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Evidence for Active Half-Molecules of α_2-Macroglobulin Formed by Dissociation in Urea†

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ABSTRACT: Urea caused dissociation of α_2-macroglobulin (α_2M) into half-molecules (two disulfide-bonded subunits) as revealed by gel electrophoresis. The fraction of whole molecules remaining decreased with increasing urea concentration. Half-dissociation occurred at about 2.2 M. The ability of α_2M to inhibit trypsin also decreased with increasing urea concentration, but the activity–urea curve was shifted to the right as compared to the dissociation–urea curve. Thus, at 3 M urea, gel electrophoresis showed only 6.6% whole molecules, whereas the trypsin inhibitory activity was 95% of that in buffer with no urea, suggesting that half-molecules retain activity. In addition, complexes formed in urea with ^{125}I-labeled trypsin were observed to migrate as half-molecules even though only 50% of such complexes were covalent. These results are surprising in light of the report by Gonias and Pizzo [Gonias, S., & Pizzo, S. (1983) *Biochemistry 22*, 536–546] that half-molecules formed by mild reduction are active; reduction is assumed to divide the molecule along an axis orthogonal to the break caused by urea. This suggests that active half-molecules can be formed by splitting either the covalent or noncovalent bonds that hold the subunits together. A model is proposed that can account for this possibility. It has the same dimensions and symmetry as a previous model of Feldman et al. [Feldman, S. R., Gonias, S. L., & Pizzo, S. V. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5700–5704] and accounts in a similar way for previous functional studies of the protein. The new model can account for some inconsistencies in the previous model, it provides a more obvious mechanism for cooperativity, and it allows for active half-molecules that can be generated either by urea or by reduction of disulfide bonds.

The plasma protease inhibitor α_2-macroglobulin (α_2M)[1] has high enzyme binding activity for a wide variety of proteases. The maximum stoichiometry is 2:1 enzymes:α_2M [for reviews, see van Leuven (1982) and Roberts (1985)]. The protein is a tetramer of identical subunits of M_r 185 000. The way in which these subunits are arranged to form the enzyme binding sites is a matter of current interest. Two pairs of the subunits are disulfide bonded to form dimers; the dimers, in turn, are held together by noncovalent forces (Figure 1). In denaturants, such as urea, $NaDodSO_4$, or guanidine hydrochloride, the dimers are separated and proteins of M_r 375 000 are observed on polyacrylamide gel electrophoresis (van Leuven, 1982; Roberts, 1985). Gonias and Pizzo (1983a) prepared half-molecules by reduction (and carboxymethylation), indicating that the noncovalent forces are of sufficient strength to maintain the subunits as pairs. Half-molecules prepared in this way retained protease-binding activity, suggesting that the enzyme binding sites are formed by the two noncovalently linked subunits. Thus, as shown in Figure 1, two different types of half-molecules are possible by various treatments of native α_2M. Dissociation along the noncovalent axis of the molecule has generally been observed to destroy activity (Barrett et al., 1979; Jones et al., 1972; Sottrup-Jensen et al., 1980; McConnell & Loeb, 1974), although there are con-

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[1] Abbreviations: α_2M, α_2-macroglobulin; $NaDodSO_4$, sodium dodecyl sulfate; STI, soybean trypsin inhibitor; pNPGB, *p*-nitrophenyl *p*-guanidinobenzoate; Tris, tris(hydroxymethyl)aminomethane.

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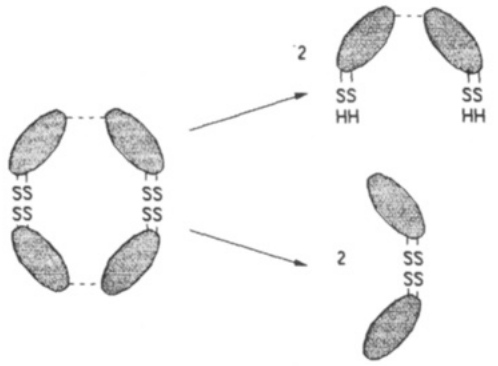

