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Thermodynamics of Protein Cross-Links†

Robert E. Johnson, Patricia Adams, and John A. Rupley*

ABSTRACT: The thermal transitions of native lysozyme and a well-characterized cross-linked derivative of lysozyme [Imoto, T., and Rupley, J. A. (1973), *J. Mol. Biol. 80,* 657] have been studied in 1.94 M guanidine hydrochloride at pH 2. The observed increase in the melting temperature from 32.4 °C for native lysozyme to 61.8 °C for the cross-linked derivative corresponds to a calculated 5.2 kcal/mol increase in the free energy of denaturation. This free-energy change is attributed to the decreased entropy of the unfolded polypeptide chain following introduction of a cross-link and is shown to compare well with theoretical predictions. The possibility that an introduction of a cross-link could also affect the enthalpy of an unfolded protein was investigated. The heats of reduction of bovine serum albumin and lysozyme by dithioerythritol in 6 M guanidine hydrochloride were determined and compared to that for the model peptide, oxidized glutathione. The near identity of the observed heats was taken as evidence that the introduction of cross-links into a random-coil protein does not, in general, introduce strain.

Many proteins contain cross-links, the function of which presumably is to stabilize the native conformation. Particular attention has been paid to intrachain disulfide cross-links, commonly found in extracellular proteins. Discussions of this topic usually emphasize the loss of entropy when a random coil is cross-linked, thus destabilizing the unfolded state. This entropy loss has not been measured experimentally, although estimates of it can be made using the models of Flory (1956), Schellman (1955), or Poland and Scheraga (1965). Also, there are few data available on the thermodynamics of disulfide bond formation for either folded or unfolded forms of proteins (Lapanje and Rupley, 1973; Creighton, 1975), though there are somewhat more data for model compounds (Sunner, 1955; Cleland, 1964; Gorin and Doughty, 1968). There have been many reports on the kinetics of disulfide reactions of proteins and small compounds (e.g., Sears et al., 1977; Creighton, 1975; Weber and Hartter, 1974).

The goals of this paper are twofold: (1) to describe a system in which the "destabilizing" effect of a cross-link in a random coil can be measured and to compare the experimental free-energy change to theoretical predictions and (2) to measure the enthalpy of disulfide bond reduction for unfolded BSA,[1] amplifying previous data obtained for lysozyme (Lapanje and Rupley, 1973).

It is helpful to use the reactions of the cycle of Figure 1 in discussing the thermodynamics of the introduction of cross-links into a single-chain macromolecule. Reactions 2 and 3 are the unfolding of the cross-linked and uncross-linked molecules, respectively. Reaction 4 is the introduction of a single cross-link into the unfolded molecule, which is assumed to be random coil whether cross-linked or not. We ignore the chemistry of the establishing of the covalent cross-link, i.e., the free energy of reaction 4 reflects only the configurational effects associated with cross-linking. These configurational effects comprise an entropy contribution to the free energy of reaction 4 and perhaps an enthalpy contribution. Reaction 1 is the introduction of the cross-link into the folded molecule. We ignore as for reaction 4 the covalent chemistry of establishing the cross-link; i.e., we assume that it is the same for reactions 1 and 4.

The thermodynamic parameters of reaction 4 are of interest for comparing with theoretical predictions and for assessing the contribution of cross-links to conformational stability. There is no straightforward way of studying reaction 4, but the thermodynamics of reactions 2 and 3 can be determined through experiment, allowing calculation of the difference in thermodynamic parameters for reactions 1 and 4. Then, if the thermodynamics of reaction 1 can be determined, those of reaction 4 can be evaluated.

It is possible to carry out the above measurements using lysozyme and a lysozyme derivative. Inactivation of lysozyme by iodine results in an ester bond being formed between the side-chain carboxylate of Glu-35 and the enol form of oxindolealanine-108 (Fraenkel-Conrat, 1950; Hartdegen and Rupley, 1973; Imoto and Rupley, 1973). This particular cross-link is appropriate to study because the effects of its introduction on the conformation of lysozyme (i.e., reaction 1) have been investigated by x-ray crystallographic (Beddell et al., 1975), ^{13}C NMR (Norton and Allerhand, 1976), and equilibrium binding studies (Imoto and Rupley, 1973).

