Localization of the Two Protease Binding Sites in Human $\alpha_2$-Macroglobulin*

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The distance between the two protease binding sites in human plasma $\alpha_2$-macroglobulin has been estimated using singlet-singlet energy transfer experiments. $\alpha$-Chymotrypsin was labeled covalently with donor (dansyl chloride) or acceptor (fluorescein isothiocyanate) groups, and the efficiency of transfer between these dyes was measured within the $\alpha_2$-macroglobulin-$\alpha$-chymotrypsin complex. The distance between the surface exterior of the protease molecules was calculated to be 4 to 11 Å, depending on the assumption made about the equivalence of the binding sites.

A catalytically active dimer of $\alpha$-chymotrypsin was prepared using the heterobifunctional reagent N-succinimidyl-3(2-pyridyldithio)propionate (Pharmacia) following manufacturer’s recommendations. An aliquot of an ethanolic solution of this reagent was added under stirring to a 0.05 mM solution of $\alpha$-chymotrypsin in 100 mM phosphate, 100 mM NaCl buffer, pH 7.5. The final molar concentration of the reagent was 10-fold that of $\alpha$-chymotrypsin. The mixture was allowed to stand at room temperature (22°C) for 20 min, after which it was filtered on Sephadex G-25 to remove excess reagent. Half of the $\alpha$-chymotrypsin derivative was dialyzed against a 100 mM sodium acetate, 100 mM NaCl buffer, pH 4.5, and treated for 20 min at room temperature with 40 parts of dithiothreitol in order to produce the free thiol conjugate. This derivative was then dialyzed against the above phosphate/NaCl buffer and freed of reductant by filtration on Sephadex G-25. The thiol and 2-pyridyl disulfide-containing $\alpha$-chymotrypsin derivatives were then mixed and reacted for an additional 3 h, yielding a heterogeneous mixture of conjugates. Unreacted $\alpha$-chymotrypsin was isolated at 4°C by Sephadex G-50 chromatography (phosphate/NaCl buffer) and discarded. The heavy fractions were collected, concentrated by ultrafiltration (Amicon YM 10 membrane), and rechromatographed on a column of Ultrogel AcA-54 in order to separate $\alpha$-CHY-CHY in a $\alpha_2$-macroglobulin-bound $\alpha$-chymotrypsin molecules is not higher than 4 Å, i.e. the two protease binding sites in $\alpha_2$-macroglobulin should be about 44 Å apart (center to center) if the anhydrous radius of $\alpha$-chymotrypsin is 29 Å.

Human plasma $\alpha_2$-macroglobulin is a large ($M_\text{r} = 725,000$), double-headed glycoprotein composed of two noncovalently bound subunits (1). Each subunit consists of two $M_\text{r} = 185,000$ peptides linked by disulfide bridges (2); these peptides appear identical from immunological, electrophoretical, and sequence determinations (1, 3, 4). $\alpha_2M^{\text{CHY}}$ exhibits a high affinity for a broad spectrum of proteolytic enzymes. It binds irreversibly 2 molecules of elastase (5), trypsin (6, 7), or $\alpha$-chymotrypsin (8) and only 1 molecule of the higher molecular weight plasmin (8). The $\alpha_2M^{\text{CHY}}$-plasmin complex binds, however, 1 molecule of trypsin (7, 9). In addition, the various proteases compete for binding to $\alpha_2M^{\text{CHY}}$ (10). $\alpha_2M^{\text{CHY}}$-bound proteases are able to react with small substrates or inhibitors at rates comparable with those of free proteases, but the degradation of high molecular weight substrates is markedly inhibited by steric hindrance (2). The localization of the protease binding sites over the $\alpha_2M^{\text{CHY}}$ surface might control these properties in part. In the following we propose an approach to this problem, using the transfer of electronic excitation energy by the Förster dipole-dipole resonance interaction (11) between fluorescent labels covalently linked to $\alpha_2M^{\text{CHY}}$.
buffer, pH 7.5, for 10 min at room temperature. The excess of protease, if any, was removed by Sephadex G-200 filtration.

**Chymotrypsin Activity Measurements**—These assays were performed at 25°C by the method of Erlanger et al. (16) using N-glutaryl-L-phenylalanine-p-nitroanilide as the substrate, except that the buffer (100 mM Tris-HCl, 100 mM NaCl, pH 7.6) did not contain CaCl₂ which precipitates CHY-CHY.

**Singlet Energy Transfer Measurements**—These experiments were done by measuring the quenching of the DNS fluorescence (λ_{emission} = 390 nm, λ_{excitation} = 470 nm) by FITC with a Furrand Mark 1 spectrofluorometer equipped with a Hamamatsu R-446 photomultiplier. Controls with FITC-free proteins were run to take specific effects into account. The average transfer efficiency δ is given by: δ = 1 - F_{DNS}^F/F_{DNS}^D, where F_{DNS}^F and F_{DNS}^D are the normalized fluorescence intensities of the donor (DNS) in the presence and in the absence of the acceptor (FITC), respectively. The fluorescence quantum yield of DNS conjugates was determined using quinine bisulfate in 0.1 N H₂SO₄. Since the concentration of protein-bound DNS or FITC was lower than 0.5 μmol, absorbance values were sufficiently low for inner filter effects to be negligible.

