Preparation and Initial Characterization of an Intermediate, Half-Cleaved Form of Human α_2-Macroglobulin†

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ABSTRACT: A form of human α_2-macroglobulin (α_2M) has been prepared that has properties intermediate to those of native α_2-macroglobulin and 2:1 protease–α_2M ternary complex by using Sepharose-linked chymotrypsin. The intermediate form has mobility on native polyacrylamide gels between the fast and slow forms of α_2M and migrates as a diffuse band. Two bait regions and two thiol esters per α_2M tetramer are cleaved, although no chymotrypsin is detectable in the modified α_2-macroglobulin species. The remaining bait regions and thiol esters can be cleaved by further reaction with other proteases. Intermediate-form α_2M can trap 1.18 mol of chymotrypsin, 0.85 mol of trypsin, and 0.65 mol of thrombin. Although both thrombin and methylamine react with intermediate-form α_2M at rates not distinguishable within experimental error from those of their reactions with native α_2M, chymotrypsin–Sepharose reacts much more slowly with the intermediate form than with native α_2M, indicating a nonequivalence of the two reactive sites on α_2M. This nonequivalence may be present initially or be induced by reaction at the first site. Comparison of ESR results obtained from spin-labeling methylamine-treated or protease-reacted α_2M with those from spin-labeling of the free SH groups in intermediate-form α_2M shows that trapped protease influences the mobility of the attached nitroxide either through direct contact or by producing a different conformation from that present in methylamine-treated or intermediate-form α_2M.

A major step toward an understanding of the mechanism by which human α_2-macroglobulin (α_2M)[1] traps proteases would be an elucidation of the organization of the four identical polypeptides in the native molecule. These four chains are disulfide linked in pairs (Jensen & Sottrup-Jensen, 1986) and are further associated through noncovalent interactions to give the native tetramer. Figure 1 illustrates two quite distinct ways in which the disulfide-linked half-molecules may associate to constitute the two protease binding sites. The second arrangement is the basis for a model of α_2M proposed by Feldman et al. (1985).

One potential means of distinguishing between the various models of α_2M is to form the 1:1 complex of protease with α_2M, rather than the maximal 2:1 complex, and to determine which of the α_2M subunits have been cleaved by the protease. The problem with such binary α_2M–protease complexes is the occurrence of slow, secondary reaction of the trapped protease with remaining uncleaved α_2M subunits. Such slow additional cleavage has been previously reported for the reaction of trypsin with α_2M (Sottrup-Jensen et al., 1983) and for the 1:1 plasmin–α_2M complex (Roche & Pizzo, 1987) and may account for the unexpectedly nonstoichiometric cleavage of subunits in 1:1 α_2M complexes with trypsin, thrombin, and plasmin isolated by immunoaffinity chromatography (Strickland et al., 1988). As a way of examining half-reacted α_2M species without the complication of such secondary proteolysis, we have exploited and extended the observation of Björk (1984) that matrix-bound protease can react with α_2M without productive complex formation. Using chymotrypsin covalently linked to Sepharose, we have produced an α_2M species which has two of the four bait regions proteolyzed and two of the four thiol esters cleaved, but which contains no trapped chymotrypsin. On native polyacrylamide gels, the species migrates at a rate intermediate between that of native α_2M and 2:1 protease–α_2M complexes. This intermediate form is capable of reacting with, and trapping, up to 1 additional mol of protease, whereupon the remaining two bait regions (Barrett & Starkey, 1973) and thiol esters are cleaved and the complex migrates identically with the normal 2:1 protease–α_2M complex on native gels. Reaction of the intermediate form with methylamine also results in opening of the remaining two thiol esters. Examination of the kinetics of reaction of α_2M with Sepharose-bound chymotrypsin shows that, while reaction with both halves of the α_2M molecule is possible, cleavage of the second pair of α_2M subunits is much slower than reaction of the first pair. The second protease binding site, at least in the half-reacted α_2M, is thus not as accessible to chymotrypsin–Sepharose as the first site, indicating either a fundamental asymmetry in the binding sites or subunit–subunit cooperativity (here negative).

The well-defined and near-stoichiometric properties of this intermediate form of α_2M suggest that it will be very useful as a means of examining subunit organization.