The thermodynamic parameters for the unfolding of cross-linked and native lysozyme (reactions 2 and 3) must be determined for the same experimental conditions. This, in principle, can be done by standard techniques, but there are experimental difficulties because the melting temperatures differ by nearly 30 °C. Thus, under experimental conditions for which the cross-linked derivative is half unfolded, an im-

† From the Department of Chemistry, University of Arizona, Tucson, Arizona 85721. *Received August 16, 1977.* This work was supported by grants from the National Institutes of Health, the National Science Foundation, and the American Cancer Society.

[1] Abbreviations used are: Gdn-HCl, guanidine hydrochloride; BSA, bovine serum albumin; DTE, dithioerythritol.

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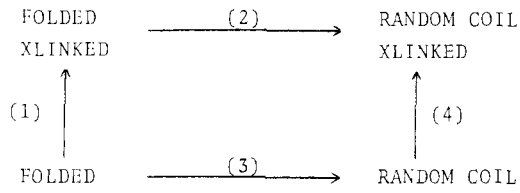

FIGURE 1.

measurably small fraction of the native protein remains folded. Tanford and Aune (1970) have developed, however, a set of equations that describe the unfolding equilibrium of lysozyme over wide ranges of temperature, pH, and Gdn-HCl concentration and that can be used to evaluate the equilibrium for lysozyme far from its melting temperature.

Our second objective, measurement of the enthalpy of reduction of unfolded BSA, is an extension of a previous report (Lapanje and Rupley, 1973), which compared the heat of reduction in 6 M Gdn-HCl of lysozyme and its limit peptic digest with the tripeptide glutatione. This type of information is essential in order to interpret the free energy of reaction 4. Is it entirely entropic in origin or is the cross-link strained even in the random coil? If the latter were true, then this strain would be reflected in an abnormally large heat of reduction.

Experimental Procedure

Materials. Salt-free hen egg-white lysozyme was obtained from Worthington Biochemical Corp. The cross-linked ester derivative of lysozyme was prepared by the method of Imoto and Rupley (1973). UltraPure Gdn-HCl, crystalline BSA, and iodoacetic acid were obtained from Schwarz/Mann. DTE and oxidized glutathione were obtained from Sigma. All other chemicals were of reagent grade, and all solutions were prepared with deionized water.

Denaturation of Native and Cross-Linked Lysozyme. The denaturation reactions of native and modified lysozyme were followed spectrophotometrically as a function of temperature in 1.94 Gdn-HCl, pH 2.00. Measurements were made on a Cary 15 instrument, using the 0.1 absorbance slide-wire and water-jacketed cuvette holders connected to separate temperature controllers. Temperature in the cell was measured with a polyethylene-encased 200 Ω Veco thermistor probe, which had been calibrated against a Hewlett-Packard quartz thermometer. Stock solutions of lysozyme and of ester derivative, 15 mg/mL in water (w/w), were made up fresh for each experiment. For both native and modified lysozyme experiments, the reference cuvette was thermostatted at 30 °C and contained 100 μL of the stock solution of native lysozyme mixed with 3 mL of 0.1 M sodium acetate solution, pH 5.0.

Native lysozyme data were obtained by mixing in the sample cuvette 100 μL of protein stock solution with 3 mL of 2 M Gdn-HCl solution, pH 2.00. After temperature equilibration, the absorbance was recorded at 300 nm, the wavelength of an extremum of the denaturation difference spectrum, relative to a 350-nm baseline value. A single sample solution was used from 5 to about 30 °C, without any sign of aggregation. Above that point a new sample solution was used for each temperature, again with no sign of aggregation, based on the absorbance at 350 nm.

The denaturation of cross-linked lysozyme was followed similarly to the native: a single solution was used to about 50 °C and fresh solutions at higher temperatures. The absorbance changes were measured at 292 nm instead of at 300 nm; the folded vs. unfolded difference spectra for the modified and native proteins are different.

