The Properties of Thyroglobulin. XIV. The Structure of Reoxidized Thyroglobulin*

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ABSTRACT: All the disulfide bonds (101) in thyroglobulin (670,000) have been reduced by mercaptoethanol in either concentrated urea or guanidine at pH 10.3. The products are linear polypeptide chains of molecular weight about 165,000 which are essentially devoid of stable cross-links.

Air reoxidation of the sulfhydryl groups largely results in two molecular species which correspond in size and shape to the 12 S subunit (335,000) and the native (670,000) form of thyroglobulin. Moreover, the products of reoxidation closely resemble the native protein in its behavior toward temperature, pH, urea, detergent, and in its immunologic activity. Minor variations in properties were observed which depended on the solvent present during reoxidation. The extent of recovery of the molecular properties is rather striking if account is taken of the complexity of the structure of the native protein.

The *in vivo* mechanism by which polypeptide chains acquire their characteristic secondary, tertiary, and quaternary structure is still imperfectly understood. Proteins which have been unfolded into polypeptide chains offer model systems to explore this process. Several proteins, composed of a single chain and a few disulfide bonds, have been shown to recover their physical and biochemical properties after reduction and reformation of their disulfide bonds (Anfinsen and Haber, 1961; Epstein and Anfinsen, 1962; Goldberger and Epstein, 1963; Steiner, 1964; Steiner *et al.*, 1965). These studies suggest that the "information" necessary for the correct pairing of the sulfhydryl groups preexists in the molecule, presumably determined by the sequence of amino acids, *i.e.*, the primary structure (Epstein *et al.*, 1963).

Native thyroglobulin of mol wt 670,000 contains 101 disulfide bonds. Complete reduction of these bonds results in the formation of four polypeptide chains of mol wt about 165,000. A few disulfide bonds serve to link the chains, the majority being intrachain (de Crombrugghe *et al.*, 1966).

Recent studies of the biosynthesis of thyroglobulin by tissue slices have demonstrated sedimenting species which correspond to single (3–8 S), double (12 S), and tetrachain (19 S) molecules. Kinetic studies indicated that the smaller units are the precursors of 19S thyroglobulin (Lissitzky *et al.*, 1964; Seed and Goldberg, 1965; Sellin and Goldberg, 1965).

The conditions necessary for the formation of linear chains, essentially devoid of structure, from native thyroglobulin have been determined (R. F. Steiner and H. Edelhoch, unpublished results). The purpose of this report is to evaluate the nature of the products formed by reoxidation of the reduced sulfhydryl groups of the structureless chains and to compare their properties with those of the native form of the protein.

Methods

All the methods used in this report have been described in preceding communications in this series (Edelhoch and Lippoldt, 1964; de Crombrugghe *et al.*, 1966). The sedimentation coefficients were obtained at room temperature and corrected to water at 20°. Viscosities were determined at 25.0°. Optical rotatory values were measured at room temperature and expressed as specific rotation $[\alpha]$. The constant b_0 was obtained from the Moffitt equation (Moffitt, 1956; Moffitt and Yang, 1956).

$$[\alpha]_\lambda \frac{3}{n^2+2}\frac{\mathrm{MRW}}{100} = \frac{a_0\lambda_0^2}{\lambda^2-\lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2-\lambda_0^2)^2}$$

λ_0 was set at 212 mμ; MRW[1] = 115. The helical content was determined also by the Shechter–Blout equation (Shechter and Blout, 1964). The relaxation time (ρ_h) was evaluated by application of the Perrin equation (Weber, 1952).

$$\left(\frac{1}{P}+\frac{1}{3}\right) = \left(\frac{1}{P_0}+\frac{1}{3}\right)\left(1+\frac{3\tau}{\rho_h}\right)$$

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[1] The following abbreviations are used: DNS, 1-dimethylaminonaphthalene-5-sulfonyl or the chloride of this dye; redox thyroglobulin, reduced and reoxidized thyroglobulin; MRW, mean residue weight.

