Bait Region Involvement in the Dimer-Dimer Interface of Human α2-Macroglobulin and in Mediating Gross Conformational Change

EVIDENCE FROM CYSTEINE VARIANTS THAT FORM INTERDIMER DISULFIDES*

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We have characterized four human α2-macroglobulin (α2M) bait region variants (G679C, M690C, V700C, and T705C) to test the hypothesis that the bait regions are involved in the interface between noncovalently associated dimers. All four variants folded correctly as judged by many normal properties. However, the presence of a cysteine resulted in disulfide formation between otherwise noncovalently associated dimers in all four variants. The extent of disulfide cross-linking varied with the location of the cysteine and gave a mixture of species that probably contained two, one, or zero interdimer disulfides in the tetramer. This was reflected in heterogeneity of conformational change upon thiol ester cleavage by methylamine, with the presence of cross-links correlating with blockage of conformational change. The stoichiometry of trypsin inhibition was less in all cases than for wild-type α2M. The M690C variant also showed evidence of some species with an intramolecular disulfide between bait regions of monomers within the same dimer. Taken together, the results are consistent with a location of the four bait regions in contact with, or in very close proximity to, one another. This suggests that they form all or part of the “cavity body” seen in the low resolution x-ray structure of transformed α2M.

Inhibition of proteinases by human α2-macroglobulin (α2M) results from a series of conformational changes that are initiated by the protease and that result in the physical sequestration of the protease within the closed cage-like structure of the conformationally altered α2M (1, 2). The initiating event is a proteolytic cleavage by the attacking protease near the center of the α2M polypeptide in a region termed the bait region (3, 4). Cleavage anywhere within the bait region also results in activation of an internal thiol ester toward cleavage by nucleophiles. From studies on the kinetics of cleavage of the thiol ester by nucleophiles and of the subsequent conformational change within the α2M, it has been shown that the conformational change occurs cooperatively after both thiol esters within one half of the α2M tetramer have been cleaved (5, 6).

Knowledge of the structural relationship between the thiol ester and the bait region and hence of the details of the activation mechanism is limited by the absence of a high resolution x-ray structure of either native or conformationally altered human α2M. From fluorescence resonance energy transfer measurements, it was shown that the four cysteines that form the four thiol esters of the human α2M tetramer are centrally located and are about 35 Å apart (7). This was subsequently confirmed by a low resolution (~10 Å) x-ray structure of conformationally altered α2M (8). NMR measurements on α2M showed that the bait region of each monomer was unusually flexible (9, 10) and that it lies close (10–17 Å) to the cysteine of the thiol ester in the transformed protein (11). However, the location of the four bait regions is still not known. Studies from this laboratory on α2M variants in which large portions of the bait region had been removed showed that bait region truncation prevented the disulfide-linked α2M dimers from associating noncovalently to give the functional tetramer (12) and that, despite normal reactivity of the thiol esters toward nucleophiles, there was no cooperativity of conformational change between subunits. This implicated the bait region in formation of the α2M tetramer and raised the possibility that the bait regions may lie in the central cavity of α2M in contact with one another. This is more plausible by the presence of a symmetrical body of electron density in the central cavity of methylamine-treated α2M, termed the “cavity body,” that is absent from proteolytically cleaved α2M (8).

To test the hypothesis that the bait region is involved in the noncovalent association of α2M dimers and in the mediation of cooperative subunit-subunit interactions that are involved in the gross trapping conformational changes of α2M, we used a scanning mutagenesis approach in which four single residue substitution variants of human α2M containing a cysteine at distal, central, or proximal ends of the bait region have been prepared and characterized (G679C, M690C, V700C, and T705C) (Fig. 1). The variants folded normally, as judged by several properties, including the formation of tetrameric α2M, the presence of thiol esters with normal reactivity toward nucleophiles, and the ability to undergo a proteinase-induced conformational change. However, the extent and rate of conformational change induced by methylamine cleavage of the thiol esters was compromised for all of the variants. Although all of the variants could still inhibit proteinase, the stoichiometries were reduced compared with wild-type recombinant α2M or plasma α2M. These perturbed properties correlated with the presence of new interdimer disulfide bonds formed between the new cysteine residues in two otherwise noncovalently associated disulfide-linked dimers. Such cross-linking resulted in covalent tetramers with either one or two new disulfides between the dimers. The extent of formation of such new disulfides was a function of the position of the cysteine within the
bait region. This suggests that the bait regions are involved both in dimer-dimer association and in mediation of trans-dimer conformational changes and that there is a requirement for unrestrained reorientation of the bait region interface. In addition, the M690C variant showed evidence of a new intra-dimer disulfide, suggesting that bait regions within a dimer are also close to one another. These findings suggest that all four of the bait regions are at the center of the αM cavity and may constitute all or part of the cavity seen in the low resolution x-ray structure of transformed αM.