The ester cross-link is labile in the unfolded state (Imoto and Rupley, 1973; Norton and Allerhand, 1976), and the absorbance at 292 nm changes rapidly and irreversibly as the oxindolealanine derivative of lysozyme is formed through hydrolysis. Therefore, in order to separate the unfolding reaction from the hydrolysis reaction, the data above 50 °C were extrapolated to the time of mixing. As shown in Figure 2, the progress curve of the hydrolysis reaction is first order, making the extrapolation straightforward.

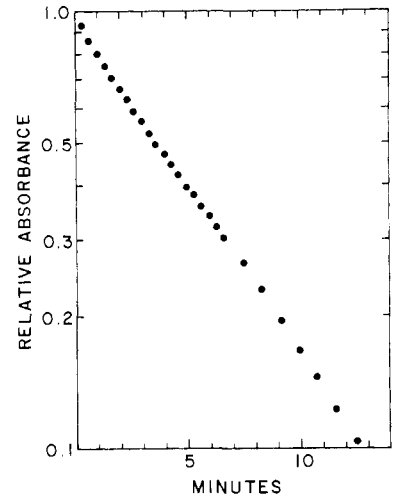

FIGURE 2: Time dependence of the absorbance at 370 nm, reflecting ester cross-link hydrolysis and formation of oxindolealanine-108 at 60 °C, pH 2.0, in 1.94 M Gdn-HCl.

Reduction of Unfolded Proteins. Heats of reduction were determined by measuring the heat of mixing of a solution of protein in 6 M Gdn-HCl with a solution of DTE in 6 M Gdn-HCl. Enough dry sample to make the final solution 0.02 M in disulfide bond (80 mg/mL for lysozyme and BSA; 12.2 mg/mL for glutathione) was dissolved in a small volume of water. This solution was added to appropriate amounts of solid Gdn-HCl and concentrated iodoacetic acid solution; after the Gdn-HCl had dissolved, the solution was adjusted to pH 9.5 with concentrated NaOH solution and was diluted slightly to give the correct final volume. A solution containing 0.02 M DTE was made by dissolving the correct weight of DTE in a stock 6 M Gdn-HCl solution and adjusting to pH 9.5. Iodoacetate (final concentration 2 to 6 mM) was added to the lysozyme and BSA solutions to prevent gel formation. The heat of reaction of iodoacetate with DTE was measured in control experiments in the absence of protein and was subtracted from the heat of the protein reaction. The corrected heats of reduction of disulfide samples did not vary systematically with iodoacetic acid concentration.

Calorimetric measurements were made with an LKB flow microcalorimeter (Monk and Wadso, 1968), thermostatted at 25 °C, and calibrated using either the internal heater or the heat of dilution of sucrose (Gucker et al., 1939). Small corrections (≤37 μcal/s) were made for the heat of dilution of the DTE solution and of the protein solutions into 6 M Gdn-HCl. Solutions were pumped into the calorimeter with a Technicon manifold peristaltic pump, the flow rate of which was determined on the day of each experiment by weighing the amount of water pumped out of a vessel in 1 h. The combined flow rate for both solutions was about 20 mL/h.

Correction was made for a decrease in the heat of reduction (10%/h) with the aging of the protein or peptide solutions, using a linear extrapolation to the time of mixing with Gdn-HCl. This phenomenon was not investigated but presumably involves the hydrolysis of disulfide bonds at alkaline pH and

