The Structure and Stability of Human Plasma Cold-insoluble Globulin*

(Received for publication, July 28, 1978)

Steve S. Alexander, Jr., Giovanni Colonna,‡ and Harold Edelhoch

From the National Institute of Arthritis, Metabolism and Digestive Diseases, Clinical Endocrinology Branch, National Institutes of Health, Bethesda, Maryland 20014

The molecular properties of cold-insoluble globulin have been investigated by velocity centrifugation, circular dichroism, and fluorescence at neutral and alkaline pH. The stability of the protein to thermal and guanidine hydrochloride has been evaluated under both conditions. The close parallelism between the properties of cold-insoluble globulin and those of the cell surface protein (fibronectin) serve to establish the essential identity of the structures of the two proteins derived from different sources. It is suggested that the cold-insoluble globulin is composed of several domains connected by flexible polypeptide segments. The large increase in the frictional ratio observed between pH 7.0 and 11.0 can be explained by an expansion of the flexible segments without significant change in the domains. These domains are stable to about 55°C at pH 7.0 but only to about 40°C at pH 11.0.

Cold-insoluble globulin is a large glycoprotein found in considerable amounts in plasma (1). It appears to exist also, in a slightly modified form, associated with the cell surface of cultured fibroblasts and other cells (2). In this form, it has been referred to as large, external, transformation-sensitive, CSP, and fibronectin and has been shown to be responsible for many of the adhesive and morphological properties of various cells (3, 4). In a recent report, CIg and opsonic α2-glycoprotein were shown to have numerous properties in common (5). A physiological function for CIg appears to exist, therefore, which is very different from that of CSP.

CIg and CSP have certain properties in common (i.e., molecular weight, subunit size, amino acid composition) but reveal a few differences, especially in solubility. In addition, the NH2-terminal group is blocked in CSP, whereas in CIg it is glutamic acid (6, 7). In order to define more precisely the relationship between these two proteins, we have investigated several spectroscopic properties since these should be very sensitive to the folding and interactions of specific chromophoric residues and should afford further criteria for establishing the similarity or identity of the conformations CIg and CSP. Since CIg is much more soluble and better behaved than CSP, it was possible to measure various properties of CIg at neutral and alkaline pH in order to evaluate the effect of pH on its structure. CSP is not readily soluble at neutral pH and has only been studied in alkaline solution (8, 9).

EXPERIMENTAL PROCEDURES

Methods

Cold-insoluble globulin from human plasma was provided by Dr. Ron Chung of the National Institute of Dental Research. The isolated protein exhibited a single band by polyacrylamide gel electrophoresis corresponding to the dimer (~440,000) (6, 10). CIg was further characterized by rocket immunoelectrophoresis. In some cases, trace amounts of the B chain of Factor XIII could be detected but were easily removed by gel filtration. The purity of the CIg preparation was >98%.

Protein concentration was determined from absorbance measurements at 280 nm with a 1-cm path length cell using the Ermyml value of 1.28 reported by Mosesson and Umfleet (1).

Fluorescence Measurements—Fluorescence measurements were performed with a Perkin-Elmer MPF3 spectrofluorometer equipped with Hitachi polarizers. Excitation and emission wavelengths were 280 and 330, respectively, for tryptophan.

The polarization was calculated using the formula P = (Zex - GZem)/(Zex + GZem) where G = Zex/Ze, I is the intensity, and the first and second subscripts refer to the plane of polarization of the excitation and emission beams.

Ultracentrifugation—Sedimentation velocity experiments were performed in a Beckman model E analytical ultracentrifuge equipped with schlieren optics at 25°C. Double sector cells were used one of which contained a wedge window.

Sedimentation coefficients were calculated according to Svedberg and Pedersen (11) and corrected to H2O at 20°C. A value of 0.72 for the partial specific volume, υ, was calculated from the amino acid and carbohydrate composition (6) according to the procedures of Cohn and Edsall (12) and Gibbons (13).

