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Proteolytic Susceptibility of Hemoglobin Synthesized in the Presence of Amino Acid Analogues

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SUMMARY

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α-Amino acid analogues with a hetero atom on the β-carbon, when incorporated into protein by a reticulocyte lysate, render the newly formed protein particularly sensitive to proteolytic hydrolysis.

Numerous reports have indicated that amino acid analogues, when incorporated into protein, render the recipient protein unstable and susceptible to denaturation and proteolysis (1–4). In an attempt to gain further insight into this problem, we have investigated the effect of several structural alterations of amino acids on the proteolytic susceptibility of hemoglobin synthesized in their presence by a reticulocyte cell-free system. Cell-free systems were employed to eliminate variations due to cellular uptake among the different analogues, and high concentrations of analogues were used to reduce differences in affinity for the aminoacyl-tRNA synthetases of the cell-free system.

Washed reticulocytes from a phenylhydrazine-treated rabbit (5) were lysed with an equal volume of water and the lysate was freed of debris by centrifugation for 15 min at 25,000 × *g*. Such lysates were rapidly frozen in small aliquots, stored under liquid nitrogen, and used immediately after thawing. Components of the incubation mixture were obtained from the Sigma Chemical Company (St. Louis, Mo.). The amino acid analogues were either synthesized in our laboratory by standard methods or were obtained from Sigma Chemical Company or Calbiochem (La Jolla, Calif.).

The incorporation system was prepared as described previously (6) and consisted of 500 μl of lysate with hemin to give a final optimal concentration of 30–60 μM, 300 μl of a master mix, and 300 μl of 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.2, either alone or containing an amino acid or its analogues. The master mix solution contained the following ingredients to yield the indicated concentrations in the total incorporation system: KCl, 75 mM; $MgCl_2$, 2 mM; ATP, 0.5 mM; GTP, 0.2 mM; creatine phosphate, 15 mM; creatine kinase, 45 units/ml; and a mixture of 18 amino acids in proportions to their content in rabbit hemoglobin, with the omissiion of both the amino acid being studied corresponding to the analogue and the amino acid used as a tracer. Of these, L-leucine, 53.5 mCi/mmole, 1 mM, was used except when leucine analogues were studied; in this case L-alanine, 20.8 mCi/mmole, 2 mM was employed. Protein synthesis proceeded for 30 min at 34° and was terminated by the addition of 100 μl of cycloheximide, 44 mM, and by cooling.

Endogenous proteolytic activity varied among the different lysate preparations. Therefore, proteolytic susceptibility of protein synthesized during the previous incubation was estimated by addition of 0.1 volume of a pronase solution, 0.13 unit/ml final concentration. Aliquots (25 μl) removed at zero time and after incubation at 34° for 30 min were added to 6 ml of a 10 mM NaCl solution containing either L-leucine or L-alanine, 40 mM, depending on the labeled amino acid used during the preceding incubation. Two milliliters of 20% trichloroacetic acid were added with vigorous agitation, the tubes were heated at 85° for 15 min and cooled to 0° for 15 min, and the precipitated protein was filtered on a Millipore membrane, 0.45-μm pore size. The precipitate was washed with 5% trichloroacetic acid, dried, and counted in a Nuclear Chicago gas flow counter with Micromil window.

The optimal level of added proteolytic enzyme was determined with the use of the valine analogue, α-amino-β-chlorobutyric acid, as a standard. A minimal proteolytic activity level was used which caused over 85% loss of incorporated leucine in trichloroacetic acid-precipitable material after a 30-min incubation with a system which had incorporated [^{14}C]leucine in the presence of a

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TABLE 1

Proteolytic susceptibility of hemoglobin synthesized in the presence of amino acid analogues

All of the amino acids and their analogues were racemic and were present in the lysate incorporation system at a final concentration of 10 mM, except when limited by solubility; *p*-chlorophenylalanine (2 mM), β-2-thhienylalanine (4 mM), tryptophan and its analogues, 1.0 mM.

Amino acid omitted	Amino acid added	Stimulation of incorporation by added amino acid	Newly formed protein lost by proteolysis
		%	%
Phenylalanine		—	1.2
	Phenylalanine	170	1.5
	Phenylserine (threo)	90	78
	Furanylserine	110	67
	β-2-Thienylserine	81	65
	2-Pyridylalanine	132	61
	2-Thiazolylalanine	130	53
	p-Chlorophenylalanine	51	20
	Phenylalanine	143	2.1
	o-Fluorophenylalanine	143	3.0
	m-Fluorophenylalanine	138	3.1
	p-Fluorophenylalanine	130	7.8
	β-2-Thienylalanine	136	3.6
	β-3-Thienylalanine	122	12.1
Leucine		—	5.2
	Leucine	170	2.7
	β-Hydroxyleucine	85	87
	4-Azaleucine	34	78
	5,5,5-Trifluoroleucine	152	13
Proline		—	3.2
	Proline	0	4.0
	Azetidine-2-carboxylic acid	−7.3	29.1
	3,4-Dehydroproline	2.0	12.2
Histidine		—	2.4
	Histidine	68	2.7
	Pyrazolylalanine	25	16
	2-Pyridylalanine	35	12
Tryptophan		—	0
	Tryptophan	65	0.9
	7-Azatryptophan	61	9.2
	6-Fluorotryptophan	69	5.8
	4-Fluorotryptophan	60	−0.2
	5-Fluorotryptophan	63	1.4

10 mM concentration of the valine analogue. This level of proteolytic activity did not reduce the precipitability of labeled protein synthesized in the absence of analogue.

The data in Table 1 show that several amino acid analogues stimulate protein synthesis in the absence of the corresponding metabolite. This may be interpreted as an indication that they are incorporated into protein. Analogues which promoted a proteolytic susceptibility of 65% or greater had a hetero atom on the β-carbon. An isosteric structure, such as azaleucine, was not required, and a β-hydroxy group substitution for hydrogen was very effective in the phenylalanine and leucine systems. Recently, Hortin and Boime (7) have reported that β-hydroxyleucine incorporation inhibited processing of preprolactin. Such a substitution may alter the tertiary structure of the substrate and thus its susceptibility to an endogenous modifying enzyme. Our results are consistent with earlier reports on the effectiveness of α-amino-β-chlorobutyric acid (8) and 4-thialysine (9) in promoting hemoglobin instability and demonstrate that amino acid analogues containing any of four hetero atoms, chlorine, oxygen, nitrogen, or sulfur, attached to the β-carbon are effective destabilizers. When hetero atoms are present on the β-position of natural amino acids, they confer an α-helix instability, apparently due to their interaction with the peptide backbone (10). Our data suggest that a similar interaction may occur following amino acid analogue incorporation, resulting in α-helix breakdown, protein denaturation, and proteolytic susceptibility.

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