Participation of Cysteinyl Residues in the Structure and Function of Muscle Aldolase. Characterization of Mixed Disulfide Derivatives*

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ABSTRACT: Cysteinyl residues in rabbit muscle D-fructose 1,6-diphosphate (FDP) aldolase (EC 4.1.2.13) were converted into mixed disulfides by treatment with a series of disulfide monosulfoxides ($RCH_2CH_2S(=O)SCH_2CH_2R$, R = carboxyl, amino, or carbamyl) at pH 7.0–7.4. Regardless of the R group in the reagent, two of the eight cysteinyl residues per subunit were reactive in the presence of the competitive inhibitor, hexitol 1,6-diphosphate (HDP) and two additional residues reactive only in the absence of HDP. The remaining four cysteinyl residues were largely unmodified whether the inhibitor was present or absent. These sulfhydryl groups of aldolase were thus divisible into three classes, exposed, protected, and buried, the classes containing two, two, and four residues per subunit, respectively. The two exposed groups were localized in the two cyanogen bromide peptides derived from the carboxyl end of the subunit polypeptide chain; the two protected groups were located one each in the amino and carboxyl CNBr peptides, and the four buried groups in the two CNBr peptides from the amino end of the subunit chain. Derivatives modified in the presence of HDP exhibited only slight changes in the kinetic parameters for FDP cleavage. Significant decreases (50–80%) in V_m and increases (30- to 100-fold) in K_m occurred when the two protected SH groups were subsequently modified, with the changes for the neutral, R = carbamyl, derivative being smaller than for the charged carboxyl and amino derivatives. The denaturation of unmodified aldolase by guanidine hydrochloride (Gd·HCl) at pH 5.5 was shown to be composed of two steps: (1) dissociation of tetramer directly to monomer, accompanied by loss of a significant portion of the ordered secondary structure, and (2) disruption of the residual structure of the dissociated subunits. All mixed disulfide derivatives were similar in exhibiting a two-step mechanism of denaturation, and were practically identical in the Gd·HCl dependence of the second step. They were different from the unmodified protein only in the Gd·HCl sensitivity of the first step, where, for each R, the order of decreasing stability was unmodified aldolase, HDP-protected derivative, then unprotected derivative. Within each class of three protected and three unprotected derivatives, the R = amino proteins were least stable to Gd·HCl, and the carbamyl and carboxyl proteins of comparable stability. At neutral pH, the disulfide cystamine modified one reactive SH group per subunit, identified as that in the CNBr peptide preceding the carboxyl-terminal CNBr peptide, but had no significant deleterious effect on the catalytic or structural properties of the protein.

The objective of many protein chemical modification studies is the identification of amino acid residues specifically involved in the biological activity of the macromolecule, its catalytic, regulatory, antigenic, or transport function (Singer, 1967). The potential disruption of protein structure by the modification is usually considered an unwanted side effect, obscuring the assignment of functional roles to the residues modified. However, in analogy to the types of functional sites mentioned, it is reasonable to propose that proteins contain structural sites, constituting clusters of amino acid residues which are essential for conformational integrity. Structurally critical residues have been directly demonstrated in hemoglobin as a result of crystallographic analysis (Perutz and Lehmann, 1968). The ability to renature rabbit muscle aldolase from acid or urea (Deal *et al.*, 1963; Stellwagen and Schachman, 1962; Penhoet *et al.*, 1966) even when mixed with extraneous denatured polypeptide chains (Cook and Koshland, 1969), demonstrates very specific self-recognition sites between the subunits, and strongly suggests the presence of structural sites in this protein. The objective of this study was to probe for such sites in aldolase by chemical modification of a single class of functional groups, the sulfhydryl groups of cysteinyl residues, and by physical characterization of these derivatives.

Fructose 1,6-diphosphate (FDP)[1]: aldolase (EC 4.1.2.13) from muscle is a tetramer of molecular weight about 160,000 (Penhoet *et al.*, 1967; Kawahara and Tanford, 1966a; Sia and Horecker, 1968; Penhoet *et al.*, 1969) whose total half-cystine content is accountable as free thiol (Westhead *et al.*, 1963). Although many determinations of the number of

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[1] The abbreviations used are: FDP, D-fructose 1,6-diphosphate; F-1-P, D-fructose 1-phosphate; Gd·HCl, guanidine hydrochloride; Gd·HBr, guanidine hydrobromide; NEM, *N*-ethylmaleimide; TEA, triethanolamine; 2-ME, 2-mercaptoethanol.; HDP hexitol 1,6-diphosphate; DTNB, 3-carboxy-4-nitrophenyl disulfide.

cysteinyl residues per 40,000 mol wt subunit have been reported (Anderson and Perham, 1970; Hartman, 1970; Eagles *et al.*, 1969; Penhoet *et al.*, 1969; Lai, 1968; Swenson and Boyer, 1957; Benesch *et al.*, 1955), at the present time there is some disagreement whether there are seven or eight. The sulfhydryl groups of aldolase were chosen as targets for a chemical modification study because a structural role for SH groups was proposed as a result of *p*-mercuribenzoate modification (Swenson and Boyer, 1957; Szabolsci and Biszku, 1961). More recently structural participation of the SH groups has been implicated by studies with 2-methylmaleic anhydride (Gibbons and Perham, 1970).

Selective modification of cysteinyl residues may be achieved *via* thiol–disulfide interchange, most commonly at rather elevated pH, when aliphatic disulfides are employed (Bradshaw *et al.*, 1967; Kowal *et al.*, 1965). To avoid the required alkaline conditions, a series of aliphatic disulfide monosulfoxide reagents were prepared, which react with thiol groups at neutral pH (Pihl and Lange, 1962). This selective modification was further limited by performing the reaction in the presence and in the absence of the competitive inhibitor, hexitol 1,6-diphosphate (HDP), permitting classifications of SH groups based upon their chemical accessibility under the chosen solvent conditions. Lacking a 2-keto group, HDP was used as a protective agent in preference to aldolase substrates, many of which have been reported to inactivate the enzyme upon prolonged incubation (Anderson and Perham, 1970; Adelman *et al.*, 1968; Lai *et al.*, 1968; Woodfin, 1967).

Experimental Procedures

Materials. Rabbit muscle aldolase, as an ammonium sulfate suspension, was purchased from Sigma (lot no. 88B-2090). Chemicals and other materials were of the highest purity commercially available, obtained as indicated for the following, or from other common sources: D-fructose 1,6-diphosphate, tetracyclohexylammonium salt, decahydrate (Boehringer); 3-carboxy-4-nitrophenyl disulfide (DTNB) and D-fructose 1-phosphate (F-1-P), barium salt (Pierce); D,L-glyceraldehyde 3-phosphate, diethyl acetal, barium salt (Sigma); Carbowax 6000 and size 18 cellulose casing (Union Carbide); *N*-ethylmaleimide and cyanogen bromide (Eastman); guanidine carbonate (Matheson Coleman and Bell); guanidine hydrochloride, Ultra Pure, and *S*-carboxymethylcysteine (Mann); peracetic acid (FMC Corp); cystamine dihydrochloride and 3,3′-dithiodipropionic acid (Aldrich); FC-43 fluorochemical oil (Beckman); and Sephadex G-75, bead form (Pharmacia).

Laboratory Preparations. Hexitol 1,6-diphosphate was prepared by reduction of FDP with sodium borohydride (24 × the stoichiometric amount), at pH 10, 5° for 95–100 hr (Ginsburg and Mehler, 1966). Barium F-1-P was converted into the soluble sodium salt with Dowex 50 (H+), followed by neutralization with NaOH. Concentrations of stock solutions of HDP and F-1-P were determined by phosphate analysis (Ames and Dubin, 1960). Guanidine hydrobromide (Gd·HBr) and guanidine hydrochloride (Gd·HCl), used in all chemical and physical studies except the FDP activity assays in Gd·HCl (for which the Mann reagent was used) were prepared from guanidine carbonate (Nozaki and Tanford, 1967; Kawahara and Tanford, 1966b) and recrystallized from methanol. The concentration of stock solutions of Gd·HCl was determined refractometrically (Kielly and Harrington, 1960), after correcting for the contribution from the buffer salts. The concentration of Gd·HBr solutions was assumed to be that prepared by weight.

TABLE I: Infrared Spectral Data and Elementary Analyses of Disulfide Monosulfoxides.

RCH_2CH_2S-$S(=O)CH_2$-CH_2R, R =	Infrared Bands[a] (cm^{-1})		Weight Percentage of: C	H	S
Carboxyl	1113	Obsd	31.78	4.48	27.92
		Calcd	31.85	4.45	28.34
Amino(hydrochloride)	1064, 1052	Obsd	20.08	5.95	26.48
		Calcd	19.92	5.85	26.59
Carbamyl	1066	Obsd	32.47	5.52	28.51
		Calcd	32.13	5.39	28.59

[a] Principal new bands observed in monosulfoxide compounds which were absent in the parent disulfides.

