ACTA CHEMICA SCANDINAVICA 22 (1968) 953–960

Ultracentrifugal Studies of the Aggregation of Human Immunoglobulin G by Freezing and by Heating

ULLA-BRITT HANSSON

Department of Clinical Chemistry, University of Lund, Malmö General Hospital, Malmö, Sweden

Fresh IgG* preparations from normal sera and from myeloma sera aggregated when frozen but not when heated.

Aggregates that formed, when IgG myeloma proteins were "frozen", were turned into 7 S molecules on subsequent heat treatment.

Aggregates that formed, when normal and myeloma IgG were "aged" could also be converted into 7 S molecules on subsequent heat treatment but in the solutions also a precipitate formed. In normal IgG 9—24 % of the molecules precipitated. In the myeloma preparations the amounts of precipitate varied from 0 to 100 %. None of the "aged" normal IgG globulins contained soluble aggregates after heating. Four of six myeloma preparations studied contained soluble aggregates in an amount varying between 0 and 24 %.

The heat aggregation was not affected by the presence of glycine but some soluble aggregates were prevented from increasing in size when glycine was present.

The precipitates formed when two myeloma IgG were "aged" and heated could be dissolved in urea solution. Reaggregation, however, occurred in urea-free solution. The precipitate from the one preparation was soluble also in 0.1 M glycine at pH 3.0. 20 % of the molecules were recovered as 7 S molecules in glycine-free buffer. None of the precipitates were soluble in 5 % NaCl.

The bonds responsible for aggregation when the globulins were heated were not identical with those that caused aggregation when the globulins were frozen.

When heated to about 60°C, immunoglobulins of type G (IgG) form aggregates with *s*-values between 9.5 and 1000 S.[1] No correlation has been found between the amount of aggregates formed on heating and the electrophoretic mobility of the original molecules.[2] Molecules that do not aggregate when heated once, aggregate only slightly when reheated.[2] Heat aggregated IgG

* Abbreviations: IgG, immunoglobulin G; "frozen", frozen and thawed; "aged", frozen and thawed and after that aged.

globulins can usually fix complement [1-4] whether heated in the presence or absence of glycine.[1]

Most of the aggregates formed by IgG when frozen have *s*-values of 10 S and 12 S.[5] The proportion of the molecules that aggregate by freezing tend to vary with the electrophoretic mobility of the original population of molecules.[5] Unaggregated molecules that are separated from those that have aggregated by the freezing procedure and refrozen aggregate to the same extent as the original population of IgG molecules.[5] The aggregation by freezing is effectively inhibited by glycine.[6]

Differences in the properties of aggregates formed by freezing and by heating immunoglobulin G may be due to differences in the denaturation of IgG molecules by heating and by freezing. This paper is concerned with a comparison of the capacity of IgG globulins to aggregate by freezing and by heating under otherwise identical conditions.

MATERIALS AND METHODS

Sera. Fresh sera from normal persons, fresh sera with M-components of type IgG (20, 21, 22, and 25) and frozen sera with M-components of type IgG were used.

Isolation of IgG from normal and from myeloma sera. Method I: 4 ml of serum was mixed with an equal volume of 0.02 M phosphate buffer, pH 8.0, and placed on a column, 3.3 in diameter and 40 cm high, containing DEAE-Sephadex A 50 equilibrated with the same buffer. Elution was done with 0.02 M phosphate buffer pH 8.0 according to Högman and Killander.[7] The fractions were dialysed against phosphate buffer pH 7.0 containing 0.5 M NaCl.

Method II. A volume of serum was diluted with three parts of 0.2 M NaCl. The solution was made 1.84 M in respect of ammonium sulphate and the pH was adjusted to 7.0. The precipitate was centrifuged down and was then dissolved and fractionated in the way described previously on DEAE cellulosa and Sephadex G 200.[8]

The globulins 12, 20, 21, and 22 were isolated from the serum according to method I. Other globulins were isolated according to method II.

Analytical methods. The protein concentration was calculated from light absorption at 280 nm in phosphate-NaCl buffer at pH 7.0. The reproducibility of the method was better than ±3 %.

Agar gel electrophoresis was run in agarose (1 %) in diemal buffer at pH 8.6.

Immunoelectrophoresis was performed according to Scheidegger.[9] As antiserum goat antihuman total serum was used.

The *s*-values and the proportions between 7 S molecules and aggregates were determined in the way described previously.[5,8] The speed of the centrifuge varied between 15 220 and 59 780 rpm.

