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VARIABLE DENATURATION OF OVALBUMIN BY INCORPORATION OF AMINO ACID ANALOGS

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SUMMARY: Denaturation of hen ovalbumin synthesized in a cell-free system was assayed by examining its sensitivity to trypsin. The native ovalbumin resisted digestion by trypsin, and it remained resistant to digestion when some amino acid analogs, including azetidine-2-carboxylic acid and meta-flourotyrosine, were incorporated into its peptide chain. However, when other amino acid analogs such as β-hydroxyleucine and 4-thiaisoleucine were incorporated during protein systhesis, ovalbumin became very lablie to trypsin. These experiments demonstrate a sensitive system for detecting protein denaturation and suggest a variable effect of different amino acid analogs on the native conformation of a protein.

Amino acid analogs have been used to investigate the types of processing which secretory proteins undergo during intracellular maturation. In some instances, these analogs inhibit or modify the course of processing reactions by incorporation directly into potential processing sites. Studies of the hydroxylation of proline (1), cleavage of the pro-forms of various secretory proteins (2-4), cleavage of signal peptides from preprolactin (5), and glycosylation of asparagine residues (6,7) are good examples. In other cases amino acid analogs affect the processing of proteins without direct incorporation into processing sites. This has been observed in proteolytic cleavage of viral polyproteins (8), protein turnover (9), asparagine-linked glycosylation (10,11), and transport of secretory proteins into the endoplasmic reticulum (12.13). In these cases the mechanism by which amino acid analogs affect processing events is unclear. Incorporation of amino acid analogs may affect protein processing indirectly by changing the protein's conformation directly by altering the local environment of its processing sites.

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In this paper we investigate the ability of various amino acid analogs to alter the native conformation of a protein. Our approach was to assess the ability of several analogs to render ovalbumin susceptible to degradation by trypsin. This was considered likely to be a sensitive assay for changes in the conformation of ovalbumin (at least of local segments within its peptide chain) because, although the protein in its native conformation is very resistant to proteolysis by trypsin, it contains many potential cleavage sites (34 lysine or arginine residues not followed by proline) (14) and because none of the analogs used here directly affected the recognition sites for cleavage by trypsin, that is, none of the analogs substituted for lysine or arginine residues. We find that some analogs did not affect ovalbumin's native resistance to trypsin degradation while incorporation of other analogs rendered it highly susceptible to trypsin digestion.

MATERIALS AND METHODS

[^{35}S] methionine (specific activity 500-1000 Ci/mmol) was purchased from New England Nuclear. Ovalbumin mRNA was graciously provided by Dr. Richard Palmiter (Department of Biochemistry, University of Washington, Seattle). β-DL-Hydroxyleucine was purchased from U.S. Biochemical. DL-4-Thiaisoleucine, L-azetidine-2-carboxylic acid, DL-p-fluorophenylalanine, and m-fluoro-DL-tyrosine were purchased from Sigma Chemical Co. L-Threo-α-amino-β-chlorobutric acid was purchased from Calbiochem.

Cell-free ascites tumor extracts and ribosomes were prepared from Krebs II ascites tumor cells propagated in mice (15). Ascites S-100 preparations were treated with $CaCl_2$ to remove endogenous membrane-associated cleavage activity (16). Translation in the presence of 0.3 μM methionine was carried out in the cell-free reconstituted ascites lysates according to Szczesna and Boime (15). To evaluate protease sensitivity, total products of reaction mixture were incubated with 5μg of TPCK-treated trypsin for 15 min. at 23 degrees C. When appropriate, 5 mM DL-4-thiaisoleucine, 2.5 mM DL-p-fluorophenylalanine, 25 mM L-azetidine-2-carboxylic acid, 2.5 mM L-threo-α-amino-β-chlorobutyric acid, 2.5 mM m-fluoro-DL-tyrosine, or 20 mM β-DL-hydroxyleucine was included in the translation mixtures which otherwise were incubated without added amino acids. The pH of all analog solutions was adjusted to 7.5 with 1M Tris(hydroxymethyl)aminomethane base prior to addition to translation mixtures.