Materials and Methods

Purification of α_2M. Human α_2M was purified from recently expired plasma (American Red Cross, Nashville Region) by zinc–chelate chromatography and gel chromatography, as previously described (Dangott & Cunningham, 1982). The purity of each preparation was checked by electrophoresis under nondenaturing conditions and also in the presence of

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[1] Abbreviations: α_2M, α_2-macroglobulin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NPGB, *p*-nitrophenyl-*p*-guanidinobenzoate hydrochloride; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; TAME, *N*-tosyl-L-arginine methyl ester; FTIR, Fourier-transform infrared spectroscopy.

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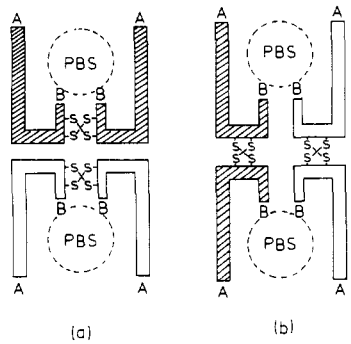

FIGURE 1: Schematic representation of two arrangements of the four subunits within human α_2M. (a) Protease binding site formed by disulfide-linked half-molecule; (b) protease binding site formed by noncovalently associated subunits [adapted from Feldman et al. (1985)]. The labels A and B on each chain are solely to demonstrate the differences between the models and do not necessarily represent the approximate locations of carboxy and amino termini. PBS indicates a protease binding site. One disulfide-linked half-molecule is hatched in each model.

SDS. Nondenaturing PAGE was performed in 5% acrylamide slabs (Davis, 1964). SDS–PAGE was carried out in 7.5% slab gels according to the procedure of Laemmli (1970). α_2M concentrations were determined spectrophotometrically by using $E^{1\%}_{280nm} = 8.9$ (Hall & Roberts, 1978; Barrett et al., 1979) and a molecular weight of 716000 based on the primary structure (Sottrup-Jensen et al., 1984).

All samples for SDS–PAGE were denatured and reduced for 45 min at 37 °C in buffer containing 2% SDS and 0.8% dithiothreitol; 1 mM PMSF was added to α_2M samples containing trapped protease prior to incubation to prevent additional cleavage of α_2M during denaturation.

Preparation of α-Chymotrypsin–Sepharose. α-Chymotrypsin (Cooper Biomedical) was dissolved in 0.1 M $NaHCO_3$, pH 8.3, to a final concentration of 9 mg mL^{-1} and coupled to 10 mL of CNBr-activated Sepharose 4B (Pharmacia) for 18 h at 4 °C. Unreacted sites were quenched with 30 mL of 0.1 M ethanolamine, pH 8.0, for 2 h at room temperature. The chymotrypsin–Sepharose was washed extensively with four cycles of alternating 0.1 M sodium acetate/0.5 M NaCl, pH 4.0, and 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.3, and stored in PBS, pH 5.7, containing 0.02% NaN_3 at 4 °C. The concentration of active chymotrypsin coupled to Sepharose was 25 nmol/mL of gel as determined by spectrophotometric assay with *N*-benzoyl-L-tyrosine ethyl ester.

Preparation of Spin-Labeled Intermediate-Form α_2M. Intermediate-form α_2M was prepared as described under Results. This was reacted with a 20-fold molar excess of 4-(2-iodoacetamido)-TEMPO that had previously been dried onto the walls of the reaction tube by evaporation of the benzene solvent. The reaction was allowed to proceed for 1.5 h at 0 °C. No free SH groups remained after this period. Unreacted spin-label was removed by ultrafiltration in an Amicon fitted with a PM30 membrane, followed by dialysis against 3 × 2 L of 10 mM Hepes/50 mM NaCl, pH 7.1.

ESR Measurements. Spectra were recorded on a Varian E-line ESR spectrometer operating at 9.41 GHz. The parameters employed were field set of 3350 G, sweep range of 100 G, modulation frequency of 100 kHz, modulation amplitude of 1 G, time constant of 0.032 s, microwave power of 10 mW, and scan time of 12 min. The sample was contained in a quartz flat cell of 370-μL volume (Wilmad WG-813). The temperature of the E-238 cavity was regulated to within ±0.5 K by an E-257 VT unit by passing precooled nitrogen into the cavity through the optical port in the front. Signal averaging by an online PDP 11/73 microcomputer was necessary to achieve an acceptable signal to noise ratio.

