**A Functional, Thioester-containing \( \alpha_2 \)-Macroglobulin Homologue Isolated from the Hemolymph of the American Lobster (Homarus americanus)**

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An \( \alpha_2 \)-macroglobulin-like protease inhibitor was isolated from the cell-free hemolymph of the American lobster (*Homarus americanus*) by ion-exchange chromatography and gel filtration. Whereas the undisassociated molecule has a molecular weight of 342,000 as determined by ultracentrifugation studies, under reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the protein has a subunit molecular weight of 180,000. On the basis of this and other evidence, we conclude that the lobster protein is a dimer consisting of two disulfide-bonded monomers. The purified protein inhibits proteolytic enzymes but protects the esterolytic activity of trypsin toward low molecular weight substrates from inactivation by soybean trypsin inhibitor. The methylamine sensitivity of this activity suggests the presence of an internal thioester bond. This was confirmed by the covalent incorporation of \([1^{14}C] \)methylamine, by the formation of \( M, 55,000 \) and 125,000 autolytic cleavage fragments in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and, more directly, by the amino acid sequence of a tryptic peptide containing the putative thioester region. Whereas the N-terminal amino acid sequence (22 residues) of the protein revealed an overall identity of only 18\% when compared with the human protein, the sequence of the thioester-containing peptide was highly conserved, both with respect to human \( \alpha_2 \)-macroglobulin and to other proteins having a thioester bond. The protein showed the "slow to fast" conformational change typical of \( \alpha_2 \)-macroglobulins in non-denaturing gel electrophoresis after treatment with trypsin, but not after incubation with methylamine.

\( \alpha_2 \)-Macroglobulin (\( \alpha_2 \)M)\(^1\) is a high molecular weight protease inhibitor found in the plasma of vertebrates (1). It has the unique property of binding and inhibiting the great majority of endopeptidases, regardless of their specificity or catalytic mechanism (2, 3). It has been proposed that \( \alpha_2 \)M inhibits the proteolytic activity of these proteases by a trapping mechanism in which the active site of the enzyme remains active and free to hydrolyze those substrates which are small enough to pass through the molecular cage with which the \( \alpha_2 \)M molecule envelops the protease. The caged protease may be bound covalently by transacylation from a sequestered thioester in \( \alpha_2 \)M. This structure is formed between cysteinyl and glutamyl side chains (4) which are separated by only two amino acids (5). In addition to \( \alpha_2 \)M and several closely related protease inhibitors (6-8), a thioester is also present in the complement proteins C3 and C4 (9). Two noteworthy characteristics of the protein thiolactone ring are the covalent incorporation of primary amines such as ammonia and methylamine (10) and its ability to mediate a denaturation-induced autolysis of the polypeptide chain (11).

The mechanism of trapping involves the cleavage of a peptide bond in the so-called "bait region" of the \( \alpha_2 \)M subunits (12, 13) which contains potential cleavage sites for most proteases and which is evidently suitably exposed. An immediate consequence of bait region proteolysis is the exposure and cleavage of the thioester bond by protease, nucleophilic amino groups, or water molecules. Thioester scission results in spectrophotometrically and electrophoretically demonstrable conformational changes (14, 15), which for \( \alpha_2 \)M, presumably results in the formation of the molecular cage around the protease. In all three human thioester-containing proteins, the same conformational changes were shown to occur when the thioester was cleaved by nucleophiles in the absence of proteolysis. The most studied \( \alpha_2 \)M is that of the human. The native inhibitor is a tetramer (\( M, \sim 720,000 \)) of identical subunits (\( M, \sim 180,000 \)). Two dimers (\( M, \sim 360,000 \)), each of which is composed of two covalently bound subunits, are noncovalently associated in the native molecule (16, 17). Each dimer is able to bind one protease molecule (18). Functional evidence for the existence of a similar protease inhibitor in the horseshoe crab *Limulus polyphemus* has been published (19); and recently (20), a protein having similar protease inhibitory properties to \( \alpha_2 \)M has been purified from the horseshoe crab. This molecule has a molecular weight by gel filtration of approximately 550,000 and was thought to be trimeric in structure.