Disulfide monosulfoxides, $RCH_2CH_2S(=O)SCH_2CH_2R$, R = carboxyl, amino, or carbamyl, were prepared by treating the parent disulfides with 1 equiv of peracetic acid (Schoberl and Grafje, 1958). Over a period of 1 hr, peracetic acid (0.025 mole), diluted to 10 ml with glacial acetic acid and mixed with 38 ml of $CHCl_3$, was added dropwise to an ice-cooled suspension of disulfide (0.025 mole) in 75 ml of absolute methanol, with continuous stirring. After an additional 4–4.5 hr at 0° followed by 2–4 hr at room temperature, the reaction mixture was filtered, and the product washed with cold ethanol. The oxidation products of cystamine dihydrochloride and 3,3′-dithiodipropionamide remained insoluble throughout, but that from 3,3′-dithiodipropionic acid dissolved, in which case the solvent was removed with a rotary evaporator, and the product recrystallized from water. The amide disulfide, 3,3′-dithiodipropionamide, was prepared from the corresponding acid (Ponci *et al.*, 1963) and recrystallized from water, mp 177–180°, lit. mp 177° (H_2O) (Luettringhaus and Schneider, 1964), 182–184° (ethanol) (Ponci *et al.*, 1963).

The disulfide monosulfoxides were characterized by infrared spectroscopy (KBr disks, Perkin-Elmer No. 337 grating infrared spectrometer) and elemental analyses (Galbraith Laboratories, Knoxville, Tenn.), summarized in Table I. The R = carboxyl compound exhibited strong absorption in the region expected for alkyl disulfide monosulfoxides (1110 cm^{-1}, Ghersetti and Modena, 1963). The absorption of the R = amino and R = carbamyl compounds at lower wave numbers may be due to hydrogen bonding between an R group hydrogen and the S=O oxygen, analogous to the lower C=O vibration frequency observed in intramolecularly hydrogen-bonded carboxylic acids (Cross and Jones, 1969). Apparently no such hydrogen bonding occurs in the R = carboxyl compound, perhaps due to loss of the carboxyl hydrogen through preferential interaction with bromide ions in the KBr disk. The strong bands characteristic of higher oxidation products (sulfones or sulfonic acids, Cymerman and Willis, 1951; Douglass and Farah, 1959; Bellamy,

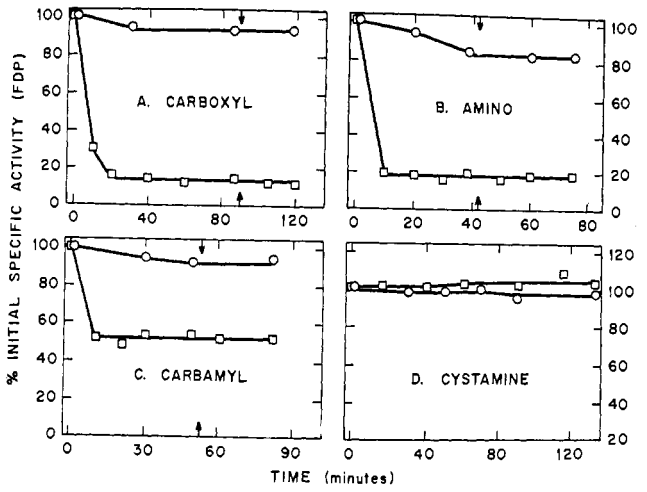

FIGURE 1: Activity monitor of modification reactions. Aldolase was treated with the indicated disulfide monosulfoxide reagent, of specified R group (A-C) or with cystamine (D) as described in the Experimental Section. Reactions were performed in the presence (○) or absence (□) of 6 mM HDP, at pH 7.0–7.4. The initial reagent concentration was 3 mM, and a second increment of 1.5 mM was added in A-C where indicated by the arrows. In the absence of any chemical reagent, native aldolase is completely stable under the conditions used. The FDP cleavage activity was measured by the spectrophotometric hydrazine method.

1958) were absent from infrared spectra of the disulfide monosulfoxides. The agreement of the elemental analyses with theoretical values demonstrated the absence of significant amounts of unreacted disulfide or doubly oxidized sulfone in the disulfide monosulfoxide preparations.

Enzymatic Assays. Aldolase catalysis of FDP and F-1-P cleavage was assayed at room temperature with a Cary 15 spectrophotometer, by the hydrazine trap procedure (Jagannathan *et al.*, 1956; Drechsler *et al.*, 1959), and specific activities were expressed as the change in A_{240} min^{-1} (mg protein)$^{-1}$. The FDP specific activity of unmodified, commercial enzyme (18–24) was somewhat lower than that reported for rabbit muscle aldolase prepared in the laboratory (*e.g.*, 30, Drechsler *et al.*, 1959). To obviate correction for a blank, both sample and reference 1-cm cuvets were filled with 1 ml of 0.05 M Tris–0.001 M EDTA–0.0023 M hydrazine sulfate, containing 0.004 M FDP or 0.013 M F-1-P for routine activity assays, and variable substrate concentrations for determination of kinetic constants. Michaelis–Menten constants were determined from linear least-squares fits to double-reciprocal plots (Lineweaver and Burk, 1934). The associated errors were approximated from lines estimated as poor fits to the data.

Aldolase activity in Gd·HCl was estimated by a modification of the standard assay for alkali-labile phosphorous (Taylor, 1955), using hydrazine rather than cyanide as a triose trap. Small volumes of protein, equivalent in activity to 100–160 μg of unmodified enzyme, were added to 0.60-ml volumes of 0.1 M hydrazine sulfate–0.05 M sodium acetate–0.001 M EDTA, pH 5.5, containing the desired guanidine concentration, and incubated at room temperature for 0.5 hr. To each sample was added 0.075 ml of 0.2 M FDP, in the same pH 5.5 buffer, and after 15 min duplicate aliquots of 0.20 ml were removed and diluted into 2.0 ml of ice-cooled 1 N NaOH. Hydrolysis was performed for 20 min at 20–23°, followed by neutralization with 0.25 ml of 8 N HCl, then phosphate analysis by the Fiske–Subbarow procedure (Leloir and Cardini, 1957). Blank-corrected absorbances at 660 nm were correlated with a relative activity using a calibration curve of absorbance *vs.* micrograms of protein, established for unmodified aldolase (3–160 μg). The calibration curve was linear only between 0 and 30 μg of unmodified protein, although even at the highest enzyme concentrations, less than 10% of the substrate was cleaved.

Chemical Modification. Chemical modifications were performed at room temperature at pH 7.0–7.4 in 0.05 M triethanolamine (TEA)–0.001 M EDTA or 0.01 M TEA–0.001 M EDTA–0.006 M HDP, with protein (3 mg/ml). The initial reagent concentration was 3 mM (5:1 mole ratio of reagent to protein) and second additions were made, increasing the concentration by 1.5 mM, where indicated by arrows along the horizontal axes of Figure 1A,B,C. Reactions were initiated by addition of the reagents in volumes equal to 1% or less of the total solution volume. Cystamine dihydrochloride and the corresponding monosulfoxide were added as solutions, and the carbamyl monosulfoxide as a suspension in 0.05 M TEA–0.001 M EDTA, originally pH 7.5. The carboxyl monosulfoxide was dissolved in methanol–TEA (7:3, v/v) containing 2 moles of amine/mole of diacid. Samples from reaction mixtures were diluted at least 20-fold into ice-cooled, pH 7.5 TEA–EDTA prior to measurement of FDP cleavage activity by the spectrophotometric hydrazine assay.

Following removal of the last aliquots for assay, the reaction mixtures were (1) dialyzed 2–4 hr *vs.* 0.01 M TEA–0.001 M EDTA, pH 7; (2) concentrated by immersing the bags 3–5 hr in a slurry of Carbowax 6000 in pH 7, TEA–EDTA; and (3) dialyzed at least 12 hr *vs.* 0.02 M TEA–0.002 M EDTA–0.2 M NaCl, pH 7.0, all operations being performed at 0–5°. All samples for enzymatic and physical studies were prepared by dilution from the resulting stock solutions, 15–25 mg/ml, stored at 5°.

Sedimentation velocity analyses of aldolase derivatives under nondenaturing conditions exhibited a slow shoulder (0.1–0.2 S), which was identified as a contaminant from the Carbowax treatment, apparently not removed in the dialysis following the concentration procedure. This contaminant had no detectable effect on the structure and activity of the native protein. When unmodified aldolase was diluted and concentrated by Carbowax, there was no effect on the protein absorbance at 280 nm, the sulfhydryl content determined by DTNB titration in sodium dodecyl sulfate, the K_m in FDP cleavage, or the optical rotation monitor of the Gd·HCl denaturation. Further evidence that the Carbowax material did not influence the enzymatic and physical measurements of aldolase derivatives, was the near identity of the cystamine modified protein (Carbowax treated) and the unmodified enzyme (untreated) in the characterizations discussed below. The contaminant did have a measurable effect on the solution viscosity, and in adjusting for this, sedimentation coefficients of Carbowax-concentrated proteins were increased by 4% for initial protein concentrations of 3 mg/ml, with proportional changes for other concentrations.