Assays. Trypsin and thrombin active-site concentrations were determined by active-site titration with NPGB (Chase & Shaw, 1970), and α-chymotrypsin, with *p*-nitrophenol acetate (Kezdy & Kaiser, 1970). α-Chymotrypsin activity was measured at 256 nm using *N*-benzoyl-L-tyrosine ethyl ester (Walsh & Wilcox, 1970). Bovine trypsin (Cooper Biomedical) and human thrombin (a generous gift from J. W. Fenton) were assayed at 247 nm with *N*-tosyl-L-arginine methyl ester in 0.1 M Tris-HCl, pH 8.0.

The appearance of sulfhydryl groups was measured by reaction with DTNB at 410 nm (Larsson & Björk, 1984).

The extent of bait region cleavage was determined from SDS gels by densitometric scanning using a Zeineh SL-504-XL scanning densitometer.

The ability of α_2M species to react further with proteases was determined by addition of a 4:1 molar excess of protease for 5 min at 25 °C in 0.1 M Tris-HCl at pH 7.8. Unreacted chymotrypsin and trypsin were inhibited by a 10-fold or 4-fold excess, respectively, of soybean trypsin inhibitor. The larger amount used for chymotrypsin inhibition was because of the lower K_d for the protease–inhibitor complex. Unreacted thrombin was inhibited with a 5-fold molar excess of human antithrombin III.

Materials. 4-(2-Iodoacetamido)-TEMPO (d_{12}) was a generous gift from Dr. Albert H. Beth. BTEE, DTNB, soybean trypsin inhibitor, and TAME were obtained from Sigma Chemical Co. CNBr-activated Sepharose was purchased from Pharmacia. Human antithrombin was prepared from pooled outdated plasma by a modification of the method of Thaler and Schmer (1975) (Gettins, 1987).

RESULTS

Reaction of α_2M with Chymotrypsin–Sepharose. Freshly prepared chymotrypsin–Sepharose appears to possess different types of chymotrypsin species, reflecting the heterogeneity of attachment sites of the chymotrypsin to the Sepharose. Thus, some protease molecules are capable of binding to α_2M in a normal manner to give covalent or noncovalent complexes; the latter can only be eluted by SDS or acid treatment. This class of attached chymotrypsin can, however, be saturated with α_2M. A second class of chymotrypsin molecules can cleave α_2M bait regions without productive complex formation. These are the sites exploited in this study. The resulting α_2M molecules remain in solution and can be separated from the matrix by centrifugation or filtration.

To follow the kinetics of reaction between this latter class of Sepharose-bound chymotrypsin and α_2M, a series of plastic vials was set up, each containing 0.62 mL of gel and 1.4 mL of 3.5 μM native α_2M in 50 mM Hepes, 0.1 M NaCl, and 2 mM EDTA, pH 7.6. These vials were slowly agitated on a nutator for different lengths of time. The reaction was stopped by centrifugation and filtration of the α_2M-containing supernatant through a 0.2-μm filter. Each time point was determined in duplicate. Figure 2 shows an SDS–polyacrylamide gel of samples reacted for different lengths of time. The band at 180 kDa corresponds to uncleaved subunits, while the pair of bands centered at 90 kDa corresponds to cleavage at the bait region. Cleavage of the first two bait regions occurs much more rapidly than cleavage of the second pair. This is clearly seen in Figure 3, which shows the number of bait regions cleaved as a function of length of time exposed to the gel. The number of bait regions cleaved was determined by comparison of the densitometric intensity of the pair of 90-kDa bands on the SDS gel as a fraction of the total intensity of 180- and 90-kDa bands. The fall-off in bait regions cleaved is not due

Table I: Properties of Intermediate-Form α_2M and Native α_2M

	free SH groups		trapped chymotrypsin (mol/tetramer)	inhibn of additional proteases (mol/tetramer)			% bait region cleaved thrombin
	initially	$+CH_3NH_2$		chymotrypsin	trypsin	thrombin	
native α_2M	0.05[a]	3.85	0	1.66	1.86	1.60	0
intermediate α_2M	1.92	3.85	0	1.18	0.85	0.65	46

[a] Errors are ±0.02 mol/tetramer.

