Effects of Temperature and Urea Concentration on the Chemical Modification and Ionization Behavior of Tyrosyl and Iodoamino Acid Residues of Porcine Thyroglobulin

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Synopsis

Acetylation of porcine thyroglobulin with N-acetyl imidazole was performed at various temperatures in the presence and absence of urea. In the medium without urea, the number of O-acetylated iodoamino acid residues formed was constantly 15 above 10°C (originating from all diiodotyrosyl and a few of monoiodotyrosyl residues), while the number of O-acetyl tyrosyl residues increased gradually from 15 at 0°C to a maximum of 70 at 70°C. All (about 25) iodoamino acid residues were acetylated at 20°C in 2M urea, while the presence of 8M urea was necessary to acetylate all tyrosyl residues at 20°C. Experiments on the nitration of thyroglobulin with tetranitromethane showed that diiodotyrosyl and thyroxyl residues were easily nitrated even at low temperatures and the low ratio of the reagent to the protein, while tyrosyl residues consisted of three groups: the first group (about 14%) which was very easily nitrated even at 0°C and the low ratio of the reagent to the protein, the second group (about 45%) which was nitrated depending on temperature and the molar ratio of the reagent, the remaining which was definitely unreactive even under extreme conditions. In addition, the ionization of tyrosyl and iodoamino acid residues was studied by spectrophotometric titration at various temperatures in the presence and absence of 8M urea. It was found that diiodotyrosyl and thyroxyl residues did not change very much with respect to ionization behaviour by the addition of urea, in contrast to clear dependency of tyrosyl residues on urea. Based on these observations, the location of these residues and the structural change of the protein with temperature are discussed.

Iodide ions taken up into thyroid glands were oxidized to be incorporated into tyrosyl residues of thyroglobulin, forming monoiodotyrosyl and diiodotyrosyl residues. Thyroxyl and triiodothyronyl residues are produced in the thyroglobulin molecule by the coupling of these iodotyrosyl residues. Evidence is accumulating to indicate that the structure, especially in the surface region, of the protein plays an important role in the iodination and coupling reactions (see for review Salvatore and Edelhoch, 1973). The nature of tyrosyl and iodoamino acid residues on the surface of the protein molecule was examined by means of ionization behaviour (Edelhoch, 1962; Edelhoch and Lippoldt, 1962; Robbins, 1963), iodination (Nunez et al., 1966; Van Zyl and Edelhoch, 1967; De Crombrugghe et al., 1967; Hosoya, 1968; Edelhoch, 1969; Lamas et al., 1974; Pommier et al., 1973; Davidson et al., 1976), acetylation (Edelhoch and Perlman, 1968) and nitration (Malan and Edelhoch, 1970) of thyroglobulin, hoping to understand the participation of protein

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structure in the formation of thyroid hormones. The present paper goes along this line, especially studying the effects of temperature and urea concentration which were not so much examined as to this problem in spite of their usefulness. A preliminary account of this work has already been presented (Itagaki and Hosoya, 1971).

Materials and Methods

Thyroglobulin preparation
Thyroglobulin was prepared from a pool of porcine thyroid glands by ammonium sulfate fractionation followed by gel filtration on Sepharose 6B column. The protein usually contained 0.9% iodine or about 45 atoms per molecule: iodine content was determined by the method of Tamura and Kondo (1963). Paper chromatographic analysis for iodoamino acids (Mandel and Bloch, 1959) gave the following values: MIT, 13 moles; DIT, 12.5 moles; T4 (T3) 1–2 moles per mole of thyroglobulin (MW = 660,000). The amount of tyrosine in acid hydrolysate of the protein preparation, determined by Millon reaction, was 115 moles per mole of the protein.

