Thiol Ester Cleavage-Dependent Conformational Change in Human α_2-Macroglobulin. Influence of Attacking Nucleophile and of Cys949 Modification†

Peter G. W. Gettins*

Department of Biochemistry, University of Illinois−Chicago, Chicago, Illinois 60612-4316

Received April 5, 1995; Revised Manuscript Received June 14, 1995®

ABSTRACT: Cleavage of the thiol ester that exists between the side chains of Cys949 and Gln952 in human α_2-macroglobulin (α_2M) destabilizes the native conformation and leads to a large-scale conformational change that results in exposure of the receptor binding domain and to changes in electrophoretic mobility and sedimentation coefficient. The basis of this destabilization of the α_2M native conformation following thiol ester cleavage is not understood. We have extended observations that chemical modifications of the newly-formed SH in thiol ester-cleaved α_2M can slow the rate of conformational change in an attempt to determine the factors that influence the kinetic stability of the native conformation. Using changes in the fluorescence of α_2M-bound 6-(*p*-toluidino)-2-naphthalenesulfonic acid, we have determined the rate constant for conformational change in human α_2M, following thiol ester cleavage by ammonia, methylamine, or ethylamine, both in the absence and in the presence of an SH-modifying group, methyl methanethiosulfate. The influence of bait region cleavage in half of the α_2M tetramer on this rate has been examined by comparing the properties of native α_2M with those of I-form α_2M. The properties of two recombinant α_2M variants, C929S and C949Q, have also been examined. We found that the stabilizing effects of Cys949 and Gln952 modification were synergistic and optimal for S-thiomethylation in conjunction with methylamine cleavage of the thiol ester. Modification of Gln952 in the absence of SH modification was destabilizing. The stabilizing effect of Cys949 and Gln952 modification was up to an order of magnitude less in I-form α_2M, indicating a significant influence of bait region cleavage in one half-molecule on the conformational stability of the other half. The two recombinant variants had properties consistent with them having conformations similar to that of reacted α_2M. This may reflect either their original conformation following biosynthesis or a relatively rapid decay to an altered conformation that is complete prior to purification. Our findings are consistent with the destabilization of the native conformation of α_2M arising from creation of charged and polar species in the region previously occupied by the thiol ester.

The thiol ester that forms between the side chains of Cys949 and Gln952 in human α_2-macroglobulin (α_2M)[1] plays a critical role in maintaining the native, active conformation of the protein and in bringing about the conformational changes that occur subsequent to reaction of the α_2M with proteinase (Sottrup-Jensen, 1989). In native α_2M, the thiol ester is relatively inaccessible to attacking nucleophiles, so that only the smallest amines, such as ammonia, methylamine, or ethylamine, react well (Larsson & Björk, 1984). In contrast, the reaction of α_2M with proteinase results in limited proteolysis in the bait region and the activation of the thiol ester to a "nascent state" in which it is very much more reactive toward many nucleophiles (Sottrup-Jensen et al., 1981). Following nucleophilic cleavage of the thiol ester, there is a further slow protein conformational change that results in a much more compact form for the protein, a reduced hydrodynamic radius, and faster electrophoretic mobility on nondenaturing polyacrylamide gel electrophoresis (PAGE). The increase in electrophoretic mobility following this conformational change is the basis for the designation of native α_2M as "slow" form (S) and reacted α_2M as "fast" form (F) (Barrett et al., 1979). The importance of thiol ester cleavage in this sequence of conformational events leading from S to F conformations is demonstrated by the essentially indistinguishable α_2M structures obtained either directly by thiol ester cleavage with nucleophile, which leaves the bait region intact, or indirectly by proteinase cleavage of the bait region, leading to thiol ester activation (Schroeter et al., 1992).

It might thus appear that the physical integrity of the thiol ester is essential for maintaining the active S conformation. However, experiments in which chemical modification of the Cys949 free SH group, immediately upon its generation by cleavage of the thiol ester, resulted in blockage of the S→F conformational change and retention of certain native-like properties for the α_2M demonstrated that the physical integrity of the thiol ester linkage *per se* is not the only factor in maintaining the S conformation. Thus, immediate cyanylation (Björk, 1985) or S-thiomethylation (Jensen et al.,

† This work was supported by Grant HD28187 and was presented in part at the 1995 meeting in San Francisco of the American Society for Biochemistry and Molecular Biology.

* Address correspondence to this author at the Department of Biochemistry, University of Illinois−Chicago, 1819−1853 W. Polk St., Chicago, IL 60612-4316. Telephone: 312-996-5534 FAX: 312-413-8769. E-mail: pgettins@gettins.bche.uic.edu.

® Abstract published in *Advance ACS Abstracts,* September 1, 1995.

[1] Abbreviations: α_2M, α_2-macroglobulin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; BHK, baby hamster kidney; PMSF, phenylmethanesulfonyl fluoride; TNS, 6-(*p*-toluidino)-2-naphthalenesulfonic acid; MMTS, methyl methanethiosulfate; DNP-SCN, 2,4-dinitrophenyl thiocyanate.

0006-2960/95/0434-12233$09.00/0

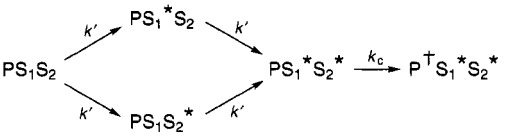
1994) of the free SH of Cys949 resulted in retention of native-like properties of human α_2M, including slow electrophoretic mobility and at least partial ability to trap proteinase. To determine the basis for such stabilization of the native conformation of α_2M in the absence of an intact thiol ester, we have examined the formation and kinetic stability of α_2M species in which there is no intact thiol ester. These forms were recombinant variants incapable of forming a thiol ester, C949S and C949Q, or thiol ester-cleaved plasma or I-form[2] α_2Ms in which the free SH group of Cys949 had been S-thiomethylated or cyanylated and/or the Gln952 residue had been re-formed, amidomethylated, or amidoethylated by use of the appropriate amine to cleave the thiol ester.

We found that the kinetic stability of the S conformation depended in a markedly nonadditive way both on the group used to modify the Cys949 SH and on the nucleophile used to attack the thiol ester (which becomes attached to the Gln952), consistent with exclusion of solvent favoring retention of the S conformation. Although the rate of decay of the S conformation could optimally be reduced by over ~1700-fold (from >0.1 s^{-1} in the absence of S-thiomethylation and with cleavage by ethylamine to 5.9×10^{-5} s^{-1} in the presence of MMTS), the slowest rate constant for the conformational change (4×10^{-5} s^{-1}) was still sufficiently high that variants that are incapable of forming a thiol ester and thus achieving indefinite stabilization of the S conformation would be expected to have decayed to the F conformation within a few hours of their biosynthesis. This was borne out by the properties of the two recombinant variants studied.