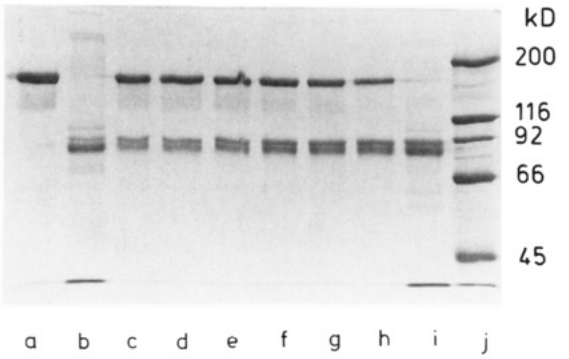

FIGURE 2: Time course of reaction of human α_2M (1.6 μM) with chymotrypsin–Sepharose followed by SDS–PAGE on a 7.5% acrylamide gel. Lane a, native α_2M; lane b, native α_2M plus trypsin; lane c, native α_2M reacted with chymotrypsin–Sepharose for 10 min; lane d, t = 20 min; lane 3, t = 30 min; lane f, t = 60 min; lane g, t = 90 min; lane h, t = 180 min; lane i, t = 180-min sample reacted further with trypsin; lane j, molecular weight standards. The band at the front in lanes b and i is soybean trypsin inhibitor and complex with trypsin.

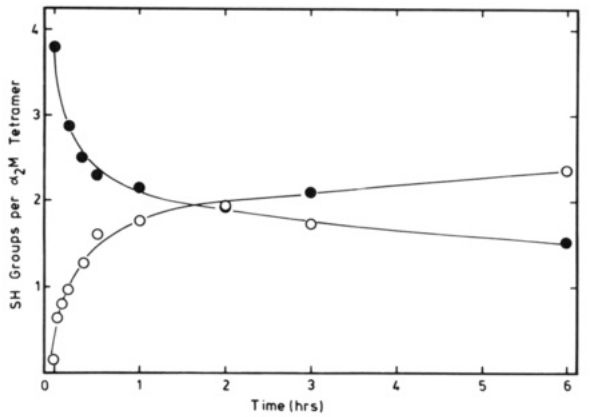

FIGURE 3: Cleavage of bait regions as a function of time of exposure of native α_2M to chymotrypsin–Sepharose. The percentage cleavage was determined from densitometric scanning of SDS–polyacrylamide gels and is the average of two separate experiments. The solid line is for visual aid only.

to inactivation of the bound chymotrypsin, since the gel retains its activity against fresh α_2M.

Part of each time point sample was assayed immediately for thiol ester cleavage and for residual, intact thiol esters by using DTNB without and with 100 mM methylamine, respectively. The results are shown in Figure 4. The curve drawn through the points representing thiol esters cleaved is that obtained from Figure 3 for bait region cleavage. Thiol ester opening thus exactly follows bait region cleavage. For each time point, the sum of cleaved and uncleaved thiol esters remains close to 4.

A native gel of α_2M reacted long enough with chymotrypsin–Sepharose to cleave 50% of the total bait regions present shows a single, rather diffuse band that has mobility intermediate between that of native α_2M and fully cleaved α_2M (Figure 5). Upon addition of trypsin to this intermediate

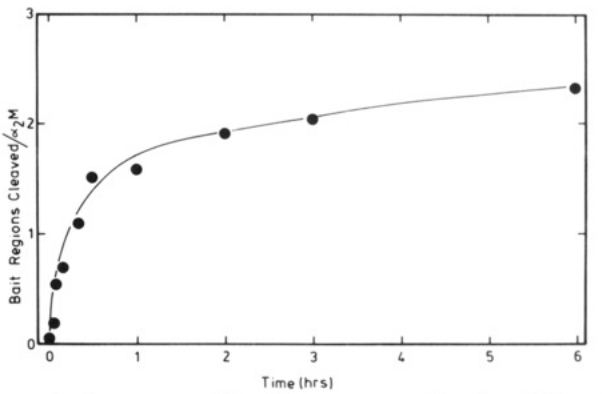

FIGURE 4: Appearance of free SH groups as a function of time of exposure of native α_2M to chymotrypsin–Sepharose. The data are for the same samples used for determination of bait region cleavage reported in Figure 3, and the solid line is taken from Figure 3. Intact thiol esters were determined as SH groups that appear upon reaction with 100 mM methylamine (●). For all samples, the sum of free SH and intact thiol esters is nearly constant.