Radiolabelled products were percipitated with trichloroacetic acid, washed with chloroform:ether (1:1), and resuspended in a buffer containing 1% sodium dodecyl sulfate. The products were analyzed on 15% polyacrylamide gels (300:1 acrylamide:bis)(17). Labelled protein was detected by autoradiography (18).

RESULTS AND DISCUSSION

Total messenger RNA from hen oviduct was translated in a cell-free system prepared from Krebs II ascites cells. As observed previously (19), ovalbumin is the predominant [^{35}S] methionine-labeled species when total products of translation are analyzed by SDS-polyacrylamide gel electrophoresis (Fig 1,

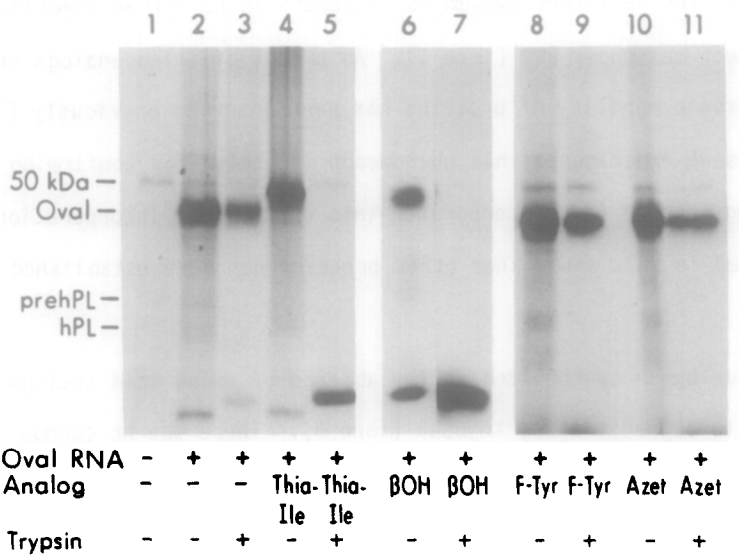

Figure 1: Sensitivity of ovalbumin synthesized in an ascites tumor cell-free system to degradation by trypsin. Total RNA from hen oviduct was translated in ascites lysates containing no exogenous amino acids (lanes 2 and 3) or one of several amino acid analogs (lanes 4-11). Total products were precipitated with trichloroacetic acid and analyzed on 15% polyacrylamide gels. Lane 1, total products of an incubation performed without added RNA. Lane 2, products of a reaction mixture containing oviduct RNA without any added amino acid analog. Lane 3, same as Lane 2, but following post-translational incubation with 5 μg of TPCK-treated trypsin. Lane 4, translation of oviduct RNA in the presence of 5mM thiaisoleucine. Lane 5, same as Lane 4, but following trypsin treatment. Lane 6, incubation in the presence of 20 mM β-hydroxy leucine. Lane 7, trypsin treatment of translation products incubated in the presence of β-hydroxy leucine. Lane 8, 2.5 mM meta-fluorotyrosine was used in the reaction mixture. Lane 9, same as Lane 8, but following post-translational exposure to trypsin. Lane 10, translation in the presence of 25 mM azetidine-2-carboxylic acid. Lane 11, same as Lane 10, but following trypsin treatment. Results are presented from 3 separate experiments--Lanes 1-5, 6 and 7, and 8-11. In each experiment there was a negative control--ovalbumin rendered susceptible to trypsin by incorporation of an analog and a positive control--ovalbumin rendered susceptible to tryspin by incorporation of an analog. Concentrations of analogs were based on a previous study (20).

lane 2). The labeled ovalbumin has an apparent molecular weight of 42,000 on polyacrylamide gels, and differs from mature ovalbumin in that it lacks oligosaccharide chains (19).

Ovalbumin remained the predominant product when the cell-free system was supplemented with various amino acid analogs, but its electrophoretic mobility was decreased by two of the analogs--β-hydroxyleucine and 4-thiaisoleucine (lane 4 and 6). The mobility change is most apparent from comparison with an internal molecular weight marker with an apparent size of 50,000 daltons

appearing just above ovalbumin. This labeled species is an endogenous component of the cell-free system as it occurs in cell-free reactions without added oviduct messenger RNA (lane 1). An effect of amino analogs on the electrophoretic mobility of proteins has been observed previously (12, 13, 20). In the present experiments this phenomenon is useful for confirming that amino acid analogs are in fact incorporated into ovalbumin. Incorporation of the analogs used in this study into other proteins has been established previously (20).