Chemical modification

a) Acetylation: AcIm was synthesized from imidazole and isopropenyl acetate by the method of Shiba and Cahnman (1962). Employing at least 50-fold molar excess of the AcIm, thyroglobulin (0.1–2.0 mg) was acetylated at various temperatures for 60 min in 1 ml of 0.2 M sodium borate buffer (pH 7.4) containing 0.05 M NaCl. The reaction mixture was passed through a Sephadex G-25 column (0.5 × 20 cm) with 3 ml of the medium to terminate the acetylation and the second 4-ml eluate which contained acetylated thyroglobulin was collected.

b) Nitration: Nitration of thyroglobulin was performed essentially by the same method as described by Sokolovsky et al. (1966). Commercial TNM was diluted to 83 mM with absolute alcohol immediately before use. A small volume of the TNM solution was added at various temperatures to 2 ml of thyroglobulin solution (2–5 mg protein in 0.2 M Tris-HCl buffer, pH 7.4, and 0.1 M KCl) to give a required ratio of moles of TNM to moles of the protein (hereafter referred to as TNM molar ratio). After 60 min, unreacted reagent and by-products such as nitroformate were separated from the nitrated thyroglobulin by gel filtration on Sephadex G-25 column (0.5 × 10 cm).

Spectrophotometric titration of thyroglobulin

Spectrophotometric titration of tyrosyl and iodoamino acid residues of thyroglobulin was performed according to essentially the same method as that described by Edelhoch (1962). Solutions were prepared containing 0.1 to 0.2% protein, 2.5 mM sodium phosphate buffer (pH 7.4), 10 mM KCl, with (8 M) or without urea. Each solution (2.0 ml) was placed in 1-cm light path cuvette which was held at a constant temperature in a water bath and stirred with a glass-enclosed magnetic needle and an external rotating magnet. The pH was measured directly in the cuvette by means of a long-thin (3 mm in diameter and 5 cm in length) glass electrode and a modified calomel electrode employing a Hitachi-Horiba F-5 pH meter. After the pH of the solution was lowered to approximately 5.1 by the addition of 0.01 ml of 1.0 M HCl, minute quantities of 1 N NaOH were added through a fine polypropylene tubing by a microsyringe to produce stepwise increments in the pH. After the pH measurement, the absorption spectrum of the solution was recorded at the constant temperature over the range between 270 and 400 nm.

The concentration of the ionized form of tyrosyl and iodoamino acid residues of the thyroglobulin at the respective pH was calculated using the following equations:

\[
[DIT^{\bullet}] = \frac{\Delta A_{295} - \Delta A_{305}}{\alpha} \quad \text{or} \quad \Delta A_{295} = \Delta A_{305} - \frac{\Delta A_{295}}{\alpha} \quad \text{at pH 5-10} \quad \text{(1)}
\]

\[
[MIT^{\bullet}] = \frac{\Delta A_{305} - \Delta A_{325}}{\alpha} \quad \text{at pH above 10} \quad \text{(3)}
\]

where \( \Delta A_{305} \) or \( \Delta A_{295} \) = change in absorbance at 305 nm or 295 nm, respectively, between the respective pH and pH 5.0.

1) Abbreviations used are: MIT, -Lmonoiodotyrosine; DIT, L-diiodotyrosine; T4, L-thyroxine; T3, 3, 3',5'-triiodothyronine; AcIm, N-acetyl imidazole; TNM, tetranitromethane.
[\text{Tyr}^\text{−}], [\text{MIT}^\text{−}], [\text{DIT}^\text{−}], \text{or} [\text{T}^\text{4−}] = \text{molar concentrations of ionized forms of tyrosyl, monoiodotyrosyl, diiodotyrosyl or thyroxyl residues, respectively.}

These equations are a modification of Edelhoch's (1962) and Robbins' (1963), but, to avoid confusion, it must be noticed that the present definition of \( \Delta A_{305} \) and \( \Delta A_{295} \), being different from their notation \( \Delta e_{305} \) and \( \Delta e_{295} \) and meaning (Robbins, 1963), takes the differences of absorbance between respective pH and pH 5 to calculate the number of the residues ionized at the pH. The contribution of ionized forms of diiodotyrosyl and, at pH above 10, tyrosyl residues to that of monoiodotyrosyl residues was also found to be not negligible and therefore subtracted as shown in Equations (2) and (3). The factor of Equation (3), \( \alpha \), was here taken as 0.8 so as to adjust the ionization curve sigmoidal.