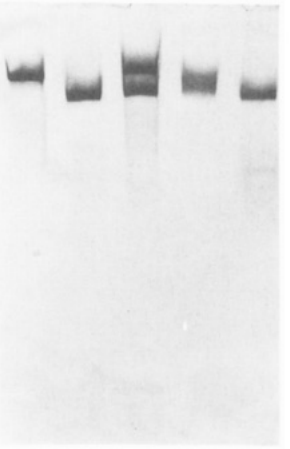

FIGURE 5: Nondenaturing gel of slow, intermediate, and fast forms of α_2M. Lane a, native α_2M; lane b, α_2M reacted with excess trypsin; lane c, mixture of samples from lanes a and b; lane d, intermediate-form α_2M; lane e, intermediate-form α_2M reacted with excess trypsin.

form, the mobility on the native gel increases to that of fully cleaved α_2M (Figure 5e).

Inhibitory Capacity of Intermediate-Form α_2M. From the results obtained above, it is clear that a distinct α_2M species can be generated by exposure to the chymotrypsin–Sepharose matrix for 1–2 h. This species migrates differently on native polyacrylamide gels from both fully reacted and unreacted α_2M but appears to be very similar in mobility and diffuseness to the binary α_2M–protease complexes reported by Strickland et al. (1988).

To determine the inhibiting capacity of this intermediate form of α_2M, the two samples of α_2M reacted with chymotrypsin–Sepharose for 2 h, for which DTNB assays and densitometric scans of SDS–polyacrylamide gels indicate that two

Table II: High-Field–Low-Field ESR Peak Separation for 4-(2-Acetamido)-TEMPO-Labeled α_2M Species

	separation (G)
methylamine treated[a]	60.6
2:1 chymotrypsin–α_2M[b]	70.5
intermediate-form α_2M	61.0

[a] Taken from Gettins et al. (1988). [b] Taken from Crews et al. (1987).

bait regions and two thiol esters have been cleaved, were assayed for their ability to trap and inhibit trypsin, chymotrypsin, and thrombin. The data are given in Table I, together with values obtained for the same native α_2M used to generate the intermediate form. For both thrombin and trypsin, the stoichiometry of trapping by the intermediate form is approximately half that of the native α_2M, while somewhat more than half-maximal amounts of chymotrypsin appear to be trapped by the intermediate form. Within the limits of experimental error (±2%), no chymotrypsin was present in the intermediate form prior to addition of free protease. A native gel of the intermediate-form α_2M and the products of reaction with methylamine, chymotrypsin, trypsin, and thrombin showed the expected conversion from intermediate to fast mobility in each case, confirming the completion of the conformational rearrangement (data not shown).

Spin-Labeled Intermediate-Form α_2M. It has previously been reported that α_2M labeled at the free SH group with 4-(2-acetamido)-TEMPO gives an ESR spectrum that is rigid glass limit if the thiol ester opening is brought about by reaction of the α_2M with chymotrypsin (Crews et al., 1987), but is characteristic of a more mobile nitroxide if the thiol ester opening results from reaction with methylamine (Gettins et al., 1988). The intermediate-form α_2M provides a useful species to help resolve the role of the chymotrypsin in affecting the environment of the cleaved thiol ester. The high-field to low-field line splitting of the ESR spectrum of intermediate-form α_2M spin-labeled with 4-(2-acetamido)-TEMPO is 61 G and is thus the same as methylamine-treated and labeled α_2M (Table II).

Reactivity of Intermediate-Form α_2M. The data shown in Figures 3 and 4 show that intermediate-form α_2M reacts much more slowly with chymotrypsin–Sepharose than does native α_2M. To investigate this difference further, the rates of reaction of native and intermediate-form α_2M with both methylamine and thrombin were determined from the appearance of free SH groups.