The ovalbumin synthesized in the absence of amino acid analogs was resistant to degradation by trypsin (lane 3). There was no change in the electrophoretic mobility of labeled ovalbumin and little change in its recovery when trypsin was added to the cell-free system following translation. Ovalbumin became highly susceptible to digestion by trypsin, however, when it was synthesized in the presence of β-hydroxyleucine or 4-thiaisoleucine, amino acid analogs which substitute for leucine and isoleucine respectively. Electrophoretograms indicated that ovalbumin, containing either of these analogs was completely degraded by trypsin (lanes 5 and 7). The increased intensity of low molecular weight radioactive material in these lanes confirms degradation of labeled protein and indicates that loss of ovalbumin did not result simply from inadequate recovery of product. Incorporation of two other analogs, meta-fluorotyrosine and azetidine-2-carboxylic acid (a proline analog) did not affect resistance of ovalbumin to trypsin (lanes 9 and 11). Analogs of phenylalanine (para-fluorophenylalanine) and valine (α-amino-β-chlorobutyric acid), also failed to alter susceptibility of ovalbumin to trypsin (data not shown).

The mobility and resistance to trypsin of the 50 kilodalton marker protein was not affected by any of the amino acid analogs. This is because the protein is not synthesized in the cell-free system. Neither cycloheximide nor other inhibitors of protein synthesis prevent the radiolabelling of the protein--which apparently results from chemical or enzymatic linkage of label to pre-existing protein in the system.

Alteration of the protease susceptibility of ovalbumin by incorporation of β-hydroxyleucine or 4-thiaisoleucine did not result from direct modification of trypsin recognition sites i.e. arginine and lysine residues within the protein. Rather, the analogs exert their effect indirectly, perhaps by altering the accessibility of lysine or arginine residues at the surface of the folded peptide chain--by producing a local conformational change or denaturation of ovalbumin. Other techniques producing conformational changes, such as heat and detergents, have also been shown to change ovalbumin's susceptibility to protease digestion (21).

The effect of incorporation of amino acid analogs on the rate of digestion of cellular protein by exogenous protease (22,23) has been examined previously by measuring the decrease in acid-precipitable protein. Unfortunately, globin, the protein studied most extensively in this regard, requires correct assembly of its four subunits and its heme moieties before it is a stable intracellular protein. It is likely that much of the effect of amino acid analogs on the stability of hemoglobin results from interference with the association of subunits rather than from direct effect on the conformation of globin chains. Globin is degraded rapidly intracellularly even without incorporation of amino acid analogs if there is unbalanced synthesis of its subunits (9). Thus, study of globin probably is not an accurate indicator of the tendency of amino acid analogs to denature proteins.

Use of electrophoresis in the present study results in a qualitatively more sensitive detection of proteolysis than studies which relied on assays of acid-precipitable protein. Since ovalbumin contains many potential trypsin cleavage sites, susceptibility to trypsin should be a very sensitive assay of its denaturation. It is not surprising then that modification of the peptide chain of ovalbumin by incorporation of amino acid analogs can enhance its sensitivity to protease digestion. It is more surprising that even with this highly sensitive assay some amino acid analogs can be incorporated into ovalbumin without detectably denaturing it. Thus, on the basis of this assay it is possible to categorize analogs as denaturing or non-denaturing with respect

to ovalbumin. This distinction is important in interpreting the many reported effects of amino acid analogs on protein processing. Further investigation will be required to establish whether amino acid analogs can be classified reliably as either denaturing or non-denaturing or whether their effects on different proteins will vary.

We are interested in the possible application of amino acid analogs to investigate of the mechanism by which ovalbumin is transported into and processed by the endoplasmic reticulum. This approach has been successfully applied to study the processing of other secretory proteins (12, 13). Understanding the processing of ovalbumin is particularly interesting because unlike other secreted proteins it does not have an excisable signal peptide (19, 24). Comparison of the effect of denaturing and non-denaturing amino acid analogs on the transport of ovalbumin and other secretory proteins into the endoplasmic reticulum may yield important clues about the structural signals in ovalbumin required for translocation across membranes.

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