (4 μl, 232 nmoles) (■); another (B), following 60 min reaction with the same amount of [^{14}C]dibromoacetone, was also treated with [^{14}C]NEM (2 μl, 246 nmoles) (□). The third (C) was treated at 0° with only [^{14}C]NEM (2 μl, 246 nmoles) (●); the fourth (D), first with [^{14}C]NEM for 60 min and then with [^{14}C]dibromoacetone (4 μl, 232 nmoles) (○). The fifth sample (E) provided a control for demonstrating the stability of the sodium tetrathionate protected enzyme, to which [^{14}C]dibromoacetone (4 μl, 232 nmoles) was added after 60 min at 0° (▲). Samples (50 μl and 40 μl for dibromoacetone- and NEM-treated enzyme, respectively) for measuring incorporation of radiocarbon were removed at the indicated times and processed as described in Methods. Arrows indicate time of addition of the second alkylating reagent.

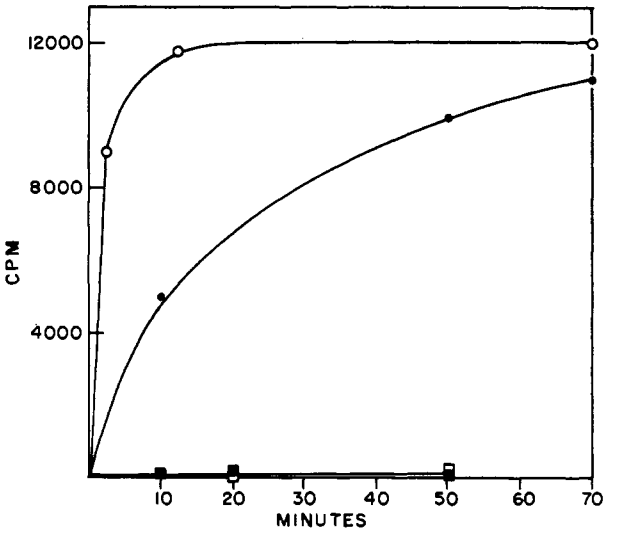

FIGURE 5: Protection of phosphoglyceroyl-enzyme from alkylation by dibromoacetone or NEM. The dehydrogenase (14.7 mg/ml, 0.5 ml, 52 nmoles) in 0.05 M Tris·HCl (pH 7.0) and 2 mM EDTA was incubated with 0.45 ml of D-glyceraldehyde 3-phosphate (16.9 mM, pH 6.3), 10 μl of sodium pyruvate (0.10 M, pH 7.0), 0.30 ml of NAD^+ (0.02 M, pH 7.0), and lactate dehydrogenase (10 μl, 50 μg) at 0° for 90 min in order to form the thiolester intermediate (final pH 6.95). This enzyme solution was separated into two 0.30-ml samples (12.4 nmoles of enzyme per sample). [^{14}C]Dibromoacetone (5 μl, 290 nmoles) was added to one sample (□); [^{14}C]NEM (2 μl, 246 nmoles) to another (■). At the times indicated 50 μl of the dibromoacetone-treated enzyme solution and 40 μl of the NEM-treated enzyme solution were removed for measuring the incorporation of radiocarbon. Controls (in which no thiolester intermediate has been formed) using the same amounts of [^{14}C]dibromoacetone (○) and [^{14}C]NEM (●) were treated in the same manner except H_2O replaced the glyceraldehyde 3-phosphate.

one of the reagents does not allow any incorporation of the other. One final experiment was performed to determine if the natural substrate glyceraldehyde 3-phosphate as the thiolester intermediate affords protection. Addition of either [^{14}C]dibromoacetone or [^{14}C]NEM causes no alkylation of the phosphoglyceroyl enzyme (Figure 5), thus implying that these reagents must be attacking a cysteine residue at, or close by, the active-site region. No alkylation occurs even at very high ratios of either reagent to the thiolester enzyme indicating that other reactive residues on the surface of the protein are not being modified. At these higher ratios, both the essential and a second cysteine residue per chain become alkylated by NEM or dibromoacetone in the native, unprotected enzyme (Figure 5).

