# EFFECT OF METHYLAMINE AND PLASMIN ON THE CONFORMATION OF HUMAN $\alpha_2$-MACROGLOBULIN AS REVEALED BY DIFFERENTIAL SCANNING CALORIMETRIC ANALYSIS

HELEN S. CUMMINGS
*Department of Chemistry, University of Notre Dame, Indiana 46556*

SALVATORE V. PIZZO
*Departments of Pathology and Biochemistry, Duke University, Durham, North Carolina 27710*

DUDLEY K. STRICKLAND
*American Red Cross Plasma Derivatives Laboratory, Bethesda, Maryland 20814*

FRANCIS J. CASTELLINO
*Department of Chemistry, University of Notre Dame, Indiana 46556*

ABSTRACT Differential scanning calorimetric analysis was used as a probe of the conformational alteration in human $\alpha_2$-macroglobulin (AM) upon its complex formation with methylamine and with the protease, human plasmin. The slow electrophoretic form of AM displayed a single thermal transition, characterized by a temperature midpoint ($T_m$) of 65.8 ± 0.3°, a calorimetric enthalpy ($\Delta H_c$) of 2,550 ± 150 kcal/mol and a van't Hoff enthalpy ($\Delta H_{vh}$) of 140 kcal/mol. In the presence of sufficient methylamine to irreversibly disrupt the four thiol ester bonds in AM, a single thermal transition was obtained, characterized by a $T_m$ of 62.8 ± 0.3°, a $\Delta H_c$ of 1,700 ± 100 kcal/mol, and a $\Delta H_{vh}$ of 169 kcal/mol. These data suggest that a major conformational alteration is produced in AM upon complex formation with methylamine. When plasmin interacts with AM, the resulting thermogram displays $T_m$ values for AM of 68–69° and 77°, also suggestive of a large conformational alteration in AM. However, this latter alteration appears dissimilar to the change induced by methylamine.

## INTRODUCTION

Human $\alpha_2$-macroglobulin (AM) is a plasma glycoprotein of molecular weight 725,000, containing four identical subunits (1, 2). This protein binds to a wide variety of proteases, usually resulting in inhibition of the protease toward large substrates and reduction of activity of the bound protease toward small substrates (3). A current hypothesis (4) suggests that upon interaction of proteases with AM, one or more peptide bonds are cleaved at a region of the inhibitor termed the bait region. Following this a conformational change and chemical reaction occurs in AM, which results in covalent entrapment of the protease (5), as well as exposure of sites on AM that are responsible for its uptake and degradation by macrophages (6). Several groups of investigators (7–9) have suggested that the covalent bonds formed with the protease involve glutamyl residues in AM, which exist in an activated state in the protease-free inhibitor as thiol ester bonds. The latter bonds are also cleaved by reaction of AM with primary amines, such as methylamine, a reaction that exposes four sulfhydryl groups (7–9) and leads to a conformational alteration in AM (10–12).

Evidence has been presented, based upon similarities in the absorption, sedimentation, fluorescence, and circular dichroic properties of the AM-methylamine and several AM-protease complexes, that the conformational alterations produced in AM by methylamine and by proteases were nearly identical (11, 13). Since this conformational alteration is of such great importance to the function of AM, we decided to evaluate the nature of the change by a technique, differential scanning calorimetry, which has been shown previously to be a sensitive measure of ligand-induced conformational alterations in proteins. This communication represents the results of these studies.

Address all correspondence to Dr. F. Castellino.

## MATERIALS AND METHODS

### Proteins

AM was purified from human plasma as described by Imber and Pizzo (14). Each mole of AM bound 2 mol of trypsin, at saturation, as revealed by assay of the concentration of bound trypsin by titration with *p*-nitrophenyl-*p'*-guanidinobenzoate (NPGB) (15), after inactivation of free trypsin with soybean trypsin inhibitor (16), in a mixture of trypsin and AM (3:1, mol/mol). AM was dialyzed against a buffer of 0.05 M Hepes/0.1 M NaCl, pH 7.4. Its concentration was determined using an E (1%, 1 cm, 280 nm) of 9.1 (17).

Bovine pancreatic trypsin was purchased from Sigma Chemical Co. (St. Louis, MO) and the concentration determined by titration with NPGB (15). The AM-methylamine complex was prepared by dissolving AM in a buffer consisting of 0.05 M HEPES/0.2 M methylamine, pH 8.0. The solution was allowed to stand at room temperature for 1 h and then dialyzed at 4° against several changes of a buffer containing 0.05 M HEPES/0.1 M NaCl, pH 7.4. These conditions have been found previously to be sufficient for incorporation of methylamine into all sites of AM (11).