Twenty milligrams of native α_2M in 8 mL of 50 mM Hepes, 0.1 M NaCl, and 2 mM EDTA, pH 7.6, was reacted with 4 mL of chymotrypsin–Sepharose by gentle agitation on a nutator. The course of reaction was followed by DTNB assay for thiol ester release. After 2.5 h of reaction, 1.91 thiols were released, and an additional 1.98 thiols could be opened with methylamine. The reaction was stopped at this point and the α_2M removed from the gel by centrifugation and filtration. This intermediate-form α_2M was used for all kinetic studies with methylamine and thrombin.

For the reaction of α_2M with methylamine, 1.38 μM native or intermediate-form α_2M was reacted with 100 mM methylamine at room temperature in 200 mM Hepes, 50 mM NaCl, and 2 mM EDTA, pH 8.0, in the presence of DTNB. Each experiment was performed in duplicate. The progress of the reaction was followed continuously by spectrophotometric assay of DTNB cleavage, with the cell blanked against a reference cell containing all the same constituents except the α_2M. A semi-log plot of the extent of reaction against time is linear for both native and intermediate-form α_2M (Figure 6), since the reaction is carried out under pseudo-first-order conditions. The pseudo-first-order rate constants for the two forms of α_2M are indistinguishable within experimental error, with a value of $(2.9 \pm 0.2) \times 10^{-3}$ s^{-1}.

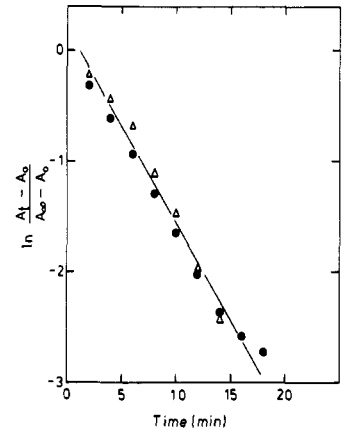

FIGURE 6: Semi-logarithmic plot of extent of reaction of α_2M species as a function of time of reaction with 100 mM methylamine. (●) Native α_2M; (△) intermediate-form α_2M.

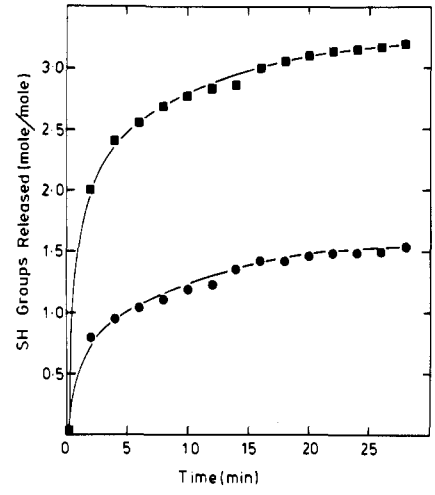

FIGURE 7: Time course of reaction of native α_2M (■) and intermediate-form α_2M (●) with thrombin at a stoichiometry of 2 mol of thrombin to 1 mol of α_2M. The reaction was performed at room temperature. Protein concentrations are given in the text. The solid lines are for visual aid.

Native and intermediate-form α_2M at a concentration of 1.38 μM were reacted with 2.76 μM thrombin at room temperature, and the reaction was followed by thiol ester release, assayed continuously, as above, with DTNB. The α_2M and thrombin concentrations used were chosen to give a relatively large change in optical density to permit accurate colorimetric monitoring of SH release. Data are thus limited for the early portion of the reaction. This, together with uncertainty about the free thrombin concentration as a function of time, precludes a rigorous kinetic analysis of the data for either native or intermediate-form α_2-macroglobulin. A plot of thiol groups released as a function of time is given in Figure 7. The difference in initial rate of reaction and in final number of thiols released is as expected for the effectively 2-fold higher α_2M reactive sites in native α_2M compared with intermediate-form α_2M. Taking this into account, and assuming second-order kinetics for the first part of the reaction (Downing et al., 1978; Dangott et al., 1983; Steiner et al., 1985), it appears that the one available site in intermediate-form α_2M reacts with thrombin with a rate constant very similar to that for native α_2M, since the time for half-reaction is in each case approximately proportional to the inverse of the initial con-

centration of α_2M reactive sites.

