Effect of Chemical Modification of Lysine Residues on the Conformation of Human Immunoglobulin G*

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SUMMARY

Human IgG (immunoglobulin G) was chemically modified by citraconic anhydride and the conformational transitions were tested by measuring CD (circular dichroism), hydrodynamic properties, and immunoprecipitation reactions. It was found that mild conversion of the lysine ε-amino groups (at pH 7.7 and room temperature) into the negatively charged citraconyl groups caused expansion and loss of the β structure of IgG. The positive CD band at 202 nm became negative, while the weak bands in the near-ultraviolet zone changed only slightly; the intrinsic viscosity increased, the sedimentation coefficient decreased, and the immunoresponse against goat antiserum was lost. The citraconylated IgG was reconverted to native IgG by dialysis against 0.05 M acetate of pH 4.0 for 72 hours. The reversal to the native conformation was achieved also by raising the ionic strength to about 1.1. Carbamylation of 88% of the ε-amino groups of IgG did not change the CD spectrum, hydrodynamic properties, or immunoprecipitation reaction. The results indicate that the conformational change on citraconylation was caused by electrostatic repulsion of the introduced carboxylate ions into the macromolecules. Moreover, according to these results the ε-amino groups of lysine residues are not essential for the immunoprecipitation reaction, whereas the native rigid conformation of the macromolecules is decisive in this reaction.

The importance of reactive groups in the side chains of immunoglobulins in maintaining the native conformation and immunological reactivity has been and is a broad subject of study. In this report we are concerned with the ε-amino groups of lysine residues. These groups have been modified previously by other authors (1–6). None of these papers, however, report a detailed study of the conformational transitions that presumably could accompany these chemical modifications, even if the modifications are accomplished avoiding extreme pH values, common denaturing agents, and high temperatures. The purpose of the present paper is to report such conformational transitions as tested by CD, circular dichroism, and hydrodynamic methods after modifying amino groups with citraconic anhydride and potassium cyanate.

EXPERIMENTAL PROCEDURE

Materials—Normal human IgG was obtained from Pentex, Kankakee, Ill. (Fraction II, lot No. 33). Myeloma IgG was the same sample prepared by Ross and Jirgensons (7). Citraconic anhydride, 1-fluoro-2,4-dinitrobenzene, and potassium cyanate were purchased from Eastman-Kodak. The latter compound was recrystallized from water-ethanol mixture as described by Stark (8). Goat antiserum to human IgG was obtained from Hyland, Los Angeles, Calif. Other chemicals used were analytical grade.

Preparation of Citraconyl-IgG In a typical experiment, approximately 3 mg per ml of IgG solution in 0.1 M sodium phosphate (pH 7.7) was prepared. The protein was modified by citraconic anhydride at room temperature with continuous stirring. A 1000-fold molar excess of citraconic anhydride was dissolved in absolute ethanol and was added to the IgG solution in small portions. The pH was maintained to 7.7 with 5 N NaOH by using a Radiometer pH-stat equipped with a SBR 2c titrigraph. The glass electrode was a Radiometer type G222B. The alcohol concentration after addition of the reagent was 2%. After 20 min of reaction the protein solution was dialyzed against 2 liters of sodium phosphate buffer, pH 7.7, and μ = 0.1, at 4° for 24 hours with one change.

To remove the modifying citraconyl groups, citraconyl-IgG was dialyzed against 2 liters of 0.05 M sodium acetate buffer, pH 4.0, at 4° for 72 hours with one change. After 72 hours of dialysis against sodium acetate buffer, the protein solution was transferred to 2 liters of sodium phosphate buffer, pH 7.7, and μ = 0.1, for 24 hours of dialysis with one change. The purpose of this buffer change was to eliminate the acetate which absorbs strongly the short wave ultraviolet light.

Carbamylation of IgG—IgG was dissolved in 0.1 M sodium phosphate buffer, pH 7.7, to make a protein concentration approximately 5 mg per ml, and recrystallized potassium cyanate was added in small portion to make final concentration of 0.5 M. The pH of the solution was monitored in a Radiometer pH-stat with 1 M acetic acid and maintained to pH 7.9. The reaction was carried out at room temperature with continuous

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stirring. The reaction mixture was dialyzed against 2 liters of sodium phosphate buffer, pH 7.7, and \( \mu = 0.1 \), at 4° for 24 hours with one change.