Human plasmin was prepared by activation of human plasminogen with urokinase, as described previously (18). The plasmin concentration was determined by active site titration with NPGB (15). The human plasmin-Am complex, free of unbound proteins, was prepared by chromatographic techniques. A 3:1 (mol/mol) ratio of plasmin/AM was allowed to incubate in 0.05 M HEPES/0.1 M NaCl, pH 7.4, for 20 min at room temperature. The plasmin was then inactivated by adjusting the buffer to a final concentration of 1 mM diisopropylfluorophosphate. Excess plasmin was removed upon passage of the solution over a 1.1 by 50-cm column of Ultrogel ACA-22 (LKB Instruments, Inc., Gaithersburg, MD), equilibrated and eluted in the same buffer. The complex, which was well-resolved from the unbound plasmin, was then placed over a 1.1 by 10-cm column of Sepharose-lysine, under conditions described by Brockway and Castellino (19). The unbound AM was virtually unretarded by this column and the AM-plasmin complex was eluted after adjustment of the buffer to 10 mM with 6-amino hexanoic acid.

### Calorimetry

Differential scanning calorimetric analyses were performed on 1-ml samples, using a calorimeter (MC-1; Microcal, Amherst, MA). Our procedures regarding use of this equipment for the purposes described herein have been published previously (20). Protein solutions for calorimetry were equilibrated by dialysis against a buffer of 0.05 M HEPES/0.1 M NaCl, pH 7.4. When analysis of the effect of methylamine on AM was desired, this reagent was added to the buffer at a concentration of 0.2 M. After 1 h at room temperature, this solution was dialyzed overnight at 4° against 0.05 M Hepes/0.1 M NaCl, pH 7.4. When plasmin additions to AM were carried out, the resulting mixtures were dialyzed against 0.05 M HEPES/0.1 M NaCl, pH 7.4, at 4°, overnight, before calorimetric analysis. The treatment of data for calculation of the calorimetric enthalpies ($\Delta H_c$) and the van't Hoff enthalpies ($\Delta H_{vh}$) was as described by Jackson and Brandts (21) and Sturtevant (22), respectively. In all cases, scan rates of 45°/h, under constant pressure of 8–10 cm Hg, were used. The calculated ΔH values are independent of the scan rate.

## RESULTS

Fig. 1 *A* illustrates a typical thermogram of AM in 0.05 M Hepes/0.1 M NaCl, pH 7.4. A single thermal transition is obtained, with a thermal transition midpoint ($T_m$) of 65.8 ± 0.3°. The calorimetric enthalpy, $\Delta H_c$, determined from integration of the peak area is 2,550 ± 150 kcal/mol (14.72 *J*/g). The example thermogram of Fig. 1 *A* was obtained at a protein concentration of 1.5 mg/ml. We have

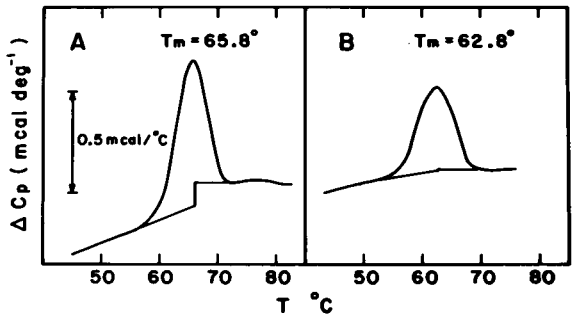


FIGURE 1 Differential scanning calorimetric thermogram of human $\alpha_2$-macroglobulin. (*A*) $\alpha_2$-macroglobulin was dialyzed against 0.05 M HEPES/0.1 M NaCl, pH 7.4 The change in heat capacity at constant pressure ($\Delta C_p$ is plotted against the temperature ($T$) in degrees C. The heat flow represented on the ordinate was determined by a calibration pulse, which is a design feature of the instrument. (*B*) $\alpha_2$-macroglobulin was first incubated in a buffer consisting of 0.05 M HEPES/0.2 M methylamine, pH 7.4, for 1 h before exhaustive dialysis against the same buffer as in *A*.