Chemicals
L-Tyrosine, L-monoiodotyrosine, L-diiodotyrosine, 3, 5, 3'-L-triiodothyronine and L-thyroxine were purchased from Wako Chem. Co., isopropenyl acetate from Tokyo Kasei Co., and TNM from Nakarai Co. Inorganic chemicals were of reagent grade. Deionized glass-distilled water was used throughout the present work.

Results

Acetylation of thyroglobulin with N-acetyl imidazole

AcIm, which reacts with the unionized forms of phenolic hydroxyl groups under mild conditions, has been used as a probe to characterize tyrosyl residues in a number of proteins (Simpson et al., 1963; Bethune et al., 1964; Riordan et al., 1965; Edelhoch and Perlman, 1968). In the present experiments, the reactivity of tyrosyl and iodotyrosyl groups of thyroglobulin for the reagents was examined at various temperatures or in the presence of various concentration of urea.

As illustrated in Fig. 1, thyroglobulin, when acetylated with AcIm, decreased in absorbance in the range of 260–290 and 300–360 nm. The absorbance change is more clearly seen in a difference spectrum, revealing two troughs at about 282 nm and 318 nm (inset of Fig. 1). Fig. 2 shows the difference spectra of thyroglobulin acetylated at various temperatures, indicating that the first trough is dependent on the acetylation temperature while the second is not greatly affected.

Since the decrease in the 280-nm region after acetylation is definitely ascribed to the formation of O-acetylated tyrosyl residues, as reported in the case of other proteins (Simpson et al., 1963; Edelhoch and...
Perlman, 1968), the number of the tyrosyl residues modified in thyroglobulin can be determined by employing \( \Delta \varepsilon_{278} = 1,160 \text{ cm}^{-1} \text{ M}^{-1} \) which was taken from the difference spectrum of \( \alpha \)-acetylated \( \alpha \)-acetyltirosine (Riordan et al., 1965). The decrease in the 300–360 nm region presented in Fig. 1 and Fig. 2 may be ascribed to the acetyl derivatives of iodoamino acid residues of thyroglobulin because difference spectra of \( \alpha \)-acetyl derivatives of MIT or DIT versus MIT or DIT themselves had troughs at 308 nm \( (\Delta \varepsilon_{308} = 1,000 \text{ cm}^{-1} \text{ M}^{-1}) \) or 312 nm \( (\Delta \varepsilon_{312} = 1,720 \text{ cm}^{-1} \text{ M}^{-1}) \), respectively. It is impossible to differentiate these iodotyrosyl residues from their difference spectra because of close overlap, and only the total number of acetyl derivatives of these iodoamino acid residues was roughly estimated together assuming the molar extinction coefficient of DIT presented above. Modified iodothyronyl residues were neglected in view of their low content.

The number of \( \alpha \)-acetylated tyrosyl and iodotyrosyl residues of thyroglobulin calculated from the data of Fig. 2 are plotted against respective reaction temperatures (Fig. 3). As will be seen from the figure, the number tyrosyl residues modified increased greatly with increase in temperature from 15 at 0°C to 70 at 70°C, whereas the number of \( \alpha \)-acetylated iodoamino acid residues remained constant (about 15) between 10°C and 60°C.

The effect of urea concentration at 20°C is shown in Fig. 4. As will be seen from the figure, the number of acetylated tyrosyl residues increased remarkably with the concentration of urea, reaching a maximum of about 120, almost equal to the total tyrosyl residues. The number of acetylated residues of iodoamino acid levelled off at 2 M urea to be about 24, which represented almost all iodoamino acid residues.

Of the 15 iodoamino acid residues acetylated without urea (Fig. 3), the proportion derived from monoiodotyrosyl and diiodotyrosyl residues cannot be determined by the method mentioned above. Spectrophotometric titration method used by Edelhoch (1962) makes it possible to estimate the number of unmodified individual residues.
and, therefore, the method was applied to a sample. The results indicate that 2–3 residues originated from moniodotyrosyl residues, and 12–13 residues from diiodotyrosyl residues. The spectrophotometric method, however, was not able to be used for the analysis of tyrosyl residues, because of the lability of O-acetylated tyrosyl residues at the high alkaline pH which is needed in this method.