TABLE I: Conditions of Reduction and Reoxidation of Thyroglobulin.[c]

Reduction				Dilution					
Redox No.	Protein Concn (%)	M	M[b] Ratio	Protein Concn (%)	M	Mercaptoethanol (M)	pH	Salt (M)	Disulfide Bonds/Mole
I	0.60	7.1 G[a]	100	0.01	5.0 G	0.003	8.1	0.025 Tris	90
II	0.60	7.1 G	100	0.01	5.0 G	0.003	8.1	0.025 Tris–0.06 NaCl	95
III	0.80	9.0 U	140	0.01	8.0 U	0.003	10.2	0.025 Tris–0.06 NaCl	94
IX	0.40	6.5 G	600	0.01	0.1 G	0.012	8.1	0.025 Tris–0.06 NaCl	
XI	0.42	6.6 G	400	0.005	0.1 G	0.006	8.1	0.025 Tris–0.06 NaCl	>85
XII	0.42	6.6 G	200	0.005	0.1 G	0.003	8.1	0.025 Tris–0.06 NaCl	

[a] U = urea; G = guanidine hydrochloride. [b] Molar ratio = moles of mercaptoethanol/mole of protein × 202. [c] Reduction was carried out at pH 10.3 for 30 min at 25°. All solutions, after dilution, were dialyzed *vs.* 0.025 M Tris buffer, pH 8.1; all solutions, except I, contained 0.06 M NaCl; the first dialysis solution for I–III also contained 0.001 M mercaptoethanol. Dialysis was at 5°.

A value of 1.2×10^{-8} sec was used for the lifetime (τ) of the conjugated dye (Steiner and Edelhoch, 1961). The Ouchterlony gel diffusion method was the same as described elsewhere (Metzger *et al.*, 1962).

Native Thyroglobulin. Thyroglobulin was prepared by a differential centrifugation procedure described recently (Edelhoch and Lippoldt, 1964). The small amount of faster sedimenting material (27S) normally present was eliminated by agar gel filtration by a procedure recently developed (Salvatore *et al.*, 1964). The protein preparation was, therefore, almost 100% 19S thyroglobulin as characterized by velocity sedimentation. Protein concentration was determined by absorbance measurements. A value of 10.5 was used for the absorbance of a 1%, pH 7 solution at 280 mμ for a 1-cm path.

Redox[1] *Thyroglobulin.* The procedure for the preparation of redox thyroglobulin consisted of four stages: (1) reduction by β-mercaptoethanol of all the disulfide bonds; (2) dilution of the protein concentration $\leq 0.01\%$ with either urea or guanidinium hydrochloride buffered water; (3) air oxidation of the sulfhydryl groups during dialysis *vs.* buffered water; and (4) concentration by negative pressure dialysis. The experimental conditions of the first two steps are enumerated in Table I.

Reduction was performed under conditions where all the disulfide bonds were reduced, *i.e.*, at pH 10.3 in concentrated solutions of urea or guanidinium hydrochloride at 25° (de Crombrugghe *et al.*, 1966). After 30 min, solutions were diluted $\leq 0.01\%$ protein concentration. In preparations I–III the diluent contained guanidine or urea (Table I); in the rest (IX, XI, XII) an aqueous solution was used. Reoxidation occurred during dialysis of the protein solution *vs.* at least 5 volumes of an aqueous buffer of 0.025 M Tris, 0.06 M NaCl, pH 8.1. The first dialysate in preparations I–III contained 0.001 M mercaptoethanol. The second and additional dialyses were *vs.* the same buffer without mercaptoethanol. It has been shown that aggregation of reduced ribonuclease can be eliminated if reducing conditions prevail during the reoxidation step (Epstein *et al.*, 1962). All procedures after reduction and dilution were carried out at 5°. In the preparations which were diluted with aqueous buffer, the buffer was added to the protein solution in a few minutes in preparations IX and XI and during 5 hr in preparation XII. The protein solution was stirred during the dilution procedure.

After several days of dialysis solutions were concentrated about 50-fold to a few milliliters by negative pressure dialysis at 5°. Finally, solutions were dialyzed *vs.* the aqueous buffer.

DNS-Redox Thyroglobulin. Redox sample II (Table I) was used for conjugation with the fluorescent dye, DNS.[1] The conjugation procedure has been described elsewhere (Steiner and Edelhoch, 1961). The conjugated protein was reprecipitated several times with half-saturated phosphate buffer at pH 6.8 in order to eliminate free dye. The precipitate was dissolved in Tris buffer (0.025 M) at pH 8.1 and dialyzed *vs.* the same buffer for 20 hr at 5°. A sedimentation experiment with the conjugated reoxidized protein showed a pattern very similar to that of the reoxidized protein prior to conjugation.

DNS-Thyroglobulin. Native thyroglobulin was conjugated with the fluorescent dye, DNS, in a similar manner as described above for redox II. A detailed account of the behavior of this molecule as evaluated by polarization of fluorescent measurements has been published (Steiner and Edelhoch, 1961). It is used presently to compare its behavior with that of conjugated redox II under identical conditions.

