Protein Synthesis by Rabbit Reticulocytes

II. INTERRUPTION OF THE PATHWAY OF HEMOGLOBIN SYNTHESIS BY A VALINE ANALOGUE*

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(Received for publication, April 10, 1959)

Many studies within the past few years have demonstrated that amino acid analogues may substitute for their corresponding natural metabolites in proteins (2-4). Indeed, selemethionine appears to satisfy all of the methionine requirements in *Escherichia coli* (5) and thus may be assumed to adequately replace the natural metabolite in all of the proteins of this species. Reports also have appeared that at least one amino acid analogue, α-fluorophenylalanine, becomes incorporated into discrete avian and mammalian cell proteins, that is ovalbumin and lysozyme of the hen ovuduct (6) and muscle aldolase and glyceraldehyde 3-phosphate dehydrogenase of rabbit muscle (7). In addition, it has been found in this laboratory† that α-fluorophenylalanine-2-C14 is readily incorporated into hemoglobin by reticulocytes in vitro. This ability of α-fluorophenylalanine to act as an unnatural substrate may account for its failure to block the incorporation into hemoglobin of amino acids other than phenylalanine (8), an observation which we have confirmed. Evidence obtained from bacterial systems indicates that some unnatural proteins formed in this manner are inactive functionally (9, 10).

Not all amino acid antagonists, however, act as competitive substrates to form unnatural proteins. Some tryptophan analogues are not activated but inhibit the activation of tryptophan (11). Also, an isoleucine analogue, α-methylthreonine, blocked incorporation of both isoleucine and other amino acids in a manner which was prevented only by isoleucine (12). The analogue therefore could not have served as coparticipant for protein synthesis.

A similar phenomenon is described in this communication; α-amino-β-chlorobutyric acid, a valine antagonist, inhibited the incorporation of valine, phenylalanine, and lysine into reticulocyte hemoglobin in vitro. The characteristics of the inhibited system furnish information on the mechanism of amino acid incorporation into protein by the intact cell.

EXPERIMENTAL

Materials—The DL-α-amino-β-chlorobutyric acids were prepared as their hydrochlorides from DL-threonine and DL-allo-threonine by the method of Fischer and Raske (13, 14) as modified by Kinoshita and Umezawa (15). The hydrochlorides were dissolved in 0.9 per cent sodium chloride solution buffered with sodium phosphate, 1 X 10-3 M, and neutralized with sodium hydrate before use.

The radioactive amino acids used were DL-valine-4,4'-C14, 5.7 X 106 c.p.m. per pmole, obtained from the Bio-organic Section of the Radiation Laboratory of this University; l-phenylalanine-uniformly labeled with C14, 4.6 X 106 c.p.m. per pmole, purchased from the Nuclear-Chicago Corporation, and dl-lysine-1-C14 hydrochloride, 4.85 X 106 c.p.m. per pmole, which was kindly furnished by Dr. M. Rothstein of this Department. In some cases, radioactive amino acids were mixed with the corresponding unlabeled material to prepare solutions of appropriate concentration and specific radioactivity.

Incubation and Isolation Procedures—Rabbit reticulocytes were prepared and incubated as described in the preceding paper (16) unless otherwise indicated. The amino acid mixture and iron however were omitted from the medium. Isolation of components (16) after incubation and the preparation of reticulocyte microsomes for incorporation of iron into hemoglobin (17) have also been described.

RESULTS

α-Amino-β-chlorobutyric Acids as Valine Antagonists—Both diastereoisomeric forms of α-amino-β-chlorobutyric acid competitively inhibited the incorporation of valine into the total protein of rabbit reticulocytes. Fig. 1 shows that the racemate prepared from DL-allo-threonine was approximately 5 times as effective in inhibiting valine incorporation as was the one prepared from DL-threonine. In the subsequent sections of this report, α-amino-β-chlorobutyric acid refers to the racemate prepared from DL-allo-threonine.

Inhibition of Phenylalanine Incorporation—In the preceding paper (16) it was shown that when reticulocytes were incubated with a tracer dose of radioactive phenylalanine in the absence of inhibitor, a marked incorporation into the microsomal protein preceded the incorporation into the soluble protein and hemoglobin of the cells. If, however, reticulocytes are preincubated for a short period with α-amino-β-chlorobutyric acid, subsequent addition of a tracer dose of radioactive phenylalanine results in changed pattern of incorporation. As is shown in Table 1, a higher level of incorporated radioactivity was found in the proteins of the particulate fractions, whereas incorporation into the soluble protein was severely inhibited. Incorporation into hemoglobin was abolished. The results indicate a block at some point of the biosynthetic pathway of hemoglobin in the particulate phase.