**Analyses of Proteins**—The myeloma IgG concentration was determined by a Beckman DU spectrophotometer at 290 nm with an extinction coefficient value of 14.0 and the molecular weight of 1.78 \times 10^5 (7). Amino acid analyses were performed following the method of Spackman et al. (9). Proteins were hydrolyzed in evacuated tubes in 6 N HCl at 110° for 22 hours. Amino acid analysis was performed by a Spinco amino acid analyzer with an accelerating system and a high sensitivity card. To determine the number of \( \epsilon \)-amino groups modified by citraconic anhydride, the dinitrophenylation method was used as described by Lenard and Singer (10). Homocitrulline was determined after carboxymethylation by the method described by Stark et al. (11).

**Determination of S Values**—The sedimentation coefficients were determined in a Beckman Spinco model E analytical ultracentrifuge at 20° and 52,640 or 59,780 rpm with an An-D rotor and a single sector cell. The buffer solution used was sodium phosphate, pH 7.7, \( \mu = 0.1 \). The protein solution was dialyzed against the same buffer for 24 hours before centrifugation. The S values were reduced to standard conditions as described by Schachman (12).

**Viscosities**—The viscosities were determined in a Cannon-Ubbelohde dilution viscometer at 28 ± 0.1°. The intrinsic viscosity, \( [\eta] \), was obtained from several determinations in the range of protein concentration between 0.3 to 0.99%, extrapolated at infinite dilution. Ionic strength was raised by adding anhydrous sodium sulfate.

**Circular Dichroism Spectra**—A Jurrum-Jasco dichrograph model CD-SP was used. Measurements were made with sensitivity scale setting 2 \times 10^{-4} or 5 \times 10^{-3} dicroic optical density difference (\( \Delta E \)) per cm on the recorder chart. All recordings were made at 23-25°. Mean residue ellipticity \( [\theta] \) was computed by using a mean residue weight of 109 for all proteins. An average \( \Delta E \) was measured from triplicate recordings. The cell having a 1.0-cm path length was used for the measurements in near-ultraviolet region (250 to 340 nm); a 0.1- or 0.05-cm cell was used in far-ultraviolet region (190 to 250 nm).

**Immunodiffusion**—An immunodiffusion plate was prepared on a polystyrene dish (dimension: 2.5 \times 7.6 cm) with 1% agar in diethylbarbital-sodium acetate buffer, pH 8.2, and \( \mu = 0.1 \). Results of the precipitation reaction were examined after 24 hours. Ionic strength on the agar plate was adjusted by adding required amounts of sodium sulfate in the buffer solution.

**Cellulose Acetate Electrophoresis**—Cellulose acetate electrophoresis was carried out in barbital buffer (pH 8.6, \( \mu = 0.075 \)) by using a Beckman Microzone electrophoresis cell, model R-101. The running conditions were constant voltage of 250 volts at room temperature for 30 min. Ponceau S was used for staining the proteins.

**RESULTS**

**Citraconyl-IgG**

**Normal IgG**—Preliminary experiments were carried out to find the optimum conditions to modify \( \epsilon \)-amino groups of normal IgG reversibly. Some typical results of citraconylation of IgG are shown in Table I. When a protein concentration of 0.72% and approximately a 2000-fold molar excess of citraconic anhydride were used, the CD spectrum of this preparation had the negative trough shifted from 216 to 208 nm. Precipitates were observed during the regeneration step and only 21% of the total protein could be recovered in solution. When the concentration of citraconic anhydride was reduced to a half of that in the previous experiment, the 216-nm negative band was shifted to 212 nm. This preparation also produced precipitates during the regeneration step but the protein recovery was improved to 49%. Finally when the reagent concentration was reduced to about 1000-fold molar excess and the protein concentration was decreased to 0.31%, 84.4 lysine residues (92%) were modified. For the modified protein, \( s_0^{\infty} \) decreased to 4.89 S and \( [\theta] \) increased to 0.227 dl per g; however, the negative trough at 216 nm did not shift and the protein was completely recovered upon regeneration. Cellulose acetate electrophoresis of the citraconyl-IgG showed a single zone which migrated toward the anode 2 times faster than the unmodified protein. After dialysis against a pH 4.0 buffer, 10 lysine residues remained covered, but the values of \( s_0^{\infty} \), \( [\theta] \), and the CD spectra were the same as that of the starting protein. It was concluded that the protein concentration of 0.3% and an approximately 1000-fold molar excess of citraconic anhydride were used, the CD spectrum of this preparation had the negative trough shifted from 216 to 208 nm. Precipitates were observed during the regeneration step and only 21% of the total protein could be recovered in solution. When the concentration of citraconic anhydride was reduced to a half of that in the previous experiment, the 216-nm negative band was shifted to 212 nm. This preparation also produced precipitates during the regeneration step but the protein recovery was improved to 49%. Finally when the reagent concentration was reduced to about 1000-fold molar excess and the protein concentration was decreased to 0.72%, 84.4 lysine residues (92%) were modified. For the modified protein, \( s_0^{\infty} \) decreased to 4.89 S and \( [\theta] \) increased to 0.227 dl per g; however, the negative trough at 216 nm did not shift and the protein was completely recovered upon regeneration. Cellulose acetate electrophoresis of the citraconyl-IgG showed a single zone which migrated toward the anode 2 times faster than the unmodified protein. After dialysis against a pH 4.0 buffer, 10 lysine residues remained covered, but the values of \( s_0^{\infty} \), \( [\theta] \), and the CD spectra were the same as that of the starting protein. It was concluded that the protein concentration of 0.3% and an approximately 1000-fold molar excess of citraconic anhydride at pH 7.7 for 20-min reaction are the most suitable conditions for reversible citraconylation of IgG.