In order to obtain more definitive information on the location in the amino acid sequence of the modified cysteine residue, two independent approaches were carried out: (1) a comparison of the trypsin and papain digests of the dehydrogenase modified only at cysteine-149 or at the unknown cysteine residue and (2) a comparison of the unmodified and modified enzyme in terms of their ability to form a specific disulfide bond with concomitant irreversible loss in enzyme activity. At the outset, it must be mentioned that the rabbit muscle enzyme has not been fully sequenced; however, preliminary reports indicate that a close similarity exists for this enzyme and the pig muscle enzyme (Harris and Perham, 1968). Previous studies by Boross (1969) and by Wassarman and Major (1969) (using pig and lobster muscle enzymes, respectively) have indicated that only cysteine-149 and cysteine-153 are available to chemical modification without a complete unfolding of the enzyme. The remaining two (or three, in the case of lobster muscle) cysteine residues appear to be buried in the native folded structure. Since cysteine-149 and cysteine-153 are on the same tryptic peptide, comparative studies should be able to decide if the second, modifiable cysteine with [^{14}C]dibromoacetone or [^{14}C]NEM is cysteine-153.

If [^{14}C]dibromoacetone (in a 5:1 ratio to the enzyme) alkylates cysteine-153 when cysteine-149 is protected by the thiosulfate group and cysteine-149 when no protection is employed, then the two radioactive peptides resulting from tryptic hydrolysis should be identical in size and charge. Separation of the tryptic peptides on the basis of size was attempted using Sephadex G-50 columns. The elution profiles indicated that the enzyme alkylated with [^{14}C]dibromoacetone in the presence or absence of sodium tetrathionate yields very similar (by size) radioisotopically labeled tryptic peptides. If the two samples (with and without sodium tetrathionate treatment) were mixed together and placed on the same column, no differences in the elution pattern could be obtained.

An alternative method for separating peptides is to utilize their charge differences. Electrophoresis at various pH values was used to separate tryptic peptides on the basis of any charge differences. At pH 6.4 the radioactive peptides from the different labeling procedures remained at (or near) the origin suggesting they are neutral at this pH, whereas electrophoresis at a much more acidic pH (1.9–2.0) caused these peptides to migrate identically toward the negative end.

Paper chromatography constitutes another valuable method for peptide comparisons (Moore and Baker, 1958). The radioactive peptides (alkylated in the absence and presence of

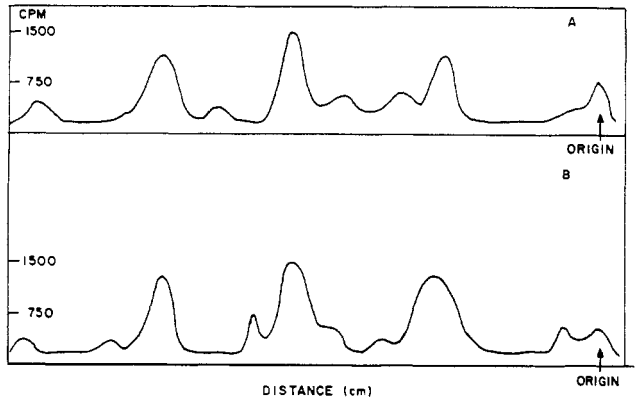

FIGURE 6: Paper chromatography of papain digest of the tryptic peptides of glyceraldehyde-3-phosphate dehydrogenase alkylated with [^{14}C]dibromoacetone in the presence (A) and absence (B) of sodium tetrathionate. Paper chromatography was performed using the solvent system composed of 1-butanol–pyridine–acetic acid–water as described in Methods. Conditions for protease digestion are described under Methods. Settings for the radiochromatogram scanner were: time constant, 30 sec; scan speed, 2 cm/min; and scan range, 3000 cpm.