Optical Absorbance. Assuming an $\epsilon_{1\,cm}^{0.1\%}$ (280 nm) of 0.91 for unmodified aldolase (Baranowski and Niederland, 1949, *cf.* 0.938, Donovan, 1964) in 0.02 M TEA–0.002 M EDTA–

0.2 M NaCl, pH 7, a value of 0.81 (*cf.* 0.78, Sia and Horecker, 1968) was determined for $\epsilon_{1\,cm}^{0.1\%}$ (276 nm) in 0.1 N HCl–0.2 M NaCl (*cf.* $\epsilon_{1\,cm}^{0.1\%}$ (277 nm) 0.832, pH 2, Donovan, 1964), which was used to determine the concentration of aldolase derivatives, acid denatured in the same solvent. No corrections were made for the contribution to the total protein absorbance of the chemically introduced disulfides, which was calculated to be maximally 2% at about 276 nm, employing the extinction coefficient of cystine (Wetlaufer, 1962). The derivatives were referred to as unprotected or protected, if modified in the absence or presence of HDP, and identified by the name of the disulfide reagent, cystamine, or the R group of the disulfide monosulfoxide employed.

Titration and Blockage of Free Sulfhydryl Groups. A small volume of protein solution was added to 1.0 ml of 0.9% sodium dodecyl sulfate–0.09 M Tris–0.01 M sodium phosphate–0.002 M EDTA, pH 8.0, containing 0.001 M DTNB. Lower sulfhydryl titers were obtained if the DTNB was added to a solution of the protein in sodium dodecyl sulfate, probably due to oxidation of exposed SH groups prior to disulfide interchange with the reagent. The total free sulfhydryl content was calculated from the observed absorbance change at 412 nm (Ellman, 1959). A subunit molecular weight of 40,000 was assumed for all proteins.

Free sulfhydryl groups were blocked with NEM (5 times the stoichiometric amount), by reaction at room temperature for 40–50 min in pH 7.0, 3.0 M Gd·HBr–0.06 M TEA–0.6 mM EDTA, at a protein concentration of 4 mg/ml. Glacial acetic acid was then added to a final concentration of 10% (v/v) followed by dialysis *vs.* water for several hours. The protein was recovered by precipitation in 5% trichloroacetic acid.

CNBr Cleavage and Peptide Fractionation. Solutions of sulfhydryl-blocked proteins (1% w/v in 70% formic acid, v/v) were treated with CNBr (one-half the weight of protein present, 63 times the stoichiometric amount, based on methionine) for 22–24 hr at room temperature, then diluted with 7 volumes of water, and lyophilized. Samples (15–25 mg) of NEM–CNBr-treated protein were separated into their constituent CNBr peptides (Lai, 1968) on Sephadex G-75 (1.8 × 80 cm, flow rate of 8–9 ml/hr), in 0.5 M formic acid (pH 2.1) at room temperature, and individual fractions detected by their absorbance at 280 nm. The peptides were labeled X1–X4, in order of their elution from G-75 (Anderson *et al.*, 1969).

Performic Acid Oxidation. Proteins and CNBr peptides were oxidized with performic acid at 0° for 3 hr (Hirs, 1956), then diluted with 7 volumes of cold water and lyophilized.

Amino Acid Analysis. Prior to amino acid analysis (Spackman *et al.*, 1958) proteins and CNBr peptides were hydrolyzed *in vacuo* (50 μ or less) in 6 N HCl, with a small crystal of phenol added to minimize destruction of tyrosine (Stark, 1968; Sanger and Thompson, 1963). The inclusion of phenol was probably responsible for the failure to detect cysteine mixed disulfides in acid hydrolysates of modified aldolase (Africa and Carpenter, 1970). The hydrolysis time, at 110 ± 5°, was 72–73 hr when *S*-1,2-dicarboxyethylcysteine was to be determined (Smyth *et al.*, 1964), and 22–24 hr in all other cases. The compositions of intact proteins and of CNBr peptides X1, X2, and X3, were calculated as the average of the normalizations to literature values for aspartic acid, alanine, and leucine (Lai, 1968); for peptide X4, glycine, alanine, and isoleucine were used.

The sulfhydryl groups of previously unmodified aldolase were blocked with *N*-ethylmaleimide, and the CNBr peptides isolated by procedures described. The color constant of *S*-1,2-dicarboxyethylcysteine was assumed to be the same as that of *S*-carboxymethylcysteine. The observed content of *S*-1,2-dicarboxyethylcysteine in the four CNBr peptides was 2.6 residues in X1, 2.0 in X2, 1.7 in X3, and 0.8 in X4. These numbers suggest integral values of 3, 2, 2, and 1, as reported by Lai (1968). Correction factors of 1.15, 0.98, 1.15 and 1.31 were used in subsequent calculations of the *S*-1,2-dicarboxyethylcysteine content of the respective CNBr peptides. For ribonuclease, reduced and treated with NEM, a correction factor of 1.14 was reported for that cysteine derivative after comparable hydrolysis conditions (Smyth *et al.*, 1964). A similar procedure was employed to determine an operational color constant for taurine (2-aminoethylsulfonic acid) from analyses of the performic acid oxidized CNBr peptides obtained from unmodified aldolase, whose sulfhydryl groups were completely blocked by reaction with the amino monosulfoxide reagent, under the conditions used for NEM blockage.

Optical Rotation Measurements. A Cary 60 spectropolarimeter was used for optical rotation measurements. The data were obtained at room temperature in a cell of 0.1-cm path length, on scales of full deflection 0.1° or 0.2°. Protein solutions (1.7–2.3 mg/ml) in Gd·HCl were incubated for 1–4 hr before measurement. Observed rotations at 234 nm were converted into reduced mean residue rotations using a mean residue weight of 109 for aldolase, and calculating the required refractive index at 234 nm ($n^{25}{}_{234}$) from the equation, $n^{25}{}_{234} = 1.3884 + (0.0252) \times$ (molarity of Gd·HCl). This equation assumes a linear relationship between $n^{25}{}_{234}$ and Gd·HCl molarity. Values for $n^{25}{}_{234}$, of 0.1 M 2-ME in 6 M Gd·HCl, and of 0.1 M 2-ME alone (1.3884, assumed to equal $n^{25}{}_{234}$ of the sodium acetate buffer used) were calculated from published information (Tanford *et al.*, 1967b; International Critical Tables, 1930) and from measured values of $n^{25}D$ for 0.1 M 2-ME and water.

Ultracentrifugation. Sedimentation equilibrium and velocity analyses were performed in a Spinco Model E analytical ultracentrifuge at constant temperatures near 20° using schlieren optics for both techniques. Photographs taken on Kodak metallographic plates were measured on a Nikon Model 6C profile projector. For equilibrium analyses, six-channel centerpieces were used (Yphantis, 1964). The fluorochemical oil was extracted with 5 M Gd·HCl–0.2 M sodium acetate–0.01 M EDTA, pH 5.5, and dried with sodium sulfate. Protein solutions were incubated at room temperature for 16 hr before centrifugation was begun. Photographs were taken after 53 hr at 27690 rpm with a column height of 2–3 mm. Without data for the apparent partial specific volume of aldolase at the Gd·HCl concentrations used, values of $\bar{v}$ for the protein under native conditions (Taylor and Lowry, 1956) and ϕ' in 3 M Gd·HCl (Reisler and Eisenberg, 1969) were used to provide limits of uncertainty in the molecular weight, calculated from plots of log $(1/r)(dc/dr)$ *vs.* r^2 (Lamm, 1929; Chervenka, 1969). For sedimentation velocity analyses double-sector boundary cells at 42,040 rpm were regularly used, and occasionally 3° single-sector cells at 59,780 rpm. Prior to the velocity runs, Gd·HCl solutions containing 1.8–3.3 mg of protein per ml were incubated 1–2 hr at room temperature. In correcting the observed sedimentation coefficients to $s_{20,w}$,

TABLE II: Extent of Total Sulfhydryl Modification.[a]

Protein	Sulfhydryl Groups (moles per mole of Subunit)		
	Number Unreacted[b]	Number Converted into Mixed Disulfide	Number Protected by HDP
Unmodified	7.9 ± 0.1		
Monosulfoxide derivatives			
Unprotected			
Carboxyl	3.6 ± 0.1	4.3	
Amino	3.9 ± 0.1	4.0	
Carbamyl	4.0 ± 0.1	3.9	
Protected			
Carboxyl	5.8 ± 0.1	2.1	2.2
Amino	5.3 ± 0.1	2.6	1.4
Carbamyl	5.6 ± 0.1	2.3	1.6
Cystamine derivatives			
Unprotected	7.1 ± 0.1	0.8	
Protected	7.1 ± 0.1	0.8	0.0

[a] The unmodified SH groups were titrated with DTNB in 0.9% sodium dodecyl sulfate, as described in the Experimental Section. [b] Each value is the average of three determinations, except the last entry (2 determinations) and the first entry (17 determinations).