Results

Sedimentation Velocity. The sedimentation patterns of redox preparations I–III were very similar. Two boundaries represented over 90% of the total schlieren

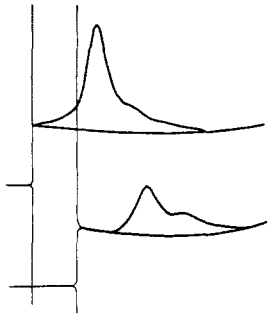

FIGURE 1: The ultracentrifugal patterns of (upper) redox III (1.2% protein, pH 8.1, 0.025 M Tris, 0.060 M NaCl) and (lower) redox I (0.63% protein, pH 8.1, 0.025 M Tris).

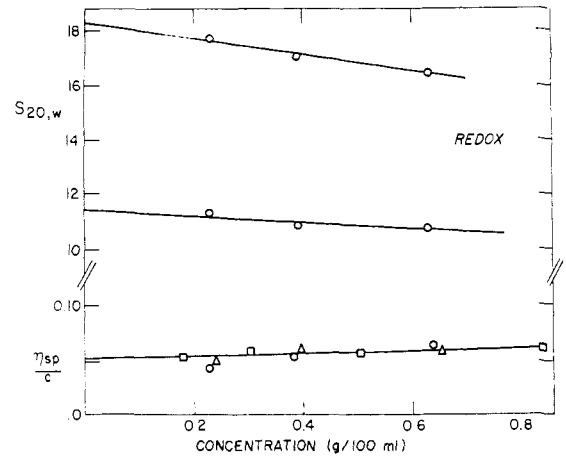

FIGURE 2: The concentration dependence of the sedimentation coefficient and reduced specific viscosity of redox thyroglobulin. ⊙, redox I; ◬, redox II; ⊡, redox III. All solutions at pH 8.1, 0.025 M Tris, and 0.060 M NaCl except redox I which did not contain NaCl.

area (Figure 1). Most of the remaining area represented material which sedimented at a slightly faster rate. The concentration dependence of the sedimentation coefficients of the two principal components of redox I at pH 8.1 is shown in Figure 2. The extrapolated values ($s^0_{20,w}$) of the sedimentation coefficients are slightly smaller than those reported for 12 and 19 S in native thyroglobulin. The relative areas of the components varied with concentration. The composition, determined by extrapolation of the schlieren areas of redox I to zero concentration, was 55% 12 S and 35% 19 S. Since the sedimentation coefficients and relative areas of redox III were close to those of redox I and II, it appears that similar results are obtained whether urea or guanidine is used in the reduction and reoxidation steps (Table I).

The properties of redox I were also examined at other pH values since characteristic molecular changes occur in native thyroglobulin at high and low pH. Little change was observed in either sedimentation coefficients or relative areas of the two main components when the pH was raised to 9.5 or reduced to 1.7. Increasing the pH to 11.5, however, decreased the sedimentation coefficients of both components by about 30% but had little effect on their relative areas. If the faster sedimenting component was a weakly bound association product of the slower one, its concentration would be expected to fall under these conditions. Dissociation of 19 to 12 S takes place in native thyroglobulin under similar conditions (Edelhoch and Metzger, 1961).

When the pH of redox I was reduced from 8.1 to 6.0, which approaches closer to the isoelectric point of thyroglobulin (~4.5), faster sedimenting boundaries were formed. The latter presumably represent higher molecular weight complexes.

It has been reported that the folding and the degree of molecular association of the fully reduced alkylated chains of thyroglobulin are different in water than in concentrated urea or guanidine (Edelhoch and de Crombrugghe, 1966). In preparations IX, XI, and XII the guanidine required for reduction was diluted to very low levels by addition of aqueous buffer (see Methods). Consequently, reoxidation now occurred while the reduced chains were in an aqueous environment.

All three preparations which were diluted with water had somewhat larger amounts of components sedimenting slightly faster than 19 S. Redox XI had 15–20%, whereas redox IX and XII had somewhat less. The ratios of the two principal boundaries were about the same as in redox I. The sedimentation coefficients of the two main components of redox XII were slightly larger than in redox I. Extrapolation to zero concentration gave sedimentation constant values ($s^0_{20,w}$) of 12.1 and 19.2. These values are in closer accord with that of each component in the native molecule than with the values of redox I[2] (Edelhoch and Metzger, 1961).