MATERIALS AND METHODS

Isolation of Plasma and Variant α_2Ms. Plasma α_2M was isolated from outdated human plasma obtained from the University of Illinois Hospital Blood Bank by chromatography on zinc chelate resin, Cibachron blue gel, and AcA22, as described previously (Dangott & Cunningham, 1982). The procedure used for establishing stably transfected BHK cells expressing C949Q or C949S variant α_2M was the same as described previously (Gettins et al., 1994). For large-scale preparation of recombinant α_2M, transfected BHK cells were grown to confluence in roller bottles and cycled every 24 h between medium devoid of fetal bovine serum and drugs and medium supplemented with these items. Only medium from the serum-free cycles was harvested. The recombinant human α_2M variants C949S and C949Q were isolated from the pooled serum-free growth medium by the protocol of Dangott and Cunningham (1982), but with omission of the Cibachron blue and AcA22 chromatography steps, which were judged to be unnecessary for isolation of pure α_2M. The α_2M was judged to be homogeneous by SDS–PAGE. Concentrations of all forms of α_2M were determined spectrophotometrically using the extinction coefficient for the plasma protein of 640 000 M^{-1} cm^{-1} (Hall & Roberts, 1978). I-form α_2M was prepared by limited reaction of plasma α_2M with agarose-immobilized chymotrypsin, as previously described (Gettins et al., 1989).

Scheme 1



Site-Directed Mutagenesis of α_2M. Site-directed mutagenesis was performed on the single-stranded M13mp18 construct containing the 2836 bp *Bsi*WI to *Xba*I fragment from plasmid p1167 (Boel et al., 1990), which covers the region coding for the residues involved in thiol ester formation. The 33 base antisense oligonucleotide 5′CAT ATT CTG CTC TCC TTG GCC ATA GGG CAT CTG3′, for which the underlined codon represents the cysteine→glutamine change at residue 949, was used for introduction of the mutation. Mutagenesis was carried out using the Amersham *in vitro* Mutagenesis System Version 2.1, which is based on phosphorothioate-modified DNA (Nakamaye & Eckstein, 1986; Taylor et al., 1985a,b; Sayers et al., 1988). After confirmation of the mutation by sequencing, the mp18–α_2M construct was digested with *Bsi*WI and *Cla*I and the 983 bp fragment containing the mutated thiol ester region ligated into the α_2M–expression vector p1167 digested with the same restriction endonucleases. Sequencing was carried out on the plasmid to confirm the presence of the altered codon at position 4309. This gave a new expression vector, p1167–C949Q, coding for C949Q variant α_2M. Preparation of the plasmid for expression of the C949S variant α_2M has already been described (Gettins et al., 1994).

TNS Fluorescence Measurements. Wavelength and kinetic measurements of TNS fluorescence were made on an SLM8000 spectrofluorometer using samples of 1.2 mL in thermostated disposable acrylic cuvettes. A TNS concentration of 50 μM was used for all measurements. Excitation was at 316 nm, with slits of 4 nm. Emission slit settings of 4 nm for emission spectra and 16 nm for monitoring the emission at a constant wavelength were used. For kinetic measurements at constant wavelength, the fluorescence intensity was monitored at 410 nm, which is close to the maximum for TNS bound to conformationally-altered α_2M. A 50 mM Hepes buffer containing 150 mM NaCl was used at the pH values indicated in the text.

Rate Constant Determination. The TNS time courses for α_2M conformational change following thiol ester cleavage were analyzed using the model shown in Scheme 1 developed by Olson (Strickland et al., 1984) as the simplest way of accounting for the conformational changes in α_2M upon thiol ester cleavage. In this scheme, "P" represents the native-like F conformation of a dimer within the α_2M tetramer and $P^\dagger$ the changed F conformation; S_1 and S_2 represent intact and S_1* and S_2* cleaved thiol esters, respectively, for the two subunits within the P dimer, k' is the rate constant for thiol ester cleavage, and is identical and independent for each subunit, and k_c is the rate constant for the major protein conformational change within the dimer detected by TNS fluorescence. The ability to determine both k' and k_c accurately from a single time course depends on

[2] I-form α_2M is a half-reacted form of α_2M in which the bait regions and thiol esters of only one of the two halves of the molecule have been cleaved, whereas those in the second half are still intact (Gettins et al., 1989). It has properties of electrophoretic mobility and proteinase inhibition consistent with it being a homogeneous population of half-reacted tetramers. It reacts with proteinase or methylamine at the same rate as tetrameric α_2M but with the expected half-stoichiometry of proteinase trapped or thiol esters cleaved (Gettins et al., 1989) and produces a structure very similar to that of "fast"-form α_2M as judged by image reconstruction electron microscopy (J. K. Stoops and P. G. W. Gettins, unpublished results).

how different the two rate constants are. If $k_c \gg k'$, the time course becomes relatively insensitive to k_c. Conversely, if $k_c \ll k'$, the time course becomes relatively insensitive to k'. Since pseudo-first-order reaction conditions were used, the value of k' could in principle be changed by altering the pH and/or the amine concentration such that the time course was dominated either by k' or by k_c.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gels were run under nondenaturing conditions in 5% acrylamide slabs according to the procedure of Davis (1964) or under denaturing conditions on 7.5% gels according to the procedure of Laemmli (1970).

DTNB Assay. Free sulfhydryl groups were measured by DTNB assay using the change in OD at 410 nm that accompanies release of the thionitrobenzoic acid group. An extinction coefficient of 13 600 M^{-1} cm^{-1} (Ellman, 1959) was used for determining the absolute concentrations of free thiol. Solutions were degassed to reduce reoxidation of the released thionitrobenzoic acid.