TABLE III: Mixed Disulfide Content of CNBr Peptides. Difference Method Analysis.[a]

Protein and CNBr Peptide	(moles per mole of CNBr Peptide)		
	S-1,2-Dicarboxyethylcysteine	Mixed Disulfide, by Difference	HDP-Protected SH Groups
Monosulfoxide derivatives[b]			
Unprotected			
X1	2.15 ± 0.06	0.85 ± 0.06	
X2	0.20 ± 0.03	1.80 ± 0.03	
X3	1.78 ± 0.07	0.22 ± 0.07	
X4	0.02 ± 0.02	0.98 ± 0.02	
Protected			
X1	2.93 ± 0.09	0.07 ± 0.09	0.78 ± 0.15
X2	0.92 ± 0.06	1.08 ± 0.06	0.72 ± 0.09
X3	1.91 ± 0.08	0.09 ± 0.08	0.13 ± 0.15
X4	0.02 ± 0.02	0.98 ± 0.02	0.00 ± 0.02
Cystamine derivative			
Unprotected			
X1	3.07 ± 0.15	0.00 ± 0.08	
X2	1.92 ± 0.05	0.08 ± 0.05	
X3	2.05 ± 0.05	0.00	
X4	0.23 ± 0.01	0.77 ± 0.01	

[a] Duplicate amino acid analyses were performed for each NEM-blocked peptide of the seven different derivatives. Values in column 3 were obtained as the difference between those in column 2 and the total number of cysteinyl residues in CNBr peptides X1, X2, X3, and X4, respectively, 3, 2, 2, and 1. [b] For these derivatives, the indicated value is the average of that for the carboxyl, amino, and carbamyl proteins.

the partial specific volume of native aldolase (Taylor and Lowry, 1956) was assumed to be unchanged by Gd·HCl.

Results

Activity Monitor of Modification Reaction. Assessed by loss of FDP cleavage activity, reaction with the three disulfide monosulfoxides (Figure 1A,B,C) was complete within 10–20 min. No further change accompanied a second addition of reagent. The inhibitor, HDP, exhibited the expected protective effect. In its presence, only 5–20% of the initial activity was lost. In its absence the loss was 80–90% (for R charged, Figure 1A,B) or 50% (for R neutral, Figure 1C). Within the time span of the experiments, little or no change in FDP activity resulted from treatment with cystamine (Figure 1D). The difference between the inactivation of the unprotected enzyme in the presence of cystamine (Figure 1D) and of cystamine monosulfoxide (Figure 1B) attested to the large increase in reactivity toward thiols, associated with oxidation of a disulfide reagent to a monosulfoxide.

Chemical Characterization of Disulfide Derivatives. The extent of cysteinyl reaction (Table II, column 3) was determined as the difference between the free SH contents of unmodified and of derivative aldolases (column 2). The number of inhibitor-protected residues (column 4) was assumed to be the difference between the free SH contents of corresponding protected and unprotected derivatives. The extents of reaction in the three protected and in the three unprotected monosulfoxide derivatives were similar, regardless of the reagent used. About one-half of the 32 sulfhydryl groups per tetramer (4 out of 8 per subunit) were reactive in the absence of HDP, and approximately one-half of these (2 per subunit) were reactive in its presence. With cystamine, the extent of modification was the same, with or without HDP.

The four CNBr peptides were obtained from each cysteinyl derivative, after blocking the remaining free SH groups with NEM. The mixed disulfide content of each peptide was calculated as the difference between its known total half-cystine content (Lai, 1968), and the content of unreacted sulfhydryl groups, determined as *S*-1,2-dicarboxyethylcysteine, the acid hydrolysis product of the adduct of NEM and cysteine. The results of such a difference method analysis were in acceptable quantitative accord with those from titration of total protein thiol with DTNB.

The three different unprotected and the three protected monosulfoxide derivatives had remarkably similar patterns of cysteinyl substitution. Thus, for each CNBr peptide, listed in Table III, the results for the carboxyl, amino, and carbamyl proteins have been averaged. Electrostatic interactions between the reagent R group and the protein did not seem to be involved in determining the extent or the pattern of

TABLE IV: Michaelis–Menten Kinetic Constants.

Protein	K_m		V_m [ΔA_{240}(min)$^{-1}$ (mg of protein)$^{-1}$]	
	FDP (μM)	F-1-P (mM)	FDP	F-1-P
Unmodified	11 ± 1	4.0 ± 0.7	25 ± 2	0.75 ± 0.07
Monosulfoxide derivatives				
Unprotected				
Carboxyl	1100 ± 100	15 ± 3	2.6 ± 0.1	0.019 ± 0.002
Amino	660 ± 120	16 ± 2	3.5 ± 0.3	0.019 ± 0.002
Carbamyl	370 ± 130	14 ± 1	12 ± 2	0.032 ± 0.001
Protected				
Carboxyl	31 ± 4	5.8 ± 0.5	27 ± 1	1.4 ± 0.1
Amino	24 ± 3	19 ± 3	14 ± 1	0.9 ± 0.1
Carbamyl	23 ± 7	7 ± 1	19 ± 1	1.0 ± 0.1
Cystamine derivative				
Unprotected	12 ± 2	3.8 ± 0.7	37 ± 2	1.2 ± 0.2

modification. The degree of reaction of a particular residue could thus be used as a measure of its accessibility. A single sulfhydryl group was almost exclusively modified in the unprotected cystamine derivative. The less than integral substitution in peptide X4 is probably a consequence of insufficient reaction time, rather than inaccessibility, because this residue was quantitatively modified by every disulfide monosulfoxide reagent. Although aldolase contains two different types of subunits (Chan *et al.*, 1967), each contains the same number of cysteinyl residues (Anderson and Perham, 1970; Koida *et al.*, 1969). The near-integral values of Table III, as averages of the extent of substitution in the two types, suggest that the three dimensional structures of the two different subunits are not markedly different in the vicinity of the accessible cysteinyl residues.

Additional samples of the CNBr peptides obtained from the protected and the unprotected amino monosulfoxide derivatives, and from the unprotected cystamine derivative were oxidized with performic acid before acid hydrolysis, to convert any mixed disulfides (of cysteine and mercaptoethylamine) into free taurine and protein-bound cysteic acid. The identification of taurine in the hydrolysate demonstrated directly the presence of some mixed disulfide in the intact protein. The approximate equality of taurine and cysteic acid (corrected for 2–5% contribution from that formed on oxidation of the NEM adduct of cysteine) demonstrated that mixed disulfides were the major or exclusive product of the modification, and the reagents had not acted as oxidizing agents (*cf.* Pihl and Lange, 1962; Chung and Folk, 1970). The agreement with the extents of modification determined from analysis of *S*-1,2-dicarboxyethylcysteine (Table III) demonstrated the validity of that indirect, difference method of analysis.

The protected and unprotected carboxyl proteins were reacted a second time, under the same protected or unprotected conditions, with the amino monosulfoxide reagent. Subsequent performic acid oxidation and amino acid analysis for taurine showed a maximum additional reaction of 0.1 residue of cysteine per subunit. This negligible difference demonstrated that these two reagents modified not only the same number of residues but also the same specific residues in the amino acid sequence.

Enzymatic Characterization. The Michaelis constant of unmodified aldolase for FDP cleavage approximates the dissociation constant of the enzyme–substrate complex (Morse and Horecker, 1968). An increase in the K_m value of an aldolase derivative thus was tentatively interpretable as a decreased ability to productively bind substrate.

The Michaelis–Menten kinetic constants for FDP and for F-1-P have been summarized in Table IV. The changes in K_m were similar for the two substrates. The values for the cystamine derivative and the unmodified enzyme were identical. The protected monosulfoxide proteins had K_m values, slightly increased, to 2–3 times that of the former two proteins. Values for the unprotected monosulfoxide derivatives were much larger, 4 times K_m (F-1-P). The K_m (F-1-P) value of the protected amino derivative was unusually large, and might indicate that the interaction with F-1-P (but not FDP) is somewhat different from that of the other protected derivatives.