**Nitration of thyroglobulin with tetranitromethane**

Several reagents, other than AcIm, have been used as the useful chemical probe for tyrosyl residues of proteins. Among them, TNM which reacts with phenolic hydroxyl group (Shiba and Cahnman, 1962; Sokolovsky et al., 1966; Riordan et al., 1966) was employed in the present study to obtain information complementary to the results described above.

The reagent also reacts with free DIT with the concomitant release of iodine atoms, suggesting that iodine atom(s) of ortho position for the phenolic hydroxyl group was substituted by nitro groups. In fact, the absorption spectra of nitrated DIT were almost the same as those of nitrated tyrosine and the peak of the difference spectrum of nitrated tyrosine or DIT against tyrosine was at 428 nm. MIT was nitrated without release of iodine atoms at low TNM molar ratio and the product showed almost similar difference spectrum to nitrated DIT. T₄ (+T₃) was also nitrated with TNM but the peak of the difference spectrum was at about 470 nm. Consequently, nitrotyrosyl residues of thyroglobulin produced by means of TNM should include some originated not only from tyrosyl but also from iodothyrosyl residues and iodothyronyl residues. The amount of these products was estimated in the mass employing the molar extinction coefficient of 3-nitrotyrosine, ε₄₂₈=4,200 cm⁻¹M⁻¹, which was obtained by us under the present conditions.

**Fig. 5.** Absorption spectra of thyroglobulin nitrated with tetranitromethane. Thyroglobulin was nitrated at room temperature for 10 min employing TNM with the molar ratio shown in the figure. A broken line shows that the peaks of these curves vary with the molar ratios. TG stands for thyroglobulin.
Fig. 6-A that, at low temperature (0–30°C), the increase in the formation of nitrotyrosyl residues is retarded at about 1,000 molar ratio of TNM, and almost levelled off at 2,000–3,000 molar ratio. In such cases, nitration is considered to occur at first in diiodotyrosyl and iodothyronyl residues because the results presented in Table 1 showed that nitration at low TNM molar ratio proceeded with concomitant release of iodine atoms. As the temperature increased, the enhancement of nitration due to the high level of TNM molar ratio became remarkable (see Fig. 6-B) and the number of nitrated residues levelled off at 3,000 molar ratio of TNM and at 70°C. It is likely that some

![Graph showing the effect of temperature and TNM ratio on the nitration of thyroglobulin.](image)

**Fig. 6.** Effect of temperature and TNM ratio on the nitration of thyroglobulin. Nitration of thyroglobulin was carried out as described in “Materials and Methods”. Number of nitrotyrosyl residues per molecule of thyroglobulin was plotted (A) against TNM ratio at a constant temperature and (B) against reaction temperature at a constant TNM ratio.

**Table 1.** Number of iodine atoms and nitrotyrosyl residues of thyroglobulin nitrated with different levels of tetranitromethane

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>TNM molar ratio</th>
<th>Nitrotyrosyl residues produced (moles/mole TG)</th>
<th>Iodine atoms remained (atoms/mole TG)</th>
<th>Iodine atoms released (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>4.6</td>
<td>46</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>250</td>
<td>15.5</td>
<td>38</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>500</td>
<td>23</td>
<td>34</td>
<td>32</td>
</tr>
<tr>
<td>5</td>
<td>750</td>
<td>28.5</td>
<td>29.5</td>
<td>41</td>
</tr>
<tr>
<td>6</td>
<td>1000</td>
<td>36</td>
<td>33</td>
<td>34</td>
</tr>
<tr>
<td>7</td>
<td>1500</td>
<td>37</td>
<td>31.5</td>
<td>39</td>
</tr>
<tr>
<td>8</td>
<td>2000</td>
<td>44</td>
<td>31.5</td>
<td>39</td>
</tr>
</tbody>
</table>