RESULTS

Comparison of Native and Modified α_2Ms. The conformational properties of α_2M species modified chemically at the thiol ester-forming residues were examined by PAGE under nondenaturing conditions. Whereas reaction of plasma α_2M with ammonia or methylamine resulted in complete conversion from the slow to the fast electrophoretic form (Figure 1, lanes b and c), similar reactions in the presence of either MMTS or DNP-SCN, which resulted in S-thiomethylation or cyanylation, respectively, of the Cys949 SH group, resulted in different degrees of blockage of the conformational changes (Figure 1, lanes d and e). This was manifested by the retention of protein intensity at the position of the "slow" form as well as at an intermediate position, corresponding to α_2M conformationally altered in only one of its two halves (Gettins et al., 1989; Larsson et al., 1985). Reaction with methylamine resulted in more blockage than did reaction with ammonia (Figure 1, lanes d−i) (ethylamine had very similar qualitative effects to methylamine, data not shown), though the differential effect was much greater for MMTS modification (Figure 1, panel A) than for DNP−SCN modification. Also, for reaction with methylamine, MMTS modification was much more effective at slowing the conformational change than was modification with DNP-SCN. The two recombinant α_2M variants, C949S and C949Q, migrated indistinguishably from fast-form α_2M (data not shown), as has already been shown for the C949S variant (Gettins et al., 1994).

As another way of examining the effects of modification of the thiol ester on the α_2M conformation, we monitored the changes in the TNS fluorescence emission spectrum caused by these α_2Ms. TNS binds noncovalently to α_2M and has been shown previously to be a sensitive monitor of conformational changes in the protein (Strickland et al., 1984, 1991). Whereas S-thiomethylated α_2M behaved similarly to unreacted S-α_2M in its effect on TNS fluorescence (Figure 2a,b), both C949S and C949Q variant α_2Ms behaved like conformationally altered F α_2M (Figure 2c,d,e). Although the effect of S-cyanylated α_2M could not be examined directly in the fluorometer, because of the absorption properties of the DNP group in the wavelength region of interest, it has previously been shown for S-cyanylated and subsequently purified α_2M that the modified α_2M affects TNS fluorescence similarly, but not identically, to S-α_2M (Björk, 1985). S-Thiomethylated α_2M thus behaved similarly to S-cyanylated α_2M in this regard (Björk, 1985), with the qualitative difference presumably arising from the partial

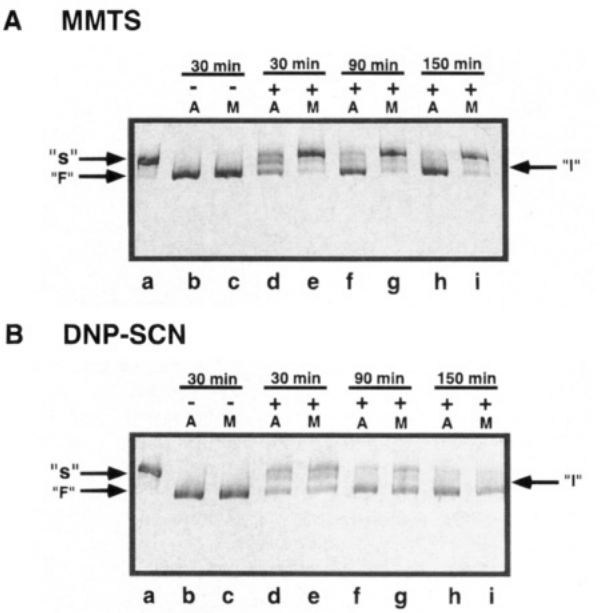


FIGURE 1: Comparison by nondenaturing PAGE (5%) of the effectiveness of S-thiomethylation and S-cyanylation as means of slowing the conformational change between "slow" and "fast" conformers of α_2M, shown for reaction with ammonia and methylamine. "−" signifies no MMTS or DNP-SCN: + indicates that each sample had 1 mM MMTS or DNP-SCN present throughout the reaction. Under these conditions, the SH groups are modified in a few seconds (Cunningham et al., 1990; Smith et al., 1972; Jensen et al., 1994). The letters A and M indicate that cleavage of the thiol ester was brought about by 0.4 M ammonia or methylamine, respectively, at pH 8.0. Reactions were carried out at 25 °C. The times indicated represent the total time between initiation of the amine cleavage reaction and loading on the gel. The arrows indicate the positions corresponding to slow ("S"), intermediate ("I"), and fast ("F") forms. Lane a in each case is native α_2M, held at 25 °C for 150 min prior to loading. Panel A, effect of MMTS; panel B, effect of DNP-SCN.

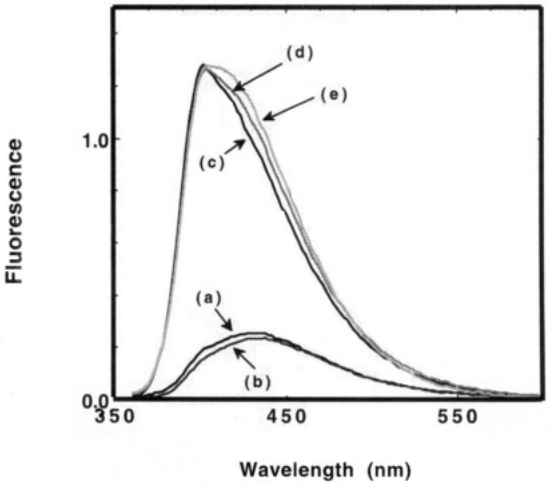


FIGURE 2: Effect of α_2M species on the fluorescence emission spectrum of TNS. (a) Native plasma α_2M; (b) plasma α_2M reacted with methylamine in the presence of MMTS to thiomethylate the liberated SH group; (c) plasma α_2M reacted with methylamine; (d) C949S variant α_2M; (e) C949Q variant α_2M. The concentration of all α_2M species was 0.4 μM tetramer. The reactions with methylamine, both in the absence and in the presence of MMTS, were allowed to proceed for 1 h prior to recording the spectra.

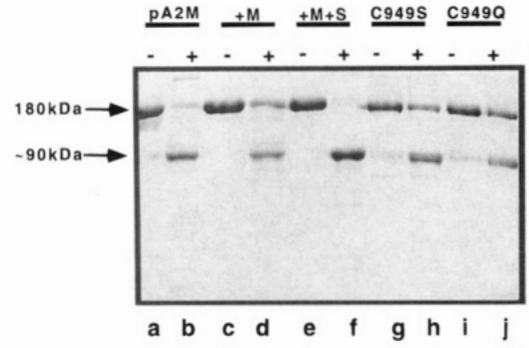


FIGURE 3: Susceptibility of bait region to tryptic cleavage in α_2M species examined by SDS−PAGE. The designations "+" and "−" indicate presence or absence of trypsin, respectively. The abbreviations pA2M, +M, and +M+S stand for native plasma α_2M, plasma α_2M treated with methylamine, and plasma α_2M treated with methylamine in the presence of 1 mM MMTS, respectively. C949S and C949Q designate the two recombinant variants. Reaction with TPCK-treated trypsin was with 2.2 equiv for 5 min before termination of the reaction by addition of PMSF to 1 mM.

decay of the cyanylated S to F conformations during the time required for purification.