Trends in the changes of V_m were somewhat different for the two substrates. The V_m values for the unprotected monosulfoxide derivatives were substantially reduced toward both FDP (by 50–90%) and F-1-P (by 96–97%). The reductions in V_m for these derivatives were not simply attributable to a mixture of completely inactive and unmodified enzymes, because their K_m values were markedly increased from that of unmodified aldolase. While the protected derivatives showed slight decreases in V_m (FDP), an activation was observed in V_m (F-1-P). The cystamine derivative showed an increased activity toward both substrates. Activation of aldolase in FDP and F-1-P cleavage has been reported (Cremona *et al.*, 1965) following treatment with limited amounts (0.5–1 mole/mole of subunit) of *p*-mercuribenzoate or 1-fluoro-2,4-dinitrobenzene. This activation was attributed to the aromatic nature of the reagents used. With the aliphatic reagents employed here, the increases in V_m (F-1-P) were somewhat smaller than the activation reported for the aro-

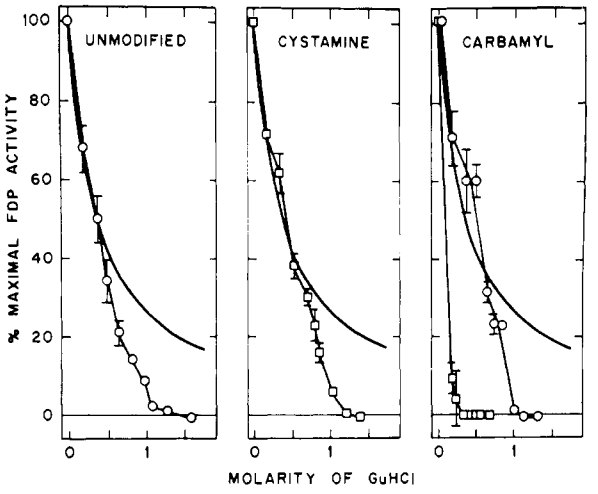

FIGURE 2: FDP cleavage activity in Gd·HCl. Activity at pH 5.5 was measured by the chemical method, determining alkali-labile phosphorus, as described in the Experimental Section. The final protein concentrations were (A) unmodified, 0.18; (B) cystamine unprotected, 0.32; (C) carbamyl monosulfoxide unprotected (□), 1.3 and protected (○), 0.45 mg/ml. The smooth curve in each case depicts activity loss expected from classical competitive inhibition, assuming K_I = 0.35 M for the denaturant.

matic reagents (about 2.5 times the native enzyme activity). However, the increases were present only in those derivatives modified to a limited extent, the singly modified cystamine derivative and the doubly modified protected monosulfoxide derivatives.

The FDP cleavage activities of aldolase and several derivatives, were measured in guanidine hydrochloride (Figure 2) Native aldolase is competitively inhibited by simple inorganic salts (*e.g.*, for NaCl, K_I = 0.23, at pH 7.0, Mehler, 1963). The apparent inactivation, due to the ionic strength of Gd.HCl, was approximated by a classical competitive inhibition curve (smooth lines in Figure 2) with K_I = 0.35 M, chosen empirically to fit the activity losses of unmodified aldolase at low denaturant concentrations. The differences in stability of FDP activity to Gd·HCl were consistent with the changes in FDP kinetic parameters accompanying modification (Table IV). The unmodified, and cystamine protein were both active up to about 1.2 M Gd·HCl, and the protected carbamyl derivative to slightly lower concentrations (about 1.0 M). In contrast, the residual activity of the unprotected carbamyl derivative was markedly less stable, being reduced to zero by 0.4 M Gd·HCl.

Physical Characterizations. The Gd·HCl denaturation of aldolase and its derivatives was monitored by both optical rotation and sedimentation velocity analysis, to assess changes in the secondary structure of individual subunits and their quaternary association in the tetramer. As shown for the unmodified enzyme, both the depth of the ultraviolet optical rotation trough, at 234 nm (Figure 3A), and the sedimentation coefficient (Figure 3B) underwent dramatic decreases within the same fairly narrow range of Gd·HCl concentration near 1.0 M. Although further increases in denaturant concentration produced only slight changes in $s_{20,w}$, there was a significant additional decrease in the depth of the rotational trough. Sedimentation equilibrium analyses were performed

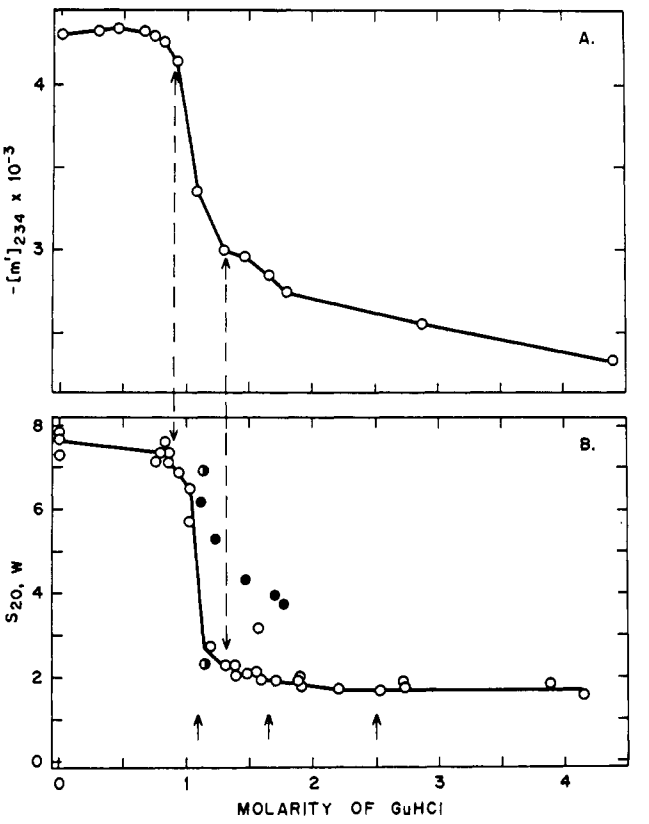

FIGURE 3: Unmodified aldolase: optical rotation, sedimentation velocity, and sedimentation equilibrium analysis of Gd·HCl denaturation. (A) The protein was 1.8 mg/ml in 0.18 M sodium acetate–0.01 M EDTA, pH 5.5. (B) Sedimentation velocity analyses were performed at pH 5.5, in 0.13–0.18 M sodium acetate–0.01 M EDTA, with initial protein concentrations of 2.8–3.0 mg/ml. In one instance near 1.1 M Gd·HCl, two schlieren peaks were seen (◑). In several instances, peaks which broadened rapidly and were sometimes asymmetric (●) suggested that oxidation of sulfhydryl groups to disulfides was occurring. In all other cases (○), a single sharp symmetrical peak was observed. At the indicated Gd·HCl molarities (arrows in B), sedimentation equilibrium analyses were performed, as described in the Experimental Section, in pH 5.5, 0.18 M sodium acetate–0.01 M EDTA–0.02 M dithiothreitol, with an initial protein concentration of 1.8 mg/ml. The following ranges of weight-average molecular weights were calculated: 1.1 M Gd·HCl, 37–44 × 10^3; 1.7 M Gd·HCl, 31–37 × 10^3; 2.5 M Gd·HCl, 29–35 × 10^3.

at the Gd·HCl concentrations indicated by arrows in the lower portion of Figure 3B. The range of weight-average molecular weights (29,000–44,000) demonstrated that S values in guanidine, between 1.7 and 2.5 S, corresponded to aldolase monomer. The values obtained at the higher Gd·HCl concentrations were somewhat lower than the expected monomeric molecular weight of 40,000. This reduction was most probably attributable to the failure to correct the values for nonideality (*cf.* Marler *et al.*, 1964).

Unlike the urea denaturation of aldolase (Stellwagen and Schachman, 1962), no aggregates were formed at intermediate guanidine concentrations. In only one instance were two schlieren peaks seen, indicated by the half-darkened circles in Figure 3B, near 1.1 M Gd·HCl. All enzymatic and physical studies of aldolase and its derivatives were performed at pH 5.5 to reduce the tendency of SH groups to oxidize (Fruton and Clarke, 1934; Stellwagen and Schachman, 1962), and in

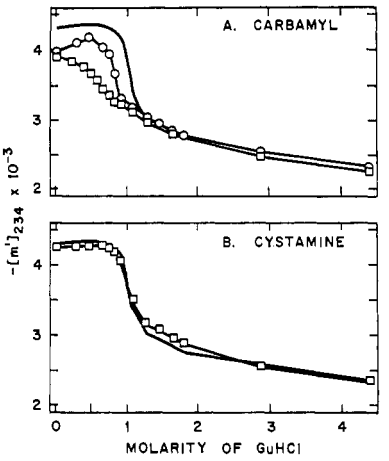

FIGURE 4: Gd·HCl denaturation of aldolase derivatives; optical rotation monitor. All measurements were made in 0.18 M sodium acetate–0.01 M EDTA, pH 5.5, at protein concentrations of 1.8 and 1.9 mg per ml, for the unprotected (□) and protected (○) carbamyl monosulfoxide derivatives, A, and 2.0 mg/ml for the unprotected cystamine derivative (□), B. The unmarked curve is that for unmodified aldolase, from Figure 3A.