In this paper, we describe the isolation and characterization of a thioester-containing protease inhibitor from the hemolymph of the lobster (*Homarus americanus*) with properties similar to human \( \alpha_2 \)M but consisting of only two disulfide-linked subunits.

**EXPERIMENTAL PROCEDURES AND RESULTS**

**Purification**—Lobster \( \alpha_2 \)M was purified in a two-step pro-
conditions yielded a subunit molecular weight for lobster α2M under nonreducing conditions (Fig. 3). Under reducing conditions, an additional band with a lower apparent molecular weight was observed for human α2M which was used as a reference protein. The results are shown in Table I. The amino acid sequence was homogeneous, and the initial yield of 60% indicates that the subunits are identical.

**Evidence for an Internal Thioester Bond** — Since autolytic fragmentation accompanying heat denaturation is one of the hallmarks of thioester-containing proteins (9), this was the initial approach used to demonstrate the presence of a thioester structure in lobster α2M. The protein was boiled for 5 min in SDS sample buffer and then reduced with β-mercaptoethanol. The SDS-PAGE analysis depicted in Fig. 4 shows that, besides uncleaved α2M (M, 180,000), two additional bands were present which were not evident when α2M was pretreated with methylvamine. The fragments had M, values of 125,000 and 55,000, respectively, which are quite similar to the human M, 120,000 and 60,000 autolytic cleavage fragments.

Further evidence for the internal thioester in lobster α2M comes from the incorporation of [14C]methylamine into the protein as revealed by SDS-PAGE and autoradiography of the gel (data not shown). Finally, the protein was treated with [14C]methylamine, and the protein was allowed to bind covalently to activated thiopropyl-Sepharose through the released sulfhydryl group. The complex was digested with an excess of trypsin, the residual bound peptide was eluted with buffer containing β-mercaptoethanol, and the 14C-labeled peptide was further purified by reversed-phase high performance liquid chromatography and sequenced. The sequence shown in Table II is consistent with that expected for an internal thioester.

**Conformational Change of Lobster α2M after Reaction with Trypsin or Methylamine** — Reaction of human α2M with proteins obtained in runs at 8,547 and 11,299 rpm, respectively, giving an average absolute molecular weight of 342,000. Taken together, these experiments suggest that lobster α2M is a dimeric structure consisting of two monomeric, M, 180,000 polypeptide chains linked by disulfide bonds.

**Amino Acid Sequence Analysis** — N-terminal amino acid sequence analysis was performed on two independent 1.6-nmol samples to determine whether more than one class of subunit was present. The results are shown in Table I. The amino acid sequence was homogeneous, and the initial yield of 60% indicates that the subunits are identical.

**Subunit Structure of α2M—SDS-PAGE** under reducing conditions yielded a subunit molecular weight for lobster α2M of approximately 180,000, a value identical to that of the human species (Fig. 3). Under nonreducing conditions, the protein moved more slowly than human α2M, suggesting a higher molecular weight estimated to be approximately 440,000. The determination of the sedimentation coefficient gave, for the lobster protein, an s20,000 of 11.6, which is significantly smaller than the s20,000 value of 18.2 that we obtained for the human protein. Making the assumption that, like human α2M, the lobster protein is globular and therefore that the sedimentation would be proportional to (M,)1/2, a molecular weight of approximately 360,000 would be predicted for lobster α2M. More accurate estimates of the molecular weight were obtained by sedimentation equilibrium ultracentrifugation measurements where values of 332,000 and 352,000 were obtained in runs at 8,547 and 11,299 rpm, respectively, giving an average absolute molecular weight of 342,000. Taken together, these experiments suggest that lobster α2M is a dimeric structure consisting of two monomeric, M, 180,000 polypeptide chains linked by disulfide bonds.

**Carbohydrate Analysis** — Periodic acid-Schiff stain of the SDS gel showed the lobster protein to be glycosylated, and gas-liquid chromatographic analysis of a hydrolyzed sample of lobster α2M showed 5.1% (w/w) carbohydrate with N-acetylglucosamine, mannose, N-acetylgalactosamine, and fucose as the major sugars (21), the lobster protein has less carbohydrate, and galactose and sialic acid appear to be absent in the lobster protein.