The ability of chemically modified and variant α_2Ms to be cleaved specifically in the bait region by proteinase was also examined. Whereas the bait region of native α_2M is completely accessible to proteinase, resulting in complete cleavage of the bait regions with the resulting loss of the 180 kDa band and appearance of the 90 kDa bait region cleavage bands (Figure 3, lane b), the bait region in methylamine-treated α_2M showed only limited accessibility (Figure 3, lane d). However, methylamine reaction in the presence of MMTS gave an α_2M that possessed the same accessibility of its bait regions to proteinase as did native α_2M, suggesting that S-thiomethylation resulted in retention of the native-like conformation (Figure 3, lane f). Both recombinant variants behaved indistinguishably from methylamine-treated α_2M, suggesting that their conformations resembled that of F-α_2M (Figure 3, lanes h and j).

Kinetics of Conformational Change Resulting from Thiol Ester Cleavage. It has previously been shown that the major methylamine-induced conformational change in α_2M, whether measured by changes in intrinsic tryptophan fluorescence or in TNS fluorescence, is a relatively slow process that occurs cooperatively in each half-molecule following thiol ester cleavage of *both* thiol esters within that half-molecule (Strickland et al., 1984; Larsson et al., 1985). To quantitiate the rate of this conformational change, we followed the change in TNS fluorescence in α_2M resulting from cleavage of the thiol ester by 0.4 M ammonia, methylamine, and ethylamine at pH 8.5 (Figure 4). Second-order rate constants for the thiol ester cleavage step of 0.27 ± 0.04, 13.9 ± 0.7, and 1.9 ± 0.02 M^{-1} s^{-1} for ammonia, methylamine, and ethylamine, respectively, were obtained. These values are in reasonable agreement with those determined directly by DTNB assay of free SH groups (Larsson & Björk, 1984). The rate constant for the conformational change (k_c) depended significantly on the amine, being slowest for ammonia and fastest for ethylamine. Because some of the values of k_c were large relative to the psuedo-first-order rate constant (k') for thiol ester cleavage, when the reactions were carried out at 0.4 M amine, the analysis of the data was not always sensitive to the value of k_c (see Materials and Methods). To obtain more reliable values for k_c, a separate

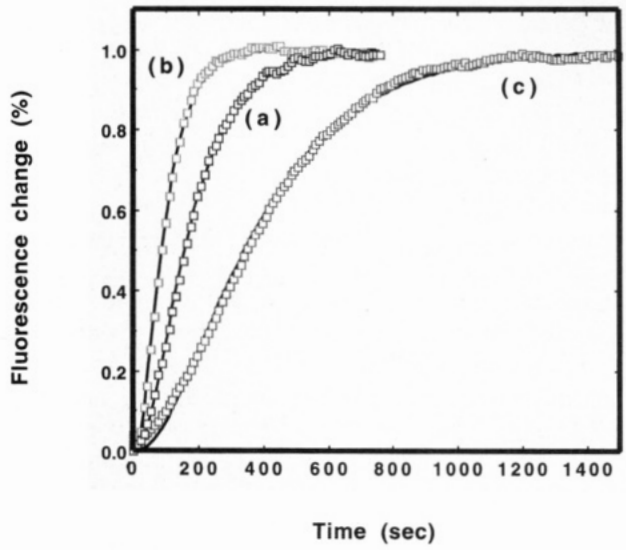


FIGURE 4: Complete time course of change in TNS fluorescence during the reaction of plasma α_2M with ammonia, methylamine and ethylamine. The solid lines represent nonlinear least-squares best fits to the data using the model of Scheme 1 and the equation derived from this by Olson (Strickland et al., 1984). (a) Reaction with ammonia; (b) reaction with methylamine; (c) reaction with ethylamine. All reactions were carried out in 50 mM Hepes buffer, pH 8.5, containing 150 mM NaCl and 50 μM TNS. Reactions were initiated by direct addition of the amine hydrochloride, to a final concentration of 0.4 M (N.B. free amine concentrations differed depending on the amine pK_a), to the cuvette. The cells were thermostated at 25 °C.

set of reactions were carried out at the same pH, but at higher amine concentration to better separate the thiol ester cleavage and conformational change steps. α_2M was incubated with 2.5 M amine at pH 8.5 for 30, 50, or 70 s and then diluted into the TNS assay buffer, to give a final amine concentration close to 0.4 M. In this way, close to 100% of the thiol esters had been cleaved for ammonia and methylamine reactions prior to the start of the fluorescence measurements, as determined by parallel reactions assayed for free SH release by DTNB assay (data not shown). Changes in TNS fluorescence should then have reflected *only* the kinetics of the conformational change step and should have given a simple exponential with a rate constant of k_c, which was found for both ammonia and methylamine (Figure 5 and Table 1). The conformational change resulting from ethylamine reaction was still too fast to follow by this method, being complete before fluorescence measurements could be started (Figure 5c). Even carrying out these reactions at pH 9.0 and 3.6 M ethylamine did not permit accurate estimation of k_c. The value of >0.1 s^{-1} for ethylamine (Table 1) is a lower limit based on the value for k' under these faster reaction conditions. That the assumption of complete thiol ester cleavage prior to assay was justified was demonstrated by the independence of the value of k_c obtained for different times of preincubation with amine (data not shown). In the absence of modification of the Cys949 SH group, attachment of methylamine to Gln952 increased the rate of α_2M conformational change by 2-fold, whereas ethylamine had a more marked effect, increasing the rate of conformational change by at least 12-fold (Table 1).