MATERIALS AND METHODS

Site-directed Mutagenesis and Expression of Recombinant αM—For circumstantial reasons, the four mutations were not all created by the same procedure. For the variants G679C and V700C, site-directed mutagenesis was performed in a single-stranded M13mp18 construct containing the 1690-base pair mutagenesis was performed in a single-stranded M13mp18 construct from plasmid p1167 (13), which covers the coding region for the bait region. Mutagenesis was carried out using the Amersham Corp. in vitro Mutagenesis System 2.1 according to the manufacturer’s protocol. The mutations were confirmed by sequencing, after which the mutated BamHI to BsiWI fragment was excised and ligated back into the p1167 expression vector. The M690C variant was created by mutagenesis using the Altered Sites II Mutagenesis System (Promega) on the 2669-base pair BamHI to ClaI fragment from p1167 subcloned into pAlter (Promega). The 28-base oligonucleotide 5'-T GAG TCA CAT GAT TTG CTT AAG AGA GGC CAT-3' was used for the mutagenesis. After confirmation of the mutation in pAlter, the mutated BamHI to ClaI fragment was excised and religated into p1167. The T705C variant was created in two stages. In the first stage, an XmaI site was introduced at base 3572 of p1167 using the Amersham Corp. in vitro Mutagenesis System 2.1 protocol. The mutation at residue 705 was then introduced by replacing the XmaI to BsiWI fragment of p1167 with a synthetic duplex containing the desired change. This duplex was formed by annealing oligonucleotides 5'-GG TG TGG AAG ACC-3' and 5'-CG TAC CTG CGT ACA C-3'. The mutation was confirmed by sequencing in p1167. Stably transfected BHK cell lines expressing each of the four αM variants were established by the co-transfection and drug resistance selection procedures previously described (14, 15).

Purification of Recombinant and Plasma αMs—Plasma αM was isolated from outdated human plasma obtained from Rush University Hospital Blood Bank. The αM was precipitated in a 4–12% cut with PEG8000 and collected as a pellet by centrifugation. The pellet was reuspended in 0.1 M sodium phosphate buffer, pH 6.5, containing 0.8 M sodium chloride and chromatographed on a zinc chelate matrix, as described previously (16). Recombinant αMs were obtained from the growth medium of the stably transfected BHK cells that were grown to confluence in roller bottles and cycled between serum-containing and serum-free medium, as described previously (16). Purification was by zinc chelate chromatography, as for plasma αM, but without the PEG8000 precipitation step.

Polyacrylamide Gel Electrophoresis—Polyacrylamide gels run under denaturing and reducing conditions (8% acrylamide) or denaturing and nonreducing conditions (3.5% acrylamide cast on Pharmacia Gel Bond film) were run according to the procedure of Laemmli (17). Nondenaturing and reducing gels (5% acrylamide) were run as described previously (18). Urea polyacrylamide gels (5% acrylamide) contained 5 M urea and were run in the same way as nondenaturing gels.

TNS Fluorescence Measurements—Both kinetic and wavelength scan fluorescence measurements were made on an SLM8000 spectrofluorometer. Kinetic measurements used excitation at 316 nm and observation of emission at 410 nm, with slits of 4 and 16 nm for excitation and emission, respectively. Wavelength scans used excitation at 316 nm and emission monitored in 2-nm steps from 360 to 600 nm. Slits of 2 nm for both excitation and emission were used.