CD Measurements—CD measurements were carried out on a Cary model 60 spectropolarimeter, equipped with a temperature controlled cell holder. Mean residue ellipticities were calculated by

\[
[\theta] = \frac{MRW (\theta_{obs})}{10 I c}
\]

where [θ] is the mean residue ellipticity at a particular wavelength λ, \( \theta_{obs} \) is the observed ellipticity, MRW is 108, the mean residue molecular weight calculated from the amino acid composition; I to the optical path length (cm) and c is the concentration in grams per ml.

Materials

Guanidine hydrochloride was ultrapure grade from Schwarz/Mann and used without further purification. All other chemicals were reagent grade.

RESULTS

Secondary Structure

The secondary structure of CIg at pH 7.0 was evaluated from the circular dichroism of the peptide group in the wavelength region of its absorption, i.e., below 240 nm. The CD spectrum contained two relatively weak extrema, a maximum at 227 nm and a minimum at 213 nm (Fig. 1). This spectrum

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address, Institute of Biological Chemistry, First Faculty of Medicine, University of Naples, Italy.

The abbreviations used are: CSP, cell surface protein; CIg, cold-insoluble globulin; CD, circular dichroism; GdmCl, guanidine hydrochloride.
CIg had almost no effect on the far ultraviolet CD spectrum of CIg. The far ultraviolet CD spectra (mean residue ellipticity) of CIg (1.02 mg/ml) in water at pH 7.0, 0.05 M phosphate, 0.10 M KCl, and 0.10 M phosphate, pH 7.0. Since no increase in sedimentation boundary was observed under all conditions. The concentration dependence of the sedimentation velocity of CIg was determined at pH 7.0, 9.0, and 11.0 in 0.05 M phosphate, 0.10 M KCl. A linear, negative dependence of the sedimentation coefficient on concentration was observed at each pH, although the negative slope increased with increasing pH (Fig. 2). The sedimentation constants (\( s_{0.5} \), \( w \)) decrease with increasing pH, especially between pH 9.0 and 11.0. Fractional ratios of 1.7, 1.9, and 2.8 were calculated from the sedimentation constants, i.e. 13.0, 11.8, and 8.0, respectively, using a molecular weight of 440,000 and a partial specific volume of 0.72. It is evident that CIg is not globular at neutral pH and undergoes considerable expansion at pH 11.0.

In order to see if there was a significant electrostatic component to the large frictional ratio at neutral pH, the effect of ionic strength on the sedimentation rate of CIg was evaluated. The sedimentation constant (\( s_{0.5} \), \( w \)) fell linearly with ionic strength from 13.6 at 0.05 M KCl to 10.8 at 0.40 M KCl, in 0.005 M phosphate, pH 7.0. Since no increase in sedimentation rate was observed with ionic strength, an electrostatic expansion of CIg at neutral pH can be ruled out as an explanation of its large frictional ratio. The reason for the substantial increase in frictional ratio with ionic strength is not evident.

Circular Dichroism—The optical activity of the aromatic chromophores of CIg was evaluated in the near ultraviolet by CD (Fig. 3). These residues are important determinants of protein structure because they are strongly hydrophobic (17), and the unusual high wavelength of the \( ^{1}L_{A} \) band centered at 299 nm probably arises from hydrogen bonding to a strong acceptor group, i.e. carboxylate or imidazole (19-21).

The spectrum shows two negative peaks centered at 291 and 299 nm and a weaker negative peak at 282 nm. The latter is reduced in half by pH 9.9 and essentially disappears by pH 11.0 (Fig. 3). The 282 nm peak is therefore probably due to undissociated tyrosyl groups which lose their optical activity when ionized in alkali. Of the two longer wavelength peaks, only the one at 299 nm changes slightly in alkali. The stability of these two peaks in alkali suggests that the environments of these optically active chromophores remain relatively constant between pH 7.0 and 11.0 at 25°C. The peaks at 291 and 299 nm can only be due to tryptophan residues and can be assigned to the \(^{1}L_{B} \) and \(^{1}L_{A} \) transitions of the indole chromophore, respectively (18). The unusual high wavelength of the \(^{1}L_{A} \) band centered at 299 nm probably arises from hydrogen bonding to a strong acceptor group, i.e. carboxylate or imidazole (19-21).