Viscosity. The concentration dependence of the reduced specific viscosity of redox preparations I–III is shown in Figure 2. Similar values were found with redox XI (Table II). The intrinsic viscosity was very near 0.055 dl/g which is close to that of the native protein. The low value of redox I is especially noteworthy since the solution contained only Tris buffer (0.025 M). It should be noted that the intrinsic viscosity of reduced alkylated thyroglobin in 5 M guanidine is 0.82 dl/g (de Crombrugghe *et al.*, 1966).

Optical Rotatory Dispersion. The specific rotation at

[2] In another preparation of redox thyroglobulin V the native molecule was reduced in 8.2 M urea, pH 10.3, at a molar ratio of 140. It was then diluted 100-fold to 0.01% protein concentration by adding it to Tris (0.025 M) buffer at pH 8.1. The final mercaptoethanol concentration was 0.003 M. The solution was not dialyzed but allowed to stand for 36 hr at 25°. Ultracentrifugal examination of the protein after concentration revealed two principal peaks, having a ratio similar to that in redox I, which comprised about 75% of the total composition. The remaining material sedimented faster. The reoxidation did not go to completion under these conditions since 20 free sulfhydryl groups were found.

TABLE II: Reoxidized Thyroglobulin.[a]

Redox No.	$[\eta]$	$s^0_{20,w}$		$[\alpha]_{550}$	% Helix $b_0/-630$	H_{193}	H_{225}
I	0.055	11.5	18.3				
II	0.055			−57	20	31	34
III	0.055			−52	22	32	33
IX				−59	~0		
X	0.055			−58	11		
XII		12.1	19.2	−61	~0		
Native	0.06	12.0	19.2	−63	10		

[a] All solutions in 0.025 M Tris, 0.060 M NaCl, pH 8.1, except redox I which did not contain NaCl. Data for native thyroglobulin taken from Edelhoch and Metzger (1961).

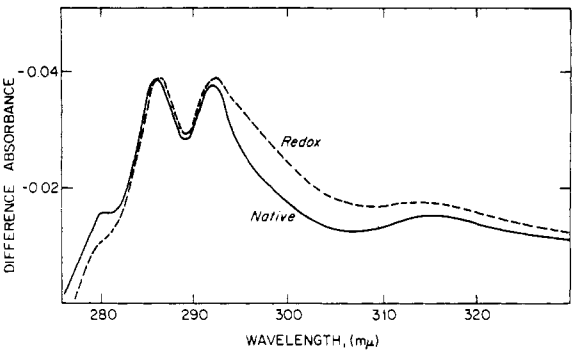

FIGURE 3: Ultraviolet difference spectra of redox I and native thyroglobulin (0.10%) in 6.4 M guanidine, pH 3.25, 0.02 M glycylglycine. Reference cell contained the same components in aqueous solution.

550 mμ and the constant of the Moffitt equation, b_0, are reported in Table II for five preparations. The specific rotation values are in approximate agreement with that of the native molecule and considerably more positive than reduced alkylated thyroglobulin (Edelhoch and de Crombrugghe, 1966). This agreement does not pertain, however, to the b_0 values. In the preparations in which urea (redox III) and guanidine (redox II) were dialyzed out of solution, the b_0 values were close to twice that of the native. When the urea and guanidine were diluted out, two of the preparations (IX and XII) had b_0 values indistinguishable from zero while redox XI had an intermediate value, *i.e.*, −70 (Table II).

The helical content of redox II and III was also evaluated by another procedure (Shechter and Blout, 1964) and the results are reported in Table II. Assuming a b_0 value of −630 for 100% helix, then a helical content of 20% is obtained by the Moffitt equation whereas the Shechter–Blout equation gives slightly higher values.

Ultraviolet Difference Spectra. The environment of the aromatic chromophores may be explored by observing the effect of the addition of either neutral or denaturing solvents to aqueous solutions of the protein. Since changes in the absorption of these residues are relatively small, the effects are usually investigated by difference spectral techniques. The difference spectrum of redox I dissolved in 6.4 M guanidine at pH 3.25 relative to that of the protein in water at the same pH is reproduced in Figure 3. The difference spectrum of the native protein observed under similar conditions is presented for comparison. The magnitudes of the tyrosyl (286 mμ) and tryptophanyl (292 mμ) peaks are approximately the same. However, a much more intense shoulder exists in redox I on the high wavelength side of the tryptophanyl peak. This shoulder can be attributed to the diiodotyrosyl residues since their difference peak in iodinated thyroglobulin (Edelhoch, 1965) and in human serum albumin (R. Perlman and H. Edelhoch, unpublished results) occurs at 301 mμ. Evidently these residues are less exposed to water in redox thyroglobulin than in the native molecule. A minor difference peak occurs with both forms at about 315 mμ and presumably represents the difference absorption of thyroxyl residues.

