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Inhibition and Reversal of Aggregation of Immunoglobulin G by Freezing

ULLA-BRITT HANSSON

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

Solutions of IgG (about 1 g/100 ml) from normal subjects form between 10 and 50 % aggregates (10 S, 12 S and precipitation) on repeated freezing and thawing. Aggregation was effectively prevented by glycine in concentrations of about 0.1 mole per liter, both at pH 7.0 and 3.0. After the IgG-glycine solution had been dialysed against a glycine-free buffer the IgG again formed aggregates upon freezing.

Aggregation did not occur in IgG solutions containing plasmin. Components smaller than 7 S were formed. Traces of aggregates were formed in a 5 % NaCl-solution and the 7 S component became unusually inhomogeneous. Proline, serine, arginine, albumin, and fibrinogen retarded the aggregation. The amount of aggregate remained unchanged when iodo-acetamide was added. An increase of percentage of aggregates was noted when IgG was dissolved in phosphate buffer containing HAc or in phosphate buffer at pH 3.0. When IgG was dissolved in acetate buffer at pH 3.0 the solution became so viscous after freezing that its composition could not be studied.

Glycine buffer at pH 3.0 had the ability to convert the aggregate to smaller molecules. A homogeneous 7 S peak persisted after the solution had been dialysed against phosphate buffer at pH 7.0. Also in urea the aggregated molecules were broken down into smaller components but reaggregation occurred in urea-free phosphate buffer. In glycine buffer at pH 5.8 part of the aggregate was broken down to 7 S molecules but after dialysis against glycine-free buffer the effect was negligible. NaCl did not affect the aggregated IgG molecules.

Aggregation and disaggregation of immunoglobulins of type G (IgG) have been observed under various conditions and in the presence of various substances. The tendency of IgG to aggregate when heated is not appreciably decreased by the addition of albumin,[1] glycine, or 0.3 M NaCl, but when IgG is dissolved in 2 M NaCl, no aggregation occurs.[2] *In vivo* aggregated myeloma protein of type IgG dissociates at high salt concentration and in glycine-HCl buffer at pH 4.0.[3] The γ-globulin complex in the sera from patients with rheumatoid arthritis dissociates in glycine-HCl buffer at pH 3.0,[4] as do the precipitates occurring when aggregated IgG is added to sera from patients

with rheumatoid arthritis.[5-7] The aggregates formed when IgG is heated do not dissociate in acid environments or in the presence of urea.[8] Myeloma IgG aggregates at pH levels below 4.[9] The γ-globulin complex in sera from patients with rheumatoid arthritis dissociates in the presence of urea.[4]

When IgG is frozen and thawed or when it is stored at -20°C, aggregation also occurs.[10] This paper is concerned with an investigation of the ability of various substances to influence the formation of these aggregates and their dissociation in order to find out whether IgG can be stored at -20°C without aggregation and to estimate the possibilities of converting the aggregates to 7 S molecules.

MATERIALS AND METHODS

Sera. Fresh sera from apparently healthy subjects.

Isolation of IgG from normal individual sera. One part of serum was diluted with 3 parts of 0.9 % NaCl. The solution was made 1.84 M in respect of ammonium sulphate and the pH adjusted to 7. The precipitate formed was dissolved and fractionated as previously described [11] on DEAE-cellulose and Sephadex G 200.

The protein concentration was determined by the method described earlier.[10]

Freezing and thawing. The sample was stored for 2 h at -20°C and afterwards allowed to stand until it reached room temperature.

Ultracentrifugation. A Spinco Analytical Ultracentrifuge with Schlieren optics was used.

Calculation of s*-values in water at 20°C* (s_{20w}). The s-values of various components of the aggregated IgG preparations were determined according to Schachmann [12] and Janke and Scholtan.[13] The components given as 10 S had s-values between 9.5 and 10.5. The components given as 12 S had s-values between 11.5 and 12.5.

Determination of ratio between 7 S molecules and aggregates was done as previously described.[10]

Addition of various substances or dialysis against various buffer systems was always done on freshly prepared IgG. The volume of the starting preparation, the preparation with addition and the dialysed preparations was adjusted to uniform IgG concentration. All the samples were frozen at the same time. They were also thawed simultaneously.

RESULTS

Table 1 shows the ability of some substances to influence the formation of aggregates in association with the freezing and thawing of two separate normal IgG preparations. No aggregation occurred in any of the IgG solutions that were 0.09 M in respect of glycine. Equally large amounts of aggregate formed in the solution containing 0.009 M glycine, in the solution containing iodoacetamide, and in the solution without any addition. Aggregation was more marked in a solution containing HAc than in one without HAc.

Table 2 shows the composition of two other normal IgG preparations that were frozen until more than 30 % of the molecules of the preparations without addition were larger than 7 S. Aggregation did not occur in solutions containing glycine at pH 3.0. Practically only 7 S molecules occurred in the solutions containing glycine at pH 7.0, but very small amounts of precipitate were seen in the solutions. The composition could not be studied in 0.1 M acetate buffer at pH 3.0 because both IgG solutions became highly viscous. 65 % of C and 54 % of D was precipitated in 0.1 M phosphate buffer solution at pH 3.0. For comparison it might be mentioned that 78 % of D consisted of 7 S mole-

Table 1. Ratio between aggregates and 7 S molecules in IgG solutions frozen in presence of various substances.