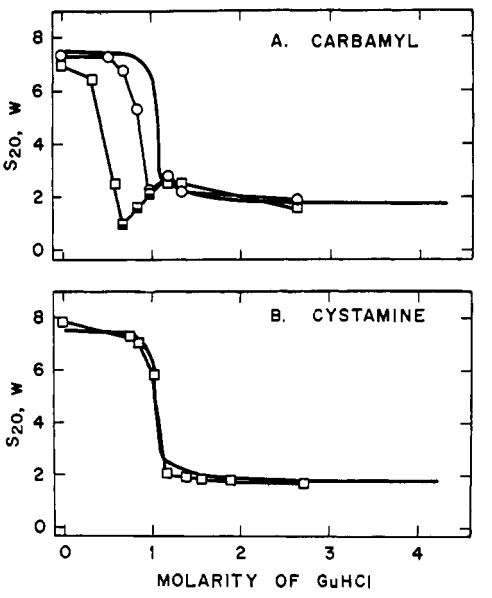

FIGURE 5: Gd·HCl denaturation of aldolase derivatives: sedimentation velocity monitor. Sedimentation velocity analyses were performed at pH 5.5 in 0.17–0.18 M sodium acetate–0.01 M EDTA, at initial protein concentrations of 3.2 and 3.4 mg per ml for the unprotected (□) and protected (○) carbamyl monosulfoxide derivatives in, A, and 3.1 mg per ml for the unprotected cystamine derivative (□), B. At some Gd·HCl concentrations, a rapidly sedimenting component (17–20 S) was observed, in addition to the slow component whose sedimentation coefficient is plotted (▣), A. The unmarked curve is that for unmodified aldolase, from Figure 3B.

the absence of reducing agents, to preserve the chemically introduced mixed disulfides. In several instances at guanidine concentrations between 1.1 and 1.8 M (solid circles in Figure 3B), the schlieren peaks broadened rapidly during sedimentation and were often asymmetric, indicating the presence of more than one component. Since this apparent inhomogeneity was probably due to cysteine oxidation, these somewhat high S values were ignored in drawing the curve shown. None of the mixed disulfide derivatives showed any such broad schlieren peaks in the 2S to 7S range, suggesting that if cysteine oxidation were responsible, the sulfhydryl groups involved were the ones accessible to the disulfide and monosulfoxide reagents, and blocked by their reaction.

Two apparent discrepancies with published reports of the Gd·HCl denaturation of aldolase may also be attributable to restrictions imposed upon the secondary structure by oxidative formation of cystine: (1) the terminal sedimentation coefficient of 1.7 S, compared to 0.73 S in 6 M Gd·HCl–0.1 M 2-ME (Tanford *et al.*, 1967a), and (2) the continuing decrease of $-[m']_{234}$, at high guanidine concentrations, compared to a constant value of the specific rotation (at 330 nm), at concentrations above 3 M (Castellino and Barker, 1968). Although disulfide bonds are asymmetric, no net contribution to the optical rotation was expected from the chemically introduced mixed disulfides. The similar patterns of substitution produced by monosulfoxides of different charge indicated that specific interactions between reagent and protein were minimal. For this reason, reactions of random orientation were expected, producing disulfide configurations of opposing chirality in equal amount.

The optical rotation and sedimentation coefficient monitors of the Gd·HCl denaturation are shown in Figures 4 and 5, respectively, for the two carbamyl monosulfoxide derivatives, and the unprotected cystamine derivative, with the smooth curve for unmodified aldolase drawn for reference in each case. The optical rotation plots for the other two pairs of monosulfoxide derivatives were similar to those of the carbamyl proteins (Figure 4A) in their general features. (1) The curves for both protected and unprotected derivatives were practically identical with that for unmodified aldolase above 1.3 M Gd·HCl. (2) At low denaturant concentrations, where the magnitude of the rotation decreased rather abruptly, quite noticeable differences were apparent in the stability of unmodified, protected, and unprotected proteins.

The optical rotation plots (Figure 4A) demonstrated: (1) that a destabilization of aldolase secondary structure accompanied the modification of the accessible sulfhydryl groups, evident even at 0.0 M Gd·HCl; (2) that the inferred disruption was limited, effecting only those transitions which occurred at low Gd·HCl molarities, and not at higher concentrations; and lastly (3) that for a particular monosulfoxide reagent, there was a decreasing order of secondary structural stability, from unmodified to protected to unprotected protein. The unprotected cystamine derivative was practically identical with unmodified aldolase with regard to both secondary and quaternary structural stability, as shown by the substantial coincidence of their plots for optical rotation (Figure 4B) and the sedimentation coefficient (Figure 5B).

The Gd·HCl dependence of the sedimentation coefficients for the two carbamyl proteins (Figure 5A) was typical of that for all monosulfoxide derivatives, whose S values underwent a very sharp decrease before 1 M Gd·HCl, and ultimately terminated at a value between 1.6 and 2.3 S. The sedimentation coefficients of the two carboxyl and the two carbamyl derivatives indicated that the dissociation proceeded directly from tetramer to monomer, assuming the correspondence between S value and molecular weight found for unmodified

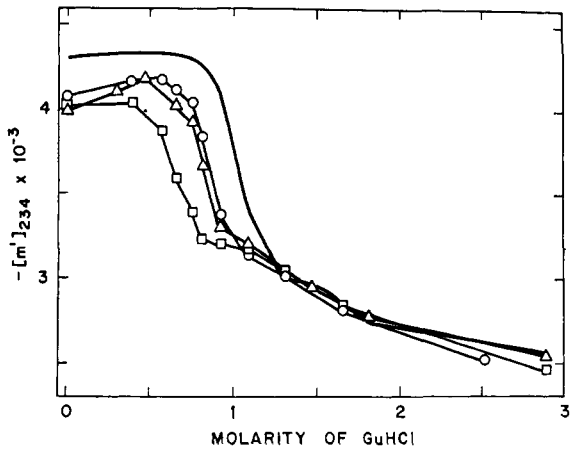

FIGURE 6: Gd·HCl denaturation of protected monosulfoxide derivatives, optical rotation monitor. All measurements were made in 0.18 M sodium acetate–0.01 M EDTA, pH 5.5, at protein concentrations of 1.8, 1.9, and 1.9 mg per ml, for the protected carboxyl (○), protected amino (□), and for the protected carbamyl (△) derivatives. The transition for unmodified aldolase, from Figure 3A, appears as an unmarked curve.

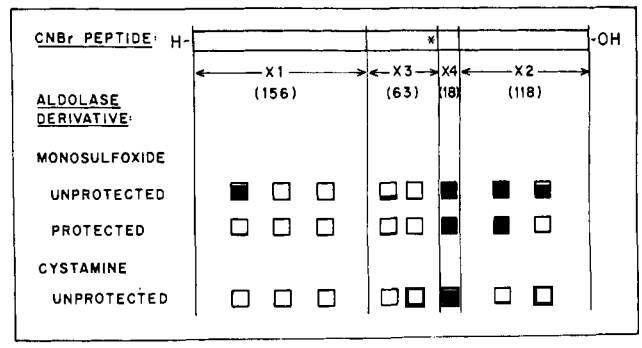

FIGURE 7: Patterns of cysteinyl modification in mixed disulfide derivatives. The length of each labeled segment is proportional to the number of amino acids in the CNBr peptide, indicated in parentheses (Anderson *et al.*, 1969), and the number of squares beneath is equal to the total number of its cysteinyl residues (Lai, 1968). The squares are darkened in proportion to the extent of *total* sulfhydryl conversion into disulfide in each peptide (Table III). Individual cysteinyl residues in a peptide were assumed to react one at a time, rather than fractional reaction occurring at several. Note that the order of the squares does not necessarily correspond to the order of modified and free SH groups in the amino acid sequence of each peptide. The approximate position of the active-site lysyl residue, in peptide ×3 (Lai and Chen, 1968), is indicated by an asterisk.

aldolase (Figure 3B). The sedimentation coefficients of the protected and unprotected amino derivatives were between 3 and 3.5 S at intermediate Gd·HCl concentrations (0.8–1.2 M), and the possibility of their dissociation from tetramer to dimer, and then to monomer, could not be excluded by these sedimentation velocity analyses alone. Only two derivatives, the unprotected carbamyl and carboxyl proteins, exhibited aggregated species (15–20 S) at Gd·HCl concentrations between 0.7 and 1 M, before dissociation was complete. The half-darkened squares in Figure 5A plot the S values of the slow component, which may be anomalously low, due to the hydrodynamic aspects of the aggregating system.