analyzed thermograms at four protein concentrations from 1.5 mg/ml to 13.1 mg/ml and the $\Delta H_c$, above, represents an average of replicate experiments at each concentration. At the higher concentrations of Am employed (9.2 and 13.1 mg/ml), a slight endothermic transition appears at 77°. This observation could indicate that aggregation of AM occurs at high concentrations and/or could represent a small population of molecules that have been isolated with bound plasma proteases (vide infra). From thermograms such as those presented in Fig. 1 *A*, we have calculated a $\Delta H_{vh}$ of 140 kcal/mol, assuming that a two state denaturation process exists. The $\Delta H_c/\Delta H_{vh}$ is 18; a value that is much larger than the value of 1.0 expected for a true two state process.

Fig. 1 *B* shows an example of a thermogram of AM, after the internal thiol ester bonds have been disrupted by methylamine. Here, a thermally destabilized form of AM exists, characterized by a lower $T_m$ of 62.8 ± 0.3°, a lower $\Delta H_c$ of 1,700 ± 100 kcal/mol (9.8 *J*/g, and a higher $\Delta H_{vh}$ of 169 kcal/mol. In this case, the $\Delta H_c/\Delta H_{vh}$ is calculated to be 10; a value significantly different from that of AM in the absence of methylamine. Here, again, the thermodynamic parameters were obtained by performing replicate analyses at four different protein concentrations, ranging from 2.2 to 10.7 mg/ml. At the higher concentrations (7.5 and 10.7 mg/ml), a small endothermic transition at 77° is obtained, originating as discussed above. The thermal denaturation curves for Figs. 1 *A* and *B* are not reversible, as cooling and reheating the samples led to significantly different thermograms.

Fig. 2 illustrates calorimetric thermograms of AM, upon titration with various levels of human plasmin. As the protease concentration is increased, there is an upward shift of the thermal transitions, with endothermic peaks at 68–69° and 77°. The 77° endotherm has been found for AM when bound to other proteases, including thermolysin,

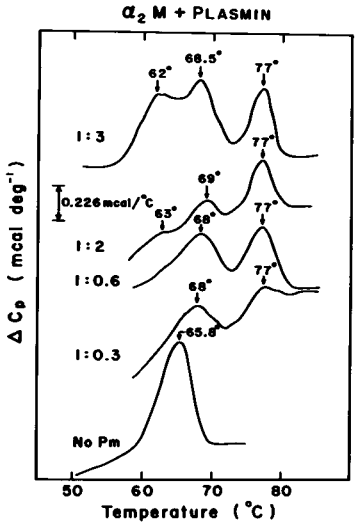


FIGURE 2 Differential scanning calorimetric thermograms of human $\alpha_2$-macroglobulin upon its interaction with human plasmin. All conditions are as in Fig. 1 *A*. The numerical insets in the graph represent the molar quantities of $\alpha_2$-macroglobulin/plasmin used for the thermogram.

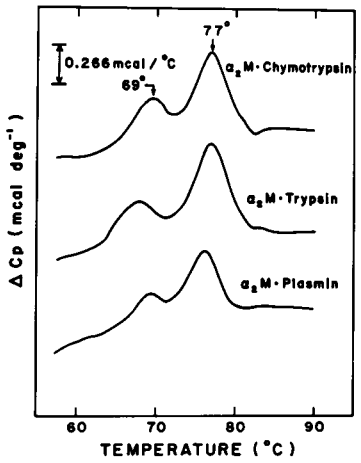


FIGURE 3 Differential scanning thermogram of the isolated $\alpha_2$-macroglobulin-plasmin complex, after removal of unbound proteins, as well as the $\alpha_2$-macroglobulin-trypsin and -chymotrypsin complexes. The conditions are as in Fig. 1 *A*.

papain, and trypsin (23). Interestingly, even at subsaturating levels of plasmin, the 65.8° transition, characteristic of native AM, does not appear to be present at sufficient intensity to suggest that AM, which is not bound to plasmin, exists in a native state. This could result from a rapid exchange of plasmin with AM, leading to modification of AM before stoichiometric incorporation of plasmin into AM. Alternatively, the plasmin-AM complex may exhibit enzymatic activity toward remaining free AM, thus altering the thermal transition of free AM. It is unlikely that at plasmin/AM molar ratios of 0.3 or 0.6, any of the thermal transitions are due to plasmin, due to the relatively small $\Delta H_c$ value of plasmin, compared with AM. However, the 63° endotherm seen when plasmin is present in excess of AM (Fig. 2) is most likely due to the plasmin present (24).