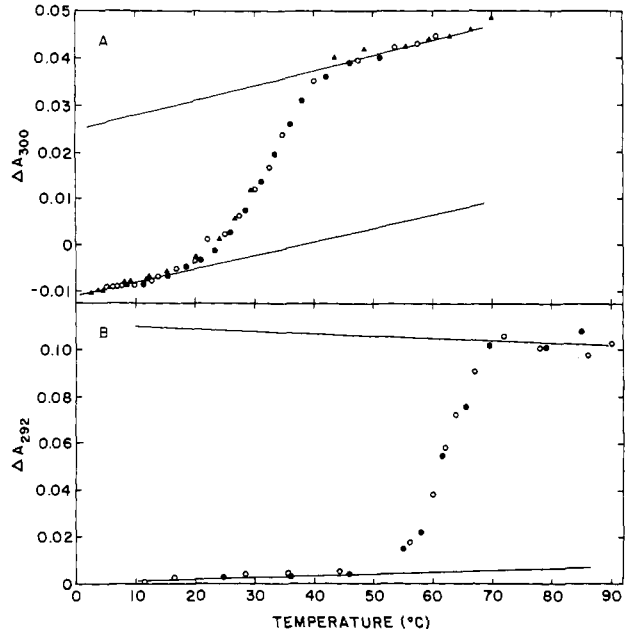

FIGURE 3: Absorbance vs. temperature for (a) native lysozyme in 1.94 M Gdn-HCl, pH 2.0, 0.5 mg/mL; (b) the ester derivative under the same conditions. Different symbols identify the individual experiments.

high salt concentration, described by Roxby and Tanford (1971) and Donovan and White (1971).

Results

Denaturation of Native and Cross-Linked Lysozyme. Figure 3 shows the melting curves of native and cross-linked oxindolealanine lysozyme in 1.94 M Gdn-HCl, pH 2.0. The high and low-temperature baselines, drawn for least-squares fits to the data points judged to be above or below the melting transition, were used to calculate apparent equilibrium constants, assuming a two-state transition between the folded and unfolded forms of the proteins. The data shown are for two sets of experiments for the cross-linked derivative and three sets for the native; there is good agreement between replicates. The scatter seen at high temperature for the cross-linked derivative reflects the error of the extrapolation procedure described under Experimental Procedures.

Table I summarizes the thermodynamic parameters of the unfolding reactions for the respective transition temperatures and for 1.94 M Gdn-HCl, pH 2.0. These parameters were derived from the data of Figure 3 and from van't Hoff analyses of the data shown in Figure 4.

The reversibility of unfolding the cross-linked derivative was demonstrated in order to ensure the validity of the derived thermodynamic parameters. Because of the rapid irreversible change in the spectrum upon unfolding, described under Experimental Procedures, this was tested as follows. An amount of protein was kept for a known period of time at 70 °C, a temperature at which unfolding is essentially complete. The protein was then diluted into solvent at room temperature, and its absorbance spectrum was measured against a reference sample which had been kept at room temperature. This procedure was carried out with varied incubation times at 70 °C. The absorbance determined by extrapolation to zero time was equal to that of the reference protein solution. Thus, despite the irreversible hydrolysis which follows the unfolding reaction, the unfolding process itself is reversible.

The equations of Tanford and Aune (1970) allow one to calculate the thermodynamic parameters for the unfolding of

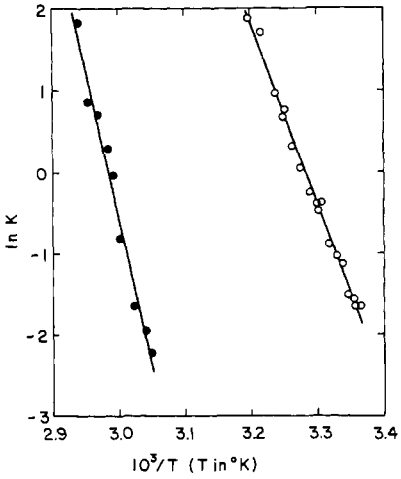

FIGURE 4: Van't Hoff plot of data from Figure 3; native lysozyme (○), ester derivative (●).

TABLE I: Thermodynamics of Unfolding of Native and Cross-Linked Lysozyme in 1.94 M Gdn-HCl, pH 2.