FIGURE 1: Subunit structure of α_2-macroglobulin and modes of dissociation. The native tetramer can be dissociated by reduction of disulfide bonds with agents such as dithiothreitol (Gonias & Pizzo, 1983a) or by breaking noncovalent bonds (dashed lines) with urea or $NaDodSO_4$.

flicting reports on the effect of urea. McConnell and Loeb (1974) reported that half-molecules, generated by dissociation in 3 M urea and dialysis against buffer, retained kallikrein inhibitory activity. Other work indicated that urea caused a loss in enzyme-binding activity even if the urea is removed by dialysis (Barrett et al., 1979). In this paper, we tried to further investigate the effect of urea on α_2M. We found that urea caused dissociation and a loss in trypsin inhibitory activity. However, these effects showed a different concentration dependence, and in agreement with the studies on the α_2M–kallikrein system, we observed high activity under conditions that showed few whole molecules, suggesting the existence of active α_2M half-molecules in urea solutions. We also found that the activity that was lost was largely regained after dialysis. These results raise the possibility that half-molecules formed either by breaking noncovalent bonds or by mild reduction as described by Gonias and Pizzo (1983a) can form an enzyme-binding site.

Feldman et al. (1985) recently proposed a model for the structure and function of α_2M based on the geometry revealed by electron microscopy (Schramm & Schramm, 1982, 1983). This model is consistent with many of the known facts about the molecule but predicts that only one of the modes of dissociation shown in Figure 1 will lead to an active subspecies. In this paper, we propose an alternative model that has similar features but, in addition, permits active molecules to be generated by either method of dissociation.

Materials and Methods

Human α_2M was prepared by a combination of affinity chromatography on Zn–Sepharose (Kurecki et al., 1979) and Cibacron Blue–Sepharose (Virca et al., 1978) as previously described (Feinman et al., 1985). Trypsin (type II), soybean trypsin inhibitor (STI), urea, and sodium dodecyl sulfate ($NaDodSO_4$) were from Sigma. ^{125}I-Labeled trypsin was prepared as previously described (Wu et al., 1981). All other reagents were of the highest grade commercially available.

The active trypsin concentration was determined by active-site titration using *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride (pNPGB) obtained from ICN Pharmaceuticals (Chase & Shaw, 1967). Thiols were monitored as described previously (Feinman et al., 1985; Grassetti & Murray, 1967) by using the reagent 4,4′-dithiodipyridine (Aldrich Chemical Co.).

The binding of enzymes to α_2M was measured by autoradiography of gel electrophoretograms of labeled enzyme–inhibitor complexes and by the ability of α_2M to protect the trypsin esterase activity from STI. The procedure is a modification of the method of Ganrot (1966) using pNPGB as the active-site titrant.

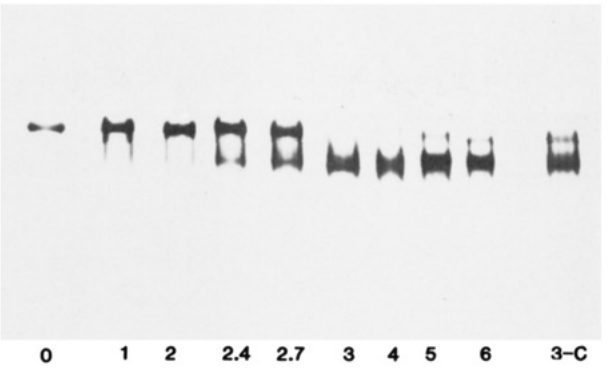

FIGURE 2: Polyacrylamide gel electrophoretogram of α_2M in urea (composite of gel electrophoretograms). Samples of α_2M (15 μg per well) containing the indicated concentration of urea were analyzed by means of electrophoresis on native polyacrylamide gels containing the same concentration of urea. The behavior of half-molecules in each urea concentration was determined by running samples containing 1% $NaDodSO_4$ as a marker. The lane marked 3-C is such a marker for 3 M urea.