Quantitation of Thiol Esters and Kinetics of Cleavage—Determination of both the number and kinetics of cleavage of thiol esters present in the variants were carried out by continuous monitoring of the free SH (sulfhydryl) released from the thiol ester by reaction with methylamine, by spectrophotometric measurement of the TNB- released by reaction of the free SH with DTNB. TNB- was quantitated from the change in absorbance at 412 nm using an extinction coefficient of 13,600 M⁻¹ cm⁻¹ (19). For kinetic measurements, the αM (final concentration, 1.0 μM) was preincubated in assay buffer containing 100 μM DTNB. The reaction was initiated by addition of amine hydrochloride stock solution (5 M, pH 8.0) to give the desired final concentration. Measurements were made in a dual beam Shimadzu UV2101PC spectrophotometer; the blank contained all components except αM. Data were fitted by nonlinear least squares analysis to a monoeponential using Student (MicroMath, Salt Lake City, UT).

Trypsin Inhibition Assay—Stoichiometry of trypsin trapping was determined by measuring the soybean trypsin inhibitor-resistant trypsin activity (20, 21) at different trypsin:αM ratios. αM (120 nM) was incubated with different molar ratios of trypsin for 5 min at 25 °C. The reaction was diluted 1:1 with a solution of soybean trypsin inhibitor (final concentration, 1.25 mM). After 1 min of incubation, residual trypsin activity, representing αM-trapped trypsin, was assayed by addition of 10 μL of the reaction mixture to 190 μL of assay buffer containing 200 μM (final concentration) N-benzoyl-t-isoleucyl-l-glutamylglycyl-l-arginine-p-nitroanilide (S-2222, Chromogenix) in a microcuvette. Change in absorbance at 410 nm, relative to a reference cell containing only substrate, was monitored using a dual beam Shimadzu 2101PC spectrophotometer. Trypsin activities were determined from the slope of the resulting time course, fitted by least squares linear fit using the program Origin 1.16 (MicroCal Inc., Amherst, MA). All reactions and assays were carried out in 20 mM sodium phosphate buffer, pH 7.4, containing 0.1 M sodium chloride, 0.1 mM EDTA, and 0.1% PEG8000.

RESULTS

Electrophoretic Mobility of Variants on Nondenaturing PAGE—Following reaction with methylamine or proteinase, human plasma αM undergoes a major change in conformation that results in an increase in electrophoretic mobility on polyacrylamide gels run under nondenaturing conditions (22). This "slow to fast" conversion is frequently used as a diagnostic for this major conformational change, with the result that αM conformations are often referred to as the slow form or fast form. In addition, there is an intermediate mobility species in which conformational change has occurred in only two of the four subunits (6, 23). We tested the ability of the four αM variants to undergo this slow to intermediate or slow to fast form interconversion upon reaction with either methylamine or proteinase. All four variants had the expected slow mobility prior to reaction (Fig. 2) that is characteristic of tetrameric αM in the native state. Reaction with a small excess of proteinase (trypsin) for 10 min was sufficient to completely convert each of the variants from slow to fast conformations (Fig. 2). However, methylamine treatment overnight produced only partial conversion to a mixture of slow, intermediate, and fast forms for all of the variants, with each variant giving different extents of conversion to intermediate and fast forms (Fig. 2). The M690C variant was least converted to fast or intermediate mobility species, whereas the other three showed the presence of about
one-third to one-half fast form and a smaller amount of intermediate mobility species. Recombinant wild-type α2M was completely converted to the fast form by methylamine treatment under the same conditions (Fig. 2), as was plasma α2M (not shown). The failure of the variants to be completely converted to the fast form was not due to incomplete cleavage of the thiol esters by methylamine (see below).

We also followed the rate of conversion of the variants from slow to intermediate or fast conformations as a function of time of reaction with methylamine at pH 8 by nondenaturing PAGE. The appearance of the intermediate and fast mobility species was complete within 2 h, after which no further changes were observed up to the longest time examined of 24 h (data not shown). Therefore, although the variants initially have uniform slow mobility, they are heterogeneous with respect to the ability to undergo change from slow to intermediate or fast mobility species.

