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"CROSS-LINKING WITH DIMETHYLSUBERIMIDATE TO STUDY THYROGLOBULIN CONFORMATION"

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Summary - The present investigation demonstrates that the cross-linking agent, dimethylsuberimidate, is an usefull tool to study thyroglobulin structure. In fact, while reproducible and discrete polymerization products are obtained in strictly controlled conditions, valuable information on the native assemblage of thyroglobulin subunits and the effects of its major post-translational modification (iodination) on its structure, are reported.

Thyroglobulin is a large glycoprotein characterized by a sedimentation coefficient of 19 S. Two similar, probably identical subunits of 330,000 form the native molecule, which contains about 10% by weight of carbohydrates. A variable amount of iodine atoms are covalently linked to some tyrosine residues forming mono and diiodotyrosines, which are the precursors of the thyroid hormones triiodothyronine (T_3) and thyroxine (T_4). The 330 KD subunit is one of the larger elementary chains known to be synthesized as a single polypeptide (1).

The assembly of the two 330,000 subunits into thyroglobulin occurs in two different ways: some are linked by weak interactions, therefore easily dissociable, whereas some others are covalently linked and can not be dissociated (2). The relative proportion of the undissociable fraction is positively correlated with the iodine content of thyroglobulin (3).

It is not known wether the two types of interactions result in thyroglobulin molecules of different conformation; however, it has been claimed that the level of iodination causes thyroglobulin to acquire different shapes (4).

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Abbreviations used: DMS, dimethylsuberimidate; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis.

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We report on the use of a cross-linking agent, dimethylsuberimidate (DMS) in order to analyze the conformation of discrete populations of 19 S thyroglobulin molecules with different, but homogeneous, iodine content.

Material and Methods

Chemicals - Dimethylsuberimidate (DMS) was purchased from EGA Chemie (W. Germany). Acrylamide monomer and N-N' methylene bis acrylamide were from Bio-Rad Laboratories. All other chemicals were the best available grade and were used without further purification.
Preparation of 19 S thyroglobulin - Thyroids were collected immediately after the animals were killed by CO_2 asphixation. Glands were minced with scissors and soluble proteins extracted (three times) with 0.1 M sodium phopsphate buffer, pH 7.2. After a fractionated (1.4-1.8 M) ammonium sulfate precipitation, 19 S thyroglobulin was finally purified by Sepharose 6B column chromatography, in Tris-HCl 0.01 M, NaCl 0.1 M, pH 7.2. Molecules of homogeneous iodine content were obtained by RbCl (34.5% w/w) centrifugation (5).The iodine throghout the gradient was measured according to Palumbo et al. (6).
Cross-linking experiments - It was carried out according to the procedure described by Coggins et al. (7) in 0.2M triethanolamine HCl, pH 8.5. Stock solutions of DMS (0.3M) were prepared immediately before use in triethanolamine HCl 0.5 M pH 8.5. Cross-linking reactions were carried out in test tubes with shaking at 25°C and quenched by the addition of 1.0 M ammonium bicarbonate. Partial unfolding of thyroglobulin was produced by exhaustive dialysis against 6.0 M urea or 6.0 M urea the in presence of 0.1 % SDS (in 0.2 M triethanolamine HCl pH 8.5). Before electrophoresis unreacted DMS and triethanolamine were removed by dialysis against Tris-HCl 0.01 M, pH 7.5.
Electrophoretic procedures (PAGE) - Polyacrylamide gels were prepared according to Laemmli (8). The buffer was 0.025 M Tris, 0.2 M glycine, 0.1% SDS, pH 8.3. Stacking gels (3.75%) of 1 cm in length were layered over resolving gels of 4.5 % or 5.0 % acrylamide. Electrophoresis was carried out at constant current of 35 mA for 1 h and then at 50 mA until the marking dye (0.001% bromophenol blue) reached the gel bottom. We have used unreducible (660,000) and reducible (330,000) thyroglobulin as standards of electrophoretic mobility and size. Reduction of denatured samples (1% SDS) was accomplished at 100°C (1 min) in 0.75 M 2-mercaptoethanol. The scans of the Coomassie stained gels were obtained by using an LKB 2202 laser densitometer.

Results

Under reducing conditions, the electrophoretic profile of thyroglobulin is quite complex. The major species consist of two closely migrating bands, whose molecular weights are both near 330,000. It has been reported that these two species are differently iodinated (9), since the halogen content of the faster migrating band is about 2.5 times higher as compared to the slower one. It has been also reported that the primary structure of these two polypeptides are very similar (10) or even identical (9). Usually other species may be detected in the electrphoretograms: this fact is related to the iodination degree of the 19 S preparation. In fact, after reduction, very iodine rich molecules give rise to several discrete low molecular weight species and some