Polyacrylamide gel electrophoresis was carried out with slabs formed on a Gelbond support. The analytical gel used for quantitating the α_2M species was a gradient of 3.5–10% acrylamide containing the appropriate concentration of urea. The stacking gel was 3.5% acrylamide with the same urea concentration. Normal Tris–glycine buffers for native gel systems were used as the electrophoresis buffer. Developed gels were stained with Coomassie Brilliant Blue and destained in a conventional manner.

Results

Dissociation of α_2M Determined on Urea Gels. We assayed the effect of urea on the structure of α_2M by incubating protein samples in urea solutions for 30 min and then subjecting the samples to electrophoresis in polyacrylamide gels containing the same concentration of urea. As markers for half-molecules, we used samples of α_2M that had been incubated in 1% $NaDodSO_4$, which is known to cause dissociation. The results are shown in Figure 2, where it is evident that increasing urea concentrations caused increasing dissociation. This effect is quantitated in Figure 3. The half-maximal dissociation occurs at a urea concentration of approximately 2.2 M. These results are consistent with previous reports (Jones et al., 1972; McConnell & Loeb, 1974) that showed, by ultracentrifugation, 3 M urea causes virtually complete dissociation of α_2M into half-molecules.

Activity of α_2M toward Trypsin in Urea Solutions. We measured the effect of urea on the activity of α_2M toward enzymes in three ways. First, we measured the thiol release from α_2M in urea solutions by added trypsin. Thiols are generated in the reaction of α_2M with enzymes by the destruction of an internal Cys-Glu thiol ester (Sottrup-Jensen et al., 1980, 1984; Howard, 1981); the glutamyl residue forms a covalent bond with lysyl groups of the enzyme or is simply hydrolyzed. Second, we measured the ability of α_2M to protect trypsin esterase activity from STI, a procedure that reflects the fact that α_2M-bound proteases are active toward small substrates but are inhibited from reaction with protein substrates or inhibitors (van Leuven, 1982; Roberts, 1985; Ganrot, 1966). In control experiments, we found that trypsin was active against small substrates in urea solutions (Figure 3) and that STI was 100% effective against trypsin at all urea concentrations (data not shown). Finally, we measured the for-

Table I: Reconstitution of Urea-Treated α_2M by Dialysis[a]

initial [urea] (M)	second treatment	% of control: whole molecules	trypsin bound*	SH release	covalent complexes (% of bound)
0	–	100.0	100.0	100.0	92.0
3	–	7.3	98.7	92.0	64.7
	dialysis; buffer		100.7	100.0	90.0
	dilution; 1.5 M urea dialysis; buffer	81.6	102.1	102.5	92.4
4	–	7.3	79.7	78.2	47.2
	dialysis; buffer		77.1	87.7	
	dilution; 2 M urea dialysis; buffer	77.0	97.4	97.3	83.5
6	–	5.5	24.0*	24.6	
	dilution; 3 M urea dialysis, 12 h	42.4	51.0	43.6	82.8
	dilution; 3 M urea dialysis, 15 h	80.0	60.6*	68.4	
	dilution; 3 M urea dialysis, 23 h	90.0	79.0*	77.1	

[a] α_2M (1 μM) was exposed to the indicated concentration of urea for approximately 30 min. Samples were dialyzed (with or without dilution as indicated) with one change of buffer after 6 h of dialysis. The amount of trypsin bound was determined by binding of ^{125}I-labeled enzyme as measured on urea gels or by protection of esterase activity by STI (indicated with an asterisk) as described under Materials and Methods. The percent of covalent binding was determined from the ratio of bound labeled enzyme seen on NaDodSO$_4$ gels to the total amount bound on urea gels.