Structural Transitions

In the absence of a high \( \alpha \) helical content for the peptide group, it is unlikely that CIg is a rod-like molecule. The large frictional ratio at neutral pH could therefore represent either a random polypeptide chain cross-linked by disulfide bonds or a structure composed of globular domains connected by random polypeptide segments. In order to determine whether globular domains are present, the effects of temperature and GdmCl on CIg were evaluated since noncovalent interactions in proteins are eliminated by these two denaturing conditions.

Temperature—The temperature dependence of polarization has been used to measure the relaxation times of proteins by application of the Perrin equation (22). The relaxation time could not be measured by this procedure since the
Human Plasma Cold-insoluble Globulin

The effect of temperature on the tryptophanyl polarization of CIg at pH 7.0 (○) and 11.0 (●). Excitation was at 280 nm and emission at 330 nm. Samples contained 0.05 M phosphate, 0.10 M KCl (0.05 mg/ml).

The lifetime of tryptophanyl fluorescence! of native CIg is too small for its size. No change in tryptophanyl polarization was observed between 20 and 50°C at pH 7.0 (Fig. 4). Moreover, 90% ethylene glycol also had no effect on the polarization. The value of the polarization of native CIg is therefore very close to its limiting value, i.e. that observed in the absence of rotational events within the lifetime of the excited state, and precludes a random polypeptide structure for CIg. The limiting value of tryptophan polarization observed in several low molecular weight polypeptide hormones known to have little or no organized structure (adrenocorticotropic hormone, calcitonin, parathyroid hormone, glucagon) is much lower, i.e. near 0.04 at 25°C. When CIg was lightly, covalently labeled at pH 8.5 with a few molecules of 1-dimethylaminonaphthalene-8-sulfonyl in order to increase the fluorescence lifetime, the solubility decreased and solutions did not remain stable during manipulations.

The polarization of tryptophanyl emission was useful, however, to follow the transition of CIg to less ordered conformational states. The polarization of CIg at pH 7.0 falls rather precipitously between 50 and 60°C (Fig. 4). The polarization-temperature transition curve did not change significantly between pH 7.0 and 9.0. At pH 11.0, however, a marked change in the transition curve was observed. The polarization began to fall at much lower temperatures than at pH 7.0 and reached a value of 0.045 at 75°C (Fig. 4). Thus, the thermal transition was much broader and much more complete at pH 11.0 than at more neutral pH values.

The reduced stability to thermal unfolding at pH 11.0 observed by polarization was also seen by the change in the optical activity of the tryptophanyl chromophores. The effect of temperature on the 299 nm peak is shown in Fig. 5 for CIg solutions at pH 7.0 and 11.0. The data at pH 9.0 were similar to those at pH 7.0. The ellipticity at pH 7.0 does not change significantly until about 50°C in accord with the polarization data. At pH 11.0, however, the ellipticity curve is shifted to lower temperatures when compared with the pH 7.0 curve. The ellipticity values could not be obtained at higher temperatures than shown in Fig. 5 since the noise to signal ratio of the CD instrument became too large. This resulted from increasing turbidity as CIg was unfolded and denatured. The development of turbidity at pH 7.0 and 60°C was observable as a time-dependent increase in absorption at 330 nm by transmission measurements. At 55°C, no time effects were observed. The melting curve of the 291 nm peak did not coincide with that of the 299 nm peak at any pH. At pH 7.0 and 9.0 the relative magnitudes of the two peaks changed only above 55°C with the one at 291 nm decreasing earlier than that at 299 nm. At pH 11.0 the two peaks diverged above 30°C with the 291 nm peak changing sooner than the 299 nm peak. Since the high temperature limits could not be obtained the transition temperatures could not be evaluated. The difference in temperature between the 291 and 299 nm peaks at ellipticity values of 50% of their low temperature values is between 5 and 10°C at either pH 7.0 or 11.0.