Treatment. IgG samples to be "frozen" were subjected 10 times to the following procedure. The sample was stored for 2 h at −20°C and then allowed to thaw and reach room temperature. The amount of the aggregate formed on exposure of the globulins to the cold, varies both with the number of times the solution is frozen and thawed and the duration of storage at −20°C.[5] Therefore when a higher degree of aggregation was desired the samples were also stored for 3−12 months at −20°C ("aged").

IgG solutions to be heat aggregated were placed in 3 ml glass tubes and heated in a water bath at 63°C for 15 min and allowed to cool to room temperature.

When IgG samples were both frozen and heated, the precipitate that formed on exposure to the cold was not removed before subsequent heating of the sample.

The precipitate formed during heat treatment was dissolved in the following way: The precipitate was centrifuged down at 5 000 rpm washed in 0.2 M NaCl and resuspended in 0.2 M NaCl to the initial volume.

RESULTS

Freezing and heating of normal IgG. 32—42 % of the molecules in 4 normal IgG (10, 11, 12, and 13) aggregated when the preparations were "aged". None of the freshly prepared globulins aggregated when heated. All of the "aged" preparations aggregated when heated.

The results of ultracentrifugal analysis of "aged" and heated preparations (14, 15, 16, 17, and 18) are given in Table 1. In all of the "aged" globulins

Table 1. Ultracentrifugal analysis of "aged" and heated normal IgG.

Step	Treatment	Sample	Distribution				
			7 S %	10 S %	12 S %	>12 S %	Precipitation %
I	Freezing and thawing and storage at −20°C						
		14	73	24	3	0	—[a]
		15	62	31	7	0	—
		16	75	21	4	0	—
		17	66	28	6	0	—
		18	68	26	6	0	—
II	Heating of I						
		14	91	0	0	0	9
		15	87	0	0	0	13
		16	76	0	0	0	24
		17	84	0	0	0	16
		18	76	0	0	0	24

[a] — not examined.

more than 25 % of the molecules formed soluble aggregates (10 S and 12 S). None of the "aged" proteins contained soluble aggregates after they had been heated. All of the "aged" preparations, however, formed precipitates when they were heated. The amount of precipitate was larger than that of the 12 S molecules, but less than the total amount of the soluble aggregates found in the preparations that had only been "aged". In this connection it might be mentioned that soluble aggregates again formed when the "aged" and heated preparations were re-frozen.

Freezing and heating of myeloma IgG. Table 2 gives the results obtained on ultracentrifugal analysis of some myeloma preparations treated in the same way as the normal globulins listed in Table 1. In all the "aged" preparations more than 13 % of the molecules had formed soluble aggregates (10 S and 12 S). The "aged" preparation 25 contained no protein in solution and preparation 26 only very little, when heat treated. The "aged" globulin 28 contained no precipitate when heated. The precipitate in the "aged" prepara-

Table 2. Ultracentrifugal analysis of "aged" and heated myeloma IgG.

Step	Treatment	Sample	Distribution				
			7 S %	10 S %	12 S %	>12 S % (S)	Precipitation %
I	Freezing and thawing and storage at −20°C						
		23	73	23	4	0	—[a]
		24	69	24	7	0	—
		25	87	13	<1	0	—
		26	72	25	3	0	—
		27	85	15	0	0	—
		28	79	18	3	0	—
II	Heating of I						
		23	82	0	0	8 (18)	10
		24	28	1	0	0	71
		25	0	0	0	0	100
		26	8	0	0	0	92
		27	68	8	2	0	22
		28	93	3	0	7 (30)	0
III	Heating of I containing 0.09 M glycine						
		23	86	0	0	18 (18)	0
		24	37	0	0	24 (54)	39
		25	0	0	0	0	100
		26	13	0	0	0	87
		27	74	7	2	0	17
		28	87	<1	0	0	3

[a] — not examined.

tion, whose amount was not measured, dissolved when the globulin was heated. Four of the "aged" preparations contained soluble aggregates after they had been heated.

Three myeloma preparations (20, 21, and 22) were used in the investigation accounted for in Table 3. In the preparations 5—9 % of the molecules aggregated when the solutions were "frozen". None of the freshly prepared globulins aggregated when heated. None of the protein solutions contained aggregates after they had been "frozen" and afterwards heated. An increased or equally large amount of aggregates formed when the globulins were first heated and then "frozen". For comparison it might be mentioned that 30—40 % of the molecules aggregated when the "frozen" protein solutions 20, 21, and 22 were allowed to age. The "aged" preparation 20 formed precipitates when heated. "Aged" preparations 21 and 22 formed no precipitates when heated.