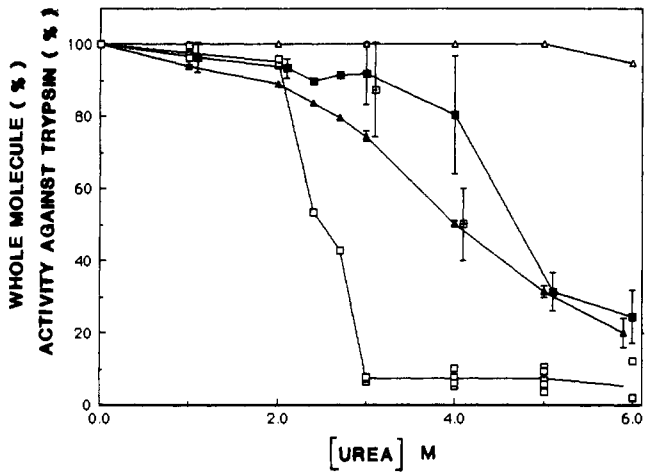

FIGURE 3: Effect of urea on the activity of α_2M toward trypsin and comparison with dissociation on urea gels. The reaction of α_2M (1 μM) with trypsin (2 μM) in the presence of the indicated concentration of urea was determined as described under Materials and Methods. Error bars show the range of experimental measurements. The parameters were as follows: (▲) protection of esterase activity (pNPGB) from STI; (■) free thiols released; (⊡) binding of ^{125}I-labeled trypsin; (△) esterase activity of trypsin in control urea solutions (no α_2M); (□) dependence of whole molecules remaining as a function of urea concentration. Gel electrophoretograms as in Figure 2 were analyzed by densitometry, and the fraction of the upper band (compared to the NaDodSO$_4$ standard) was determined. Each point is the average of at least duplicate determinations. For each integral value of urea concentration there are at least three separate points, some of which overlap.

mation of complexes of α_2M with ^{125}I-labeled trypsin by gel electrophoresis. The results from these methods of measuring the reaction of α_2M with trypsin are shown in Figure 3. This figure shows that there was a decrease in the activity as the urea concentration was increased. The activity vs. urea curves, however, when compared to the dependence of remaining whole molecules on urea concentration, are shifted markedly to the right. Examination of Figure 3 reveals that, at 3 M urea, more than 90% of the protein on a urea gel is attributable to half-molecules, but in this concentration of urea, the α_2M is 75% active as measured by protection of trypsin from STI and 82–100% active in terms of release of sulfhydryl groups by trypsin. The decrease in trypsin-releasable thiols at the higher urea concentrations was not due to the destruction of the thiol ester since remaining thiols could be released by methylamine or, as discussed below, by trypsin after removal of urea by dialysis. These results suggest that half-molecules created by urea treatment have intact thiol esters and are active with respect to enzyme binding and reaction. These results are consistent with studies of McConnell and Loeb (1974) but in disagreement with the report of Barrett et al. (1979), who found that urea-treated α_2M was inactive, although there is no obvious explanation for the differences in these results.

Polyacrylamide Gel Electrophoresis of Trypsin–α_2M Complexes in Urea. We measured the binding of trypsin to α_2M in 3 or 4 M urea by carrying out the reaction with ^{125}I-labeled enzymes and measuring incorporation into complexes seen on gel electrophoresis in urea and in NaDodSO$_4$. The results are shown in Figure 4. In urea, the bands attributed to the half-molecule contain substantial radioactivity. When we compared the number of counts in each band to those for controls in the absence of urea, we found that the percent bound was similar to the values for enzyme bound as determined by measurements of activity described above (Figure 3). The good correlation between the results of direct measurement of binding and the measurement of protection from STI is somewhat surprising since we would expect a reduction in the steric hindrance characteristic of the complexes of enzymes with native α_2M in buffer. A loss in the ability of half-molecules to protect enzymes from STI was, in fact, found by Gonias and Pizzo (1983a) for α_2M samples dissociated by reduction. By comparing the behavior of our samples on NaDodSO$_4$ gels (known to cause dissociation of noncovalently bound enzyme) to the behavior on urea gels, we determined that the binding measured in 4 M urea was approximately 50% covalent (Table I). Thus, although urea treatment is sufficient to break the intersubunit bonds in α_2M, it does not affect the noncovalent enzyme–α_2M interaction. Figure 4 also shows that the reaction product of trypsin and α_2M in urea does not have the characteristic band attributed to a bivalent cross-linked product, referred to previously as band 3 (Feinman et al., 1985, Wang et al., 1983, 1984). A similar result was found by Gonias and Pizzo (1983a) for the reduced half-molecules. Surprisingly, the very highest bands (bands 4 and 5) do persist in these samples. These are more poorly characterized than band 3, and we had previously attributed them to *inter*molecular cross-links of enzymes and