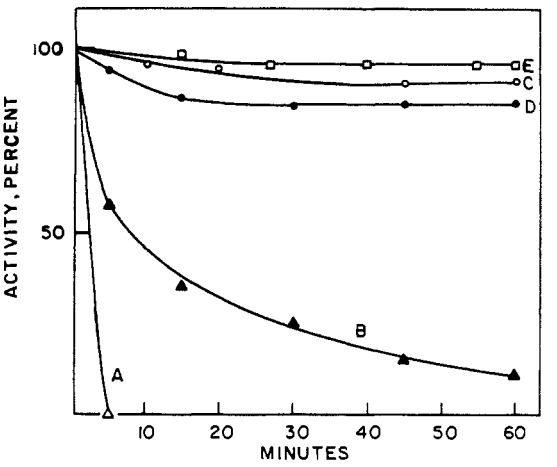

FIGURE 7: Determination of dehydrogenase activity of glyceraldehyde-3-phosphate dehydrogenase modified by dibromoacetone or NEM in the presence and absence of sodium tetrathionate. Five 1.0-ml samples of the enzyme (7.3 mg/ml, 52 nmoles) in 0.05 M Tris-HCl (pH 7.0) and 1 mM EDTA were placed in separate tubes (A–E). To one (A) was added [^{14}C]dibromoacetone (10 μl, 580 nmoles) (△), and to another (B) [^{14}C]NEM (10 μl, 1230 nmoles) (▲). Sodium tetrathionate (5 μl, 200 nmoles) was added to each of the three remaining samples. After several minutes at 0° (0% activity), [^{14}C]-dibromoacetone (as before) (○) was added to one of these samples (C); [^{14}C]NEM (as before) (●), to a second (D). The final sample (E) was treated only with sodium tetrathionate (no addition of alkylating agents) (□). Before assaying, an aliquot (20 μl) was placed into 0.18-ml solution of 0.05 M Tris·HCl (pH 7.0) containing 1 mM NAD^+–1 mM EDTA–70 mM mercaptoethanol. After incubating in this medium at 0° for 30 min, the enzyme in a 30-μl portion was assayed for dehydrogenase activity.

sodium tetrathionate) had identical mobilities when chromatographed in the 1-butanol–acetic acid–water solvent system, indicating the similarity in chemical composition of the two peptides. Results identical with these were also obtained using [^{14}C]NEM.

These three methods suggest that the two peptides (alkylated by dibromoacetone or *N*-ethylmaleimide in the absence and presence of sodium tetrathionate) are very similar in size, charge, and chemical composition. However, before we can conclude that the two peptides are virtually the same (the only difference being the location of the modifying group on either cysteine-149 or -153), we must consider the structures of other possible cysteine-containing tryptic peptides. Harris and Perham (1965) have isolated and sequenced the three tryptic peptides that contain cysteine from pig muscle glyceraldehyde-3-phosphate dehydrogenase. Because of the observed similarity in sequence of the pig and rabbit muscle enzymes (Harris and Perham, 1968), we assume the same peptides will arise from trypsin treatment of rabbit muscle enzyme. The peptide containing cysteine-281 is much larger (36 amino acids) than the peptides containing cysteine-244 (14 amino acids) and cysteines-149 and -153 (17 amino acids) and should be easily separated on the Sephadex column. In addition the charge at pH 6.5 of the largest tryptic peptide is much more negative than the others (−4 compared to −1), suggesting the feasibility of electrophoretic separation. Since the two peptides from unprotected and sodium tetrathionate protected enzyme appear very similar by all criteria, the peptide containing cysteine-281 can be eliminated as being a possible site for alkylation. Resolution of the two remaining peptides containing cysteine-244 and cysteines-149 and -153 is more difficult due to their similarity in charge and size. In an attempt to distinguish between these two alternatives, another experiment utilizing papain treatment after the tryptic proteolysis was performed. Papain is used to cleave the larger peptides into smaller units yielding several radioactive peptides which could be used as a fingerprinting technique. Comparison of these peptides by chromatography suggests that the original peptides were similar (Figure 6A and B). Therefore, on the basis of these structural relationships it would appear that only the active-site peptide has been modified by dibromoacetone or NEM in the presence and absence of the thiolsulfate group, which protects only the essential cysteine-149 (Pihl and Lange, 1962). However, it must be stressed that, to determine unambiguously the position of alkylation in the sodium tetrathionate protected enzyme with these reagents, the radioactive peptide should be isolated and its amino acid composition determined.

