The Thiol Groups of Thyroglobulin

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The principal component of thyroglobulin has mol.wt. 670 000 and sedimentation coefficient 19s. Piez (1964) showed that this molecule contained 202 half-cystine residues. De Crombrughe, Pitt-Rivers & Edelhoeh (1966) confirmed this finding by Ellman's (1959) method after complete reduction of thyroglobulin at pH 10 in 8M-urea, using 15 equiv. of β -mercaptoethanol/half-cystine residue. These authors were unable to detect any thiol groups with Ellman's reagent.

We have reinvestigated this problem by three different methods: (1) carboxymethylation of thyroglobulin followed by hydrolysis and determination of carboxymethylcysteine in the amino acid analyser (Guidotti & Konigsberg, 1964); (2) an adaptation of the method of Zwann (1966) whereby proteins are separated by electrophoresis in polyacrylamide gel, treated with the Barnett & Seligman (1952) reagent, DDD,[†] and stained with Fast Black K salt; (3) solutions of thyroglobulin were tested for thiol groups with 4,4'-dithiopyridine by the method of Grassetti & Murray (1967).

Materials and methods. Thyroglobulin was prepared by the method of Salvatore, Salvatore, Cahnmann & Robbins (1964), with Sephadex G-200. The 19s protein was prepared by polyacrylamide-gel electrophoresis and did not show more than one component in the ultracentrifuge or in the polyacrylamide gels. Human thyroglobulin was a gift from Mr G. H. Turner of the Wellcome Research Laboratories (Beckenham, Kent) and was prepared by the method of Derrien, Michel & Roche (1949). Crystalline ovalbumin was obtained from the Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.) and β -lactoglobulin (from milk) from British Drug Houses Ltd. (Poole, Dorset).

DDD was obtained from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.) and Fast Black K salt from G. T. Gurr Ltd. (London, S.W. 6). 4,4'-Dithiopyridine was prepared from 4-thiopyridone (Kodak Ltd., Kirkby, Liverpool) by the method of Koenigs & Kinne (1921); it had m.p. 75–76°, as found by Comrie & Stenlake (1958).

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[†] Abbreviation: DDD, 2,2'-dihydroxy-6,6'-dinaphthyl disulphide.

[Koenigs & Kinne (1921) give m.p. 155°.] Cyanogum 41 and iodoacetic acid came from British Drug Houses Ltd. The latter was recrystallized from carbon tetrachloride-petroleum.

(1) Carboxymethylation. Carboxymethylation of thyroglobulin was carried out in 0.2M-tris-acetate buffer, pH 8.0, for 2 hr., with 100 equiv. of iodoacetic acid/mole of protein. A similar carboxymethylation was carried out in 8M-urea, pH 8.0.

(2) DDD reaction. Electrophoresis was carried out in columns (1 cm. $\times$ 5 cm.) of 5% Cyanogum 41 in 0.04M-glycine-0.022M-tris, pH 8.7. Approx. 1 mg. of thyroglobulin, accurately measured, was used in each run. The standard compound was ovalbumin, which has 3 thiol groups/mol. of mol.wt. 46 000 (Cecil, 1963), and was submitted to electrophoresis at several concentrations. β -Lactoglobulin, which has 2 thiol groups/mol. of mol.wt. 36 000 (Cecil, 1963), was analysed to test the method. After electrophoresis, the gels were cut to give a slice 2 mm. thick and incubated in DDD solution as described by Zwann (1966). The washing times recommended by Zwann (1966) were doubled and the final reaction with Fast Black K salt took 30–40 min. After being washed in water, the gel strips were scanned in a Joyce-Loebl microdensitometer, and the areas under the peaks were integrated by planimetry. Pearce (1961) found that histidine also reacted with DDD, but indicated that the colour obtained did not interfere significantly with the estimation of thiol groups. However, we found that histidine blanks must be performed, by carrying out the reactions on completely alkylated proteins. No detectable thiol groups were found in ribonuclease or insulin by this procedure.

This method was unsuitable for estimations of thyroglobulin thiol groups in urea, since in most of the electrophoretic runs the protein did not penetrate into the gels.

(3) 4,4'-Dithiopyridine. Reduced glutathione was used to prepare a standard curve, which was linear for thiol concentrations between 0.01 and 0.15M. Protein solutions in tris-glycine buffer as above were prepared to contain thiol concentrations in this range. Readings of E_{324} were taken at different time-intervals to ensure maximum reaction.

Table 1. *Estimation of thiol groups in water and in 8M-urea by carboxymethylation and by their reactivity towards DDD and 4,4'-dithiopyridine*

Crystalline ovalbumin (3 thiol groups/mol. of mol. wt. 46000) was used as the standard in the DDD estimation. Except where indicated, the 4,4'-dithiopyridine reaction was carried out for 15 min.

Protein	Thiol groups/mol. of protein					
	Carboxymethylation		DDD		4,4'-Dithiopyridine	
	Water	Urea	Water	Urea	Water	Urea
β -Lactoglobulin (mol.wt. 36000)	—	—	2.2 1.9	—	0.5 1.45 (2 hr.) 1.75 (4 hr.)	—
Thyroglobulin (mol.wt. 670000)						
Pig (19s)	1.8	5.2	2.6 2.3	5.5	3.7	4.6
Sheep	—	—	2.1	—	3.4	3.8
Calf	—	—	2.3	—	4.1	4.5
Human	—	—	2.2	—	4.4	4.4

Results. The results are shown in Table 1. Thyroglobulin gave a maximal reaction with 4,4'-dithiopyridine in 15 min., and was stable for 2 hr. β -Lactoglobulin, however, only showed 1.75 thiol groups/mol. of protein after 4 hr. Phillips, Jenness & Kalan (1967) found a similar lack of reactivity of the thiol groups of β -lactoglobulin B when using Ellman's (1959) reagent.

Comment. It is evident from these results that the hydrophilic reagents, iodoacetic acid and DDD, do not react with more than 2 thiol groups/mol. of thyroglobulin in water. In the two experiments performed with these reagents in urea, the thiol content was increased to 5 groups/mol. of protein. The less hydrophilic reagent, 4,4'-dithiopyridine, could react with as many thiol groups/mol. in water as in urea. It appears that thyroglobulin contains 5 thiol groups/mol. of mol.wt. 670000; 3 of these are inaccessible to the hydrophilic reagents mentioned above and all are apparently inaccessible to 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) (De Crombrughe *et al.* 1966).

No species differences in thiol content of thyroglobulin were found in the four preparations studied.

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