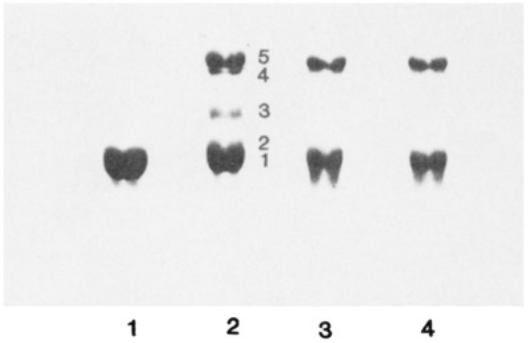

FIGURE 4: Comparison of covalent complexes derived from native α_2M and α_2M treated with urea ($NaDodSO_4$–polyacrylamide gel electrophoresis). (Lane 1) α_2M control (M_r 375 000); (lane 2) α_2M–trypsin complex prepared in buffer before application to gel; (lanes 3 and 4) α_2M complexes treated with 3 and 4 M urea, respectively. The molar ratio of enzyme to α_2M in each case was 2:1.

α_2M. Such an intermolecular interaction is not excluded between half-molecules but more study will be required to determine if this is the explanation.

Regeneration of Urea-Treated α_2M. There is ambiguity in the literature about the extent to which any α_2M activity lost in urea can be regained. One report showed the lost trypsin inhibitory activity was not regained after dialysis (Barrett et al., 1979) whereas another showed that kallikrein inhibitory activity was lost (McConnell & Loeb, 1974). The results of our work, summarized in Table I, show that activity could be restored although it was somewhat sensitive to the method by which the dialysis was carried out. Samples subjected to 4 M urea can be almost completely reconstituted with respect to trypsin activity if dialysis is carried out in stages: first, dilution to 2 M urea, and then, after 6 h, dialysis against buffer. Similar results are seen in 6 M urea although, here, the time of dialysis was found to be important. As shown in Table I, 60.6% of the activity was recovered after 15 h (final dialysis) as compared to 79% after 23 h. This low rate of reassociation is consistent with the report of isolation of half-molecules of α_2M, which were stable for 2–3 h after dialysis of 3 M urea solutions against buffer (McConnell & Loeb, 1974).

Discussion

In this paper, we have provided evidence that active half-molecules of α_2M exist in urea solutions of concentrations up to 4 M. Activity was measured by direct binding, thiol release, and protection of bound protease from STI. The evidence that half-molecules were the active species is that (1) enzyme complexes migrate as half-molecules in urea gels even though only 50% of the complexes are covalent and (2) enzyme complexes formed in urea do not show the slowly migrating bands attributed to cross-linked subunits. Additional evidence from the literature comes from demonstration of kallikrein inhibitory activity of half-molecules dissociated by urea but assayed after dialysis (McConnell & Loeb, 1974). Thus, our data, in combination with the results of Gonias and Pizzo (1983a), support the idea of active species generated by two different modes of dissociation. To reconcile this idea with our current knowledge of α_2M behavior, we propose the model shown in Figure 5.

The Model. The model for the α_2M structure shown in Figure 5A is similar to the one proposed by Feldman et al. (1985). Their structure is also shown (Figure 5C) along with the computer-averaged electron micrographs of α_2M from which both models are derived (Figure 5B). In addition to providing a molecular basis for the electron micrographs, the