Without having more data points for the early part of the reaction in each case, it is not possible to state with any certainty that the reaction is biphasic. For low ratios of thrombin to α_2M, simple second-order kinetics are observed (Downing et al., 1978; Dangott et al., 1983; Steiner et al., 1985) whereas biphasic behavior is only seen when thrombin is in excess (Steiner et al., 1985) and is thought to result from free thrombin reacting slowly with thrombin–α_2M complex.

Discussion

Björk (1984) reported the use of trypsin–Sepharose as a means of cleaving α_2M bait regions without detectable trapping of protease. He did not, however, report formation of an intermediate-form α_2M, as reported here using chymotrypsin–Sepharose, but found complete reaction of all bait regions. The present findings are thus interesting from the point of view of possible differences between trypsin–Sepharose and chymotrypsin–Sepharose, but are also practically useful in being a means of producing a half-reacted form of α_2M without the complication of trapped protease that may still react with remaining bait regions. As a species for investigation of subunit organization, the present intermediate form of α_2M is superior to the binary protease–α_2M complexes isolated by Strickland et al. (1988) using an antibody affinity column. The presence of protease in the latter complexes probably accounts for the higher percentage of bait region cleavage (60–75%) than is reported here (50%). In turn, the lack of cleavage of additional bait regions probably explains the ability of the present intermediate form of α_2M to inhibit greater amounts of protease than the binary α_2M complexes. The latter can bind and inhibit only 0.3–0.4 mol of trypsin, whereas the present intermediate can inhibit 0.85 mol of trypsin, 0.65 mol of thrombin, and 1.18 mol of chymotrypsin. A value of up to 1 is expected if the second site on α_2M is still fully functional, which seems to be the case here.

Despite the variation in extent of bait region cleavage for binary–α_2M complexes and intermediate-form α_2M, both proteins behave similarly in native gels. They have mobilities intermediate between native and fully reacted α_2M, and both give much more diffuse bands than either of the two former species. Although the point was not previously addressed (Strickland et al., 1988), the very existence of an intermediate migratory form, whether generated by binary complex formation or exposure to chymotrypsin–Sepharose, is at the same time implicitly expected (Björk et al., 1984) and experimentally contradicted (Gonias & Pizzo, 1983) by earlier reports. Gonias and Pizzo (1983) reported nondenaturing gels of α_2M reacted with trypsin at different molar ratios of protease to inhibitor ranging from 0 to 2.0. Only slow and fast forms are seen, and by assuming equivalent binding sites such that a statistical distribution of protease is obtained (Pochon & Bieth, 1982), it was concluded that the binary α_2M complex migrates as the fast form. This is clearly at variance with the reported properties of isolated binary trypsin–α_2M (Strickland et al., 1988). Furthermore, Björk et al. (1984) performed a titration of trypsin into α_2M and followed reaction by bait region cleavage, appearance of free thiols, and assessment of conformational change monitored by α_2M tryptophan fluorescence. Each of these measures of reaction showed a linear change up to 2 mol of protease per α_2M tetramer. These studies, which did not involve native gels, do not distinguish between noncooperativity or cooperativity between half-molecules of α_2M but clearly show that degree of reaction or conformational change is proportional to extent of protease incorporation. This implies that a binary complex should have a conformation intermediate between native α_2M and the 2:1 protease–α_2M complex, as has been recently reported (Strickland et al., 1988). It might also be expected that the half-reacted α_2M reported here should have the observed intermediate mobility. The means of reconciling these findings with those of Gonias and Pizzo (1983) is not clear, though it is possible that additional bait region cleavage occurred in the binary complexes reported by Gonias and Pizzo such that all reacted species migrated as fast form.