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tease or methylamine results in a faster migrating species in polyacrylamide gel electrophoresis under nondenaturing conditions. This phenomenon is known as the "slow to fast" conformational change (15). Lobster αM migrates faster than the human species in nondenaturing gels with a migration rate corresponding to that of human half-molecules (Fig. 7). Pretreated with methyleamine, lobster αM does not show any change in migration. However, either native or methyleamine-treated lobster αM does show the slow to fast conformational change after incubation with trypsin.

**Inhibitor Activity of Lobster αM**—Two assays were carried out to show the inhibitor activity of lobster αM. In the amidolytic N-benzoyl-DL-arginine-p-nitroanilide assay, as described in the Miniprint Section, a corrected value for trypsin binding of 18.1 μg of αM/μg of trypsin was found. This corresponds to 0.79 mol of trypsin/mol of dimeric αM, assuming molecular weights for lobster αM and trypsin of 342,000 and 23,800, respectively.

In the [14C]casein assay, the amount of αM which protects [14C]casein from cleavage by 1 μg of trypsin was about 17 μg. When corrected, this corresponds to a molar ratio of 0.75 mol of trypsin/mol of dimeric αM, which is in good agreement with the result obtained from the amidolytic assay. Table III shows results obtained for a variety of proteases tested in the [14C]casein assay. Lobster αM inhibits all the proteases tested.

**DISCUSSION**

Assays of lobster hemolymph with trypsin and soybean trypsin inhibitor using large and small molecular weight substrates indicated the existence of protease inhibitor activity with properties similar to those of human αM. The purification of this invertebrate protease inhibitor was achieved with ion-exchange chromatography and gel filtration. The concentration in hemolymph was estimated by radial immunodiffusion to be about 60 mg/100 ml. Harpel and Brower (22) have commented on the seemingly high concentration of αM in human serum (250 mg/100 ml), pointing out that, because of the large molecular weight, this represents a concentration of only 3.5 μM, close to that of most other serum protease inhibitors. The molar concentration of αM in lobster hemolymph is about 1.8 μM. Measurements on several batches of lobsters purchased at different times showed this concentration to be fairly constant.

SDS-PAGE under reducing conditions gave a subunit molecular weight of 180,000, identical with that of the human protein. Attempts to determine an accurate molecular weight of the native form using gel filtration and SDS-PAGE with human αM and standard proteins as references did not give results which would permit a designation of a di-, tri-, or tetrameric structure composed of M, 180,000 monomer subunits. We therefore determined the molecular weight of the native inhibitor by ultracentrifugation. The molecular weight value obtained (342,000) favors a dimeric structure for the lobster inhibitor composed of disulfide-bonded monomers. This result is consistent with the sedimentation coefficient of 11.6 S, which is close to the value reported for dimeric species of human αM prepared by acid dissociation or reduction (23, 24) and for dimeric αM of the plaice (25). This is probably the first description of a natural dimeric αM homologue with disulfide-bonded monomers since, in the plaice and the southern grass frog (26), the subunits are noncovalently associated. Apart from the anomalously high molecular weight estimate of 440,000 from nonreducing SDS-PAGE, which might be due to different glycosylation, no evidence was found for a trimer species such as that which occurs in the horseshoe crab (20).

N-terminal amino acid sequence analysis of the inhibitor gave a single sequence, and the initial yield of 60% indicated that the dimers are composed of identical monomers. Comparison of the sequence of the lobster protein with known NH2-terminal sequences of vertebrate inhibitors revealed striking differences (Table I). Only 4 residues out of 22 are identical. The conserved proline in position 6 is located in position 7. The conserved tyrosine in position 8 is absent. Met-9 is conserved; but there is a tryptophan, usually a conservative residue, which is not found in the vertebrate proteins in position 10. With the exception of Ser-14 and Thr-20, the residues following Trp-10 differ from those in other species, including the normally conserved proline in position 13. The overall homology is therefore about 18%, which is perhaps an indication that this region is not important for the function of the inhibitor. Differences in the primary structure can be expected considering the fact that we compare proteins from species which diverged c considerable time ago. On the other hand, the partial sequence analysis of a peptide containing the thioester region which is important for the inhibitor activity of the protein is seen to be highly conserved when compared with the corresponding region in human αM (Table II). Besides the amino acid residue in position 12, all the residues are identical, including the 2 critical amino acid residues, cysteine and, presumably in the nascent chain (9), glutamine, from which the thioester is formed. We did not confirm position 8 as glutamic acid because the yield of the phenylthiohydantoin-derivative was too low for a back hydrolysis and subsequent amino acid analysis. But the circumstantial evidence provided by the [14C]methyleamine label and the elution position provides a strong argument for glutamine in position 8 of the peptide.