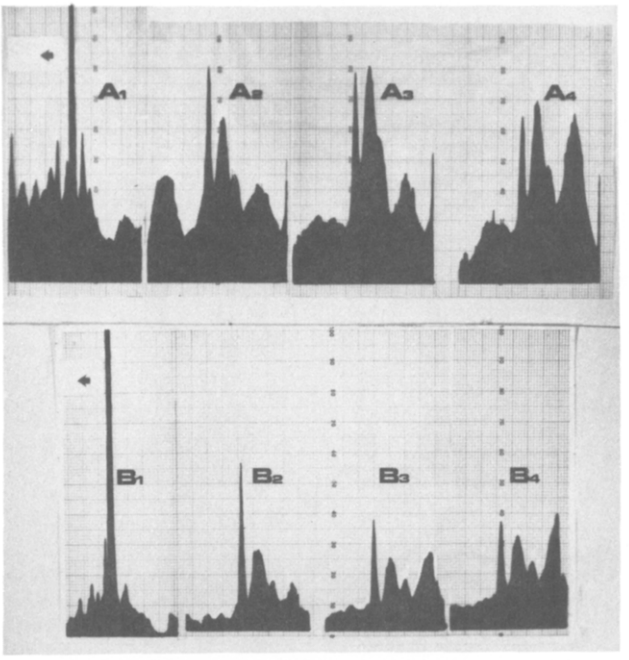

Fig.1

Panel A : Cross-linking of thyroglobulin with DMS: 0.4 mg of thyroglobulin (200 ul of a 2 mg/ml solution) were reacted (3 h) at 25°C with increasing volumes of the stock DMS solution (0.3 M) and analyzed by SDS PAGE under reducing conditions. The amount of DMS used in each experiment was: A1, 0 (control); A2, 2 mg; A3, 4 mg; A4, 8 mg.

Panel B : Cross-linking of thyroglobulin with DMS: 0.4 mg of thyroglobulin (200 ul of a 2 mg/ml solution) were reacted at 25°C for increasing periods of time with a fixed amount of DMS (8 mg): B1 is reduced 19 S thyroglobulin in absence of DMS (control); B2, 10 min; B3, 40 min; B4, 60 min.

Scans of the Coomassie blue stained gels were obtained by using a Laser densitometer (see Methods).

The arrow indicates the direction of electrophoresis.

covalently linked, undissociable material (660,000).

Fig.1A shows the effect of increasing concentrations of DMS on the cross-linking reaction with highly iodinated thyroglobulin molecules (60 iodine atoms per thyroglobulin molecule), as revealed by PAGE in SDS 1% under reducing conditions. It is evident: i) the progressive decrease of the faster migrating band and the concomitant increase of the slower migrating component of the 330,000 doublet; ii) the progressive disappearance of all small peptides which are known (3) to be present in highly iodinated thyroglobulin preparations and iii) the appearance, and the parallel increase, of a new discrete high molecular weight species, whose size is very similar to that of

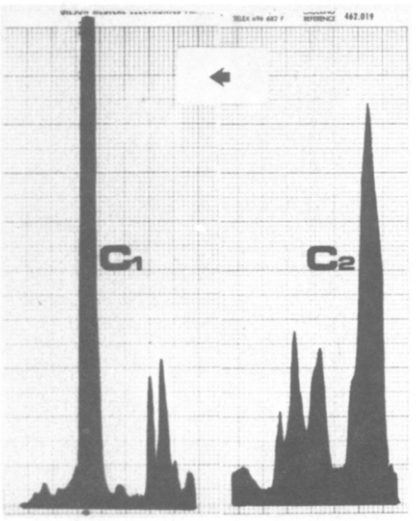

Fig.2

Electrophoretogram of unreduced thyroglobulin before (C1) and after (C2) the reaction with DMS. In this experiment 0.4 mg (200 ul of a 2mg/ml solution) of thyroglobulin were reacted with 8 mg of DMS at 25°C for 1 h. It is evident the formation (C2) of a molecular species having the same electrophoretic properties of the covalently linked 19 S (C1).

Scans of the Coomassie blue stained gels were obtained by using a Laser densitometer (see Methods).

The arrow indicates the direction of electrophoresis.

the unreducible, covalently linked 19S described above (660,000). The latter point is better demonstrated by electrophoresis in absence of reducing agent (fig.2C): in this case a remarkable amount of high molecular weight material is observed. Similar results have been obtained studying the kinetics of the cross-linking reaction with poorly iodinated thyroglobulin molecules (20 iodine atoms per thyroglobulin molecule) at constant (20 mg/ml) DMS concentration (Fig. 1B). In this case according to our previuos observations (3) no low molecular species are detectable in the pattern of the control (B1).

To verify whether the possibility of forming discrete species reflects structural features and not merely the presence of reactive residues, we have studied the cross-linking reaction in 3 M urea or 3 M urea in presence of 0.1% SDS. Indeed, while in 3 M urea a significant loss of specificity is observed (no discrete species being produced), further addition of SDS totally abolishes the possibility of forming cross-links (fig.3D, upper panel). The partial or total failure to observe discrete linkages in these conditions suggests