		T (°C)	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (eu)
Native	Exptl	32.4 ± 1.0	0	43.6 ± 1.0[c]	143
	Calcd[a]	32.4	0.3	38.3	125
	Calcd[b]	32.4	0.5	40.0	130
Cross-linked Native	Exptl	61.8 ± 1.0	0	73.3 ± 4.4[c]	219
	Calcd[a]	61.8	−5.2	75.2	240

[a] Calculated using eq 1-2 and 8-11 (Tanford and Aune, 1970), eq 10 (Aune and Tanford, 1969a), eq 2 and 15 (Aune and Tanford, 1969b). [b] Calculated from results of Pfeil and Privalov (1976) for 2 M Gdn-HCl, pH 2. Data from their Table II was extrapolated from 36 to 32.4 °C. [c] These experimental enthalpies and uncertainties are from the least-squares best fits of the data plotted in Figure 4. Due to reasons discussed in the text, the actual uncertainties will be greater than this.

the native lysozyme over ranges of temperature (5 to 70 °C), Gdn-HCl concentration (1.5 to 4 M), and pH (1.5 to 5.5). The parameters included in Table I were calculated for our experimental conditions at the observed melting temperatures for native and modified lysozyme, assuming that the "D" and "X" forms described by Tanford and Aune (1970) have the same UV spectrum.

The agreement between our native lysozyme data at 32.4 °C and the results of the calculation according to Tanford and Aune (1970) is good in terms of free energy but there is a deviation of about 5 kcal between the experimental and calculated enthalpy changes. Also included in Table I is an extrapolation to 32.4 °C of the calorimetric data of Pfeil and Privalov (1976). The free-energy change is close to zero, and the enthalpy change is lower than reported in this paper, although greater than that calculated according to Tanford and Aune (1970).

The principal conclusion from the data of Table I is that there is a nearly 30 °C increase in the melting temperature of lysozyme upon cross-linking. At 61.8 °C, where the free energy of unfolding of the cross-linked derivative is zero, the unfolding of native lysozyme shows a 5.2 kcal/mol change in free energy.

The discrepancy between $\Delta H°$ determined in this work and the previously reported values could reflect either the method used to draw the low- and high-temperature baselines, a flatter

TABLE II: Enthalpy of Reduction of Disulfide Bonds of BSA, Lysozyme, and Glutathione by Dithioerythritol[a] in 6 M Guanidine Hydrochloride at 25 °C, pH 9.5.

Protein	Protein concn (mg/mL) before mixing	Iodoacetate[b] concn (mM) before mixing	Obsd signal (μcal/s)	Signal corrected for iodoacetate reaction	ΔH (kcal/mol of disulfide bond)
BSA	80	2.15	243	122.0	−2.29
		4.30	332	76.9	−1.45
		4.30	340	85.4	−1.59
		8.60	569	77.9	−1.41
					−1.69 ± 0.30[c]
Lysozyme	80	2.15	248	127.0	−2.35
		4.30	339	83.9	−1.55
		8.60	570	79.7	−1.47
					−1.79 ± 0.37[c]
Glutathione	12.2	0	113	113.0	−2.10
		0	106	106.0	−1.91
		4.30	336	80.7	−1.50
		4.30	368	113.0	−2.03
		8.60	573	82.0	−1.53
		8.60	599	109.0	−1.96
					−1.84 ± 0.22[c]

[a] The final concentration after mixing was 0.01 M in both disulfide bond and dithioerythritol. Doubling the dithioerythritol concentration did not change the observed signal. [b] Iodoacetate was present to prevent gelation of the protein solutions in 6 M Gdn-HCl. [c] Average deviation.

low-temperature baseline giving exactly the $\Delta H°$ predicted by Tanford and Aune's equations, or the presence of an "X" form of unfolded lysozyme which was described by Tanford and Aune (1970) and which will be discussed in the next section. Both of these sources of uncertainty would affect $\Delta H°$ strongly and would have little effect on the melting temperature.

Reduction of Unfolded Proteins. Table II gives the heats of reaction of DTE with lysozyme, BSA, and glutathione in 6 M Gdn-HCl and the calculated enthalpies of reduction per mole of disulfide bond. The enthalpies are calculated on the assumption that the reaction has gone to completion before the mixed solutions leave the calorimeter which, under the conditions of the experiment, takes 80 ± 3 s (Johnson and Biltonen, 1976). The validity of this assumption was checked by following the reaction in a spectrophotometer at 315 nm (Iyer and Klee, 1973). Using either lysozyme, BSA, or glutathione, the reaction was 90 ± 5% complete in 80 s.