Effect of Modification of α_2M Cys949 on the Rate of Conformational Change. S-Thiomethylation of the Cys949 SH group gave large, amine-dependent reductions in k_c (Figure 6 and Table 1). The reduction was greatest with

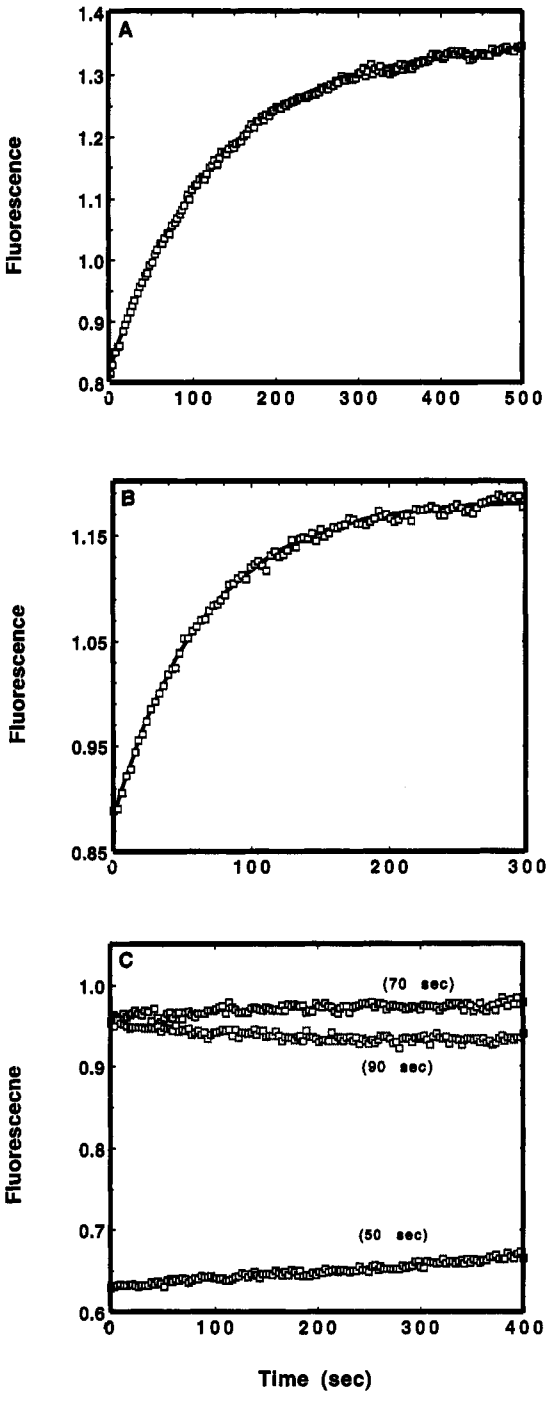


FIGURE 5: TNS-monitored conformational change step in α_2M resulting from cleavage of the thiol ester with different amines. Incubation of α_2M with pH 8.5 amine at a final concentration of 2.5 M was carried out for 50 s, except as noted, before dilution into the sample cuvette (final amine concentration about 0.4 M) and monitoring of fluorescence. The cleavage of the thiol esters was complete for ammonia and methylamine prior to the start of data collection. The kinetics therefore represent only the conformational change step and are well fitted by a single exponential (solid line). Values of k_c are given in Table 1. For ethylamine, the thiol ester cleavage reaction is slower and is thus not complete after 50 s, resulting in a smaller fluorescence change after 50 s reaction than after 70 s reaction. Panel A, ammonia; panel B, methylamine; panel C, ethylamine showing data for preincubations with amine for 50, 70, and 90 s, as indicated.

ethylamine (>1700-fold), less for methylamine (~400-fold), and least for ammonia (~7-fold) (Table 1). The effect could be reversed by subsequent addition of dithiothreitol, which displaced the S−CH_3 group. The TNS fluorescence showed an immediate increase, which levelled off at close to the value seen for the fast-form conformation (data not shown).

Two other nonabsorbing, cysteine-modifying groups, Hg^{2+} and iodoacetamide, gave no significant alteration of the rates of the conformational change for any of the amines (data not shown). This could be due to the inability of these groups to effect a stabilization of the native conformation or to too slow a reaction of the reagents with the newly-formed free SH group.

Blockage and Kinetics of Conformational Change in I-Form α_2M. Scheme 1 treats the conformational changes in each half-molecule as independent. However, rapid kinetic studies of the reaction of α_2M with limiting amounts of proteinase suggest that bait region cleavage within one half-molecule may affect the properties of the second half-molecule, promoting decay and limiting further reaction with proteinase (Strickland et al., 1991). To determine the effect of bait region cleavage in one half of α_2M, such as would occur in binary α_2M−proteinases complexes, on the rate of thiol ester cleavage-induced conformational change in the second half, we therefore examined the properties of I-form α_2M.

The effect of I-form α_2M on TNS fluorescence was intermediate between those of native α_2M and fully methylamine-reacted α_2M (Figure 7a,b,d). After reaction with methylamine, however, the TNS spectrum closely resembled that produced by methylamine treatment of native α_2M (Figure 7d,e). A major difference in the effect of MMTS on the rate of conformational change in I-form versus normal α_2M was that after 1 h of reaction with methylamine in the presence of MMTS the TNS fluorescence indicated partial conversion (~30%) to the F conformation (Figure 7c), whereas normal α_2M showed no detectable change during the same time period (Figure 2a,b). This suggested that the effect of MMTS on reducing the rate of conformational change following thiol ester cleavage was less in I-form α_2M than in normal α_2M. Rate constants for the conformational change step were therefore determined for I-form α_2M, both in the presence and in the absence of MMTS, using changes in TNS fluorescence. In the absence of MMTS, reactions were carried out at high amine concentration for 30 s prior to dilution into the TNS-containing assay buffer (Figure 8A,B), analogously to determinations for native α_2M shown in Figure 5. As with native α_2M, the conformational change when the reaction was carried out with ethylamine was too fast to measure by this method (Figure 8C), so that only a lower limit is given in Table 1. However, side-by-side comparisons of complete time courses for reaction of native α_2M or I-form α_2M with 1 and 2 M ethylamine showed indistinguishable kinetics for the two α_2M species (data not shown). The qualitative difference in the effect of MMTS on stabilizing the S conformation was confirmed by quantitative measurements of k_c, determined from TNS-monitored complete time courses of reaction with 0.4 M amine at pH 8.5 (Figure 6B and Table 1). Although MMTS caused a comparable small reduction in k_c for reaction with ammonia, the reduction in k_c for reaction with either methylamine or ethylamine was an order of magnitude smaller for I-form α_2M than for native α_2M (Table 1), confirming the observation made from the complete TNS emission spectra (Figure 7) and showing that the presence of cleaved bait regions in the first half of the molecule interfered with the stabilizing effect of the Cys949 and Gln952 modifications.