*a* Thyroglobulin (2 mg/ml) was nitrated with TNM of 100–2,000 molar ratio at 19°C for 1 hr. An aliquot of the samples was analyzed for nitrotyrosyl residues and the remaining samples were dialyzed against water for 2 days to determine the iodine contents.
loosening of protein structure facilitated the nitration of hitherto buried tyrosyl residues. The conformational change caused by thermal agitation seemed to be reversible at least below 40°C, because the number of tyrosyl residues modified at 20°C did not vary with or without preincubation at 40°C. From the curves shown in Fig. 6, the maximum number of nitrotyrosyl residues appeared to be around 90. This means that about 60% of tyrosyl residues in the original thyroglobulin, in addition to almost all iodoamino acid residues, might be nitratred under the extreme conditions used.

Phenolic hydroxyl ionization of tyrosyl and iodoamino acid residues of thyroglobulin

Spectrophotometric titration of thyroglobulin was performed as described under "Materials and Methods" in the presence and absence of urea at various temperatures. Fig. 7 shows the ionization curves of monoiodotyrosyl, diiodotyrosyl and thyroxyl residues obtained at 25°C in 0.01M KCl. The ionization curves of diiodotyrosyl residues are broad, extending over 6 pH units, both in water (0.01M KCl) and in 8M urea, and shifted from the right to the left with increasing temperature. The degree of the shift was very similar to each other in these cases and fairly small (0.4 pH unit/30° in water and 0.05 pH unit/30° in urea) as will be seen from the pH values of half ionization shown in Table 2. When these values are plotted against 1/T, two straight lines with a similar slope were obtained as shown by open circles (in water) and closed circles (in urea) of Fig. 8. Although the calculation of apparent heat of ionization3, $\Delta H_{app}$, from the lines may be regarded to be a kind of over-simplification for the reason described in footnote 2, it may be said that ionization behaviour of...
Table 2. pH Values of half ionization of iodoamino acid residues of thyroglobulina.

<table>
<thead>
<tr>
<th>Amino acid residues</th>
<th>Solventb</th>
<th>10°C</th>
<th>20°C</th>
<th>30°C</th>
<th>40°C</th>
<th>50°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoiodotyrosyl residues</td>
<td>W</td>
<td>10.1</td>
<td>9.9</td>
<td>9.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Diodotyrosyl residues</td>
<td>W</td>
<td>8.3</td>
<td>8.1</td>
<td>8.0</td>
<td>7.8</td>
<td>—</td>
</tr>
<tr>
<td>Thyroxyl residues</td>
<td>U</td>
<td>8.1</td>
<td>7.8</td>
<td>7.75</td>
<td>7.5</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>W</td>
<td>9.0</td>
<td>8.8</td>
<td>8.7</td>
<td>8.3</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td>U</td>
<td>—</td>
<td>7.4</td>
<td>7.3</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

a. Ionization curves were obtained as described in Materials and Methods and the pH values of 50% ionization are presented.
b. W or U stands for 2.5 mM sodium phosphate buffer (pH 7.4) containing 0.01 M KCl without or with 8 M urea, respectively.

diodotyrosyl residues are very similar to each other in the presence and absence of urea.

The ionization curve of thyroxyl residues also showed a similar shift with increasing temperatures. The pH values of half ionization in water are presented in Table 2. Plots of these values against 1/T also give a straight line with slightly smaller slope compared with those described above. Although the shift of ionization curve by the addition of urea seems to be rather great compared with the case of diiodotyrosyl residues, the slope of plots of the half ionization pH versus 1/T appears to be similar to that in water.

In the case of monoiodotyrosyl residues, the slope of sigmoidal ionization curves varied with temperature, being sharper at elevated temperatures in a high pH region. This may be due to a kind of loosening of monoiodotyrosyl residues hitherto fixed, and only the pH values of half ionization are presented in Table 2.

As for tyrosyl residues, the forward and reverse curves of spectrophotometric titration in water did not coincide above pH 11.5, due to denaturation of the protein as reported previously (Edelhoch and Lippoldt, 1962), and the ionization curves shown in Fig. 9 are taken from the forward titration. The figure also shows the ionization curves in 8 M urea, which are all located in the pH 7.4 to 12.5 range.