The autolytic cleavage of αM which occurs at the thioester site gave, under reducing conditions on SDS-PAGE, two fragments with molecular weights which differed slightly from those of the fragments obtained from the autolytically cleaved human inhibitor. The thioester seems to be shifted by about 5000 daltons, corresponding to about 45 amino acid residues, toward the C-terminal end of the subunits. Alternatively, these differences might be related to the differences in the glycosylation of the two proteins.

Quigley and Armstrong (20) reported that the inhibitory activity of horseshoe crab αM is lost after methylene treatment. This indication that the thioester bond occurs in proteins in invertebrates has been fully realized with the demonstration of the sequence and of the autolytic cleavage in lobster αM, as well as through the incorporation of methylene and the concomitant release of a free sulfhydril which binds to activated N-isopropyl-Sepharose. Experiments with lobster αM on gel electrophoresis under nondenaturing conditions confirmed the fact that the lobster protein is smaller than the human protein. The faster migration of the invertebrate protein is not due to differences in the isoelectric points as both proteins focused in the same region of a pH 5.0–7.0 gradient gel (data not shown). Methylene or protease treatment leads, in human αM, to an increase in electrophoretic mobility. The lobster protein shows the slow, fast change only after protease treatment. Methylene treatment does not produce a conformational change detectable with nondenaturing gel electrophoresis (similar to bovine αM (27)). In addition, methylene-treated αM has a tendency to aggregate and remain at the origin, and hence the bands in Fig. 7 appear faint.

Inhibition assays (N-benzoyl-DL-arginine-p-nitroanilide and [14C]casein) show the ability of lobster αM to inhibit a broad variety of proteases. The N-benzoyl-DL-arginine-p-ni-
troanilide and $^{[14C]}$casein assays gave values of 0.79 and 0.75 trypsin molecules bound per $\alpha_2$M molecule, respectively. It can therefore be concluded that the ratio is one protease molecule/inhibitor molecule and therefore different from the stoichiometry found for human $\alpha_2$M half-molecules (24) and for the plaice and southern grass frog (25, 26) where values in the range of 0.45 and 0.55 mol of trypsin/mol of macroglobulin were found.

In human $\alpha_2$M, methylation treatment produces a slow to fast conformational change, and the protein loses completely its ability to inhibit trypsin. In the case of lobster $\alpha_2$M, methylation treatment does not induce a similar conformational change, but its ability to inhibit proteases is nevertheless lost. This behavior is in marked contrast to that found for bovine $\alpha_2$M, which also shows no shape change but remains at least partially active following methylation treatment. The kinetics of the conformational change accompanying proteolytic cleavage of human $\alpha_2$M, as well as of C3 and C4, appears to be rate-limited by the proteolytic event per se. By contrast, the presence of an uncleaved polypeptide chain has been shown to retard, to variable degrees, the transitional transition at 30 °C in C3, 0.45 mol of trypsin/mol of macroglobulin. For bovine azM, the transition does not induce a similar conformational change but re-6.2 min in C4 and 111 in C3 at 37 °C.

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Invertebrate Thioester Protein α2-Macroglobulin

**Materials and Methods**

A functional expression system for α2-macroglobulin in yeast was established from the isolate of the American Lobster (Homarus americanus) by P.L. Scherfler, S. Arna, E.N. Leman, and R.H. Painter.

**Results**

Neurotensin (NT) and neuropeptide Y (NPY) in vitro effects were studied using the human neuroendocrine cell line PC12. For analysis, aliquots of media and conditioned media were analyzed by reverse-phase HPLC (Yale University, CA), or by radioimmunoassay (RIA; Amersham, IL). The antibody used for RIA was raised against a synthetic peptide corresponding to the sequence of human NT (1-35). The antibody was affinity-purified and used at a final concentration of 1:50,000. The specificity of the RIA was confirmed by the absence of cross-reactivity with other peptides.