One particularly intriguing property of intermediate-form α_2M is its further reactivity to various species. When probed with methylamine or even thrombin, there seems to be no significant difference between the rate of reaction of intermediate α_2M and of native α_2M. In contrast, when probed with chymotrypsin–Sepharose which can completely cleave two bait regions per tetramer of native α_2M in 2 h, reaction of the intermediate-form α_2M has a half-time of many hours. Although it is suprising that a protease as large as thrombin does not appear to be discriminated against by the two binding sites on α_2M, it is clear that, when tested with the right probe, the protease binding site of intermediate-form α_2M is not as reactive as the first site in native α_2M. A similar finding has been reported earlier from this laboratory for the reaction of α_2M with trypsin followed by stopped-flow fluorescence measurements (Dangott et al., 1983). In contrast, Pochon and Bieth (1982) claimed to have established equivalent and independent binding sites from the partitioning of chymotrypsin between native, binary, and ternary α_2M species. At present, it is not possible to distinguish between an asymmetry present in native α_2M (Bretaudiere et al., 1988) and the asymmetry generated by subunit–subunit interactions at the half-reacted stage.

A final point of interest from the present studies is the apparently similar and relatively unhindered environment for the liberated SH groups in methylamine-treated α_2M and chymotrypsin–Sepharose-treated α_2M, that, however, differs from this constrained condition in 2:1 chymotrypsin–α_2M complex, as judged by the mobility of nitroxide labels attached to the sulfhydryl (Table II). This may arise for two distinct reasons. Either the protease has a direct effect on the mobility of the nitroxide, through close proximity required by the confines of the trap, or the conformation of the cleaved subunit of intermediate-form α_2M differs from that of the chymotrypsin–α_2M complex and is closer to that of methylamine–α_2M. There is evidence from FTIR measurements that the latter two do differ in structure (Lee & Chlebowski, 1988).

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Registry No. Chymotrypsin, 9004-07-3; trypsin, 9002-07-7; thrombin, 9002-04-4.

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Activation Energy of the Slowest Step in the Glucose Carrier Cycle: Break at 23 °C and Correlation with Membrane Lipid Fluidity†

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ABSTRACT: Glucose transport in the rat erythrocyte is subject to feedback regulation by sugar metabolism at high but not at low temperatures [Abumrad et al. (1988) *Biochim. Biophys. Acta 938*, 222-230]. This indicates that temperature, which is known to alter membrane fluidity, also alters sensitivity of transport to regulation. In the present work, we have investigated a possible correlation between the effects of temperature on rate-limiting steps of glucose transport and on membrane fluidity. The dependences of methylglucose efflux and influx on cis and trans methylglucose concentrations were studied at temperatures between 17 and 37 °C. Membrane fluidity was monitored over the same temperature range by using electron paramagnetic resonance spectroscopy. External sugar did not affect efflux, and the K_m and V_{max} of sugar exit were respectively the same as the K_m and V_{max} of equilibrium exchange. These K_m's were relatively temperature independent, but the V_{max}'s increased sharply with temperature. The K_m and V_{max} of methylglucose entry were respectively much lower than the K_m and V_{max} of exit and exchange. Consistent with the above, intracellular sugar greatly enhanced sugar influx, and did so by increasing the influx V_{max} without affecting the influx K_m. Both lines of evidence indicated that the conformational change of the empty sugar-binding site from in-facing to out-facing orientation is the rate-limiting step of sugar entry into the rat erythrocyte. This was the case at all temperatures; however, the discrepancies of coefficients declined significantly with increasing temperature. The temperature dependence of the slowest step (change from in- to out-facing empty carrier) was evaluated. An Arrhenius plot showed that this step had a much greater activation energy below 23 °C than above: 48.5 ± 6.0 kcal/mol compared to 24.1 ± 1.8 kcal/mol. The temperature dependence of membrane fluidity, monitored by electron paramagnetic resonance spectroscopy of the probe 5-nitroxylstearate, also exhibited a transition near 23 °C. Our data indicate that membrane fluidity may be a factor influencing ease of carrier conformation change.

The relationship between the ordering of membrane lipids and the function of the membrane glucose carrier remains uncertain. In the human red blood cell (RBC), Lacko et al. (1973) showed a change in the activation energy for the glucose-entry V_{max} at around 20 °C. They suggested that the break could be related to the membrane structural transitions. Several such transitions have been documented (Forte et al., 1985). Lowe and Walmsey (1986) demonstrated a break in the Arrhenius plots of entry and exchange V_{max}'s for glucose transport at 35 °C, but without a change in the activation energy of the rate-limiting step of the carrier cycle. In the rat adipocyte, Amatruda and Finch (1979) compared the effect

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