Results similar to those presented in Figure 3 were obtained with redox II dissolved in 8.0 M urea at pH 3.5. With both native and redox thyroglobulin in 8 M urea, the tyrosyl difference peak is about 15% less than that of the tryptophanyl residue. In guanidine the peaks have approximately the same intensity.

The difference spectrum of the native protein was reduced by about 20% when the pH was lowered from 3.25 to 2.65. A similar change in pH had essentially no effect on the difference spectrum of redox I. However, a further decrease in pH to 1.9 produced the same change in redox I as was found with the native protein at pH 2.65.

Electrophoresis. Electrophoresis of redox III in 0.05 M bicarbonate at pH 10.0 was performed by the moving-boundary method. After traversing 80% of the cell in 6.5 hr, both limbs showed only a single, symmetrical boundary.

Polarization of Fluorescence. Another method of assessing the degree of intramolecular interactions is that of polarization of fluorescence. If a molecule contains structures which can rotate independently of each other, then it will have a relaxation time that is smaller than that which the molecule would have if it were completely rigid. The greater the independence of the segments of the polypeptide chain the smaller will be the relaxation time.

The variation of the polarization of DNS-redox II with temperature is shown in Figure 4. A linear dependence of the reciprocal polarization on T/η was observed between 13 and 46° from which a relaxation time of 2.4×10^{-7} sec was computed from the Perrin equation. This value is slightly higher than has been found recently for the native form (H. Edelhoch and

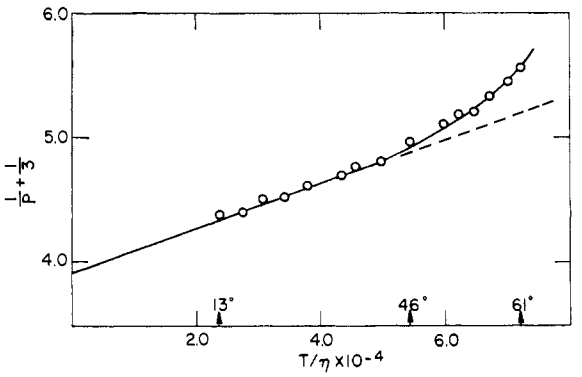

FIGURE 4: Perrin plot of the effect of temperature on the polarization of DNS-redox II (0.012%) at pH 8.1, 0.025 M Tris, 0.10 M NaCl.

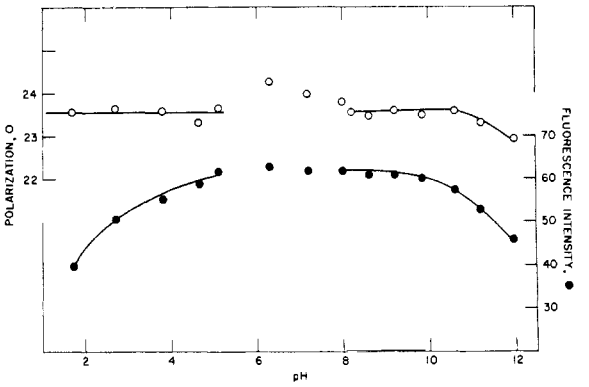

FIGURE 5: The pH dependence of the polarization, O, and fluorescence, •, of DNS-redox II (0.012%), between pH 1.9 and 12.0. Lysine (0.01 M) was present between pH 6.3 and 12.0 and 0.025 M acetate from pH 5.1 to 1.9.

R. F. Steiner, unpublished data) and close to the computed value of a rigid sphere of 330,000 mol wt (Steiner and Edelhoch, 1962). At higher temperatures, deviations from linearity occurred in a direction which indicate smaller relaxation times.

The effect of pH on the stability of DNS-redox II was evaluated by polarization measurements. As seen in Figure 5, the polarization was constant between pH 8 and 11 and from pH 5.1 to 1.8. A small increase occurred in the neutral pH region which probably represents molecular association, since faster sedimenting components were observed in the ultracentrifuge when the pH was reduced from 8.1 to 6.0.