Table 3. Ultracentrifugal analysis of IgG M-components after combined freezing and heating.

Step	Treatment	Sample	Distribution			
			7 S %	10 S %	12 S %	Precipitation %
I	Freezing and thawing					
		20	94	6	0	<1
		21	91	9	0	0
		22	95	5	0	0
II	Heating					
		20	100	0	0	0
		21	100	0	0	0
		22	100	0	0	0
III	Heating of I					
		20	100	0	0	0
		21	100	0	0	0
		22	100	0	0	0
IV	Freezing and thawing of II					
		20	73	24	3	<1
		21	89	11	0	0
		22	93	7	0	0

Table 4 gives the results of an investigation of the reproducibility of the results obtained by methods used for heat aggregation and ultracentrifugal analysis. Heat treatment and a subsequent ultracentrifugal analysis of the "aged" myeloma preparations given in Table 2 were repeated once more with another aliquot of the same frozen sample. The table shows that the reproducibility was good.

Table 4. Reproducibility of results obtained by methods used for heating and for ultracentrifugal analysis.

Sample	% 7 S		% 10 S		% 12 S		% > 12 S		Precipitate	
	Test I	II	Test I	II	Test I	II	Test I	II	Test I	II
23	82	82	0	0	0	0	8	11	10	7
24	28	28	1	0	0	0	0	<1	71	72
25	0	0	0	0	0	0	0	0	100	100
26	8	11	0	0	0	0	0	0	92	89
27	68	68	8	8	2	2	0	0	22	22
28	93	94	3	3	0	0	7	7	0	0

Effect of glycine. The effect of glycine on aggregation by heating is given in Table 2. Glycine was added to the "aged" myeloma preparations before they were heated. The composition of preparations 25, 26, 27, and 28 was not appreciably affected by the presence of glycine during heat treatment. Globulin 23 contained the same number of 18 S molecules when it was heat treated in the presence of glycine as of 18 S molecules plus precipitate when heated in the absence of glycine. Globulin 24 contained more 7 S, many more 54 S molecules, and a smaller amount of precipitate after heat-treatment in the presence than in the absence of glycine.

The aggregation of myeloma globulins 20, 21, and 22 after addition of glycine was also studied (*cf.* Table 3). After freezing and thawing of the globulins in the presence of glycine extremely small amounts of aggregates appeared in preparation 20 and only 7 S molecules in the other preparations. The composition was not changed by subsequent heating. No aggregation occurred when the protein solutions containing glycine were heated nor when they were afterwards "frozen".

Solubility of precipitate. Precipitates formed by the heat treatment of "aged" myeloma proteins 25 and 26 were found to be soluble in 6 M urea. Dissolution of precipitates of globulin 25 resulted in only 3 S molecules, which again formed a precipitate when the urea solution was dialysed against phosphate buffer. Dissolution of the precipitate from globulin 26 resulted in a series of components with s-values of 7, 6, 5, 3, and less than 3. All components broke down to molecules of less than 3 S when the urea solution was dialysed against phosphate buffer. Precipitates from preparation 25 were not soluble in glycine buffer, pH 3.0, but precipitates from preparation 26 were. 7 S molecules and soluble aggregates larger than 12 S formed. When glycine solution 26 was dialysed against phosphate buffer 21 % persisted as 7 S molecules. None of the precipitates proved soluble in 5 % NaCl or glycine at pH 7.0.

DISCUSSION

None of the three myeloma globulins nor of the four normal globulins isolated from sera according to two methods aggregated when heated directly after they had been prepared. That the freshly prepared myeloma globulins did not aggregate might perhaps be explained by the fact that they belonged to the group of myeloma IgG which according to Laurell and Nilsson [3] and Morse [2] do not aggregate when heated. The inability of freshly prepared normal IgG globulins to aggregate could, however, not be explained in this way because all of the normal IgG globulins studied by Laurell and Nilsson and by Morse aggregated to 15—20 % when heated. The difference between the results obtained in this investigation and those reported by other workers in this field may be due to differences in preparative methods used or conditions for storage of fractions. Most of the myeloma IgG and all of the normal globulins aggregated when they were heated after first having been "aged".