Discussion

We believe that the free-energy difference of 5.2 kcal/mol, from the comparison of the unfolding reactions of the cross-linked derivative and native lysozyme, can be equated to the free-energy change that follows the introduction of this particular cross-link into the random-coil protein and that there is no appreciable corresponding enthalpy change.

Two major assumptions were made in drawing this conclusion. First, it assumed that for reaction 1 of Figure 1 there is no configurational contribution to the free energy, making ΔG_1 equal to zero. The principal supporting evidence is the crystallographic analysis of Beddell et al. (1975), who showed that establishment of the cross-link resulted in no significant changes in the structure of the molecule except for rotations about the side-chain bonds of the two cross-linked residues. This study revealed no perturbations which would be expected to change the enthalpy of the system. Also, since the two residues involved in the cross-link were already immobilized in the native molecule, cross-link formation does not change the number of configurations accessible to the folded molecule.[2] The crystallographic results are supported by the results of Norton and Allerhand (1976), whose ^{13}C NMR study of iodine-treated lysozyme showed no evidence of a conformational change associated with cross-link formation.

Secondly, it is assumed that the covalent chemistry of the cross-link can be ignored. This, since it would hold for both reactions 1 and 4, is equivalent to assuming the chemistry of the two reactions to be exactly the same and to cancel in the cycle of Figure 1. Since the solution conditions are identical for both reactions (i.e., pH 2, 1.94 M Gdn-HCl, 61.8 °C), the only possible source of change in the covalent chemistry would be from a change in the local environment due to unfolding. Because the reaction involves residues that are on the surface of the native molecule and that have no unusual interactions with the surrounding residues (Beddell et al., 1975), we expect any such perturbation to be within the error of our measurements.

Tanford and co-workers have shown that both native (i.e., disulfide cross-linked) and reduced uncross-linked lysozyme are random coils in 6 M Gdn-HCl at 25 °C (Tanford et al., 1967; Tanford, 1968). Tanford and Aune (1970) interpreted their optical rotation data as indicating, however, the presence of an "X" form of unfolded lysozyme at high temperature and moderate Gdn-HCl concentration, which at higher Gdn-HCl concentration is converted to the completely denatured form "D". They estimated the "X" form to be approximately 70% unfolded based upon the relative changes in ORD (68%) and number of bound Gdn-HCl molecules (65%). Under our experimental conditions, native lysozyme would be 70% in the X form and 30% in the fully unfolded D form. However, Pfeil and Privalov (1976) found no evidence for the X form of lysozyme in isothermal and scanning calorimetric studies of lysozyme denatured in Gdn-HCl solution. Since scanning calorimetry more directly measures the thermodynamic state of a system than optical rotation, we believe that unfolded lyso-

[2] Sturtevant (1977) has suggested that low-frequency vibrational motions that involve large regions of the protein contribute to the heat capacity and entropy. Introduction of a cross-link could change such vibrational motions, but too little is known about the low frequency contributions to allow speculation about effects on the entropy.

zyme under the conditions used in this work can be considered a random coil. The equations of Tanford and Aune (1970) of course remain an accurate description of the denaturation process regardless of the model on which they are based. At 61.8 °C and 1.94 M Gdn-HCl, D and X differ by only 3 kcal in enthalpy and 0.5 kcal in free energy.

We now consider the source of the observed free energy of cross-link stabilization, i.e., whether it is entirely entropic or whether the introduction of a cross-link into a random-coil protein creates strain due to local interactions around the cross-link, the enthalpy of cross-link formation thus being nonzero. There are two lines of evidence suggesting that there is no significant enthalpy change associated with reaction 4. First, the near equality between the ΔH values for reactions 2 and 3 at 61.8 °C, shown in Table I, indicates that if there is an enthalpy contribution it is no more than a few kilocalories. Secondly, Lapanje and Rupley (1973) measured the heats of reduction of lysozyme in 6 M Gdn-HCl. Any strain due to disulfide cross-link formation would be relieved and appear as an abnormally large heat of reduction. They found no difference between the heat of reduction of lysozyme and its limit peptic digest.