Table 1: First-Order Rate Constant (k_c) for the Conformational Change Step in α_2M That Follows Reaction with Ammonia, Methylamine, and Ethylamine at pH 8.5 and Results in a Change in TNS Fluorescence

	k_c ($\times 10^3$ s^{-1})[a]					
	NH_3		CH_3NH_2		$CH_3CH_2NH_2$	
α_2M	−MMTS	+MMTS	−MMTS	+MMTS	−MMTS	+MMTS
p-α_2M	8 ± 0.5	1.2 ± 0.1	15 ± 0.2	0.038 ± 0.01	>100	0.059 ± 0.02
I-α_2M	7.3 ± 0.2	2.1 ± 0.05	24 ± 0.8	0.21 ± 0.01	>100	0.47 ± 0.03

[a] All rate constants were determined by nonlinear least-squares fits to time courses covering all or most of the complete reaction. The slower rate constants were determined from continuous monitoring of the reactions at 0.4 M amine. The fastest rate constants were determined by prereacting the α_2M and amine at high concentration prior to dilution into the TNS-containing assay buffer. The rate constants for ethylamine reaction in the absence of MMTS represent lower limits based on $k_c \gg k'$ and therefore the conformational change being primarily determined by k', which could be readily measured. All errors represent one standard deviation. All time courses were carried out at least twice and showed less than 10% variation in the fitted rate constant.

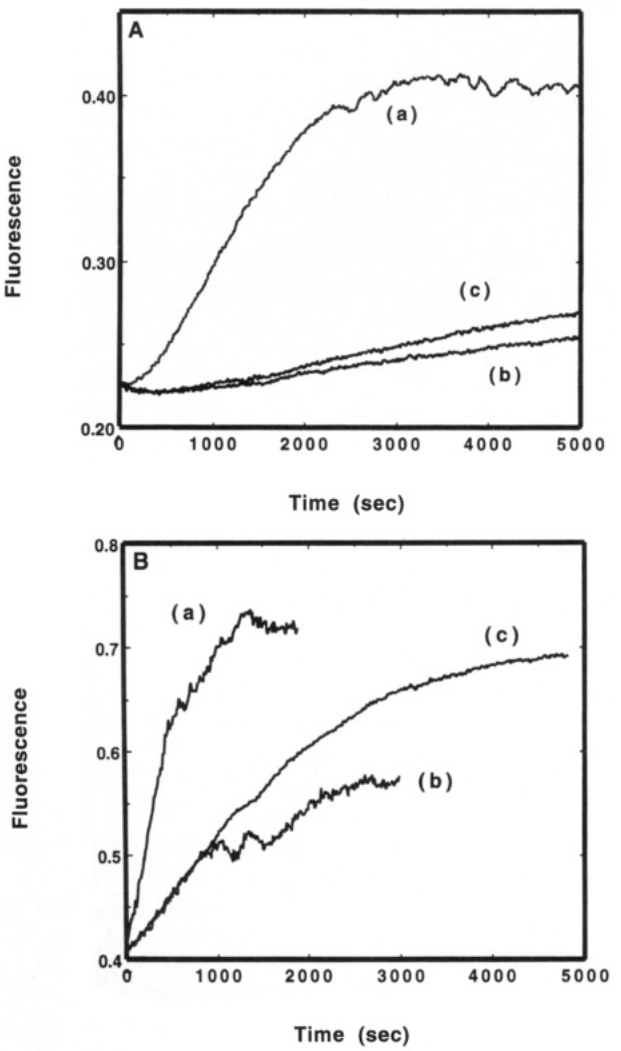


FIGURE 6: Time course of TNS fluorescence change during the reaction of plasma and I-form α_2Ms with amines in the presence of the sulfhydryl modifying group MMTS (1 mM). (a) Reaction with ammonia; (b) reaction with methylamine; (c) reaction with ethylamine. Reactions were carried out at 25 °C and pH 8.5 using 0.4 M amine. Values of k_c for the reactions are given in Table 1. Only 5000 s of the reaction is shown for the reactions of plasma α_2M with methylamine and ethylamine. These reactions were, however, followed for 15 000 s to obtain data for a large fraction of the reaction. Panel A, plasma α_2M; panel B, I-form α_2M.

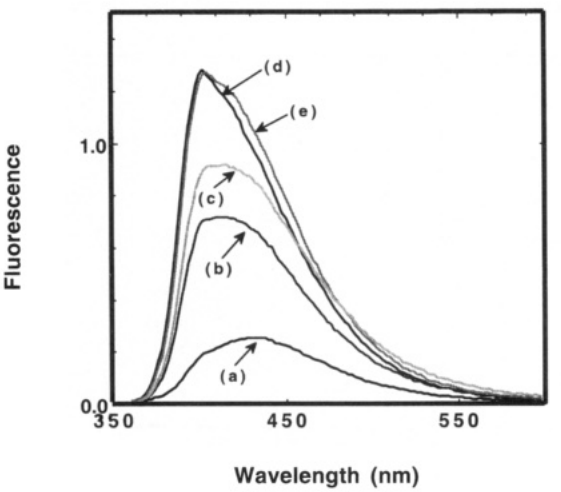


FIGURE 7: Comparison of the effects of I-form α_2M with those of "slow"- or "fast"-form α_2Ms on TNS fluorescence. (a) Native "slow" form α_2M; (b) I-form α_2M; (c) I-form α_2M reacted with methylamine in the presence of 1 mM MMTS for 1 h prior to recording the spectrum; (d) "fast form", methylamine-treated α_2M; (e) I-form α_2M reacted with methylamine for 1 h in the absence of MMTS.

DISCUSSION

In this work, we set out to determine the basis for the stabilization of the native-like S conformation of α_2M after the thiol ester has been cleaved, by examining the effects of separate and joint modifications of the Cys949 SH and Gln952 amido groups on properties of the α_2Ms. We found that, in the absence of modification of the Cys949 SH the effect of alkyl substituents on the attacking amine was to *destabilize* the native-like conformation. Whereas the smaller amine, methylamine, had only a 2-fold effect, the larger ethylamine caused at least a 12-fold destabilization compared with cleavage by ammonia (Table 1). In the absence of modification of Gln952 (i.e., by using ammonia for the cleavage), modification of the Cys949 SH group had a modest *stabilizing* effect. Much more impressive were the joint effects of S-thiomethylation of the Cys949 SH and alkylation of the Gln952 amido group. Thus, compared to the rate of conformational change when neither group was modified (8×10^{-3} s^{-1}), methylamine and thiomethylation resulted in a 210-fold reduction in the rate of conformational change, and ethylamine and S-thiomethylation caused a 140-fold reduction. When the intrinsic *destabilizing* effect of methylation or ethylation of the Gln amide was also taken into account, the stabilizing effect of S-thiomethylation was greater than 1700-fold (from >0.1 s^{-1} to 5.9×10^{-5} s^{-1}), suggesting a synergistic effect of the alkylamido and thiomethyl groups. This synergy was sensitive to the nature of the two groups, since cyanylation, although stabilizing the native conformation to about the same degree as S-thiomethylation when Gln952 was unmodified (Figure 1, lanes d of panels A and B), showed little additional