Similar to the optical rotation analysis, the sedimentation coefficient curves were successively shifted toward lower Gd·HCl molarities (Figure 5A), in the progression from unmodified to protected carbamyl to unprotected carbamyl protein. The same relative shifts were evident in the transitions of the other two pairs of monosulfoxide derivatives, and demonstrated that modification of the accessible sulfhydryl groups of aldolase affected the tertiary and quaternary stability of subunit association as well as the secondary structural stability. Within each series of three protected and three unprotected monosulfoxide modified aldolases, the amino derivatives were the least stable, while the carbamyl and carboxyl derivatives were of comparable stability. This dependence of stability to Gd·HCl on the R group of the reagent was evident in both optical rotation and sedimentation coefficient analyses, and is shown for the former by the protected monosulfoxide derivatives in Figure 6.

Discussion

Total Number and Accessibility Classifications of Aldolase Cysteinyl Residues. Discrepancies exist about the total number of sulfhydryl groups in muscle aldolase. Recent determinations of 7 per 40,000 mol wt subunit (Hartman, 1970; Eagles *et al.*, 1969) conflict with the previously accepted value of 8 per subunit (Koida *et al.*, 1969; Penhoet *et al.*, 1969; Lai, 1968; Swenson and Boyer, 1957; Benesch *et al.*, 1955). Anderson and Perham (1970) have conclusively demonstrated seven cysteinyl residues, and suggested that an eighth per subunit may be present. In this study the DTNB titration data (Table II) and the uncorrected yields of *S*-1,2-dicarboxyethylcysteine in the four CNBr peptides (Experimental Section) clearly suggest 8 cysteinyl residues per subunit.

Slightly different experimental procedures may account for the disagreement with one DTNB result showing a total of only seven sulfhydryl groups per subunit (Hartman, 1970). In that instance, the protein was incubated with a denaturant prior to addition of DTNB, a procedure which was found to produce lower SH titers than that used in this study.

The patterns of aldolase cysteinyl modification, by the disulfide monosulfoxides and cystamine, have been pictorially summarized in Figure 7. Based upon their extent of reaction, the eight SH groups per subunit were divisible into three accessibility classes. Four of the five cysteinyl residues in the amino terminal portion are "buried," being largely unreactive. The two SH groups whose reaction was significantly prevented by the binding of HDP were classed as "protected." One protected residue was in the amino-terminal (X1) and another in the carboxyl-terminal CNBr peptide (X2). The two sulfhydryl groups reactive in the presence of the inhibitor composed the third class of "exposed" residues, located in the carboxyl-terminal peptides X4 and X2.

The failure to label four active site lysyl residues with dihydroxyacetone phosphate has been attributed to the presence of 1 mole of covalently bound organic phosphate per mole of aldolase tetramer (Kobashi *et al.*, 1966). A similar explanation might be proposed for the protection of about 0.75 rather than a full cysteinyl residue in CNBr peptides X1 and X2 (Figure 6 and Table III, column 3).

One of the exposed residues, that in CNBr peptide X4,

was highly reactive, as shown by its almost exclusive modification by cystamine. The presence of a single reactive sulfhydryl group per aldolase subunit has been detected in modification studies with other chemical reagents (Cremona *et al.*, 1965; Eagles *et al.*, 1969; Anderson and Perham, 1970). It has not previously been demonstrated that the cysteinyl residue involved was that in peptide X4, although this has been suggested (Anderson and Perham, 1970).

Four accessible sulfhydryl groups per subunit (two exposed plus two protected) were found in this study. This value is in accord with an earlier report using a variety of chemical reagents (Kowal *et al.*, 1965), but conflicts with other chemical modification investigations (Anderson and Perham, 1970; Eagles *et al.*, 1969; Swenson and Boyer, 1957) which find three, and results of a spectrophotometric titration (Donovan, 1964) which reveal two accessible cysteinyl residues. Chemical modification studies do not provide absolute criteria of side chain accessibility. Measures of accessibility may be expected to depend upon the nature of the chemical reagent, and may not necessarily be in accord with those determined by other methods.

Structure and Activity of Unmodified Aldolase in Gd·HCl. The mechanism of Gd·HCl denaturation of unmodified aldolase appears to involve two gross steps (Figure 3). The first occurs near 1.1 M Gd·HCl, within a rather small increase in denaturant concentration of 0.15 M. In this step, the tetramer dissociates directly into monomers, accompanied by loss of a substantial portion of the ordered secondary structure, as shown by coincident changes in optical rotation and the sedimentation coefficient. This parallel disruption of secondary and quaternary structures indicates that the conformation of each subunit is very dependent on its multimeric associations in the tetramer. In contrast to the dissociation of aldolase by acid (Blatti, 1969), there is no evidence of a dimeric intermediate. The second step involves disruption of the residual secondary structure of individual subunits. This disruption is apparent in ultraviolet optical rotation spectra (300 to 225 nm), between 1.3 and 1.8 M Gd·HCl. The observed continuous decrease in depth of the characteristic rotatory trough near 234 nm corresponds to the shoulder of the $-[m']_{234}$ plot (Figure 3A). By 2.9 M Gd·HCl, the trough is abolished, and only a shoulder remains in the 230-nm region of a rotation spectrum of otherwise continuously increasing negative amplitude. Above about 2 M Gd·HCl, rotational changes cannot unambiguously be attributed to structural transitions; solvent effects upon asymmetric chromophores may be involved.

The partially ordered monomer, existing at Gd·HCl concentrations of 1.2–1.8 M, was completely devoid of FDP cleavage activity (Figure 2). However, even after adjusting for ionic strength inhibition, substantial reductions in activity were evident at 0.6–0.7 M Gd·HCl, concentrations at which only slight changes in optical rotation and sedimentation coefficient were apparent (Figure 3). These observations suggest: (1) that aldolase catalytic activity requires a virtually intact tetramer: and (2) that even the slight molecular expansion, preceding the precipitous changes in the physical monitors, is sufficient to cause significant inactivation. This proposed tetrameric requirement for activity is consistent with the demonstrated inactivity of aldolase dimer, which is formed as an intermediate in acid dissociation (Blatti, 1969).

Structural Roles of the Cysteinyl Residues. The mechanism of Gd·HCl denaturation of all cysteinyl derivatives appeared to be similar to the two-step process of unmodified aldolase. The combined sedimentation and optical rotation monitors (Figures 4 and 5) showed that an initial dissociation of tetramer to partially ordered monomers (possibly dimers of the amino monosulfoxide derivatives) was followed by further loss of secondary structure of the dissociated species. For all derivatives, both protected and unprotected, a major portion of the second stage (Gd·HCl concentrations above 1.3 M) was practically identical with that of unmodified aldolase, in the changes of both sedimentation coefficient and optical rotation.

Differences between the various derivatives were apparent only in the first step of Gd·HCl denaturation. Modification of the accessible sulfhydryl groups selectively destabilized aldolase, and altered the cooperativity of this denaturation evident in the shifts and changes of slope of curves of the first transition region.

If any sulfhydryl groups are involved in maintaining the core secondary structure of subunits, they must be the buried SH groups, since modification of aldolase did not affect the second step of the denaturation. The cystamine derivative, with nearly exclusive modification of the exposed SH group in CNBr peptide X4, was virtually identical with unmodified aldolase in its physical properties (Figures 4B and 5B). That single residue thus seemed to be of little structural importance in itself. Since the unmodified enzyme, and the protected and unprotected monosulfoxide derivatives all showed progressive destabilizations in the first step of Gd·HCl denaturation, the second of the exposed cysteinyl residues and the two protected residues per subunit appeared to be involved in maintaining the quaternary structure of the tetramer, and those secondary and tertiary structures associated with the multimeric state. Of the four accessible sulfhydryl groups of aldolase, these three may be located at structural sites, small regions, critical for the integrity of the macromolecule.

All protected monosulfoxide derivatives were modified at the X4 cysteinyl residue, and the second exposed residue, located in CNBr peptide X2; all were destabilized with respect to the cystamine derivative and unmodified aldolase in the first step of the Gd·HCl denaturation. This stability difference was attributable either (1) exclusively to modification of the exposed SH group in peptide X2, or (2) to interactions between the RCH_2CH_2S side chains of the exposed X2 and X4 cysteinyl residues. If such an interaction occurred, it would not seem to be electrostatic in nature because the neutral, carbamyl-protected derivative was also destabilized with respect to unmodified aldolase. The flexibility permitted by the ethylene groups made steric interference of the X4 and X2 cysteinyl substituents seem unlikely. The first alternative thus seemed favored, assigning a predominant structural role to the exposed SH group in peptide X2.