The decrease in the transition temperature between pH 7.0 and 11.0 observed by tryptophanyl CD activity was also apparent in the far ultraviolet CD transition. Since the concentration of CIg was much lower there was almost no interference from turbidity effects. The effect of temperature on the 227 nm positive maximum of CIg at pH 7.0 and 11.0 is

2 H. Edelhoch, unpublished experiments.
ters, polarization and emission maximum, is shown in Fig. 7. The CD spectrum of the peptide group in a /I conformation. As observed by thermal denaturation, the two tryptophanyl CD peaks also do not melt-out at the same rate with GdmCl (Fig. 8). The 299 nm curve melts-out more slowly than the 291 nm curve and even shows some residual activity in 6 M GdmCl. The 291 nm peak is eliminated by 4 M GdmCl. The two peaks clearly represent tryptophanyl residues in different environments. Each of the peaks evidently monitors a different domain of the protein since their transitions are very distinctive. The transition curve of the 227 nm peak has also been plotted in Fig. 8 in order to compare it with the two aromatic peaks. It is evident that the curve for the 227 nm peak is very similar to that of the 291 nm peak at least above 1.6 M GdmCl.

It is of interest that the negative 291 nm peak increased in intensity by about 20% between 0 and 1.6 M GdmCl while that at 299 nm did not appear to change (see inset, Fig. 8). The increase in negative ellipticity could represent some minor rearrangement, i.e., refolding of one domain of the protein prior to the major unfolding that starts at 1.6 M GdmCl.

**DISCUSSION**

The various spectroscopic parameters of CIg that we have examined are unaffected by pH between 7.0 and 9.0. In contrast, there is a small but definite increase in frictional ratio. Since the spectroscopic properties depend on organizational constraints, i.e., secondary or tertiary structure, and the frictional properties depend on the dimensions of the protein, the change in frictional ratio should represent an expansion of the protein without loss in structure. This concept fits in with the structure proposed for CSP in which highly organized domains are linked by random polypeptide segments which results in a molecule with considerable flexibility and a high frictional ratio (8, 9).

The small increase in frictional ratio between pH 7.0 and 9.0 could be due to a slight electrostatically induced expansion at the molecule. Increasing the pH to 11.0 results in a much larger increase in frictional ratio but relatively little change in spectroscopic parameters at 25°C. However, CIg is significantly destabilized at this pH as seen by the marked alteration in the thermal transition curves at pH 11.0 compared to pH 9.0 or 7.0. Evidently internal interactions are lost at pH 11.0 which do not affect the spectroscopic parameters of CIg but are evident in the reduced stability to denaturing conditions.

Direct evidence of molecular domains in CIg is available from the marked difference in the transition curves of the two tryptophanyl peaks at 291 and 299 nm with temperature or GdmCl. Different transition curves were also observed with CSP for these two CD peaks with temperature. One of the clearest examples of a molecule containing independent domains is fibrinogen. It has been shown to be composed of three globular regions connected by random polypeptide chains by electron microscopy (23). Two of the three domains are readily isolated as globular molecules by trypsin or plasmin digestion. The independent thermal unfolding of each domain has been observed by calorimetry for both native fibrinogen and a trypsin digest (24). Most small globular proteins show a single thermal transition by calorimetry and fit a two-state process of denaturation (25). Several large molecular weight fragments of CIg have been observed by plasmin digestion (26). We have found similar fragments after trypsin digestion of CSP. The large fragments appear to contain most or all of the organized structure of the parent native molecule since the transition temperature of this trypsin digest of CSP at pH 9.0 was very similar to that observed with the native molecule (26). The increase in negative ellipticity could represent some minor rearrangement, i.e., refolding of one domain of the protein prior to the major unfolding that starts at 1.6 M GdmCl.

As observed by thermal denaturation, the two tryptophanyl CD peaks also do not melt-out at the same rate with GdmCl (Fig. 8). The 299 nm curve melts-out more slowly than the 291 nm curve and even shows some residual activity in 6 M GdmCl. The 291 nm peak is eliminated by 4 M GdmCl. The two peaks clearly represent tryptophanyl residues in different environments. Each of the peaks evidently monitors a different domain of the protein since their transitions are very distinctive. The transition curve of the 227 nm peak has also been plotted in Fig. 8 in order to compare it with the two aromatic peaks. It is evident that the curve for the 227 nm peak is very similar to that of the 291 nm peak at least above 1.6 M GdmCl.