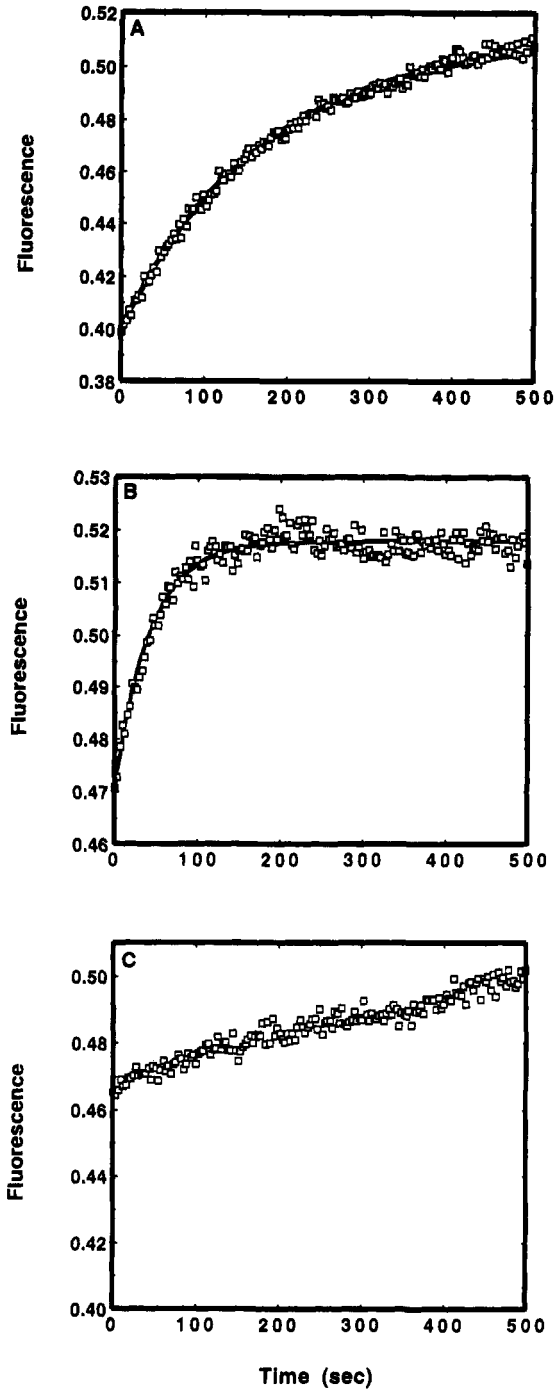


FIGURE 8: Time course of change in TNS fluorescence during the reaction of I-form α_2M with ammonia (panel A), methylamine (panel B), and ethylamine (panel C). The reactions were carried out in 50 mM Hepes buffer, pH 8.5, containing 150 mM NaCl and 50 μM TNS. Incubation of I-α_2M with pH 8.5 amine at a final concentration of 2.5 M was carried out for 30 s for ammonia and methylamine at 25 °C before dilution into the sample cuvette (final amine concentration about 0.4 M) and monitoring of fluorescence. The cleavage of the thiol esters was complete in each case prior to the start of data collection. The kinetics therefore represent only the conformational change step and are well fitted by a single exponential (solid line). Values of k_c are given in Table 1. The solids lines represent the nonlinear least-squares best fit to the data using the model of Scheme 1 and the equation derived from this by Olson (Strickland et al., 1984). For ethylamine, the rate of the conformational change was again too fast relative to the k' that was obtained during the reaction. The data in panel C represent TNS fluorescence after preincubation of I-α_2M with ethylamine for 50 s. The small increase in fluorescence with time results from incomplete cleavage of the thiol esters even after 50 s incubation (cf. Figure 5, panel C).

synergistic effect when methylamine was used (Figure 1, lanes e of panels A and B).

The other major finding was that although thiol ester cleavage in I-form and native α_2M resulted in a conformational change at very similar rates in the absence of modification of either Cys949 or Gln952 side chains, the ability of S-thiomethylation and amidoalkylation to stabilize the native-like conformation was an order of magnitude less than for native α_2M (Table 1). This shows a clear effect on the conformation of half of the α_2M tetramer on the properties of the other half, at least when the bait regions are cleaved in the first half, and demonstrates that the immediate environment of the thiol ester can be influenced by factors other that the chemical nature of the side chains of the 949 and 952 residues.

The properties of the two recombinant α_2M variants, C949S and C949Q, which can be considered as wild-type α_2M "chemically modified" at the cysteine residue, were comparable to those of reacted F conformation plasma α_2M. Thus, their effects on TNS fluorescence, their electrophoretic mobilities, and their susceptibility to proteolysis in the bait region were all comparable to those of conformationally-altered plasma α_2M, implying either that the S conformation was never formed or that, if it did form, there was no long-term stabilization of this conformation.

The way in which the chemical modifications of Cys949 and Gln952 side chains brought about kinetic stabilization of the slow-form conformation of α_2M suggests that the synergistic effect may arise from the increased ability to exclude solvent from the volume previously occupied by the intact thiol ether, and conversely that entry of solvent is normally responsible for destabilization of the slow-form conformation and bringing about the large-scale conformational changes in α_2M. Thus, alkylation of Gln952 in the absence of modification of the free SH actually destabilizes the slow-form conformation, whereas, once the SH is suitably modified [with SCH_3 being more effective than CN, and CN being much more effective than many other SH-modifying groups that have been qualitatively examined by others (e.g., see Van Leuven et al., 1982)] amidoalkylation can have a very beneficial effect in stabilizing the slow-form conformation. Taken together with the inverse correlation between size of attacking amine and rate of thiol ester cleavage, which implies very restricted access to the thiol ester, and the restricted mobility and hydrophobic environment of spin-labels and fluorophores attached to the Cys949 SH in cleaved α_2M (Zhao et al., 1988; Gettins et al., 1988), this suggests that the thiol ester is located at the bottom of a narrow hydrophobic cleft with limited accessibility.

