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Enthalpy of Reduction of Disulfide Cross-Links in Denatured Lysozyme†

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ABSTRACT: Calorimetric measurements were made at pH 8, 30°, and 6–8 M guanidinium chloride for the reduction of disulfides with dithiothreitol or dithioerythritol (disulfide exchange). The enthalpy changes were −1.5 kcal/mol of disulfide for lysozyme and −1.1 kcal/mol for an exhaustive peptic hydrolysate of lysozyme. These results support the general understanding that the thermodynamics of cross-link formation in a random-coil polymer are dominated by the entropy, and that the cross-linked chain in strong denaturing solvents is essentially a random-coil structure. The enthalpy of disulfide exchange for oxidized glutathione is −0.4 kcal/mol in guanidinium chloride solution and −0.5 kcal/mol in water. Cyclic and noncyclic disulfides have nearly equal enthalpies.

Intramolecular covalent cross-links are generally understood to favor folded over unfolded conformations of protein molecules (Kauzmann, 1959; Scheraga, 1963). This contribution is believed to be an entropy effect which arises from the few configurations accessible to the cross-linked compared with the linear polymer. Because introduction of a cross-link into a random-coil molecule in a good solvent may require that one or more bonds be brought into a conformation with energy higher than obtains for the unconstrained molecule, it is possible that the enthalpy of cross-link formation is not zero. In order to evaluate the enthalpy contribution, the heat of reduction (*i.e.*, of disulfide interchange) of disulfide cross-links in a random coil polypeptide chain was determined in calorimetric experiments that compared in concentrated guanidinium chloride solution the heat of reaction of lysozyme, a peptic hydrolysate of lysozyme, and glutathione. In this solvent, lysozyme has been shown by optical rotatory dispersion (Tanford *et al.*, 1967), viscosity (Tanford *et al.*, 1967), and nuclear magnetic resonance (nmr) experiments (McDonald *et al.*, 1971) to behave as a random coil when the four disulfide cross-links are present or absent.

Materials and Methods

Salt-free hen egg-white lysozyme (six-times crystalline, lot 7102) was obtained from Miles Laboratories; dithiothreitol, dithioerythritol, and oxidized disodium glutathione from Sigma; ultra pure guanidinium chloride (GdmCl)[1] and urea, from Mann Research Laboratories. Other chemicals were analytical grade. Deionized water was used.

Protein solutions were prepared by dissolving weighed amounts of lysozyme in the appropriate solvent. The water content of the protein was taken into account, and it was determined by drying under vacuum at 107° to constant weight and by absorbance of aqueous solutions of weighed

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[1] Abbreviation used is: GdmCl, guanidinium chloride.

samples ($E_{280}^{1\%}$ 26.3; Imoto *et al.*, 1972) with results that agreed within experimental error (0.3%). Solution pH was adjusted to the desired value using concentrated sodium hydroxide.

Peptic digestion was carried out at pH 2 and 50° for 16 hr, using 1:100 pepsin. Ninhydrin analyses showed the half-time to be *ca.* 1 hr. Other experiments (T. Shimoda, private communication) have shown the products of peptic digestion to be small peptides. For calorimetric experiments the pH was adjusted to 8 after digestion and solid GdmCl was added to obtain a 6 M solution.

Solutions of oxidized disodium glutathione were prepared similarly to the protein solutions, except the pH was above 9 and concentrated HCl was added to obtain the desired pH.

Dithiothreitol or dithioerythritol was dissolved in the appropriate solvent and the pH was adjusted with 1 M sodium hydroxide. Dithiothreitol and dithioerythritol were used in reduction of the disulfide bridges in preference to mercaptoethanol because the former compounds are nonvolatile and are more effective considering both rate and equilibrium in the disulfide exchange reaction. In most experiments dithiothreitol was the reductant. Several experiments with dithioerythritol (see Table I) showed no significant difference in the measured heats of reaction.