To exclusively assign the 68–69° and 77° endothermic transitions to AM, in complex with plasmin, we have isolated the complex, free of unbound AM and plasmin, and have subjected the material to calorimetric analysis. The results are presented in Fig. 3 where two clearly defined transitions, occurring at 69° and 76° are present. We obtained virtually the same thermograms when similar experiments were performed with trypsin and chymotrypsin in complex with AM (Fig. 3). Thus, clearly a large degree of thermal stability in AM results when AM is present in complex with proteases, compared with AM in its native state.

## DISCUSSION

In the presence of sufficient methylamine to disrupt the four thiol ester bonds in AM, the $T_m$ of the thermal denaturation of this protease inhibitor is decreased by 3°, as compared with intact AM. This finding argues for a significant conformational alteration of the molecule, upon cleavage of the thiol ester bonds. Furthermore, the $\Delta H_c$ of thermal denaturation of native AM is 2,550 kcal/mol; a large value compared with other proteins examined. This parameter decreases to 1,700 kcal/mol because of incorporation of methylamine by AM.

Because the magnitude of the $\Delta H_c$ for thermal denaturation reflects the difference in the number of noncovalent bonds broken and formed in proceeding from the native to the thermally denatured state, the differences in AM and methylamine-AM could result from dissimilarities in the native structure of AM, or differences in the thermally denatured state of these proteins. Based solely upon the data obtained here, no distinction could be made between these possibilities. However, in either case, the argument for conformational differences between the two forms of the protein is strong. One should exercise caution, however, in attempting a more detailed comparison. Because of the irreversibility of the thermal denaturation, the $\Delta H_c$ terms may not represent true equilibrium values. However, the comparison of the $\Delta H_c$ for the protein under each set of conditions is indeed revealing. Conformational differences between AM and methylamine-AM have been monitored by other methods. Compared with AM, the methylamine-AM complex possesses an increased $S_{20,w}$ (11), a decreased Stokes radius (11), a decreased helical content (11), and an increased intrinsic fluorescence (12).

If the thermogram obtained in Fig. 1 *A* is symmetrical in shape, a comparison with the $\Delta H_c$ or $\Delta q_c$ (3.5 cal/g) to $\Delta H_{vh}$ provides some information as to the steps involved in the thermal denaturation of AM. Because $\Delta H_c/\Delta H_{vh}$ is

much larger than 1.0, obviously a two-state process is not involved.

The data of Fig. 2 show that when the protease, plasmin, is mixed with AM, there is progressive growth of endotherms at 68–69° and 77°. A previous study of AM in complex with other proteases (23), showed only the 77° transition. We find both the 68°–69° and 77° transitions present in AM complexes with trypsin, chymotrypsin, and plasmin, and conclude that both of these endothermic transitions characterize AM, when complexed to proteases. More importantly, however, the data of Figs. 1 and 2 strongly suggest that the conformational alterations in AM, as a result of complex formation with methylamine and plasmin, are indeed different. Previous studies (11, 13), using probes of the gross conformation of AM, suggest that the conformational alterations produced in AM by proteases and methylamine are very similar. Apparently, the techniques used were not sufficiently sensitive to differentiate between the protease effect and the methylamine effect on AM conformation. However, differential scanning calorimetry is capable of differentiation of the two conformations.

H. Cummings is the Paul A. Nicoll Fellow, American Heart Association, Indiana Affiliate.

These studies were supported by grants HL-13423 and HL-19982 (to F. Castellino), and HL-24066 (to S. Pizzo) from the National Institutes of Health.