It is of interest that the negative 291 nm peak increased in intensity by about 20% between 0 and 1.6 M GdmCl while that at 299 nm did not appear to change (see inset, Fig. 8). The increase in negative ellipticity could represent some minor rearrangement, i.e., refolding of one domain of the protein prior to the major unfolding that starts at 1.6 M GdmCl.

**DISCUSSION**

The various spectroscopic parameters of CIg that we have examined are unaffected by pH between 7.0 and 9.0. In contrast, there is a small but definite increase in frictional ratio. Since the spectroscopic properties depend on organizational constraints, i.e., secondary or tertiary structure, and the frictional properties depend on the dimensions of the protein, the change in frictional ratio should represent an expansion of the protein without loss in structure. This concept fits in with the structure proposed for CSP in which highly organized domains are linked by random polypeptide segments which results in a molecule with considerable flexibility and a high frictional ratio (8, 9).

The small increase in frictional ratio between pH 7.0 and 9.0 could be due to a slight electrostatically induced expansion at the molecule. Increasing the pH to 11.0 results in a much larger increase in frictional ratio but relatively little change in spectroscopic parameters at 25°C. However, CIg is significantly destabilized at this pH as seen by the marked alteration in the thermal transition curves at pH 11.0 compared to pH 9.0 or 7.0. Evidently internal interactions are lost at pH 11.0 which do not affect the spectroscopic parameters of CIg but are evident in the reduced stability to denaturing conditions.

Direct evidence of molecular domains in CIg is available from the marked difference in the transition curves of the two tryptophanyl peaks at 291 and 299 nm with temperature or GdmCl. Different transition curves were also observed with CSP for these two CD peaks with temperature. One of the clearest examples of a molecule containing independent domains is fibrinogen. It has been shown to be composed of three globular regions connected by random polypeptide chains by electron microscopy (23). Two of the three domains are readily isolated as globular molecules by trypsin or plasmin digestion. The independent thermal unfolding of each domain has been observed by calorimetry for both native fibrinogen and a trypsin digest (24). Most small globular proteins show a single thermal transition by calorimetry and fit a two-state process of denaturation (25). Several large molecular weight fragments of CIg have been observed by plasmin digestion (26). We have found similar fragments after trypsin digestion of CSP. The large fragments appear to contain most or all of the organized structure of the parent native molecule since the transition temperature of this trypsin digest of CSP at pH 9.0 was very similar to that observed with the native molecule when measured by fluorescence polarization.3

The positive CD peak observed at 227 nm is not seen in polypeptides and proteins which do not contain aromatic residues. The 227 nm CD peak is eliminated by GdmCl in a transition which parallels the major transition of the 291 nm peak.

3 G. Colonna, S. S. Alexander, Jr., and H. Edelhoch, unpublished observations.
tryptophanyl peak. It is likely, therefore, although by no means certain, that the 227 nm band can be identified with the same tryptophanyl group as the 291 nm band. Woody has recently reported that a positive band observed between 225 to 230 nm for a number of proteins may arise from aromatic chromophores (27). The thermal transition curves of the 227 nm and 291 nm peaks are not in agreement since some \( \beta \) structure is formed at higher temperatures and modifies the 227 nm transition. This structural change does not occur in GdmCl solutions where the two bands show the same transition curve.

All the data reported here for human CIg agree within experimental errors with other data obtained by us for the CSP dimer isolated from chick fibroblasts (8, 9). The sedimentation rate of the CSP dimer was indistinguishable from that of CIg at pH 11.0. We could not obtain the sedimentation rate of CSP at neutral pH due to its limited solubility. The similarity or identity of the secondary and tertiary structures of the two proteins is, however, primarily established by their CD spectra. These spectra are sufficient to reach this conclusion since they have very distinctive features in both the near and far ultraviolet which do not resemble those of other proteins (14). Moreover, the close resemblance between CIg and CSP in their thermal transition curves by two different methods, i.e., fluorescence and CD, strongly support the similarity in structure seen by the CD data.