Calorimetric measurements were carried out at 30° using an LKB batch microcalorimeter Model 10700-2 (Wadsö, 1968). This instrument is a twin calorimeter based on the heat conduction principle. The equipment gave literature values for the heat of neutralization of Tris and for the heat of dilution of sucrose. The calorimeter cells were filled using Hamilton syringes that had been calibrated and frequently checked by weighings before and after delivery of solutions. In a typical experiment, one compartment of the reaction cell was filled with 2.00 ml of lysozyme solution and the other with 4.00 ml of dithiothreitol or dithioerythritol solution. Protein concentrations were 1–3% and the reductant was in 2–10-fold excess. The two compartments of the reference cells were filled with 2 ml of solvent and 4 ml of dithiothreitol or dithioerythritol solution. Immediately before or after each protein run a control experiment was carried out in which identical volumes of solvent and reductant solution were mixed in both reaction and reference cells. The heat of autooxidation of the thiol reductant is large. The blank runs were necessary because of differences between the cells in thermophile response and reaction of oxygen with the reductant. Heats of reaction in these controls were several millicalories at pH 8. Heats of dilution of the protein were negligible (Lapanje and Wadsö, 1971). The pH of the reactants was measured and adjusted immediately before a run and was measured immediately after. There was consistently a small drop in pH of 0.01–0.05 for protein reactions and of *ca.* 0.1 for glutathione reactions. The same pH changes were observed in blank experiments (in which protein and glutathione were absent). Thus heat changes due to pH shifts should cancel when comparing blank and protein reactions. The pH of guanidine solutions was measured after 5-min equilibration, when the rate of pH drift was slow although not zero as in dilute salt.

TABLE I: Enthalpy of Reduction of Disulfide Bonds of Lysozyme by Dithiothreitol at 30°.

pH	Solvent	Lysozyme (mg/ml)[b]	Reductant (M)[b]	Measured Heat (mcal)	ΔH (kcal/mol of Disulfide Bond)
8.0	6 M GdmCl	4.2	0.03	3.2	−1.34[a]
		9.6	0.03	7.8	1.45
		11.3	0.03	9.5	1.52
		14.8	0.04	12.8	1.54[a]
		17.1	0.04	14.6	1.52
		22.7	0.04	20.4	1.61
		25.1	0.05	22.1	1.58[a]
		38.9	0.05	34.2	1.49
				Average	−1.51 ± 0.06[c]
8.0	8 M GdmCl	9.0	0.03	7.6	−1.51
		9.8	0.04	9.1	1.68
		12.5	0.03	9.8	1.40[c]
		16.6	0.04	11.2	1.20
				Average	−1.45 ± 0.15[c]
6.0	6 M GdmCl	17.8	0.04	12.4	−1.27
		21.5	0.04	14.4	1.19[a]
		30.9	0.04	18.2	1.05
		34.4	0.05	23.2	1.21
				Average	−1.18 ± 0.07[c]
8.0	9 M Urea	27	0.05		+4.5 ± 0.5[d]
8.0	Water	14.6	0.04		+18.0 ± 1.5[d]
	16-hr Peptic Digest of Lysozyme				
8.0	6 M GdmCl	20.3	0.03	12.2	−1.01
		20.4	0.04	12.6	1.04[a]
		21.5	0.03	13.3	1.17
		21.5	0.04	12.8	1.12
				Average	−1.09 ± 0.07[c]

[a] Reducing agent was dithioerythritol. [b] Initial concentrations are given; 2 ml of protein solution was reacted with 4 ml of thiol. [c] Average error. [d] Estimated error.

Results and Discussion

Table I gives enthalpies of reaction of lysozyme and its peptic digest with dithiothreitol or dithioerythritol in several solvents. In 6 M GdmCl solution of pH 8, in which solvent lysozyme is fully unfolded, the enthalpy of the disulfide exchange process is −1.5 kcal/mol of disulfide bond for lysozyme and −1.1 kcal/mol for the peptic digest. The heat of reduction is not significantly affected by change in GdmCl concentration to 8 M or in pH to 6. At pH 6, the reduction is much slower than at pH 8 (Cleland, 1964), and accordingly the voltage–time curve in the calorimetric experiment was broad and reaction, although near completion, was not complete in the time of measurement (*ca.* 1 hr). This accounts for the 0.3-kcal lower value obtained at pH 6 than at pH 8.