The myeloma preparations 20, 21, and 22 that did not aggregate when heated formed largely equal amounts of soluble aggregates when "frozen". When they were heated before they were "frozen" the amounts of soluble

aggregates formed was larger or unchanged. The results showed that heat treatment can affect IgG molecules without aggregating them. If myeloma IgG consist of relatively homogeneous components the result suggests that individual IgG molecules are affected in different ways.

The freshly prepared myeloma proteins aggregated when frozen but not when heated. This suggests that different types of bonds are affected or that the molecules are not affected to the same extent when frozen as when heated.

Soluble and insoluble aggregates formed on freezing of the three myeloma preparations were converted entirely to 7 S molecules when heated. This showed that intermolecular bonds formed on denaturation by freezing can be broken by heat treatment and that such bonds need not cause aggregation when the globulins are heated.

Even when the three myeloma preparations were "aged" and thus denatured to a greater extent then when only "frozen", two of them showed the same tendency to convert soluble and insoluble aggregates into 7 S molecules on subsequent heating. One of them (No. 20), however, formed a precipitate when heated. This showed that the bonds causing aggregation by heating and that are exposed only after subsequent severe freezing do not occur in all myeloma IgG or are more inaccessible in some. They do, however, occur in all normal IgG, because all "aged" normal globulins formed precipitates when heated.

Aggregates formed by heating were often insoluble. After being heated some "aged" myeloma preparations contained small amounts of soluble aggregates as well as precipitates. This is in agreement with the results obtained by Laurell and Nilsson.[3] The aggregates formed when the globulins were frozen always consisted mainly of 10 S and 12 S molecules and the amount of material precipitated was insignificant. IgG contains unusually large amounts of amino acids with nonpolar side chains.[10,11] Hydrophobic bonds are presumably not the main cause of aggregation when globulins are frozen.[6] Intramolecular hydrophobic bonds could thus be exposed after freezing but are not broken by this treatment. This would be possible only when the preparations are heated.

That the bonds, which initially caused aggregation when the preparations were heated differed in type from those that caused aggregation when the preparations were frozen was shown by the examination of the effect of glycine on aggregation of globulins when heated. Equal amounts of aggregates formed when the IgG solutions were heated in the presence and in the absence of glycine. Globulins, which formed aggregates larger than or as large as 18 S when glycine was absent also formed such aggregates when glycine was present. The amount of 18 S molecules increased, however, at the same time as the amount of precipitate decreased. The results agree with those reported by Frommhagen and Fudenberg [1] and showed that some of the bonds that take part in the growth in size of the aggregates can be affected by glycine and may be of the same type as those that caused aggregation on freezing and whose formation is inhibited in the presence of glycine.[6]

The amount of aggregates formed when myeloma preparations were heated varied. Individual differences were also found in two myeloma globulins which formed about the same amount of aggregates when heated because the

precipitates from two such globulins differed in solubility in glycine at pH 3.0 and formed different components when dissolved in 6 M urea.

Acknowledgement. This investigation was supported by grants from the *Swedish Medical Research Council* (Project No. K 67—13X—581—03B) and *Anna-Lisa o. Sven E. Lundgrens Stiftelse.*

REFERENCES

1. Frommhagen, L. H. and Fudenberg, H. *J. Immunol.* **89** (1962) 362.
2. Morse, J. H. *J. Immunol.* **95** (1966) 722.
3. Laurell, A.-B. and Nilsson, U. *Acta Pathol. Microbiol. Scand.* **59** (1963) 79.
4. Cooper, R. G. *J. Lab. Clin. Med.* **63** (1964) 404.
5. Hansson, U.-B. *Acta Chem. Scand.* **22** (1968) 483.
6. Hansson, U.-B. *Acta Chem. Scand.* **22** (1968) 490.
7. Högman, C. F. and Killander, J. *Acta Pathol. Microbiol. Scand.* **55** (1962) 357.
8. Hansson, U.-B., Laurell, C.-B. and Bachmann, R. *Acta Med. Scand. Suppl.* **445** (1966).
9. Scheidegger, J. J. *Intern. Arch. Allergy Appl. Immunol.* **7** (1955) 103.
10. Brand, E. *Ann. N. Y. Acad. Sci.* **47** (1946) 187.
11. Kauzmann, W. *Advan. Protein Chem.* **14** (1959) 37.

Received October 30, 1967.