Quantitation of Free Cysteine and of Intact Thiol Esters and Determination of the Kinetics of Thiol Ester Cleavage—Because of the limited quantities available for some of the variants, the number of free thiols present initially in each of the variants was estimated by reaction with 5-iodoacetamido fluorescein rather than by DTNB assay because this gave higher sensitivity and allowed fluorescence quantitation. When allowance was made for a small amount of nonspecific labeling, which was also present in recombinant wild-type α2M, there was little evidence of a significant fraction of the new cysteine residues being in unconjugated form (Table I), suggesting that the new cysteines introduced into the bait region were all involved in disulfide linkages, either to other cysteines within the tetrameric protein or to small thiols, such as glutathione.

![Fig. 2. Conversion of recombinant wild-type and variant slow form to faster mobility species on polyacrylamide gel run under nondenaturing conditions following treatment with methylamine or trypsin. For each α2M, there are three consecutive lanes corresponding to native protein (N), protein incubated with 0.2 M methylamine overnight (M), and protein reacted with 2.2 equivalents of trypsin (T), based on active site titration for 10 min. The α2Ms are as indicated above each set of lanes. S, I, and F indicate the positions of the slow, intermediate, and fast forms of the tetramer, respectively; Dimer indicates the position of small amounts of dimeric α2M.](image)

![Fig. 3. Near normal rates of appearance of free thiols in variant α2Ms as a result of thiol ester cleavage by methylamine, monitored by release of TNB - . Reactions were carried out at pH 8.0 with 0.2 M methylamine. The curves represent the nonlinear least squares fits to the data to a monoeponential. The rate constants representing these curves are given in Table I.](image)

### Table I

| α2-Macroglobulin | Free SH<sup>a</sup> | Thiol esters<sup>b</sup> | Trypsin trapped<sup>c</sup> | Trypsin activity<sup>d</sup> | k<sub>1</sub><sup>e</sup> | k<sub>c</sub><sup>f</sup> |
|-----------------|---------------------|--------------------------|---------------------------|-----------------------------|----------------|-----------------|
| Plasma          | ND                  | 2.9 ± 0.07               | 1.5                       | 0.69                        | 4.9 ± 0.1      | 0.022 ± 0.004   |
| Wild-type recombinant | 0.39                | 3.7 ± 0.2               | 1.3                       | 1.00                        | 3.8 ± 0.6      | 0.018 ± 0.0002  |
| G679C           | 0.62                | 2.6 ± 0.06              | 0.5                       | 0.42                        | 2.4 ± 0.9      | 0.013 ± 0.001   |
| M690C           | 0.26                | 3.0 ± 0.9              | 0.6                       | 0.20                        | 2.5 ± 1.0      | 0.0074 ± 0.0001 |
| V700C           | 0.73                | 2.6 ± 0.2              | 0.4                       | 0.40                        | 2.8 ± 0.9      | 0.0094 ± 0.0015 |
| T705C           | 0.57                | 2.0 ± 0.3              | 0.5                       | 0.15                        | 3.0 ± 0.8      | 0.010 ± 0.004   |

<sup>a</sup> Free SH present after purification, quantitated by reaction with 5-iodoacetamido fluorescein.

<sup>b</sup> Thiol esters present quantitated by DTNB assay of free SH generated by reaction to completion with methylamine.

<sup>c</sup> Moles of trypsin trapped by the α2-macroglobulin tetramer.

<sup>d</sup> Activity of trapped trypsin relative to that for trypsin trapped by recombinant wild-type α2M.

<sup>e</sup> Second-order rate constant for cleavage of thiol esters by methylamine.

<sup>f</sup> First-order rate constant for rapid phase of the conformational change detected by change in TNS fluorescence.
The number of intact thiol esters present was also determined by DTNB assay, which involved reaction of the variants with methylamine and quantitation of the free SH generated (Fig. 3). From analysis of the time dependence of the change in absorbance at 412 nm, both the total number of methylamine-cleavable thiol esters and the kinetics of their cleavage were determined (Table I). By both criteria, all four of the variants were similar to both plasma and recombinant wild-type α₂Ms, having between two and four intact thiol esters that could be cleaved at rates that were 63–79% that of recombinant wild-type α₂Ms. These results indicated that the mutations had not greatly altered either the ability of the α₂M subunits to form thiol esters or the subsequent reactivity of the thiol esters toward small nucleophiles. The lower stoichiometry of thiol esters for the V700C and T705C variants correlated with the presence of a small amount of intermediate mobility material prior to reaction (Fig. 2). This form is likely to have only two intact thiol esters. However, the major species had slow electrophoretic mobility and probably contained three or four intact thiol esters.