The fluorescence intensity of the conjugated dye was constant in the neutral pH zone, decreased above pH 10, and was quenched in acid (Figure 5). The pK of the bound dye, determined from the pH of 50% quenching of its fluorescence, is below pH 1.8. Since the pK of the dye is close to 4.0 when conjugated to glycine (Klotz, 1960), it is evident that the basic form of the dye is strongly stabilized by interaction with the

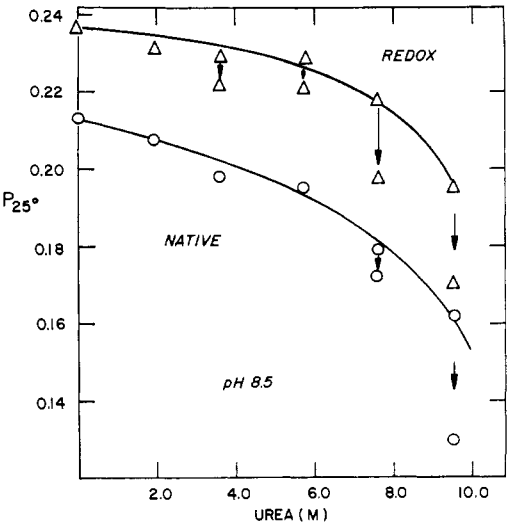

FIGURE 6: Effect of urea on the polarization of DNS-redox II and DNS-native thyroglobulin (0.012%) at pH 8.5, 0.050 M Tris. Arrows indicate time effects. Lower points were polarization values recorded after 3 hr. Values are uncorrected for changes in viscosity with urea concentration.

protein. Laskowski has shown that DNS conjugated to serum albumin is incompletely exposed to the aqueous solvent (Laskowski, 1966).

The stability of DNS-redox II and DNS-native thyroglobulin was compared by dissolving them in solutions containing reagents which modify protein structure. The influence of urea on the polarization of the two forms of thyroglobulin at pH 8.5 is shown in Figure 6. The arrows indicate those experiments in which time-dependent changes were observed. The larger values were obtained about 1 min after adding the protein to the urea solution. Time effects were followed for 3 hr except in 9.5 M urea where reaction was complete in about 2 hr. The smaller polarization values were those observed after 3 hr.

It can be seen in Figure 6 that the polarization values decrease with increasing levels of urea. The changes are relatively small until high levels of urea are reached. The variation of polarization with urea and the time effects are seen to be similar in the two proteins. The gradual fall in polarization reflects a reduction in relaxation time since the viscosity of the solutions increases and the lifetime of the dye decreases with increasing urea concentration. Both of these factors would increase the polarization if the structure remained invariant.

The effect of alkaline pH on the time-dependent changes in polarization of native and redox protein in 7.6 M urea is depicted in Figure 7. After about 2 hr, mercaptoethanol was added to each solution. A rapid fall in polarization occurred in all solutions (Figure 7). It is clear that the rates and magnitudes of the polarization changes approximately parallel each other in the two forms of the protein. The significance of the changes

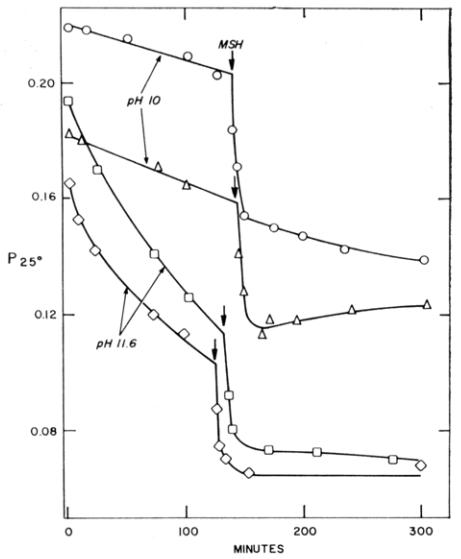

FIGURE 7: Kinetics of polarization change of DNS-redox II and DNS-native thyroglobulin (0.012%) at pH 10.0 and 11.6 in 7.6 M urea, 0.05 M glycine. Early times, in the absence of β-mercaptoethanol. Later times, after addition of 0.001 M β-mercaptoethanol to give a molar ratio of 28. Times of addition indicated by vertical arrows. ○, □ = redox; △, ◇ = native.