The relative stability of derivatives with different R groups must be a consequence of specific interactions between the side-chain substituents (RCH_2CH_2S) and the immediate protein chemical environment, because the pattern and the extent of substitution were practically identical. In each series of three protected and three unprotected monosulfoxide derivatives, the amino proteins were the least stable (*e.g.*, Figure 6). Since the ammonium ion is the smallest of the three R groups, destabilization may be associated with its positive charge. Short-range electrostatic interactions may play a role

in the structural changes accompanying modification. Such interactions could be operative in the high ionic strength in the guanidine denaturations, as well as under native solution conditions, and have been invoked in interpreting the pH dependence of the Gd·HCl denaturation of lysozyme (Aune and Tanford, 1969). Such proposed interactions did not affect the extent of modification, although they may have influenced the rate of reaction at individual cysteinyl residues. It is apparent from Figure 1 that, under the conditions used, the reaction rate was too rapid to reveal differences between the different disulfide monosulfoxide reagents.

All cysteinyl derivatives, whether protected or unprotected, and irrespective of the charge of R in the mixed disulfide showed the same type of structural destabilization, a shift of the curves in the first step of the denaturation of lower Gd·HCl concentrations. The four accessible sulfhydryl groups may not be as freely available on the enzyme surface as charged side chains commonly are (*e.g.*, Kendrew, 1962), because of their reactivity and the tendency of free thiols to oxidize. Conceivably the cysteinyl side chains are sequestered in somewhat hydrophobic crevices in the surface. In such a case, a similar structural effect might be expected from the different types and degrees of modification, as each served to weaken the hydrophobic bonding of the protein, as the disruptive equivalent of a low molarity of Gd·HCl.

Catalytic Roles of the Cysteinyl Residues. In spite of numerous chemical modification studies (reviewed in Morse and Horecker, 1968), the question of a sulfhydryl group requirement for aldolase activity seems to have evaded a conclusive answer. The present study does not resolve this question of catalytic involvement, but suggests that the answer is not an affirmative or negative, but one requiring qualification of the degree and the type of participation. In the following discussion, cysteinyl residues are described as contact, auxiliary, contributing or noncontributing according to their proposed catalytic involvement (after Koshland, 1960).

The similarity of unmodified aldolase and the cystamine derivative enzymatically (Figure 2, Table IV) and physically (Figures 4B and 5B) demonstrated that the exposed and highly reactive cysteinyl residue in peptide X4 was noncontributing, essential neither for the catalytic process nor the associated protein conformation. This cysteine is quite close in the primary structure to the lysyl residue which forms a schiff base with the substrate (Lai and Chen, 1968); however, the properties of the cystamine derivative suggest that this residue may be some distance from the substrate binding site in the three-dimensional structure. In the protected monosulfoxide derivatives, the modification of the reactive X4 cysteinyl and of the second exposed residue proposed only slight changes in kinetic parameters (Table IV). However, substantial destabilization of the protein structure was evident, at least in the absence of substrate (Figures 4A and 5A). The second exposed cysteinyl residue, in CNBr peptide X2, could thus be classified as one contributing to the catalytic process, but definitely not a contact or auxiliary amino acid.

Assigning a catalytic role to the protected sulfhydryl groups from studies of the unprotected monosulfoxide derivatives, was somewhat less certain. The activity of the unprotected carbamyl derivative was much more sensitive to Gd·HCl than that of either the protected carbamyl or the unmodified protein (Figure 2). This sensitivity might suggest a selective destabilization of the catalytic site accompanying modification of the protected SH groups. However, physical studies of the Gd·HCl denaturation of unprotected monosulfoxide derivatives (Figure 4A and 5A) revealed that modification had pronounced and probably generalized structural effects. It was thus unjustified to attribute the activity losses in guanidine (Figure 2) to a specific disordering of the catalytic or binding site by modification of nearby residues. The protective effect of an inhibitor (Figure 1), in itself, is not sufficient evidence for catalytic involvement (Singer, 1967). Structural disruption in the unprotected derivatives could reasonably be responsible for the observed changes in kinetic parameters (Table IV); modification of catalytically involved, protected sulfhydryl groups need not be invoked.

Neither the reductions in V_m nor the increases in K_m of the three unprotected monosulfoxide derivatives were proportional to their extent of sulfhydryl modification. Such a correlation would be anticipated if an SH group were intimately involved in the catalytic process, as in glyceraldehyde 3-phosphate dehydrogenase, papain, and ficin. All three unprotected monosulfoxide derivatives exhibited identical patterns of cysteinyl substitution (Table III), but quite different kinetic parameters (Table IV). It thus seems clear that the protected residues are not contact amino acids. As free SH, the protected sulfhydryl groups are not required for interactions with the substrate, although such interactions have been predicted from model studies (Mel'nichenko *et al.*, 1969). Probably the two protected residues per subunit possess an auxiliary catalytic function. They may be required for flexibility of the catalytic or binding site or may be involved in the substrate-induced conformational changes of aldolase (Szabolsci and Biszku, 1961; Adelman *et al.*, 1968; reviewed in Morse and Horecker, 1968).

The increasingly frequent X-ray crystallographic solutions of protein three-dimensional structure are proving to be panaceas to the ailments and ambiguities intrinsic to the methodology of chemical modification in solution. Most certainly when the current X-ray analysis (Eagles *et al.*, 1969) provides the crystal structure of aldolase, valuable insights will become possible into the questions incompletely answered by this study. What is the catalytic role of the two protected cysteinyl residues? Are any of the four accessible sulfhydryl groups per polypeptide chain located in intersubunit contact regions or other critical structural sites? What is the physical–chemical basis of the profound changes in secondary, tertiary, and quaternary structure which accompany conversion of the accessible SH groups into mixed disulfides?

Acknowledgments

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Isolation and Characterization of Bovine Lactate Dehydrogenase X*

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ABSTRACT: Lactate dehydrogenase X (LDH X) from bovine testis has been purified to apparent homogeneity. Its kinetic properties, such as pH optima, temperature stability, and degree of substrate and product inhibition, were found to be different from those of bovine heart and muscle LDH. In addition, ATP had a considerably greater inhibitory effect on LDH X than on LDH isoenzymes 1 and 5. Other metabolites, such as pyrophosphate and citrate, selectively inhibited isoenzyme X, but only at concentrations far above physiologic levels. Since the inhibitory effect of ATP on LDH X occurred at relatively low concentrations, it may be significant in sperm cell metabolism.

In spermatozoa of a number of animal species, most or all of the lactate dehydrogenase (LDH),[1] activity is the property of a protein that is electrophoretically distinct from the LDH fractions in somatic tissues. This enzyme, named LDH X by Blanco and Zinkham (1963), is also present in small quantities in mature testis, but absent from the immature organ (Allen, 1961). It appears to be synthesized first in spermatocytes before the meiotic cell division and subsequently in all stages of sperm development (Goldberg and Hawtrey, 1967). Homogenates from testis also contain the common five LDH isoenzymes which are tetramers of the two polypeptides M or A and H or B (Cahn *et al.*, 1962; Markert, 1962). In an electrophoretic pattern from any tissue, homopolymer H_4 (or isoenzyme 1, which predominates in heart) is the most anodal, homopolymer M_4 (or isoenzyme 5, which predominates in muscle) is the least anodal band, and the three heteropolymers with intermediate mobility represent all possible hybrid forms. LDH X from all species so far studied is found relatively close to the point of application. The bovine enzyme, for instance, has electrophoretic mobility intermediate between isoenzymes 4 and 5. The most obvious questions—namely, whether this enzyme functions as a lactate dehydrogenase and whether its structure is similar to that of the known five isoenzymes—have been the subject of several publications. The specificity of crudely separated LDH X has been studied with different substrates and coenzyme analogs (Blanco and Zinkham, 1963; Zinkman *et al.*, 1963). Subsequent studies have dealt with kinetic properties of partially purified preparations from human sperm and rabbit testis (Stambaugh and Buckley, 1967; Battellino *et al.*, 1968). Very recently, highly purified enzyme was obtained from rat testis (Schatz and Segal, 1969)[2] and studied in respect to several of its properties. The results indicate that NAD^+ and lactate are indeed the preferred substrates of LDH X, although its affinity spectrum is generally broader than that of LDH 1 and LDH 5. LDH X has a molecular weight similar to that of the other isoenzymes (Zinkham *et al.*, 1968; Schatz and Segal, 1969). It also appears to contain four characteristic subunits, since *in vitro* experiments of crude extracts have revealed a tendency for hybridization with subunits M and H (Zinkham *et al.*, 1963; Goldberg, 1965).

In this paper the purification of LDH X from bull testis to apparent homogeneity is described and a number of kinetic properties of the enzyme are compared to the corresponding properties of purified bovine LDH 1 and LDH 5.

Experimental Section

Materials. NAD^+, Na_2NADH, Na_2ATP, NaADP, AMP (muscle adenylic acid), cyclic AMP (free acid), $NaNADP^+$, and Na_4NADPH were purchased from P-L Biochemicals, Inc., Milwaukee, Wis. D,L-Lactic acid (85–90%) was from Merck and Co., Rahway, N. J. Sodium pyruvate and ammonium sul-

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[1] Abbreviation used is: LDH, lactate dehydrogenase.

[2] This paper appeared while our manuscript was in preparation.