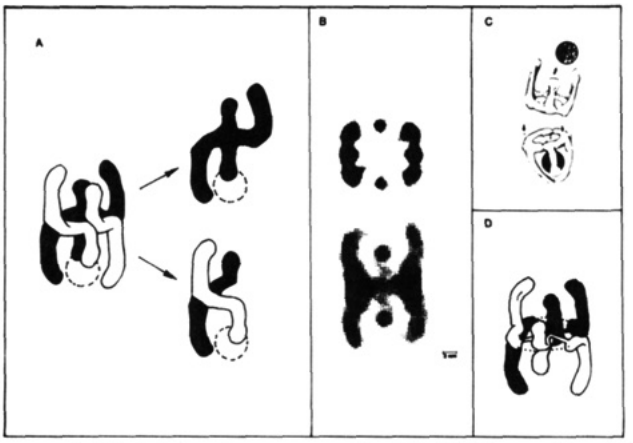

FIGURE 5: Models for the structure of α_2M. (A) Proposed model for the α_2M structure. The two modes of attachment, disulfide and noncovalent (see Figure 1), are represented by the dark–dark/light–light or the light–dark pairs although it is not known which is which. The dotted line represents bound enzyme. The model is intended to conform to the dimensions of the structure shown at the bottom of (B) and other physical data summarized in Feldman et al. (1985). (B) Computer-averaged electron micrographs of α_2M from the work of Schramm and Schramm [(1983), used with permission]. The top figure is referred to as the "open" form and the bottom, as the "closed" form. Models in (A) and (C) are based on the shape of the closed form. The model in (D) assumes that they represent the same molecule; see text. (C) Model of the α_2M structure of Feldman et al. [(1985), used with permission]. The structure shown is separating along the disulfide-bonded axis. (D) Variation of the model in (A). The open and closed forms in (B) are assumed to be the top and side views of the same structure. Each subunit contains a long and short arm as in (A) connected by the "spokes" observed in the open form in the electron microscope.

model of Feldman et al. (1985) was able to rationalize an impressive amount of data in the literature. Our model has the same symmetry and is based on antiparallel arrangement of subunits consistent with the primary structure (Jensen & Sottrup-Jensen, 1986), and many of the arguments applied to their model apply to ours. The essential feature of our model is that the subunits are arranged sequentially in an antiparallel manner around the main axis rather than in a bilateral pattern as proposed by Feldman et al. (1985). The subunit is a Z-shaped structure with a short arm and a long arm, corresponding to the arms of the whole molecule. We postulate that any active form requires that contacts be made between the enzyme and at least one of each of the arms. The half-molecules created either by urea or by reduction fulfill this requirement. An alternate description of the model is that the enzyme binding sites are composed of parts of all four subunits and contributions from any two pairs are sufficient to retain some binding properties. Because the subunit polypeptide chains are antiparallel, the different half-molecules do not present identical faces to the central axis, but depending on the orientation of critical side chains, a bound enzyme may see the same groups in the same orientation in both types of half-molecules. An additional feature of our model is that since a bound enzyme is in proximity to all four subunits of the protein, there is an obvious mechanism for the negative cooperativity, which has been suggested for α_2M reactions (Howell et al., 1983; Gonias & Pizzo, 1983b; Steiner et al., 1985).

We also propose a variation of this model as shown in Figure 5D. This variation, although speculative, reconciles the two different forms seen in electron micrographs (Schramm & Schramm, 1982, 1983; Figure 5B). Although it has been suggested that the two conspicuous forms seen in electron microscopy apply to different states of the α_2M molecule

(Schramm & Schramm, 1982), the samples used were almost entirely the "fast" (reacted) form of the molecule, and both types of geometrical forms, "open" and "closed", were seen in this sample. We suggest that the open form represents molecules oriented so that a "top" view is visible in the electron microscope and the closed form, the side view of the same molecule. In this way one could explain how a single molecule shows two different forms. The characteristic features of both forms, the spokes of the open form and the arms of the closed form, are included in a single model.

The model we propose can accommodate the idea of enzyme-binding sites formed by two different modes of dissociation. In addition, it can resolve some inconsistencies in the previous model of Feldman et al. (1985), particularly with regard to the modes of binding of enzymes.