The changes in the CD spectra on citraconylation in the non-specific (normal) human IgG were similar to those observed in the individual myeloma IgG (Table I, Fig. 1 and Fig. 3). Be-

**Table I**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>IgG concentration</th>
<th>Molar ratio of reagent</th>
<th>No. of lysine residues modified</th>
<th>After modification</th>
<th>After regeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG conc.</td>
<td></td>
<td></td>
<td>( s_0^{\infty} )</td>
<td>( [\eta] )</td>
</tr>
<tr>
<td>1</td>
<td>0.72</td>
<td>1000</td>
<td>90.7</td>
<td>4.55</td>
<td>( dl/g )</td>
</tr>
<tr>
<td>2</td>
<td>0.72</td>
<td>1000</td>
<td>90.7</td>
<td>4.55</td>
<td>( dl/g )</td>
</tr>
<tr>
<td>3</td>
<td>0.31</td>
<td>1000</td>
<td>84.4</td>
<td>4.69</td>
<td>( dl/g )</td>
</tr>
</tbody>
</table>

Cellulose acetate electrophoresis was carried out in barbital buffer (pH 8.6, \( \mu = 0.075 \)) by using a Beckman Microzone electrophoresis cell, model R-101. The running conditions were constant voltage of 250 volts at room temperature for 30 min. Ponceau S was used for staining the proteins.
FIG. 1 (left). CD spectra of myeloma IgG at various ionic strengths. For the near ultraviolet (250 to 310 nm), the light path length was 1.0 cm with the protein concentration of 0.09 to 0.16%. For the far ultraviolet (190 to 250 nm), the optical path was 0.1 or 0.05 cm and the protein concentration was 0.015 to 0.020%. The protein was dissolved in sodium phosphate (pH 7.7, $\mu = 0.1$) ($\text{--}$); the ionic strength was increased to 0.4 with sodium sulfate ($\bullet$); the ionic strength was adjusted to 1.1 ($\Delta$). FIG. 2 (right). Ultraviolet absorption spectra of native and modified myeloma IgGs. Myeloma IgG solutions were dialyzed against 2 liters of sodium phosphate buffer (pH 7.7, $\mu = 0.1$) at 4°C for 24 hours. The protein concentrations were 0.28 mg per ml. Control IgG ($\bullet$); citraconyl IgG ($\circ$); carbamylated IgG ($\triangle$).

TABLE II

Modification of lysine residues of myeloma IgG by citraconic anhydride

<table>
<thead>
<tr>
<th>Myeloma IgG</th>
<th>No. of modified lysine residues</th>
<th>$\mu = 0.1$</th>
<th>$\mu = 0.4$</th>
<th>$\mu = 1.1$</th>
<th>Precipitation in immunodiffusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>5 ($87.9$)</td>
<td>6.54</td>
<td>0.070</td>
<td>0.065</td>
<td>0.074</td>
</tr>
<tr>
<td>Modified</td>
<td>88.4</td>
<td>5.56</td>
<td>0.173</td>
<td>0.064</td>
<td>0.080</td>
</tr>
<tr>
<td>Regenerated</td>
<td>9.6</td>
<td>6.68</td>
<td>0.070</td>
<td>0.072</td>
<td>0.080</td>
</tr>
</tbody>
</table>

* Free lysine residue obtained after dinitrophenylation.  
Control IgG was hydrolyzed in 6 N HCl at 110°C for 22 hours.  
The result of immunodiffusion was positive in an agar plate of $\mu = 1.1$.

cause of the multiplicity of molecular species in the normal nonspecific IgG, the individual myeloma IgG is preferred as a homogeneous object. Further studies on the myeloma IgG are described in the following section.