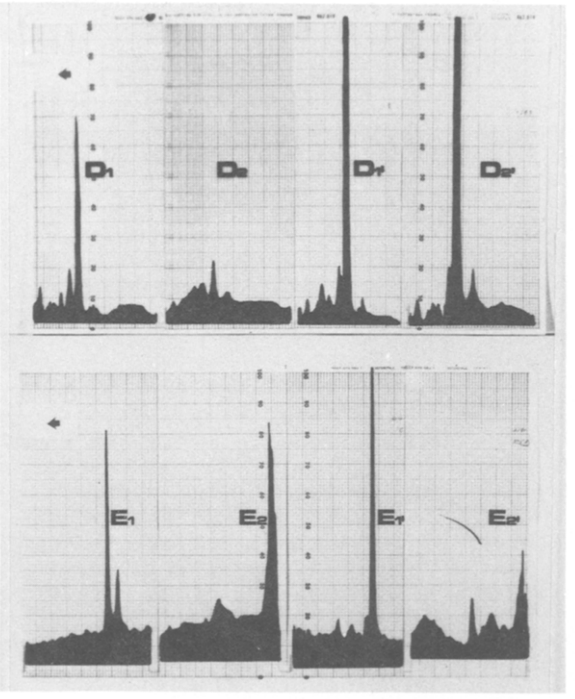

Fig.3

Panel D: Cross-linking of thyroglobulin in 3 M urea or in 3 M urea and 0.1 %SDS with DMS. Electrophoretogram of dehatured (D1) and cross-linked thyroglobulin with DMS (D2), in 3 M urea. Note the dramatic loss of specificity since no discrete species are detectable. Electrophoretic profile of denatured (D1', control) and of cross-linked thyroglobulin with DMS (D2'), in 3 M urea and 0.1% SDS. Note the total lack of polymerization products. In both experiments 0.4 mg (200 ul of a 2mg/ml solution) of thyroglobulin were reacted with 8 mg of DMS at 25°C for 1 h.

Panel E: Cross-linking of differently iodinated thyroglobulin molecules. Electrophoretogram, under reducing conditions of highly iodinated (60 iodine atoms per thyroglobulin molecule) (E1, control) and DMS-cross-linked (E1') thyroglobulin. Electrophoretic pattern, under reducing conditions, of poorly iodinated (20 iodine atoms per thyroglobulin molecule) (E2, control) and DMS-cross-linked (E2') thyroglobulin. Note that highly iodinated mole- cules produce larger amounts of high molecular weight species.

Scans of the Coomassie blue stained gels were obtained by using a Laser densitometer (see Methods).

The arrow indicates the direction of electrophoresis.

that the two thyroglobulin subunits, in the native state, are not randomly but, rather stereospecifically oriented.

The effect of different degree of iodination in thyroglobulin on cross--linking reaction is shown in Fig.3E (lower panel). Two types of thyroglobulin molecules, having high (60) and low (20) iodine atoms per molecule have been prepared by RbCl gradients. Highly iodinated molecules produce larger amounts of high molecular weight species as compared with iodine poor molecules. This

fact implies, therefore, that thyroglobulin is a molecule where the tertiary and quaternary structures are strongly dependent on the degree of iodination of the protein.

Discussion

The most important post-translational modification of thyroglobulin is iodination. It was of interest to test if a chemical method, based on the use of DMS, is suitable to reveal possible structural modifications induced in thyroglobulin by the iodination process. Actually, DMS or similar reagents have already been successfully employed to analyze the subunits of multimeric proteins, their spatial arrangement and hormone-receptor interactions (7, 11-14). In this work, we show that the reaction of thyroglobulin with DMS produces discrete, reproducible and specific species providing that the native folding of thyroglobulin (fig.3D) is entirely preserved.

More information about the spatial arrangement of the two subunits of thyroglobulin have been obtained by comparing the cross-linking reaction using native or denatured (3 M urea or 3 M urea in 0.1% SDS) thyroglobulin.

To gain information about the effect of iodination, the 19 S thyroglobulin was fractionated according to its density, by means of RbCl gradients. We have studied two fractions, containing 60 and 20 iodine atoms per thyroglobulin molecule respectively. These two families of molecules differ significantly in their electrophoretic pattern (under reducing conditions) (fig.3E), since, while the iodine rich molecules display the typical 330,000 doublet, the iodine poor molecules result essentially in one species of comparable size. More interesting is the electrophoretic pattern observed after the reaction with DMS. In fact the iodine rich molecules produce a significantly larger amounts of material having a size of 660,000 as compared to that obtained by cross-linking of the iodine poor molecules. Early investigations (3) have shown that an higher amount of covalently linked 19 S is obtained in iodine rich thyroglobulin preparations. This observation is in excellent agreement with our present finding since a larger amount of cross-linked 19 S is found with highly iodinated thyroglobulin molecules. This suggests that, as a

consequence of iodination, the two subunits come in closer contact fulfilling the steric requirements for the formation of new interchain S-S bridges (in vivo) or cross-linkages with DMS (in vitro).

Indeed, beside every effort, the formation of a molecular species having a mass higher than 660,KD, has never been observed in our hands. This failure may confirm to the hypothesis that the "mature" 19 S is not, both in vivo (15) and in vitro (16) a precursor of the 27 S iodoprotein, the naturally occurring covalently linked dimer of thyroglobulin (M_r= 1,200KD). It appears that the steric conditions required for dimerization, are not satisfied by the particular spatial arrangement of native thyroglobulin.

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