Modes of Binding. Enzymes bind both covalently and noncovalently to α_2M. The covalent binding is not required for inhibition (van Leuven, 1982; Roberts, 1985; Barrett & Starkey, 1973; Wu et al., 1981; Salvesen et al., 1981; Gonias & Pizzo, 1983b; Steiner et al., 1985). It is generally believed that binding of enzymes is accompanied by a conformational change that causes the enzyme to be geometrically "trapped" (Barrett & Starkey, 1973) in an irreversible way although several native and derivatized enzymes have been shown to be dissociable (Wu et al., 1981; Steiner et al., 1985; van der Graaf et al., 1984). The model of Feldman et al. (1985) identifies movements of the arms of the inhibitor with the trapping of proteases. Their model is bilateral, each half-molecule containing a trap. The prediction is, then, that active half-molecules would be capable of trapping proteases and should not allow dissociation or reaction of bound enzymes with STI. The results of Gonias and Pizzo (1983a) show that for the reduction-dissociated α_2M, this is not the case; although covalent binding takes place, no irreversible noncovalent binding was found and about one-third of the bound enzyme reacted with STI. The model proposed here produces half-molecules capable of binding protease covalently or noncovalently, but any trapping will require reassembly of the tetrameric form. In this regard, it is interesting that our results suggest that half-molecules formed in urea do provide some protection from STI for the bound protease. Thus, if our model is correct, the two types of enzyme-binding sites formed from different modes of dissociation may not be identical.

Bivalent Cross-Linking of α_2M by Enzymes. We previously proposed that the high reactivity of the thiol esters of α_2M can lead to structures in which more than one subunit is covalently bound to a single enzyme (Feinman et al., 1985; Wang et al., 1983, 1984). We offered this as an explanation of slowly migrating species frequently observed on $NaDodSO_4$ gel electrophoresis and suggested they were due to cross-linking of the two (covalently bound) half-molecules producing a whole molecule that could not dissociate in $NaDodSO_4$. Gonias and Pizzo (1983a) showed similar high molecular weight structures that disappeared during the reaction of reduced half-molecules. This means that cross-linking of this type can occur across the disulfide-bonded subunits as well as across the noncovalently bound subunits. The model of Feldman et al., by restricting enzyme–subunit interactions to one half-molecule, precludes such an effect. In the model proposed here, because the subunits are staggered, bivalent cross-linking will prevent dissociation by reduction or denaturants.

Limitations of the Model. The unusual feature of the proposed enzyme-binding sites in the model is that dissociated half-molecules will have four total sites per mole of whole molecule. This predicts the possibility of an increase in the level of enzyme binding as a consequence of dissociation. We found that a 4-fold molar excess of trypsin showed no greater binding to α_2M in 4 M urea than a 2-fold molar excess under the same conditions (data not shown). There may be several effects of urea or reduction, of course, and an increase in available sites may be offset by the reduced effectiveness of each site. In the absence of evidence of such a phenomenon, however, this would have to be considered a defect in the model.

Summary. The enzyme-binding activity of α_2M is maintained in high concentrations of urea. Evidence from gel electrophoresis run in urea shows that α_2M is dissociated into half-molecules at these concentrations. In combination with previous work in the literature showing active half-molecules dissociated by reduction, this work suggests that the enzyme-binding site can be maintained under different methods of dissociation. A model to accommodate this idea has been presented.

Registry No. Urea, 57-13-6; trypsin, 9002-07-7.

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Identification of a New in Vitro Substrate of Tyrosine Protein Kinase†

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ABSTRACT: Recent studies in our laboratory [Tokuda, M., Khanna, N. C., Aurora, A., & Waisman, D. M. (1986) *Biochem. Biophys. Res. Commun. 139*, 910–917] have identified in membranes of rat spleen two tyrosine protein kinases named TPK-I and TPK-II. In this paper the identification of the Ca^{2+} binding protein CAB-48 as a major in vitro substrate of TPK-II is reported. TPK-II catalyzed the incorporation of 0.73 mol of phosphate/mol of CAB-48. Phosphoamino acid analysis revealed that phosphorylation of CAB-48 was specific for tyrosine residues. Phosphorylation of CAB-48 by TPK-I (rat spleen), protein kinase C, casein kinase I, casein kinase II, cAMP-dependent protein kinase, or calcium calmodulin dependent protein kinase was not observed.