Myeloma IgG—Analyses of the native myeloma IgG are shown in Table II. The $\varepsilon_{280}$ was 6.54 S and the intrinsic viscosity was 0.070 dl per g. It contained 88 lysine residues, and 5 residues were not susceptible to dinitrophenylation in the native state. The effect of ionic strength on the intrinsic viscosity was small. The CD spectra are shown in Fig. 1. Slightly increased molar ellipticity values were observed at very high ionic strength in some parts of the CD spectrum (Fig. 1). However, these changes are small in comparison to those of citraconyl-IgG (Fig. 3).

To modify myeloma IgG, the optimal conditions described above were applied. After modification, ultraviolet absorptions of modified and control myeloma IgG's were compared. No change of absorbance at 278 nm was observed as shown in Fig. 2. This indicates that no acylation took place on tyrosyl groups. Further, no change at 278 nm was observed by adding hydroxyl-
amine as described by Riordan and Vallee (13). The absorbance minimum at 250 nm shifted to 260 nm upon modification, as observed previously with pepsinogen (14). Dansylation followed by thin layer chromatography (15) indicated no free amino terminals present in the modified IgG. The value of $\theta_{\text{obs}}/\eta$ decreased to 5.56 S and [a] increased to 0.173 dl per g upon citraconylation. The citraconyl-IgG did not react with goat antiserum. The modified IgG gave a single fast moving electrophoretic band on cellulose acetate. Drastic changes in e-amino terminals present in the modified IgG. The value of $\theta_{\text{obs}}/\eta$ decreased to 0.054 dl per g; and it slightly increased when ionic strength was raised to 1.1. The CD spectra of citraconyl myeloma IgG are shown in Fig. 3. In the near-ultraviolet region, the CD bands were slightly enhanced. In the far-ultraviolet region the prominent positive peak at 202 nm became negative. The effect of ionic strength observed in CD spectra resembles the spectrum of native IgG, as shown in Fig. 4. Ionic strength variation had no effect on the CD spectra and viscosity. Immunochemical reactivity tested with goat antiserum showed positive precipitation reactions that were independent of the number of modified lysine groups.

**DISCUSSION**

In this paper the studies deal with conformation of IgG modified by citraconic anhydride and potassium cyanate. The former modification introduces acidic groups into e-amino groups reversibly, and the latter compound converts the e-amino group to an electrostatically neutral one. The changes are shown schematically as follows:

![Diagram showing the conversion of lysine residues into negatively charged groups upon citraconylation](image)

According to our experimental findings, conversion of the e-amino groups of lysine residues into negatively charged groups on citraconylation resulted in expansion and partial disorganization of the immunoglobulin molecules. The ability to form precipitate with goat antiserum also was lost on such conversion. However, the precipitability and the conformational features of the native globulin were restored in two ways: either by in-

![FIG. 4: CD spectra of carbamylated myeloma IgG at various ionic strengths. The conditions are identical with those of Fig. 1.](image)
creasing the ionic strength or by cleaving off the citraconyl residues at pH 4.0. Moreover, we observed that carbamyl-
ation of the ε-amino groups of the lysine residues did not affect the CD spectra, viscosity, and immunological precipitability. Hence we concluded that (a) the native conformation rather than the presence of free ε-amino groups is essential for the immuno-precipitation reaction, and (b) that the conformational transition incited by citraconylation is caused by electrostatic repulsion.

A detailed specification of the reversible conformational transition on citraconylation is presently impossible, because of the limited information that is available on conformation of native immunoglobulins. In spite of the many efforts, the three-dimensional structure (conformation) of the immunoglobulins is only partially elucidated. Attempts to obtain good immunoglobulin crystals suitable for x-ray structural analysis have been disappointing; and the low resolution results on imperfect crystals have not revealed much about conformation (16–18). The limited information presently available on the secondary structure of these proteins is from ORD (7, 18 21) and CD data on solutions (22–24). ORD and CD are quite useful methods in detecting changes in conformation, especially if supplemented to other methods. Expansion of the serum γ-globulins in strongly acidic and alkaline solutions has been detected as early as 1954 (25–29). However, more interesting are the conformational transitions which occur in nearly neutral solutions, especially if one wants to consider immunochemical reactivity. For this reason, attempts have been made to study the conformational transitions in the absence of strong acids, alkalies, concentrated urea, or guanidine salts. One of the ways is to modify the side chains under mild conditions. This approach allows also to test the importance of the various side chains in the immunoreactive reactions. Expansion of the macromolecules of human γ-globulin on mild succinylation has been described by Habeeb (2); also he observed that this conformational transition was accompanied by a moderate loss of reactivity toward antibody. More recently, similar observations were reported on maleylation of IgG (6). In none of these reports, however, were the conformational changes tested by chiroptical methods.