Proteinase- and Methylamine-induced Change in Conformation Detected by TNS Fluorescence—The conformational changes that accompany reaction with proteinase were complete within the 10 min used for the reaction, so the TNS emission spectrum was stable after this time. Comparison of the TNS emission spectra for unreacted and proteinase-reacted variants with those for plasma α₂M showed significant differences for both the starting and the finishing conformations (Fig. 4). TNS in the presence of plasma α₂M gave an emission spectrum with λ<sub>max</sub> at about 440 nm. Upon reaction with proteinase, the TNS fluorescence intensity increased more than 2-fold, and the wavelength maximum blue shifted from 440 to about 410 nm (Fig. 4). The same behavior was seen for wild-type recombinant α₂M (Fig. 4). In contrast, TNS in the presence of any of the four variants already showed enhanced intensity, and wavelength maxima between those of native and methylamine-treated α₂M (Fig. 4). Reaction of the variants with either chymotrypsin or trypsin produced very little additional increase for the M690C and T705C variants, and a reduction in intensity for the G679C and V700C variants. Almost no change in emission maximum was seen for the M690C and T705C variants after reaction with either trypsin or chymotrypsin. Although small blue shifts were observed upon proteinase treatment of the G679C and V700C variants, the wavelength maxima of the proteinase-treated variant was still not as much blue shifted as wild-type or plasma α₂M. Methylamine treatment resulted in almost no change in wavelength maximum for any of the variants and much smaller percentages of enhancement for any of the variants, particularly T705C, than for recombinant wild-type or plasma α₂Ms.

Ability of Variants to Inhibit Trypsin by Trapping—The ability of each variant to inhibit trypsin by the normal bait region cleavage-induced conformational change was determined by a trapping assay in which increasing amounts of trypsin were incubated with each α₂M variant, and any nontrapped trypsin was inhibited by addition of a large excess of soybean trypsin inhibitor. The residual trypsin activity was then determined by chromogenic assay. The stoichiometry of trapping was determined from the turnover point of a plot of residual activity against the trypsin:α₂M ratio (not shown). Each variant was able to trap trypsin, but the stoichiometry was greatly reduced compared with plasma and wild-type recombinant α₂Ms (Table I), despite complete and specific cleavage of all four of the bait regions (Fig. 5). The activity of trypsin trapped by each of the
variants was also reduced from that trapped by wild-type α₂M (Table I). The largest reductions in specific activity were seen for the M690C and T705C variants.

**Origin of Aberrant Properties of Cysteine Variants**—From the above characterization of the four variants, it is clear that whereas they behave normally in forming tetramers with intact thiol esters that are cleavable by methylamine at normal rates and bait regions that are still specifically cleavable by proteinase, their ability to undergo rapid cooperative conformational changes and to efficiently trap 1–2 mol of proteinase has been compromised by the introduction of cysteine into the bait region. We therefore determined whether the aberrant properties arose from the presence of new disulfides within the tetramer.

Plasma α₂-macroglobulin is a tetramer, made up of two noncovalently associated disulfide-linked dimers (24). Upon treatment with either urea (Fig. 6A) or SDS (Fig. 6, B and C), the noncovalent interactions can be disrupted, and the protein migrates as an ~360-kDa dimer. We found that all of the cysteine variants showed evidence of the presence of additional disulfide bonds between the dimers, from the presence of nondissociable 720-kDa tetramers (Fig. 6). Such behavior could result from either one or two additional disulfides between the dimers. In each case, a minority of the protein lacked such additional interdimer disulfides and was still dissociable to dimer. This varied among the variants and was the greatest for the T705C variant (Fig. 6).