The following model can be envisioned to account for the behavior of α_2M following thiol ester cleavage and may serve as a basis for further testing of the effects of modifications on kinetic stability. (i) In native α_2M, the intact thiol ester serves to physically exclude solvent from residues immediately adjacent to it and/or underlying it. (ii) Following cleavage of the thiol ester by an amine (in the absence of bait region cleavage to serve to activate the thiol ester), there are two potentially ionizable side chains in the place where the thiol ester previously existed (Glu951 and Cys949). These may favor entry of solvent, which in turn may open up the cleft leading to the thiol ester region and lower the activation energy to the thermodynamically favored fast-form conformation. (iii) If the Cys949 is not modified, the side chains

of large amines may serve to displace the Glu951 and Cys949 side chains, resulting in further destabilization. (iv) If the Cys949 is modified to block development of negative charge, though without introducing a large or polar group (such as CN, Hg^{2+}, or CH_2CONH_2), then an alkyl side chain on Gln952, rather than disrupting the now-occupied thiol ester hole, might lie in the cleft that forms the approaches to the thiol ester-forming residues and prevent access by solvent. This would account for CH_3 or CH_3CH_2 being much more effective than no modification of the glutamine. Interestingly, if thiol ester cleavage is brought about by activation of the thiol ester following bait region cleavage by proteinase, such as occurs *in vivo* as a result of reaction of α_2M with proteinase, the native-like S conformation cannot be significantly stabilized by modification of Cys949 and Gln952 side chains. This is consistent with the proposed model, since the activation of the thiol ester consequent to bait region cleavage by proteinase may result from a small conformational change in the approaches to the thiol ester that greatly increases access to the thiol ester. Under such circumstances, once cleavage of the thiol ester has occurred, it would be much more difficult to prevent entry of solvent through the more open access channel.

In the absence of an intact thiol ester, the rate of conformational change to the thermodynamically favored F conformation depends critically on the immediate environment of the cysteine and glutamine residues. In human α_2M, this environment can be modified chemically or by mutagenesis. In other species, the scope for stabilization may be very different Thus, it has been reported that, in the absence of bait region cleavage, opening of the thiol ester in bovine α_2M does not result in a conformational change until bait region cleavage occurs (Dangott & Cunningham, 1982). This may again not be an all-or-nothing effect, but a manifestation of an even greater stabilization of the native conformation arising from particular residues in the vicinity of the thiol ester-forming residues. In ovostatin, an egg macroglobulin, there is no thiol ester (asparagine replaces the cysteine) yet there is still a bait region cleavage-induced conformational change (Nielsen & Sottrup-Jensen, 1993). Both of these systems merit detailed examination to determine the kinetics of conformational change and the factors that can enhance or reduce this rate. If a common mechanism of destabilization (solvent entry) occurs in all of these, it should be possible to show a common pattern of effects of modifications.

ACKNOWLEDGMENT

I thank Brenda Crews, Dr. Bingqi Fan, and Dr. Kwangho Hahn for preparation of the C949S and C949Q variants, Dr. Esper Boel for the generous gift of the α_2M expression plasmid p1167, Dr. Steven Olson for access to his SLM8000 spectrofluorometer, Dr. Philip Patston for helpful and critical comments on the manuscript, and Scott Suda and Lingshi Qiu for technical assistance.

REFERENCES

Barrett, A. J., Brown, M. A., & Sayers, C. A. (1979) *Biochem. J. 181*, 401−418.
Björk, I. (1985) *Biochem. J. 231*, 451−457.
Boel, E., Kristensen, T., Petersen, C. M., Mortensen, S. B., Gliemann, J., & Sottrup-Jensen, L. (1990) *Biochemistry 29*, 4081−4087.
Cunningham, L. W., Crews, B. C., & Gettins, P. (1990) *Biochemistry 29*, 1638−1643.
Dangott, L. J., & Cunningham, L. W. (1982) *Biochem. Biophys. Res. Commun. 107*, 1243−1251.
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404−427.
Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70−77.
Gettins, P., Beth, A. H., & Cunningham, L. W. (1988) *Biochemistry 27*, 2905−2911.
Gettins, P., Crews, B. C., & Cunningham, L. W. (1989) *Biochemistry 28*, 5613−5618.
Gettins, P. G. W., Boel, E., & Crews, B. C. (1994) *FEBS Lett. 339*, 276−280.
Hall, P. K., & Roberts, R. C. (1978) *Biochem. J. 17*, 27−38.
Jensen, P. E. H., Shanbhag, V. P., & Stigbrand, T. (1994) *Ann. N.Y. Acad. Sci. 737*, 451−452.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Larsson, L.-J., & Björk, I. (1984) *Biochemistry 23*, 2802−2807.
Larsson, L.-J., Olson, S. T., & Björk, I. (1985) *Biochemistry 24*, 1585−1593.
Nakamaye, K. L., & Eckstein, F. (1986) *Nucleic Acids Res. 14*, 9679−9698.
Nielsen, K. L., & Sottrup-Jensen, L. (1993) *Biochim. Biophys. Acta 1162*, 230−232.
Sayers, J. R., Schmidt, W., & Eckstein, F. (1988) *Nucleic Acids Res. 16*, 791−802.
Schroeter, J. P., Kolodziej, S. J., Wagenknecht, T. Bretaudiere, J.-P., Tapon-Bretaudiere, J., Strickland, D. K., & Stoops, J. K. (1992) *J. Struct. Biol. 109*, 235−247.
Smith, D. J., Maggio, E. T., & Kenyon, G. L. (1972) *Biochemistry 11*, 766−771.
Sottrup-Jensen, L. (1989) *J. Biol. Chem. 264*, 11539−11542.
Sottrup-Jensen, L., Petersen, T. E. & Magnusson, S. (1981) *FEBS Lett. 128*, 123−126.
Strickland, D. K., Bhattacharya, P., & Olson, S. T. (1984) *Biochemistry 23*, 3115−3124.
Strickland, D. K., Larsson, L.-J., Neuenschwander, D. E., & Björk, I. (1991) *Biochemistry 30*, 2797−2803.
Taylor, J. W., Ott, J., & Eckstein, F. (1985a) *Nucleic Acids Res. 13*, 8765−8785.
Taylor, J. W., Schmidt, W. Cosstick, R., Okruszek, A., & Eckstein, F. (1985b) *Nucleic Acids Res. 13*, 8749−8764.
Van Leuven, F., Marynen, P., Cassiman, J.-J., & Van den Berghe, H. (1982) *Biochem. J. 203*, 405−411.
Zhao, B., Musci, G., Sugawara, Y., & Berliner, L. J. (1988) *Biochemistry 27*, 5304−5308.

BI950763C