That tyrosine phosphorylation may be involved in the virus-induced malignant transformation of cells and in the regulation of cellular growth and proliferation has been suggested by the observation that tyrosine protein kinase activity appears to be intrinsic to several retroviral transforming proteins (Bishop, 1983) as well as certain growth factor receptors (Ushiro & Cohen, 1980; Ek & Heldin, 1982; Kasuga et al., 1982). High levels of tyrosine protein kinase activity have been demonstrated in the membranes of rat spleen (Swarup et al., 1983). Solubilization and partial purification of the tyrosine kinase activity of the 30000*g* pellet of rat spleen have identified two tyrosine protein kinases (Brunati et al., 1985; Tokuda et al., 1986), which have been named TPK-I and TPK-II.[1] Both TPK-I and TPK-II have been characterized as oncogenic and growth factor independent tyrosine protein kinases (Tokuda et al., 1986). In contrast, bovine spleen has been shown to contain a single tyrosine protein kinase activity (Kong & Wang, 1987). Fundamental to our understanding of the cellular function of the oncogenic and growth factor independent tyrosine protein kinases is the identification of the substrates of these kinases.

Recent studies in our laboratory (Waisman et al., 1983a, 1985) have used the combined techniques of $^{45}Ca^{2+}$ autoradiography and Chelex Ca^{2+} binding assay to identify the complete spectrum of Ca^{2+} binding proteins of bovine brain 100000*g* supernatant. Two of these proteins, CAB-27 (Waisman et al., 1983b) and CAB-48 (Waisman et al., 1985; Tokuda et al., 1987), have been shown to be novel Ca^{2+} binding proteins of unknown function. CAB-48 was shown to bind 1.0 mol of calcium/mol of protein with an apparent K_d of 15 μM (in the presence of 150 mM KCl and 3.0 mM $MgCl_2$). One kilogram of bovine brain was found to contain about 100 mg of CAB-48. On the basis of the concentration of CAB-48 in bovine brain tissue we have suggested that this protein represents a major brain calcium binding protein.

In this paper, the bovine brain M_r 48 000 Ca^{2+} binding protein is identified as an in vitro substrate for bovine and rat spleen tyrosine protein kinases. Phosphorylation is shown to approach stoichiometry and to be specific for tyrosine residues.

Experimental Procedures

Materials. [γ-^{32}P]ATP (10 Ci/mmol; 1 Ci = 3.7 × 10^{10} Bq) was purchased from Amersham. Phosphoserine, phosphothreonine, and phosphotyrosine were purchased from Sigma. Casein kinase I and casein kinase II were generous gifts from Dr. T. J. Singh (University of Waterloo). cAMP-dependent protein kinase was purchased from Sigma. Protein kinase C was purified from bovine brain as described (Kikkawa et al., 1982). Ca^{2+}-calmodulin-dependent protein kinase was purified from bovine brain as described (Sharma & Wang, 1986).

Purification of CAB-48. CAB-48 was purified from bovine brain by the procedures described by Tokuda et al. (1987).

Purification of Tyrosine Protein Kinase. Bovine spleen tyrosine protein kinase was purified as described (Kong & Wang, 1987). Rat spleen tyrosine protein kinases (named TPK-I and TPK-II) were purified by the procedures described previously (Tokuda et al., 1986). Enzyme solutions containing 25 mM HEPES (pH 7.5), 1 mM EDTA, 10% glycerol, 0.1% NP40, and 1 mM DTT were stored at −70 °C.

In Vitro Phosphorylation Assay. The phosphorylation reaction was performed as described earlier (Tokuda et al., 1986). The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 10 μM sodium vanadate, [γ-^{32}P]ATP (50 μM, 1–2 μCi), and

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[1] Abbreviations: CAB, calcium binding protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; NP40, Nonidet P-40; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; TPK-I, tyrosine protein kinase I; TPK-II, tyrosine protein kinase II; Tris, tris(hydroxymethyl)aminomethane.

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