It has been pointed out (J. Hermans, personal communication) that some strain might develop when a loop containing no glycine residues is formed by cross-linking a polypeptide chain, even though a glycine-containing loop is strain-free. All disulfide loops of lysozyme have glycine. Therefore, in this work, we have measured the heat of reduction of the disulfides of BSA, a protein which contains several small loops having no glycine residues (Brown, 1975). As shown in Table II, the apparent heats of reduction of BSA, lysozyme, and glutathione are the same.[3] We feel this to be strong evidence that the cross-linking process does not introduce strain into the unfolded proteins but only affects configurational entropy.

Comparison of Experiment with Models. Several models have been used to estimate the entropy loss of reaction 4 in Figure 1, i.e., the introduction of a cross-link into a random-coil protein. The model of Flory (1956) is the most appropriate one for comparison with the present work, because he considered the introduction of a cross-link into a polymer which already contains cross-links. In the case of lysozyme there are four preexisting cross-links, with half-cystine residues 30 and 64 bracketing Glu-35 and residues 94 and 116 bracketing Trp-108. According to this model, the introduction of the Glu-35 to Trp-108 cross-link is equivalent to bringing the ends of four Gaussian chains together inside a small volume element $\Delta\tau$, the chains being the polypeptide segments from the new cross-link to the first half-cystine. The entropy change associated with cross-link formation is related to the probability of the chain ends meeting within $\Delta\tau$, divided by the probabilities that they will pair up correctly to give the original unsevered chains. We can use eq 62 of Flory (1956) to estimate the entropy change associated with cross-link formation, setting $\Delta\tau$ equal to the volume of a sphere of 7.5-Å diameter, the distance between the C_α atoms of residues 35 and 108, and the length of the statistical element of the chain as 3.8 Å, the C_α–C_α distance of a polypeptide chain. With these values, we calculate for reaction 4 $\Delta S = -11.9$ eu and $\Delta G = 4.0$ kcal/mol.

The choice of $\Delta\tau$ as a sphere of 7.5 Å diameter, however, is only an upper bound, a more realistic model perhaps being a shell instead of a sphere. Poland and Scheraga (1965) suggest the use of a shell with a diameter of one half of a statistical element and with a thickness 3% of a statistical element. When these values are used, $\Delta G = 4.6$ kcal/mol.

The agreement between the above theoretical estimates and the 5.2 kcal/mol we have measured is excellent.

The consistency between experiment and prediction is of particular interest, since in general it is not possible to determine by experiment the changes in thermodynamic properties associated with cross-linking. The results of this work support the use of theoretical predictions, following Flory's and related approaches, to estimate the importance of cross-linking in biologically important macromolecules such as collagen, immunoglobulins, and small peptide hormones.

Acknowledgment

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[3] The heat of reduction we have reported in Table II for glutathione at pH 9.5 is different from the value reported for pH 6 and 8 by Lapanje and Rupley (1973), -1.80 ± 0.2 kcal vs. -0.40 ± 0.1 kcal, respectively. Because the pK of thiol ionization is near 9.5, a difference between enthalpies of reduction comparing pH 9.5 with the lower pH is to be expected.

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5-[^{125}I]Iodonaphthyl Azide, a Reagent to Determine the Penetration of Proteins into the Lipid Bilayer of Biological Membranes†