**RESULTS**

**Properties of Dimeric Chymotrypsin (CHY-CHY)**—This a-chymotrypsin dimer was prepared in order to complete the energy transfer data obtained with monomeric a-chymotrypsin (see below). Its R_max value on sodium dodecyl sulfate-polyacrylamide gel electrophoresis corresponded to a M₉ = 44,000 protein. Its K_m value (0.83 mM) for N-glutaryl-L-phenylalanine-p-nitroanilide was the same as that of a-chymotrypsin while its k_cat value was lower (0.016 s⁻¹ compared to 0.022 s⁻¹ for a-chymotrypsin). In practice, not more than 2 molecules of FITC could be bound per molecule of CHY-CHY because the disulfide bridge of the spacer arm of CHY-CHY was partly cleaved upon reaction with this dye. Attempts at producing CHY-CHY from heavily FITC-labeled a-chymotrypsin resulted in poor yields, due probably to the substitution by the dye of surface lysine residue(s) being essential to reaction with the substrate, hydroxysuccinimide ester of the coupling reagent.

**Stoichiometries of the Dye-labeled Chymotrypsin-a₂M Complexes**—In order to interpret correctly the singlet energy transfer data, it was necessary to know exactly the stoichiometries of the various complexes used for the transfer experiments. For FITC-labeled a-chymotrypsin, the stoichiometry was determined by measuring the absorbance at 280 nm (proteins) and 495 nm (dye) and using the appropriate ε values (see "Experimental Procedures"). The absorption of the dye at 280 nm was taken into account. The a-chymotrypsin-a₂M binding ratio was found to be 2.1:1 and 2.0:1 using two batches of labeled a-chymotrypsin whose µ values were 0.45 and 3.1 (see also Footnote 2).

The absorption band of DNS being ill-defined in the presence of a₂M, the binding of DNS-labeled a-chymotrypsin was also monitored by measuring the fluorescence intensity of the dye at 510 nm (λ_{emission} = 340 nm) and comparing it to the absorbance at 280 nm. The a-chymotrypsin-a₂M binding ratio was found to be 2.1:1 for µ_{DNS} = 1.1 and 1.9:1 for µ_{DNS} = 2.2.

1 Labeling with DNS or FITC decreases the activity of a-chymotrypsin. For µ_{DNS} = 1.8 and µ_{FITC} = 3.1, the residual activity on N-glutaryl-L-phenylalanine-p-nitroanilide is 81% and 51%, respectively. If the binding of the labels obeys a Poisson distribution, part of the a-chymotrypsin molecules will be poorly labeled or even unlabeled. Thus, they will have a higher enzymatic activity than the more heavily labeled molecules. If a₂M reacts faster with native (i.e. 100% active) than with labeled a-chymotrypsin, the binding stoichiometry might therefore be underestimated. To rule out this possibility, we have compared the enzymic activities of free and a₂M-bound labeled and unlabeled a-chymotrypsin. We have found that a₂M does not change the specific activity of unlabeled a-chymotrypsin. On the other hand, the activity of FITC-labeled a-chymotrypsin (µ_{FITC} = 3.1) per A_{280} unit was found to be exactly the same whether the protease is free or complexed to a₂M. This rules out the possibility that a₂M binds more than 2 chymotrypsin molecules.

2 These assumptions are (a) R₀ = 33 Å (R₀ is the donor-acceptor distance for which δ = 0.5), (b) a-Chymotrypsin is an isotropic sphere with an anhydrous radius of 20 Å, (c) the probability of finding bound labels over the protein surface obeys a Poisson distribution.

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**Table I**

| CHY²⁺\(a₂M\) | CHY²⁺\(a₂M\) + CHY\(α\) | CHY²⁺\(a₂M\) + CHY-CHY\(α\) |
|----------------|--------------------------|-----------------------------|
| µ₀ | µ₁ | δ | µ₀ | µ₁ | δ | µ₀ | µ₁ | δ |
| 1.8 | 2.6 | 0.72 | 1.8 | 3.1 | 2 x 0.125 | 2.2 | 0.7 | 0.12 |

The CHY-CHY dimer was labeled with FITC and its binding stoichiometry was determined in the same way as that of monomeric a-chymotrypsin. The stoichiometry of the CHY-CHY-a₂M complex was 1:1 for µ_{FITC} = 0.7 and 0:9:1 for µ_{FITC} = 2. Reaction of this complex with FITC-labeled a-chymotrypsin gave a ternary complex formed of 1 mol of a₂M, 1 mol of CHY-CHY, and 1 mol of a-chymotrypsin.

Experiments were also undertaken to see whether CHY-CHY would be able to cross-link a₂M molecules. Constant amounts of a₂M were reacted with increasing amounts of CHY-CHY chosen such as the CHY-CHY/a₂M ratios were 0.2, 0.5, 1.0, and 2.2. The various mixtures were all eluted from Ultragel A-4 at the same volume as free a₂M with no trace of heavier fractions. Therefore, no cross-linking occurs.