The linear random coil is favored over the cross-linked random coil by 2–5 kcal of free energy, estimated from the statistics of polymer chains (Kauzmann, 1959; Scheraga, 1963). The magnitude of the estimate depends on the loop size in the cross-linked molecule and reflects the fewer number of configurations accessible to a cross-linked chain. Considering only configurational restrictions, there should be no enthalpy difference between cross-linked and linear molecules. It is possible that loop formation could change the enthalpy through introducing strain into one or more bonds, and for disulfide cross-links this would be reflected in a more negative heat of reduction. Strain associated with loop formation should be absent in a peptic digest of a cross-linked protein because intrachain cross-links are absent. Thus, the small difference (less than 0.4 kcal/mol of disulfide) observed

TABLE II: Enthalpy of Reduction of the Disulfide Bond of Oxidized Glutathione by Dithiothreitol at 30°.

pH	Solvent	Concentration: Glutathione (mg/ml)	Concentration: Dithiothreitol (M)	No. of Expts	ΔH^a (kcal/mol)
8.0	6 M GdmCl	9–13	0.06	3	−0.43 ± 0.10
6.0	6 M GdmCl	9–19	0.06	3	−0.34 ± 0.10
8.0	8 M GdmCl	7–10	0.06	3	−0.44 ± 0.12
8.0	9 M Urea	22	0.06	2	−0.24 ± 0.05
8.0	Water	15	0.05	3	−0.51 ± 0.05

[a] Average errors are given.

comparing lysozyme with its peptic hydrolysate suggests that the presence of a loop is not associated with appreciable strain and that the free-energy change for loop formation is determined by configurational restrictions only. This observation extends previous descriptions of the cross-linked molecule in strongly denaturing solvents as a fully disordered structure. Optical rotation measurements (Tanford *et al.*, 1967) have shown the absence of ordered backbone structure, and nmr measurements (McDonald *et al.*, 1971) the absence of perturbation of side-chain environment. The absence of a significant disulfide enthalpy difference between lysozyme and its peptic digest in guanidinium chloride shows that the average values of bond parameters (conformational angles, etc.) for main chain and side-chain elements are the same in the cross-linked and linear cases.

At pH 8 in water the enthalpy change for reaction of lysozyme with dithiothreitol is positive (Table I), as expected from the large positive contribution associated with unfolding of structured regions. At pH 6 in water, the reaction of dithiothreitol with lysozyme was too slow to observe. In 9 M urea cross-linked lysozyme is not fully unfolded but reduced lysozyme is (Warren and Gordon, 1970), which explains the small positive heat of reduction observed in this solvent (Table I).

Table II gives enthalpies of reaction of oxidized glutathione with dithiothreitol under conditions comparable to those of Table I. The enthalpy of reduction of glutathione is *ca.* 0.5 kcal more positive than for the peptic digest. This might reflect either different chemistry for the oxidized glutathione disulfide compared with the protein disulfide or differences in strain or solvation. In this regard a different change in optical rotation is found for reduction of the glutathione disulfide compared with protein disulfides.[2]

The near zero value for the disulfide interchange reaction between glutathione and dithiothreitol is noteworthy. Cleland (1964) has estimated that the cyclic disulfide is favored by *ca.* 5 kcal in free energy over the intermolecular disulfide. The near equality of the enthalpies for the cyclic and noncyclic disulfides shows that the relative stability of the disulfide in the cyclic molecule is an essentially pure entropy effect.

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[2] Measurements of optical rotatory dispersion of glutathione in 6 M GdmCl at pH 8 and 25° gave values for the difference ($[m']_{CysH} - [m']_{Cys}$) slightly larger than literature values for nondenaturing solvents: at 300 nm, 1880 compared with the value 1300 of Coleman and Blout (quoted by Tanford *et al.*, 1967); at 589 nm, 270 compared with the value 225 of Wurz and Haurowitz (1961). The rotation change for glutathione is about twice the size of the changes for disulfide reduction found for protein disulfides (Tanford *et al.*, 1967; Tanford, 1968).