T. Bercovici and C. Gitler*

With the Technical Assistance of A. Bromberg

ABSTRACT: 5-[^{125}I]Iodonaphthyl 1-azide is shown to be a useful reagent for the determination of the extent of penetration of proteins into the lipid bilayer of biological membranes. The label can readily be made highly radioactive and stored for reasonable times or repurified and then used. In the dark it has a high partition coefficient into membrane lipids. It has a high extinction coefficient for the light-mediated conversion of the azide into the reactive nitrene. It can be activated by short periods of light at wavelengths which membrane proteins and lipids do not absorb so that their radiation damage is minimal. The light-generated nitrene inserts covalently with very high efficiencies into the membrane components. With different membrane preparations, 20 to 55% of the added label inserts into the membrane proteins and lipids. It appears to insert from within the lipid bilayer mainly into intrinsic membrane proteins; very little, if any insertion occurs into extrinsic proteins. In rabbit skeletal muscle sarcoplasmic reticulum, the main insertion of the nitrene occurs into the Ca^{2+}-sensitive ATPase. The ATPase activity is not affected by the labeling procedure. Mild tryptic cleavage of the 100 000 molecular weight Ca^{2+}-ATPase results in the formation of two fragments of molecular weight 52 000 and 46 000. The two fragments are equally labeled, suggesting that the protein is in contact with the bilayer by at least two segments of its polypeptide chain. In intact erythrocytes and hemoglobin-free erythrocyte membranes, little, if any, label is inserted into bands 1, 2, or 5 (spectrin and erythrocyte actin, respectively). Label occurs in the region of bands 3, periodic acid-Schiff 1, 2, and 3 and in band 7.

Previous communications have described a new method for labeling those portions of membrane proteins that are in contact with the lipid bilayer (Klip & Gitler, 1974, 1976; Klip et al., 1976). The method was based on the following properties of the labeling agents as studied with rabbit skeletal muscle sarcoplasmic reticulum (SR).[1] Firstly, when the apolar radioactive azides [^{3}H]-1-azidonaphthalene and 4-[^{125}I]-iodobenzene 1-azide were added to the membrane suspension in the dark, they partitioned rapidly and nearly quantitatively into the liquid hydrocarbon regions of the membrane lipids. Furthermore, the azides effectively quenched the fluorescence of perylene present in liposomes and in the SR membranes. Since the quenching was due to molecular encounter, a significant portion of the azides must dissolve in sites equivalent to those of the perylene (Klip & Gitler, 1974). Secondly, exposure to light after the partition step converted the aromatic azides into reactive nitrenes which were capable of covalent incorporation into both the membrane proteins and the membrane lipids.

That the labeling occurred within the bilayer was suggested by the majority of the evidence. Thus, the nitrene products were incorporated to an extent greater than 88% into the fatty-acyl chains of the SR membrane phospholipids. Furthermore, Pronase digestion of the SR resulted in a significant release of peptides from the membrane proteins without appreciable release of radioactivity (Klip & Gitler, 1974; Klip, 1974). Even though the incorporation of the label was low, only the "integral" and not the "peripheral" proteins appeared to be labeled.

The aromatic azides used in these studies had several limitations. Relatively high concentrations of the labels had to be used because of their low specific radioactivities. Furthermore, the iodo derivative had an absorption maximum (258 nm) which overlaps with that of the membrane components. It therefore had to be excited in the edge of its absorption band (>300 nm) where the efficiency of nitrene formation is low. Initial studies with [^{3}H]-1-azidonaphthalene with a specific radioactivity of 3 Ci/mmol showed that radiation damage of the compound was extensive during storage. Evidence also was obtained that on irradiation it formed artefactual polymeric products.

The above limitations have been overcome by the use of the label 5-[^{125}I]iodonaphthyl 1-azide (INA). The radioisotope can be incorporated in the final step of the synthesis and the azide can be stored for reasonable periods of time without degradation. Its absorption maximum is at 310 nm with an extinction coefficient of 21 400 M^{-1} cm^{-1}. No polymeric products have been detected upon irradiation and it is covalently inserted into the membrane components to the extent of 20 to more than 50%. It does not appear to label "peripheral" proteins significantly and may therefore be a useful reagent

† From the Department of Membrane Research, Weizmann Institute of Science, Rehovot, Israel. *Received July 18, 1977.* Supported in part by a grant from the Israel National Academy of Sciences.

[1] Abbreviations used: INA, 5-iodonaphthyl 1-azide; [^{125}I]INA, 5-[^{125}I]iodonaphthyl 1-azide; PAS, periodic acid-Schiff; $NaDodSO_4$ gel electrophoresis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SR, rabbit skeletal muscle sarcoplasmic reticulum.

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