IgG	Medium			Distribution [c]		
Prep.	pH	Solvent	Added subst.	7 S %	10 S %	12 S %
A [a]	7.0	0.05 M phosphate NaCl [d]	0	85	15	0
»	7.0	»	0.009 M glycine	88	12	0
»	7.0	»	0.09 »	100	0	0
»	7.0	»	0.12 mM iodoacetamide	88	12	0
»	6.7	»	0.09 M acetic acid	77	21	2
B [b]	7.0	»	0	84	16	0
»	7.0	»	0.09 M glycine	100	0	0

[a] Protein conc. 1.2 g/100 ml = 7×10^{-3} mM.
[b] » » 0.8 g/100 ml = 4.8×10^{-3} mM.
[c] After the solutions had been frozen and thawed once.
[d] 0.5 M in respect of NaCl.

cules, 12 % of 10 S molecules, and 10 % of 12 S molecules in a clear solution before it was frozen in phosphate buffer at pH 3.0. D contained 100 % 7 S molecules in a non-viscous solution before it was frozen in acetate buffer at pH 3.0. The aggregate formation of D was almost entirely prevented by serine but only reduced in C, from 42 % to 14 %. Arginine reduced the amount of aggregates formed in D from 38 to 12 % and in C from 42 to 15 %. In a proline-containing solution C formed 12 % molecules larger than 7 S. Albumin retarded the aggregation of both C and D. Fibrinogen retarded the formation of aggregates in C but to a less extent than albumin. In a plasmin-containing solution there were no aggregated molecules but, instead, about 10 % of a component with an s-value of less than 7. In NaCl-solution C formed no measureable amounts of aggregate, but the solution was opalescent and on ultracentrifugation a distinct asymmetry of the 7 S peak was noted. In urea C formed 98 % of a component with an s-value of less than 7 and only 2 % persisted as 7 S molecules.

The investigation included also a study of the capacity of four other individual normal IgG preparations to form aggregates in the presence of glycine at pH 7.0. After the addition of glycine the IgG preparations were frozen and thawed 10 times. After this treatment three of the preparations contained only 7 S molecules in clear solution, while the fourth contained 7 S molecules in a slightly opalescent solution. When frozen and thawed in the absence of glycine the four IgG preparations formed between 12 and 26 % aggregates.

A frozen IgG solution (E) containing glycine contained no aggregates after dialysis against a glycine-free buffer. After the dialysed solution had been frozen and thawed 10 times and stored for 2 months at −20°C, it contained 40 % aggregates. A solution of E that had never contained glycine and that was frozen and thawed 10 times and then stored at −20°C for 6 months contained 49 % aggregates.

Table 2. Ratio between aggregates and 7 S molecules after addition of various substances to an aggregated IgG solution.

	Medium				Distribution [c]				
IgG prep.	pH	Solvent	Added subst.		< 7 S %	7 S %	10 S %	12 S %	Precipitation [d]
C [a]	7.0	0.05 M phosphate NaCl [f]	0		0	58	32	10	Traces
»	7.0	»	0.09 M	glycine	0	100	0	0	»
»	7.0	»	0.09 M	serine	0	86	14	0	0
»	7.0	»	0.09 M	arginine	0	85	15	0	Traces
»	7.0	»	0.09 M	proline	0	88	12	0	0
»	7.0	»	1.5×10^{-2} mM	albumin	—	86	14	0	0
»	7.0	»	5×10^{-3} mM	fibrinogen	0	76	23	1	0
»	7.0	»	0.6 mM	plasmin	10	90	0	0	0
»	3.0	0.1 M glycine-HCl	0		0	100 [e]	0	0	0
»	3.0	0.1 M acetate	0		—	—	—	—	0
»	3.0	0.1 M phosphate	0		0	26	6	3	65
»	—	0.85 M NaCl	0		0	100	0	0	Traces
»	—	6 M urea	0		98	2	0	0	0
D [b]	7.0	0.05 M phosphate NaCl [f]	0		0	62	33	5	0
»	7.0	»	0.09 M	glycine	0	100	0	0	Traces
»	7.0	»	0.09 M	serine	0	100	0	Traces	0
»	7.0	»	0.09 M	arginine	0	87	13	0	0
»	7.0	»	1.5×10^{-2} mM	albumin	—	93	7	0	0
»	3.0	0.1 M glycine-HCl	0		0	100	0	0	0
»	3.0	0.1 M acetate	0		—	—	—	—	Traces
»	3.0	0.1 M phosphate	0		0	40	6	0	54

— Not performed.
[a] Protein conc. 0.6 g/100 ml = 3.7×10^{-3} mM.
[b] Protein conc. 0.8 g/100 ml = 4.8×10^{-3} mM.
[c] After the solutions had been frozen and thawed some times.
[d] Traces = opalescent; numeral = % precipitate.
[e] Inhomogeneous.
[f] 0.5 M in respect of NaCl.