*Received for publication 19 May 1983 and in final form 7 October 1983.*

## REFERENCES

1. Swenson, R. P., and J. B. Howard. 1979. Structural characterization of human $\alpha_2$-macroglobulin subunits. *J. Biol. Chem.* 254:4452–4456.
2. Swenson, R. P., and J. B. Howard. 1980. The temperature sensitive bond in $\alpha_2$-macroglobulin is the alkylamine-reactive site. *J. Biol. Chem.* 255:3820–3823.
3. Rinderknecht, H., R. M. Fleming, and M. C. Geokas. 1975. Effect of $\alpha_2$-macroglobulin on some kinetic properties of trypsin. *Biochim. Biophys. Acta.* 377:158–165.
4. Barrett, A. J., and R. M. Starkey. 1973. The interaction of $\alpha_2$-macroglobulin with proteinases. *Biochem. J.* 133:709–724.
5. Salveson, G. S., and A. J. Barrett. 1980. Covalent binding of proteinases in their reaction with $\alpha_2$-macroglobulin. *Biochem. J.* 187:695–701.
6. Debanne, M. T., R. Bell, and J. Dolovich. 1975. Uptake of proteinase-$\alpha_2$-macroglobulin complexes by macrophages. *Biochim. Biophys. Acta.* 411:295–301.
7. Sottrup-Jensen, L., T. E. Petersen, and S. Magnusson. 1980. A thiol ester bond in $\alpha_2$-macroglobulin is cleaved during proteinase complex formation. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 121:275–279.
8. Howard, J. B. 1981. Reactive site in human $\alpha_2$-macroglobulin. Circumstantial evidence for a thiol ester bond. *Proc. Natl. Acad. Sci. USA.* 78:2235–2239.
9. Slaveson, G. S., C. A. Sayers, and A. J. Barrett. 1981. Further characterization of the covalent linking reaction of $\alpha_2$-macroglobulin. *Biochem. J.* 195:453–461.
10. Barrett, A. J., M. A. Brown, and C. A. Sayers. 1979. The electrophoretically slow and fast forms of the $\alpha_2$-macroglobulin molecule. *Biochem. J.* 181:401–408.
11. Gonias, S. L., J. A. Reynolds, and S. V. Pizzo. 1982. Physical properties of $\alpha_2$-macroglobulin following reaction with methylamine and trypsin. *Biochim. Biophys. Acta.* 705:306–314.
12. Straight, D. A., and P. A. McKee. 1982. Effect of protease binding by $\alpha_2$-macroglobulin on intrinsic fluorescence. *Biochemistry.* 21:4550–4556.
13. Bjork, I., and W. W. Fish. 1982. Evidence for similar conformational changes in $\alpha_2$-macroglobulin on reaction with primary amines or proteolytic enzymes. *Biochem. J.* 207:347–356.
14. Imber, M. J., and S. V. Pizzo. 1981. Clearance and binding of two electrophoretic fast forms of $\alpha_2$-macroglobulin. *J. Biol. Chem.* 256:8134–8139.
15. Chase, T., and E. Shaw. 1970. Titration of trypsin, plasmin, and thrombin with *p*-nitrophenyl-*p'*-guanidinobenzoate HCl. *Methods Enzymol.* 19:20–27.
16. Ganrot, P. O. 1966. Determination of $\alpha_2$-macroglobulin as trypsin-protein esterase. *Clin. Chim. Acta.* 14:493–501.
17. Dunn, J. T., and R. G. Spiro. 1967. The $\alpha_2$-macroglobulin of human plasma. I. Isolation and composition. *J. Biol. Chem.* 242:5549–5555.
18. Morris, J. P., S. Blatt, J. R. Powell, D. K. Strickland, and F. J. Castellino. 1981. Role of the lysine binding regions in the kinetic properties of human plasmin. *Biochemistry.* 20:4811–4816.
19. Brockway, W. J., and F. J. Castellino. 1972. Measurement of the binding of antifibrinolytic amino acids to various plasminogens. *Arch. Biochem. Biophys.* 151:194–199.
20. Ploplis, V. A., D. K. Strickland, and F. J. Castellino. 1981. A calorimetric evaluation of the existence of separate domains in bovine prothrombin. *Biochemistry.* 20:15–21.
21. Jackson, W. M., and J. F. Brandts. 1970. Thermodynamics of protein denaturation. Calorimetric study of the reversible denaturation of chymotrypsinogen and conclusions regarding the accuracy of the two state approximation. *Biochemistry.* 9:2294–2301.
22. Sturtevant, J. M. 1974. Some applications of calorimetry in biochemistry and biology. *Annu. Rev. Biophys. Biomed. Eng.* 3:35–51.
23. Chlebowski, J. F., and K. Willliams. 1983. Differential scanning calorimetry of $\alpha_2$-macroglobulin and $\alpha_2$-macroglobulin-proteinase complexes. *Biochem. J.* 209:725–730.
24. Castellino, F. J., V. A. Ploplis, J. R. Powell, and D. K. Strickland. 1981. The existence of independent domain structures in human $lys_{77}$-plasminogen. *J. Biol. Chem.* 256:4778–4782.