**Energy Transfer Experiments**—Preliminary experiments were performed on a-chymotrypsin labeled with both DNS (µ = 1.8) and FITC (µ = 2.6). Batches of a-chymotrypsin labeled with one single label served as controls. Measurement of the quenching of DNS fluorescence at 470 nm (λ_{emission} = 360 nm) yielded δ = 0.72. The theoretical value of δ was obtained by the method of Gennis et al. (17-19) by assuming the same parameters as these authors. The good agreement between the calculated (0.74) and the experimental (0.72) value of δ indicates that the calculations and assumptions of Gennis and Cantor (17) can be used safely for inferring donor-acceptor distances from singlet energy transfer data.

Measurements were then performed on a donor- and acceptor-labeled a₂M-(CHY)₂ complex prepared by reacting equimolar amounts of a₂M and DNS-labeled a-chymotrypsin (µ = 1.8); this 1:1 a-chymotrypsin-a₂M complex was then reacted with a 3-fold molar excess either of a-chymotrypsin or of FITC-labeled a-chymotrypsin (µ = 3.1). Another complex was prepared by reacting equimolar amounts of a₂M and a-chymotrypsin; this 1:1 complex was subsequently reacted with a 3-fold molar excess of FITC-labeled a-chymotrypsin (µ = 3.1) (Table I). In each case, the excess of protease was removed by gel filtration. The two a₂M-(CHY)₂ complexes containing only donor or acceptor labels served as controls for the (donor + acceptor)-labeled complex. Absorption measurements indicated that 1 mol of FITC-labeled a-chymotrypsin was present per mol of a₂M within the two acceptor-containing complexes. δ was measured as before and was found to be 0.125. As is shown below, this result may need to be corrected. If the two binding sites of a₂M are equivalent or if the occupancy of one site does not affect the binding capacity of the other site, the (donor + acceptor)-labeled a₂M-(CHY)₂ complex will be composed of 25% of the DNS-labeled complex, 25% of the FITC-labeled complex, and only 50% of the (DNS + FITC)-labeled complex. Hence δ_corrected = 0.25. On the other hand, if the two binding sites are not equivalent or if the occupancy of one site decreases the binding capacity of the other site, the bulk of...
the α₂M-(CHY)₂ complexes will occur as mixed (DNS + FITC)-labeled complexes. Hence, ε needs no correction. Using the same calculations as before, we found that the distance between the 2 α-chymotrypsin molecules is either 4 Å (ε = 0.25) or 11 Å (ε = 0.125).

In order to confirm this result in a more straightforward manner, we have prepared complexes formed of 1 mol of CHY-CHY and 1 mol of α₂M. One mol of α₂M was reacted with either 1 mol of unlabeled CHY-CHY or 1 mol of FITC-labeled CHY-CHY (μ = 0.7 or μ = 2.0). One mol of the former complex was reacted with either 1 mol of free CHY or of DNS-substituted α-chymotrypsin (μ = 2.2) and 1 mol of the latter complex was reacted with either 1 mol of free CHY or of DNS-labeled CHY (μ = 2.2). Since the α₂M:CHY-CHY stoichiometry is 1:1, each molecule of α₂M contains 1 molecule of CHY-CHY and 1 molecule of α-chymotrypsin. Hence, ε needs no correction. The two complexes containing only donor or acceptor molecules served as controls for the (donor + acceptor)-labeled complex. In this case, the acceptor molecules present on the two monomers of CHY-CHY, thus leaving the second binding site undamaged and ready to bind small endoproteases. The kinetic analysis of accessibility, proximity, and symmetrical localization. On the other hand, an asymmetrical binding of proteases such as α-chymotrypsin in α₂M would fit our results but appears unlikely from what is known about the proteolytic cleavage of the M₆ = 185,000 quarters which parallels the trapping of enzymes by α₂M (4). The present findings indicate that the binding of protease to α₂M can be controlled by steric hindrance. In fact, α₂M appears unable to trap 2 molecules of our synthetic CHY-CHY dimer (as previously observed for plasmin (8)), despite the presence of a CO₂CH₂CO₂- spacer arm between the α-chymotrypsin moieties. We would suggest the M₆ = 185,000 to M₆ = 85,000 proteolytic cleavage (4, 25, 26) does not occur on both halves of the α₂M molecule when using sterically hindered proteases such as plasmin or CHY-CHY, thus leaving the second binding site undamaged and ready to bind small endoproteases. The kinetics of protease binding should allow this problem to be elucidated.

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FIG. 1. Two-site model for the interaction of α₂-macroglobulin with the α-chymotrypsin and CHY-CHY molecules, as deduced from energy transfer measurements.

Moles of FITC/mol of dimeric chymotrypsin. The dye molecules are assumed to be equally distributed among the two CHY-CHY subunits.
α2-Macroglobulin-α-Chymotrypsin Interactions

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