Table 3. Ratio between aggregates and 7 S molecules in IgG solutions frozen in presence of various substances.

Sample		Treatment	Distribution				
			<7 S %	7 S %	10 S %	12 S %	Precipitation [b]
I		Original solution in 0.05 M phosphate NaCl [a] pH 7.0	0	75	22	3	Traces
		After dialysis of I against					
II	a	6 M urea	100	0	0	0	0
	b	5 % NaCl	0	75	22	3	Traces
	c	0.1 M glycine pH 5.8	0	87	8	5	»
	d	0.1 M » pH 3.0	100	0	0	0	0
		After dialysis of II against 0.05 M phosphate NaCl [a] pH 7.0					
III	a		0	52	0	0	48
	b		—	—	—	—	—
	c		0	82	18	0	Traces
	d		0	100	0	0	»

— Not performed. [a] 0.5 M in respect of NaCl. [b] Traces = opalescent.

Table 3 shows the ability of some substances to convert aggregates to 7 S molecules. An aggregated IgG (F) containing 76 % 7 S-, 22 % 10 S-, and 3 % 12 S molecules and traces of insoluble aggregate in phosphate-NaCl buffer was used as a starting material. After dialysis against urea there were only molecules with s-values below 7, and the solution was clear. When the urea solution had been dialysed against phosphate-NaCl buffer, 52 % of the sample consisted of 7 S molecules and 48 % of insoluble aggregates. Dialysis of the aggregated immunoglobulin F against glycine buffer at pH 3.0 gave a clear solution containing only molecules with an s-value below 7. When these returned to the original medium the solution contained only 7 S molecules but was opalescent. Glycine at pH 5.85 also tended to convert aggregates to 7 S molecules. The effect was, however, only slight and whether it persisted after the IgG had been returned to the original medium was doubtful. NaCl had no ability at all to convert formed aggregates to 7 S molecules.

DISCUSSION

In our investigation it was found that glycine and proline (amino acids with non-polar side chains), serine (amino acid with polar side chain), and arginine (diamino-monocarboxylic acid) can inhibit the tendency of frozen IgG to form aggregates. Glycine inhibited the formation of aggregates most effectively. No difference in this respect was found between the other amino acids. Immunoglobulin G contains unusually large amounts of amino acids with non-polar side chains.[14] That glycine and proline were able to retard

aggregation could be explained by the stabilising effect of non-polar groups on hydrophobic bonds.[15]

The stabilising effect of amino acids might also depend on salt effects. That electrolytes stabilised the types of bonds responsible for the formation of aggregates upon freezing was apparent from the fact that no aggregates were formed when IgG was frozen in 5 % NaCl and that aggregates formed in association with freezing did not convert to 7 S molecules in this medium. But the amino acids are weak electrolytes and their concentrations were almost only one-tenth of the concentration of NaCl in the above-mentioned NaCl solutions.

If the freezing process breaks hydrogen bonds between the oxygen atom of carboxyl groups and hydrogen atoms of amino-groups the amino- or carboxyl groups in the added amino acids would be able to form hydrogen bonds with groups in the protein, thereby preventing the formation of intermolecular bonds. Also the proteins, albumin and fibrinogen, suppressed aggregation of IgG. Albumin was roughly just as effective as proline, serine, and arginine, whereas fibrinogen was somewhat less effective. The effect of the proteins might be explained in the same way as that of the amino acids. The lower stabilising effect of fibrinogen might be explained by the fact that it occurred in a lower concentration than the other substances. The difference in proportion of formed aggregates in an IgG solution without addition and in one containing amino acid or protein was about 30 %. The difference between an IgG solution to which amino acid (not glycine) or protein had been added and one containing glycine was about 15 %. Glycine is a smaller molecule than the others. One might therefore imagine the glycine to be bound at points not accessible to larger molecules. This, however, did not appear likely because intermolecular bonds could occur between two or more IgG molecules also in the presence of small amino acids and protein molecules.

The investigation also showed that IgG when frozen in the presence of glycine at pH 7.0 formed traces of aggregates of such a size that they appeared as a precipitate. When IgG was frozen in the presence of glycine at pH 3.0, on the other hand, no precipitation occurred and the solution contained only 7 S molecules. The type of bond that caused aggregation at pH 7.0 but not at pH 3.0 was not affected by glycine. It might be the same as that causing precipitation in the 5 % NaCl solution. It might also be of the same type as that causing aggregation of isolated heavy IgG chains, because the above-mentioned precipitates were found to possess the same properties as purified heavy chains from IgG.[16]

The examination also showed that iodo-acetamide did not affect the aggregation of IgG and that aggregation did not occur in an IgG solution containing plasmin. In the IgG-plasmin solution only degradation products of IgG occurred. This was in agreement with the known fact that proteins in native form usually are more resistant to proteolytic enzymes than denatured proteins are.[17]

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