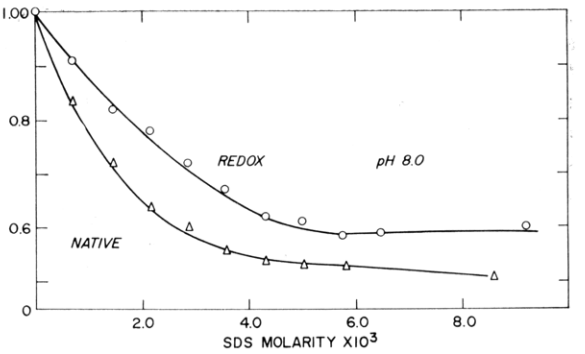

FIGURE 8: The effect of sodium dodecyl sulfate (SDS) on the polarization of DNS-redox II, ○, and DNS-native thyroglobulin, △ (0.012%), in pH 8.0, 0.01 M Tris, 0.01 M NaCl at 25°. The polarization values (vertical axis) are shown relative to their values in the absence of sodium dodecyl sulfate.

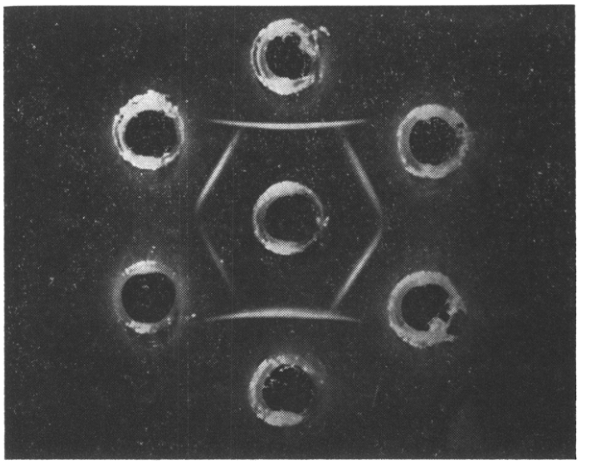

FIGURE 9: Ouchterlony gel diffusion, pH 8.6, 0.05 M Veronal. North and south wells contained native thyroglobulin; the next two wells, moving clockwise, contained redox I; the remaining two wells contained redox II. The center cell contained rabbit antibovine thyroglobulin antiserum.

in polarization of the native protein has been elaborated on elsewhere (Steiner and Edelhoch, 1961).

In contrast to the high concentrations of urea and guanidine needed to unfold proteins, detergents are effective at very low concentrations. Profound changes in viscosity and sedimentation rate of thyroglobulin are produced by sodium dodecyl sulfate (Edelhoch and Lippoldt, 1960). The fall in polarization resulting from the interaction of native and redox II with sodium dodecyl sulfate is reported in Figure 8. The polarizations are given relative to their values in the absence of detergent. It is apparent that the behavior of the two forms resemble each other reasonably closely.

Immunodiffusion. By the Ouchterlony gel diffusion technique, all the redox samples gave lines of partial identity similar to those shown in Figure 9 when measured *vs.* rabbit antibovine thyroglobulin antiserum. It should be noted that no reaction occurred with reduced alkylated thyroglobulin (Edelhoch and de Crombrugghe, 1966).

Disulfide Reductions of Reoxidized Thyroglobulin. The disulfide content, determined by the procedure of Ellman (1959), of several redox preparations is listed in Table I. Essentially the full complement of disulfide groups were found in redox I and II under conditions where all the groups in the native protein are reduced, *i.e.*, 8 M urea, pH 10.3, 0.07 M mercaptoethanol, and 30-min reduction time at 25°. Only half the disulfide groups were reduced in redox III under these conditions. Increasing the mercaptoethanol concentration to 0.70 M and the temperature to 50° resulted in the reduction of 94% of the disulfide groups. The value reported for XI was obtained under the latter set of conditions. Determination of the sulfhydryl groups of XI showed that none were present.

Discussion

Thyroglobulin has at least a dual function in the regulation of thyroid activity since the thyroid is the site of both the synthesis and release of the thyroidal hormones, triiodothyronine and thyroxine. These two functions apparently are controlled by different cellular mechanisms (Rall *et al.*, 1964). The structure of the

protein will obviously play an important role in each since the hormones are bound by peptide bonds and form part of the polypeptide backbone. At present biochemical assays which measure these activities are not available. Consequently, we must rely on physical and chemical criteria in order to characterize the properties of the reoxidized form of the molecule.