Before proceeding with the additional evidence to support the proposition that cysteine-153 is the residue specifically alkylated by these reagents, we must consider an important property of the modified enzyme protected at the catalytically essential cysteine-149. Although iodoacetate is an irreversible inhibitor of the enzyme, sodium tetrathionate and sodium iodosobenzoate are well-known reversible inhibitors of the enzyme (for example, see Parker and Allison, 1969). However, under the conditions employed in these experiments, inactivation by sodium iodosobenzoate is not completely reversible. A loss in 15–20% of the original activity occurs when the enzyme is incubated first in the presence of sodium iodosobenzoate (a 6:1 molar ratio of sodium iodosobenzoate: enzyme) for 60–90 min at 0° and then in a reducing solution containing mercaptoethanol. The use of sodium tetrathionate is preferred, since all reactions (sodium tetrathionate inhibition and subsequent alkylation) are performed at 0° and only 2 min are required for complete inactivation (using a stoichiometric amount of sodium tetrathionate per active-site sulfhydryl group). The enzyme, when inactivated in this manner, regains 94–96% of its original activity if mercaptoethanol is added at the end of 60 min. Figure 7 reveals that incorporation of [^{14}C]dibromoacetone or [^{14}C]NEM (equivalent to 3.8–3.9 moles/mole of enzyme) into the sodium tetrathionate protected enzyme does not greatly inhibit the enzyme. Re-

covery of dehydrogenase activity is over 80% for the NEM-modified enzyme and approximately 90% for the dibromoacetone-modified enzyme. The greater loss in activity in the NEM-modified enzyme is undoubtedly related to our earlier observation concerning its thermal lability. Nevertheless, it is possible to obtain an enzymatically active preparation, in which one cysteine residue per monomer has been modified.

To pinpoint further the position of this cysteine residue advantage may now be taken of a specific reaction involving disulfide bond formation between cysteines-149 and -153 (Vas and Boross, 1970; Wassarman and Major, 1969). Following formation of this linkage, the protein undergoes substantial structural change leading to an irreversible loss in activity. Alkylation at cysteine-153 should protect against this form of inactivation. If the native enzyme is inactivated with a 4:1 molar ratio of sodium tetrathionate:enzyme at 37° and pH 8.0, it loses 70% of its original activity in 60 min. However, if the enzyme has been previously alkylated with dibromoacetone at the second cysteine and then inactivated as above with sodium tetrathionate, the enzyme loses only 10% activity in the same period of time (Figure 8). This observation that a molecule of dibromoacetone attached to a second reactive cysteine residue protects the essential cysteine residue from disulfide bond formation with subsequent inactivation strengthens the notion that it is attached to cysteine-153 in the primary sequence.