Another difference between variants was that the M690C variant showed a different mobility for its dimer compared with the dimers of the other variants when dissociation was carried out in SDS (Fig. 6C), but less so when carried out with urea (Fig. 6A). This may be due to the presence of a new intradimer disulfide in a fraction of the subunits that may constrain the conformation of the SDS-unfolded protein so that it has a different mobility from the equivalently unfolded dimers that do not contain such an additional disulfide. SDS-PAGE run under reducing conditions confirmed that all of the variants were composed of the expected 180-kDa monomers and that the bait regions were all accessible to proteinase and could be cleaved upon exposure to trypsin (Fig. 5).

**Kinetics of Fast Phase Conformational Change**—The time-course of change in mobility of the variants on nondenaturing PAGE showed that only a fraction of each variant showed conversion at rates similar to that of wild-type or plasma α₂M when reacted with methylamine (see above). To test whether there was an equivalent fraction that also showed normal kinetics of conformational change, we followed the time-dependence of the TNS fluorescence change upon reaction with methylamine (Fig. 7) and determined the rate constant for the change. For all four variants it appeared that there was a rapid phase of smaller magnitude than for wild-type α₂M, that was equivalent to the normal conformational change step of recombinant wild-type and plasma α₂Ms, because the fluorescence changes were well fitted to a monoexponential, as expected for the conditions of the reaction, and gave rate constants (kₐ) no more than about 3-fold slower than those for plasma and wild-type recombinant α₂Ms (Table I).

**DISCUSSION**

We have examined four variants of human α₂M, each of which contains a single cysteine at a different location within the bait region, to test the involvement of the bait region in dimer-dimer association and in the mechanism of conforma-
nutional change and proteinase inhibition by determining the
tendency of these variants to form new interdimer disulfides and
thereby alter the properties of the $\alpha_2M$. We found that a
major fraction of each of the four variants formed new inter-
dimer cross-links, suggesting a very close approach of bait
regions in separate dimers to one another in the tetramer and
consequently a location for the bait regions at the interface
between these dimers. These new cross-links blocked the con-
formational change that occurs upon cleavage of the thiol es-
ters by nucleophiles and suggests that the bait regions not only
are in contact with one another across the dimer-dimer inter-
face but also mediate the cooperative conformational changes
that constitute the slow to fast form interconversion, perhaps
through a rearrangement of their contact surfaces. Formation
of a new disulfide cross-link between pairs of bait regions
across the interface may lock the interface so that conforma-
tional changes can only occur very slowly or not at all compared
with the unconstrained bait regions of wild-type recombinant
or plasma $\alpha_2M$. Consistent with this is the greatly lowered
ability of the variants to trap trypsin, even though trypsin
completely and specifically cleaved all bait regions in each of
the variants. The finding that cysteine at different positions
within the bait region is able to cross-link to a bait region of
the other dimer with different efficiencies suggests that the inter-
face is more complex than a parallel alignment of bait regions.
Instead, different parts of the bait region must approach the
equivalent residue in the other dimer to greater or lesser ex-
tents. In addition, the possible formation of a new disulfide
between monomers of the same dimer in the M690C variant
suggests that the bait regions within a dimer may approach one
another at this point. This suggests an organization of the four
bait regions of human $\alpha_2M$ in the central cavity of the tetramer,
perhaps as the cavity body of the x-ray structure, with exten-
sive interaction between pairs of bait regions from different
dimers and less extensive contact between pairs from the same
dimer (Fig. 8).

A possible concern in trying to deduce structural properties
of plasma $\alpha_2M$ from the properties of these variants is that
formation of a new disulfide between dimers might have
grossly distorted the interface that results in the normal dimer-
dimer association and thus either precluded tetramer forma-
tion or driven the tetramer into a distorted structure that is not
representative of native $\alpha_2M$. However, by several criteria, this
appears not to be the case for these variants, because they
possess a number of structurally dependent properties that are
very similar to those of plasma $\alpha_2M$. Thus, all four variants
form tetramer as the nearly exclusive species, with the excep-
tion of M690C and V700C, which also form a small amount of
dimer. This contrasts with more drastically modified bait re-
gion variants that we have previously examined, which had
large truncations within the bait region and that consequently
formed exclusively dimers that could not associate to tetramer
(12). The present variants also form close to the full com-
plement of thiol esters that can be cleaved by nucleophiles at rates
very similar to plasma or recombinant wild-type $\alpha_2M$ (Table I).
They are also capable of undergoing complete slow to fast
conformational change upon reaction with proteinase. The
presence of new interdimer disulfides is therefore likely to
result from the juxtaposition of the new cysteines across the
dimer-dimer interface as a consequence of initial dimer-dimer
association in the normal manner. This then requires the bait
regions to be directly involved in dimer-dimer interactions in
plasma $\alpha_2M$.