Perhaps the most interesting result is the resemblance between the reoxidized and the native protein in molecular composition. Native 19 S thyroglobulin is readily but incompletely dissociated into 12 S subunits. Very mild reduction with mercaptoethanol dissociates the remaining 19 S and also converts 12 S into two smaller molecules, which appear to be the basic polypeptide chains. The hydrodynamic properties of redox thyroglobulin are sufficiently close to those of the native to permit the identification of the two principal components with the 12 and 19 S of the native. Essentially no monomeric material was found. Moreover, the composition did not change significantly at either high or low pH, suggesting that these molecules are not loose complexes of the monomer.

Preliminary data from polyacrylamide gel electrophoresis indicate that the two chains in 12 S, derived from native thyroglobulin, may be different (Edelhoch, 1965). In this case the 12 S species that is formed by reoxidation would also be composed of two dissimilar chains since only one boundary was observed by electrophoresis. There appears to be a strong tendency for the single chains to form dimers since reduced alkylated thyroglobulin, which is devoid of disulfide bonds, is also largely dimer and migrates as a single electrophoretic boundary in aqueous solution.

It has been shown that the structure of fully reduced thyroglobulin is very different in aqueous medium from that in concentrated urea or guanidine solutions (Edelhoch and de Crombrugghe, 1966). It is not surprising therefore that differences in the properties of redox thyroglobulin were observed which depended on the solvent present during the reoxidation of the reduced chains. Dialysis of urea or guanidine solutions resulted in a greater yield of 12 and 19 S components. Small amounts of faster sedimenting components were found when dilution of the urea or guanidine solution was accomplished by the addition of water. In the latter case reoxidation of the sulfhydryl groups took place in an aqueous environment. In addition, the helical content, as evaluated by current theories of analysis of optical rotatory dispersion data, was found to depend on the method of dilution. In two samples prepared by dialysis, a helical content about twice that of the native was found (Table II). In two of the samples diluted with water the helical content was indistinguishable from zero. The native protein has about 10% of its residues in helices.

The small intrinsic viscosity and large relaxation time of reoxidized thyroglobulin are indicative of a very compact and rigid structure with little internal or external hydration. The difference spectral and optical rotational data are in accord with this point of view. The strong blue shift in guanidine results from an increase in exposure of the absorbing chromophores to the solvent. Consequently they must be incompletely exposed to the solvent in aqueous media and therefore involved in tertiary interactions. The specific rotation values also suggest that the asymmetric centers are at least partially excluded from contact with the solvent, since the more unfolded forms of thyroglobulin, such as reduced alkylated in water (Edelhoch and de Crombrugghe, 1966) or native in urea or guanidine (Edelhoch and Metzger, 1961), have considerably more negative values.

The compactness and rigidity of globular proteins depend on the extent of intramolecular bond formation between segments of the polypeptide chain. The resistance of a protein to reagents or conditions which destroy these bonds furnishes an estimate of the stability of the intramolecular interactions. The effect of urea in disorganizing the structure of redox thyroglobulin was determined by polarization measurements. There was no important difference between the behavior of redox and native thyroglobulin in (a) the shape of the urea–polarization curve, (b) the influence of pH and mercaptoethanol in concentrated urea solutions, and (c) the effect of detergent. It is evident, therefore, that the two forms of thyroglobulin resemble each other in their stability and, presumably, in their internal organization. However, specific differences in structure and behavior were observed by other methods.

The antigenicity studies show that redox thyroglobulin has not recovered all the immunologic determinants of the native protein. The solubility properties of the two forms were somewhat different in acid solutions. Neither protein is soluble near the isoelectric point (~pH 4.5). The native form is denatured near pH 5 and does not recover its properties at more acid pH values. The reoxidized protein had the same sedimentation and polarization value near pH 2 as at neutral pH values. In addition, a difference in the reducibility of the disulfide groups was noted. Redox III (but not I and II) was more resistant to reduction than the native protein. This result is being explored further.

Reoxidation leads to the recovery of the major features of the internal organization of native thyroglobulin. The medium in which reoxidation occurs appears to produce only minor variations in structure. It is rather surprising that the differences were not greater than observed in view of the profound difference in structure of the reduced chains in water compared to urea or guanidine solution (Edelhoch and de Crombrugghe, 1966). The similarities in the properties of the final products may, of course, result from S–S exchanges which occur as the sulfhydryl groups are oxidized and the concentration of urea and guanidine is reduced during dialysis.

Since a precise biochemical or biological test of thyroglobulin activity does not exist it is impossible to assess the significance of the minor variations in properties that have been observed. If the requirements for biological activity are as exacting as is the structure of enzymes for their activity, then minor differences in properties may be of the utmost importance.

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