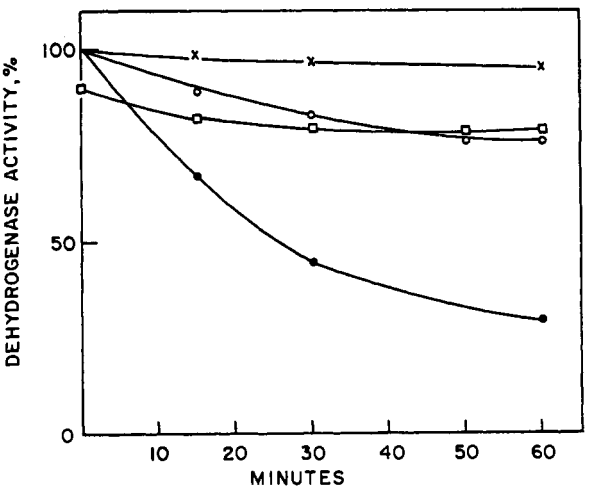

FIGURE 8: Inactivation of sodium tetrathionate treated glyceraldehyde-phosphate dehydrogenase at 37°. The dehydrogenase (8.35 mg/ml, 59.6 μM) in a 4.0-ml solution of 0.05 M Tris·HCl (pH 7.0) and 1 mM EDTA was divided into four 1.0 ml aliquots. Two aliquots were inactivated with sodium tetrathionate (25 μl, 250 nmoles) at 0°. At the end of 2 min, [^{14}C]dibromoacetone (5 μl, 290 nmoles) was added and allowed to react at 0° for 60 min before the addition of mercaptoethanol (10 mM). All four samples were dialyzed against 0.05 M Tris·HCl at pH 8.0 (500 volumes per sample). One untreated sample acted as the control for the 37° inactivation studies (×). The other unalkylated sample was inactivated with sodium tetrathionate (25 μl, 250 nmoles) (●). One of the two [^{14}C]dibromoacetone-alkylated samples was again treated with tetrathionate (25 μl, 250 nmoles) (○), while the other acted as another control (□). An aliquot (20 μl) was removed at the designated times and placed into 0.18 ml of a solution containing 0.05 M Tris·HCl (pH 8.0)–1 mM EDTA–2 mM NAD^+–80 mM mercaptoethanol. Incubation time was 30 min at 0° before removing an aliquot (30 μl) for assaying.

Discussion

The evidence that we have presented clearly shows that rabbit muscle glyceraldehyde-3-phosphate dehydrogenase can be selectively modified at cysteine-153 in the amino acid sequence without greatly affecting the ability of cysteine-149 to function in enzyme catalysis. The position of this modification has been established by indirect, but nevertheless compelling, evidence: (1) complete protection from incorporation by the thiolester enzyme intermediate derived from the natural substrate, (2) comparative peptide studies, and (3) protection from irreversible inactivation caused by intrachain disulfide-bond formation (between cysteines-149 and -153).

The presence of carboxymethyl, thiosulfate, and phosphoglyceroyl groups covalently attached to the essential cysteine residue and of the coenzyme noncovalently bound to the active site produces intriguing effects on the ability to modify this second, proximal cysteine residue. For example, the tetrathionate-treated enzyme reacts with dibromoacetone or NEM, while the phosphoglyceroyl-enzyme is inert to these reagents. Studies using proteolysis and immunochemical techniques suggest that the sodium tetrathionate treated enzyme has a less tightly folded structure than the native enzyme (Allison and Kaplan, 1964). Although corresponding investigations have not been conducted with the phosphoglyceroyl- and *S*-carboxymethyl-enzymes, the available evidence on the latter indicates there is very little structural change (compared to the native enzyme) (Wassarman and Watson, 1970). On the other hand, NAD^+ bound to the enzyme leads to a more compact structure as determined by various approaches (Hoagland and Teller, 1969; Conway and Koshland, 1968; Krimsky and Racker, 1963); however, the effect of NAD^+ on the alkylation of cysteine-153 is the same as that of tetrathionate, which has the opposite structural effect. It can be argued that environmental changes for specific amino acid residues may proceed in a direction unrelated to that detected at the gross structural level. Nevertheless, the observations made here will require further study.