4) A tentative value for $\Delta H_{\text{app}}$ is calculated to be 5.2 kcal.
region where free tyrosine dissociates (pK'a = 10.2 at 20°C). When the pH values of half ionization in 8 M urea are plotted against 1/T, a straight line is obtained with a slope similar to that of thyroxyl residues. Plots of the half ionization pH in water versus 1/T also give a straight line, but its slope is much sharper (Fig. 8).

Discussion

Acetylation with AcIm and nitration with TNM of thyroglobulin were previously examined at one temperature in the absence of urea (Edelhoch and Perlman, 1968; Malan and Edelhoch, 1970). In the present report, the chemical modification of the protein has been explored in a wide range of temperature and urea concentration. The results showed that diiodotyrosyl residues were easily accessible to AcIm even at low temperatures and without urea and to TNM at low temperatures and low TNM molar ratios. The results of spectrophotometric titration also showed that the ionization behaviour of these residues were not greatly affected in the presence of urea. These findings may be explained if these residues are located on the surface or near the surface of the protein. This is favorable for these residues to couple each other to form thyroxyl residues. In fact, thyroxyl residues thus formed seem to show easier reactivity to TNM (Fig. 5) and ionization (Fig. 8) compared with those of diiodotyrosyl residues.

It was shown previously by acetylation with AcIm at 25°C that more than half of monoiodotyrosyl residues were unreactive (Edelhoch and Perlman, 1968). The present report demonstrates that most of monoiodotyrosyl residues remain unreactive even at high temperatures (Fig. 3) but become reactive with the reagent in a high urea concentration (Fig. 4). Experiments on iodination of thyroglobulin performed both chemically and enzymatically with high concentration of iodide (Hosoya, 1968; Edelhoch, 1969) also showed that the

![Fig. 9. Fractional ionization of phenolic hydroxyl group of tyrosyl residues of thyroglobulin. Titration were carried out in 0.01 M KCl (full lines) and in 8 M urea (broken lines) at the temperature specified.](image-url)
formation of monoiodotyrosyl residues did not increase above a maximum value, about 15. These facts indicate that most of monoiodotyrosyl residues remain unreactive both at the hydroxyl group and at the ortho position to the hydroxyl group so far as the protein is not denatured.

The results of chemical modification with AcIm revealed that, under the conditions without urea, about 45 to 115 tyrosyl residues were resistant to the acetylation even at 70°C, in contrast to easy acetylation in the presence of high concentration of urea. A similar percentage of tyrosyl residues was found to be resistant to nitration with TNM even at high TNM ratios and at high temperatures. These facts indicate that a certain proportion of tyrosyl residues (about 40%) is different from that of others, being unaccessible for the reagents. On the other hand, about 15 residues (14%) are easily acetylated and nitrated even at 0°C, and the remaining (about 45%) are dependent on temperature in reactivity to the reagents. The increased reactivity of the 45% group to AcIm at high temperatures cannot be accounted by the change in the number of acceptors, because the reagent reacts with the unionized form of phenolic hydroxyl group, the number of which decreases as the temperature increases, as shown in Fig. 9. The effect of temperature may be rather explained by assuming that an equilibrium may exist between two (or more) forms of the proteins, in which the residues are unexposed in the native forms but exposed in the reactive forms, as seen in the case of a number of proteins (Foss, 1961; Herscovits and Laskowski, 1962; Hvidt and Nielsen, 1966; Bello, 1969).

The other group (40% of the total tyrosyl residues) which was not reactive to the reagents was found to become reactive in the presence of high concentration of urea. These groups may be related to the tyrosyl groups (30% of the total) which were reported to be iodinated only with modification in protein structure (Edelhoch and Lippoldt, 1962). Most of monoiodotyrosyl residues, as described above, are also resistant to acetylation and iodination without urea. It appears that these tyrosyl and monoiodotyrosyl residues form a rigid part of the protein, say "core", the reactivity of which hardly varies with temperature.

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References