Such involvement of the bait regions of different dimers in
the noncovalent interface of $\alpha_2M$ is not surprising, given the
cooperative nature of the conformational changes that result
from bait region or thiol ester cleavage. Earlier studies by
Roche et al. on chemical cross-linking of subunits of $\alpha_2M$ (25,
26) had already shown that covalent cross-links could lead to a
rigidification of the $\alpha_2M$ interface such that it no longer re-
sponded normally to bait region or thiol ester cleavage. How-
ever, unambiguous interpretation of these earlier studies was
not possible because numerous cross-links were always present
and the sites of cross-linking were unknown. In contrast, it was
seen in the present study that the introduction of one or two
interdimer cross-links between residues within the bait region
leads to a blockage of the thiol ester cleavage-induced confor-
mational change, as evidenced both by incomplete conversion
from slow to fast forms after cleavage of the thiol esters by
methylene and by the small fraction of each variant that shows
near-normal conformational change reported by changes
in TNS fluorescence. Even when conformational change is
brought about by proteinase cleavage of the bait region, there is
evidence from the greatly lowered efficiency of proteinase trap-
ping (Table I) that the structural rearrangements may have been
slowed down. Such changes in efficiency of proteinase trapping
as a function of the relative rates of cleavage and conformational change have been documented for plasma $\alpha_2M$ and trypsin (27, 28). This suggests that when the thiol esters alone are cleaved, the conformational change that occurs coop-
ervative between noncovalently interacting subunits across the
dimer-dimer interface involves a reorganization of the bait
region-bait region contact area. By introducing a disulfide be-
tween such pairs of bait regions, as in any of the four variants
considered here, this reorganization is either halted or greatly
slowed down. The observation that this blockage of movement
is much less when the bait region is cleaved by proteinase, such

![Fig. 8. Schematic representation of the location and degree of contact between bait regions in human $\alpha_2M$.](image)
that all of the $\alpha_2$M is in the fast form in less than 10 min, is quite consistent with and even supportive of such a model, in that the cleavage by proteinase, which is specifically restricted to the bait region, could isolate the disulfide cross-link from the parts of the bait region that need to move relative to one another, thereby freeing them to reorient and thus abrogating the blockage of conformational change.

Our proposed location of the four bait regions at the center of the $\alpha_2$M tetramer in the position of the cavity body found in the crystal structure of methylamine-transformed $\alpha_2$M is also consistent with the changes in TNS fluorescence caused by proteinase treatment of the variants and of the reduced activity of trypsin in complex with these $\alpha_2$M variants. The location and size of the cavity body in the crystal structure of methylamine-transformed $\alpha_2$M is such that it would have to be removed in whole or in part to accommodate one or two proteinase molecules in the central cavity. If the bait regions constitute all or part of the cavity body, their cleavage by proteinase and the consequent reorientation of the interface, as suggested by results presented here, could constitute such a restructuring. For the four variants, we found that TNS fluorescence spectra not only were different from either native or transformed plasma $\alpha_2$M but also were much less responsive to change upon reaction with proteinase than was plasma $\alpha_2$M. This was particularly so for the M690C and T705C variants (Fig. 4). These are the variants that also show the lowest specific activity for trapped trypsin and also give TNS fluorescence spectra for the proteinase-treated forms that are higher specific activity for trapped trypsin and also give TNS

interface, either by disulfide formation, as described here, or by chemical cross-linking, as suggested elsewhere (25, 26), slows or prevents this reorientation with consequent reduction in rate of conformational change and in efficiency of proteinase trapping.

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