**Discussion**

The functional expression system for α2-macroglobulin was functional in vitro and the potential for further studies was promising. The results of the study were encouraging and suggest that the functional expression system could be used for further studies on the potential use of α2-macroglobulin in various biological systems.

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Invertebrate Thioester Protein α2-Macroglobulin

Fig. 6. Purification of the thioester containing tryptic peptide from lobster αM. Immunoactive HPLC fraction of the trypsin digest treated protein on an Altex C18 10 × 4 column (4.6 × 250 mm). Effluents: (A) 0.1% trifluoroacetic acid in water; (B) 0.1% trifluoroacetic acid in 5 M LiCl aqueous, flow rate 0.5 ml/min.

![Image of a gel with bands](image)

**TABLE I:** Sequence of thioester containing peptide of lobster αM compared with other relevant protein sequences (derived from cDNA sequences).

| Sequence                          | Source     |
|----------------------------------|------------|
| Lobster αM                       |            |
| Ser-Tyr-Ile-Thr-Thr-Pro-Lys-Glu-Ala-Ser-Pro-Ala-Ser-Pro-Ala-Thr-Ile-Val-... |            |
| Human αM (41)                    |            |
| Ser-Val-Ser-Glu-Pro-Glu-Tyr-Ala-Val-Leu-Pro-Val-Leu-Val-Kys-Leu-Val-... |            |
| Rat αM (42)                      |            |
| Ser-Ala-Pro-Glu-Pro-Pro-Ile-Tyr-Met-Ala-Glu-Pro-Ser-Leu-Leu-Val-... |            |
| Human pregnancy protein αM       |            |
| Thr-Glu-Pro-Glu-Ile-Tyr-Met-Leu-Pro-Ser-Leu-Leu-Ile-Val-Glu-Ala-Val-... |            |

Note: The C-terminal sequence indicates Glu in position 6 of the sequence of rat αM (42).

The average repetitive yield was 99%, based on the third cycle, the initial yield was 99%.

The absolute yield of αM-αM with from 1600 pmol of cDNA (determined by enzymatic activity) from step 4 to 16 in a pool was 50, 65, 155, 165, 165, 155, 72, 82, 134, 93, 45, 39, 31, 39, 19, 95, 19, 13, 5.

The [14C]labelled residue in step 8 contained a major [14C]radioactivity. The corresponding PTH amino acid was eluted on reversed-phase column in a different position to the standard PTH amino acid between PTH glutamine and PTH glutamic acid. Such a profile is not inconsistent with the expected profile for a modified protein. The labelled peaks corresponding to the peaks indicated with an arrow in the Fig. 6 appear to be degradation products of the sequenced peptide arising as a result of chymotryptic activity in the trypsinic preparation used to generate the thioester containing peptide. Sequence analyses of the labelled peaks preceding the designated peak showed that they either lacked a terminal or C-terminal residues consisting of chymotryptic cleavage of the peptide indicated. For example the sequence analysis of the peak eluting immediately before the designated peak showed the same sequence. The labelled peak is therefore attributed to chymotryptic activity in the enzyme preparation. The labelled peak in the C-terminal region presumably as a consequence of a cleavage of the Phe-Ala peptide bond between position 13 and 14.

**TABLE II:** Addition of [14C]methionine of various proteolytic enzymes and the effect of pretreatment of αM with methionylase.

| Enzyme Tested | Addition αM | [14C]Sar αM | [14C]Sar αM | [14C]Sar αM |
|---------------|-------------|-------------|-------------|-------------|
| Ns enzyme     | -           | 530         | -           | -           |
| Chymotrypsin  | -           | 1500        | -           | -           |
| Cysteine      | -           | 1500        | -           | -           |
| Trypsin       | -           | 1500        | -           | -           |
| Trypsin       | -           | 1500        | -           | -           |
| Trypsin       | -           | 1500        | -           | -           |

* αM pretreated with methionylase

1 µg of each of the different proteolytic enzymes was incubated with [14C]Sar in the absence or presence of 30 µg of αM treated with methionylase.