One means for studying these effects as well as others related to enzyme catalysis, ligand binding, subunit interactions, etc., is to capitalize on the availability of a selectively modified, catalytically active glyceraldehyde-3-phosphate dehydrogenase by introducing environment-sensitive probes at cysteine-153. The two alkylating reagents studied are sufficiently different to suggest that various types of reporter groups might be covalently attached at this site. For example, Kirtley and Koshland (1970), using inactive rabbit muscle enzyme produced by alkylating the essential cysteine residue with 2-bromoacetamido-4-nitrophenol, have demonstrated that binding of NAD^+ causes a structural change with the active site leading to a less polar environment. In preliminary studies we have prepared a catalytically active enzyme containing the nitroxide radical from 3-(2-bromoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl at cysteine-153. Further utility of this modification might be demonstrated in the X-ray diffraction studies (Wassarman and Watson, 1970). The inability to cross-link the cysteines-149 and -153 using the bifunctional reagent 1,3-dibromoacetone (present study; Moore and Fenselau, 1972) suggests that the two sulfhydryl groups are not in close proximity in the stable, folded structure of the enzyme; thus, the same approach described in this paper should lead to an active enzyme bearing a covalently bonded heavy atom, and possibly isomorphous, derivative at position 153 (McMurray and Trentham, 1969).

Finally, the physiological significance of the invariant cysteine-153 can possibly be tested by employing this modification procedure. One plausible control mechanism in-

volving the second cysteine residue is suggested by the instability of the inactive disulfide enzyme. Oxidation of the enzyme under aerobic conditions could produce the inactive enzyme, which, in effect, would terminate glycolytic activities. This possible role among others can be studied in yeast cell-free systems, by employing the yeast dehydrogenase which contains only cysteines-149 and -153.

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Circular Dichroic Studies of the Interaction of Dihydrofolate Reductase with Substrates, Coenzymes, and Inhibitors†

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ABSTRACT: Streptococcal dihydrofolate reductase shows a marked aromatic side-chain Cotton effect in the 260–310 nm region of its circular dichroic spectrum. This effect consists of at least three distinct ellipticity bands with maxima centered at 305, 292, and 270 nm. Interaction of the enzyme with TPNH results in the generation of an extrinsic Cotton effect at *ca.* 340 nm and a decrease in the magnitude of the aromatic side-chain Cotton effect. Titration of the enzyme with TPN^+ results in a sixfold lower decrease in the side-chain Cotton effect of the protein as compared to that observed in the TPNH complex. Binding of dihydrofolate, folate, or amethopterin generates a large enhancement of the molar ellipticity of the protein in the 270–315 region but does not alter the far-ultraviolet dichroic spectrum of the enzyme. The increase in ellipticity is proportional to the extent of complex formed. The maximum total change in molar ellipticity at 290 nm for the dihydrofolate–enzyme complex is +828,000 (deg cm^2) $dmole^{-1}$. This enhancement is, to our knowledge, the largest change in magnitude observed in an enzyme–substrate complex. From enzyme–ligand *vs.* enzyme difference circular dichroic spectra the dihydrofolate–enzyme complex exhibits a single maximum at 290 nm whereas the oxidized compounds, folate and amethopterin, show double maxima in the 290 to 315 nm region. The enhancement of molar ellipticity at 290 nm is 30–40% smaller in the folate and amethopterin complexes as compared to that of the dihydrofolate complex. The binding of amethopterin at pH 5.9 appears to be stoichiometric from circular dichroic titration studies, whereas the affinity of the enzyme for the inhibitor is reduced at pH 7.5. The ellipticity differences observed in the various folate analog complexes are considered to reflect different modes of attachment of these ligands to dihydrofolate reductase. The possible involvement of aromatic amino acid residues in the binding of these folate analog and pyridine nucleotide coenzymes is discussed in terms of a mutually facilitated orientation of the ligand with functional groups on the enzyme.

There have been an increasing number of communications describing Cotton effects and dichroic bands associated with optical activity in the aromatic side chains of proteins (reviewed by Beychok, 1968). The presence of such effects

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