We have shown that the shape, spectroscopic parameters, and thermal stability of CIg are very similar or identical to those properties of CSP. The current concept of protein folding is that the three-dimensional structure is controlled by the amino acid sequence (28). We therefore conclude that the amino acid sequence (28). We therefore conclude that the amino acid sequence (28).

REFERENCES

1. Mosesson, M. W., and Umfleet, R. A. (1970) J. Biol. Chem. 245, 5728-5736
2. Rusolaiti, E., and Vaheiri, A. (1975) J. Exp. Med. 141, 497-501
3. Hynes, R. O. (1976) Biochim. Biophys. Acta 458, 73-107
4. Yamada, K. M., Olden, K., and Pastan, I. (1978) Ann. N. Y. Acad. Sci. 321, 256-277
5. Blumenstock, F. A., Saba, T. M., Weber, P., and Laffin, H. (1978) J. Biol. Chem. 253, 4267-4291
6. Mosesson, M. W., Chen, A. B., and Hushey, R. M. (1975) Biochim. Biophys. Acta 386, 609-634
7. Yamada, K. M., Schlesinger, D., Kennedy, D. W., and Pastan, I. (1977) Biochemistry 16, 5552-5559
8. Alexander, S. S., Jr., Colon, G., Yamada, K. M., Pastan, L., and Edelhoch, H. (1978) J. Biol. Chem. 233, 5820-5824
9. Colon, G., Alexander, S. S., Jr., Yamada, K. M., Pastan, L., and Edelhoch, H. (1978) J. Biol. Chem. 233, 7787-7790
10. Keski-Oja, J., Mosher, D. F., and Vaheri, A. (1977) Biochem. Biophys. Res. Commun. 74, 689-706
11. Svedberg, T., and Pedersen, K. O. (1940) The Ultracentrifuge, Oxford University Press, New York
12. Cohn, E. J., and Edsall, J. T. (1943) Proteins, Amino Acids and Peptides as Dipolar Ions, p. 370, Reinhold, New York
13. Gibbons, R. A. (1972) in Glycoproteins (Gollschalk, A., ed) p. 78, Elsevier, New York
14. Chen, Y.-H., Yang, J. T., and Chau, K. H. (1974) Biochemistry 13, 3350-3359
15. Greenfield, N., and Fasman, G. D. (1969) Biochemistry 8, 4108-4116
16. Mosesson, M. W. (1978) Ann. N. Y. Acad. Sci. 312, 11-30
17. Nozaki, Y., and Tanford, C. (1971) J. Biol. Chem. 246, 2211-2217
18. Strickland, E. H. (1974) CRC Crit. Rev. Biochem. 2, 113-175
19. Strickland, E. H., Horwitz, J., Kay, E., Shannon, L. M., Vilcek, M., and Billups, C. (1971) Biochemistry 10, 2631-2638
20. Strickland, E. H., Billups, C., and Kay, E. (1972) Biochemistry 11, 3657-3662
21. Edelhoch, H., Lipoldt, R. E., and Vilcek, M. (1968) J. Biol. Chem. 243, 4799-4805
22. Chen, R. F., Edelhoch, H., and Seiner, R. F. (1969) Physical Principles and Techniques of Protein Chemistry (Leach, S. J., ed) pp. 172-244, Academic Press, New York
23. Hall, C. E., and Slayter, H. S. (1959) J. Biol. Chem. 234, 11-15
24. Donovan, J. W., and Mihalyi, E. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 4125-4128
25. Privalov, P. L., and Khechinashvili, N. N. (1974) J. Mol. Biol. 86, 665-684
26. Jilek, F., and Hormann, H. (1977) Hoppe-Seyler Z. Physiol. Chem. 358, 130-136
27. Woody, R. W. (1978) Biopolymers 17, 1451-1467
28. Anfinsen, C. B. (1973) Science 181, 223-230
The structure and stability of human plasma cold-insoluble globulin.
S S Alexander, Jr, G Colonna and H Edelhoch

J. Biol. Chem. 1979, 254:1501-1505.

Access the most updated version of this article at http://www.jbc.org/content/254/5/1501

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/254/5/1501.full.html#ref-list-1