While the increase in intrinsic viscosity and decrease of sedimentation coefficient are clear indications of the expansion of IgG, as we observed on citraconylation, the changes in the secondary structure cannot be interpreted with the same degree of confidence. The positive (CD) band centered near 200 nm and the negative band at 216 to 218 nm are indicative of the β structure (30–32) and replacement of the positive band with a negative one indicates loss of the β form and disorganization of the polypeptide backbone. However, the amount of the pleated sheet β conformation cannot be very high in these globulins; neither is there any indication of a high α helix content, as it has been concluded by several groups of investigators (7, 22, 23, 29, 33, 34). Although some parts of the IgG macromolecule are flexible (35), the major portions are rigid. Thus it is likely that the major secondary structure in the immunoglobulins is neither the β form, α helix, nor the flexible random coil but some unspecified aperiodic conformation, as it now is disclosed by x-ray structural analysis in many proteins (e.g. in chymotrypsin by Matthews et al. (36)). From the changes we observed in the far-ultraviolet CD spectra (Fig. 3) we can conclude that some of the secondary structure was lost on citraconylation, and that the disorganization probably was due to the hydrogen bond disruption of the pleated sheet conformation. It is, however, impossible to state that all β structures were disrupted and to what extent the unspecified other rigid conformations were involved in the conformational transition.

We did not examine in detail the 230- to 250-nm spectral zone, but others (22, 37) have paid much attention to a positive band centered near 232 nm. In comparison to the previously described bands (observed at 200 and 216 nm), this band is very weak as indicated by a hump in the CD curve in our Fig. 3. According to Cathou et al. (22) and Dorrington and Smith (37), this band should be attributed to tyrosine chromophores in asymmetrical protein environment.

The changes of the CD in the near-ultraviolet spectral zone also are difficult to explain in detail, chiefly because of overlap of the CD bands caused by the vicinal effects on tyrosine, tryptophan, and phenylalanine side chains and the inherent asymmetry of the disulfide bonds. The bands near 284 and 291 nm can be attributed to tryptophan (37, 38), but the effects of the other aromatic side chains cannot be fully excluded in this zone. The CD bands in the near-ultraviolet zone, depending on the protein asymmetrical environment, can be either positive or negative. Thus any of the weak bands we observed in the 250- to 280-nm spectral zone (Fig. 3) may be caused by cancellation of bands of opposite sign as it has been concluded for other instances by other authors (39, 40). Increased freedom of rotation of the aromatic chromophores upon disorganization of a rigid conformation should result in decrease in magnitude of the CD effects, as it often is observed in denaturation. The fact that the bands became more positive on citraconylation (Fig. 3) may be due to the unequal cancellation of the positive and negative CD in the same zone, i.e. that the disorganization presumably resulted in weak cancellation of the positive effects and strong cancellation of the "hidden" negative bands in the 260- to 290-nm spectral region.

A detailed analysis of the near-ultraviolet zone would be extremely difficult at present because of the complexity of structures and large number of the chromophoric groups in the various parts of these macromolecules. Work is in progress on the CD effects of chemically modified Fragment Pab and Fc, as well as on light chains of definite antigenic type (Bence-Jones proteins). It is hoped that conformational analysis of these macromolecules will yield more meaningful results about the CD bands in the near-ultraviolet zone than the present data on the whole IgG.

Previously lysine residues were converted to amidic which causes no electrostatic change (1) and it was found that amidation of ε-amino groups did not change physical and immunological properties. In the present work, carbamyl groups were introduced onto the ε-amino groups and the resulting protein also showed no change in physical, hydrodynamic, and immunological properties (Table III and Fig. 4). Singer and Thorpe (41) have shown that tyrosine is involved in the active site of immunoglobulin. Although the tyrosyl residues of the citraconyl-IgG were not modified, no precipitation reaction against goat antiserum was observed. Thus neither the intact tyrosine residues nor lysine side chains secure the immunoprecipitation if the specific native conformation is not maintained. The finding that native IgG, citraconyl-IgG at high ionic strength, and carbamyl-IgG in a wide range of salt concentration yielded practically identical CD spectra in the far- and near-ultraviolet spectral zones is noteworthy. It shows that the chemical modification of a significant number of lysine side chains has no effect on the conformation of these macromolecules.
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