*Supported by a grant from the Cancer Research Funds of the University of California. A preliminary report of this work has been presented (1).
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‡ M. Rabinovitz and H. McGrath, unpublished observations.
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Fig. 1. Double reciprocal plot showing competitive inhibition of valine incorporation into total rabbit reticulocyte proteins by \( n\-\alpha\-\text{amino-}\beta\-\text{chlorobutyric acids: } 1 \), inhibition by analogue prepared from allothreonine, 0.001 M; 2, inhibition by analogue prepared from threonine, 0.001 M; 3, inhibition of analogue prepared from allothreonine, 0.001 M; 4, uninhibited control. Warburg flasks contained DL-\(\alpha\-\text{amino-}\beta\-\text{chlorobutyric acid} \) and DL-valine-\(4,4\'-\text{Cl}^{14} \) in 1.5 ml. of buffer in the main compartment, and 0.50 ml. of reticulocyte suspension, 1 volume of cells to 2 volumes of buffer, in the side arm. After temperature equilibration the side arms were tipped and the incubation allowed to proceed for 15 minutes. The incubation was terminated by addition of trichloroacetic acid from the second side arm.

The data in Fig. 2 show that the inhibition of lysine incorporation into hemoglobin caused by \( \alpha\-\text{amino-}\beta\-\text{chlorobutyric acid} \) is completely prevented by valine when it is present at \( \frac{1}{2} \) the concentration of inhibitor (mole ratio, inhibitor/valine = 2). At a mole ratio of 15, the incorporation is inhibited by 50 per cent. The accumulation of incorporated radioactive lysine in the microsomal and coarse particulate fractions is also prevented by valine. The effect of various valine concentrations upon the incorporation into these fractions is inversely related to that found for hemoglobin.

The inhibition caused by \( \alpha\-\text{amino-}\beta\-\text{chlorobutyric acid} \) can be relieved by valine after the block has been established. Indeed, addition of radioactive lysine and valine to cells preincubated with inhibitor results in a greater incorporation of lysine than when the addition of these amino acids is made to cells preincubated in buffer alone. This effect is shown in Fig. 3. The stimulation found upon the relief of a pre-existing inhibition resembles the overcompensation phenomena frequently encountered in physiological processes, and may be due to the accumulation of intermediates which are limiting in the normally functioning cell. A similar stimulation of amino acid incorporation was found upon the addition of glutamine to Ehrlich ascites tumor cells in which the synthesis of this amino acid had been blocked by methionine sulfoximine (18).

Exchange of Phenylalanine Incorporated into Inhibited System—

When nonradioactive phenylalanine is added to cells which had been previously incubated with \( \alpha\-\text{amino-}\beta\-\text{chlorobutyric acid} \) and radioactive phenylalanine, the specific activity of the total cell proteins decreased rapidly. This phenomenon, illustrated in Fig. 4, is completely dependent upon the previous incubation with the inhibitor and subsequent addition of nonradioactive phenylalanine.

The above pattern of inhibition was also observed when the amino acid was incorporated at a concentration adequate for saturating the protein synthetic system. This pattern, together with the prevention of inhibition by various concentrations of added valine, is shown in Fig. 2 with lysine-1-C\(^{14} \) as the incorporated amino acid.

Fig. 2. Prevention by valine of both \( \alpha\-\text{amino-}\beta\-\text{chlorobutyric acid} \) inhibition of lysine incorporation into hemoglobin and the associated increase of lysine incorporation into particulate protein. Points at the extreme left of each curve represent the uninhibited control. A preliminary incubation of the cells with the inhibitor and valine was carried out for 20 minutes, then DL-lysine-1-C\(^{14} \), 1.9 \( \times 10^{4} \) c.p.m., was added to give a final concentration of 1 \( \times 10^{-4} \) M and the incubation was continued for an additional 30 minutes.

## Table I

Inhibition of phenylalanine incorporation into hemoglobin and its accumulation in particulate protein

Reticulocytes were incubated in buffer with or without DL-\(\alpha\-\text{amino-}\beta\-\text{chlorobutyric acid} \), 5 \( \times 10^{-3} \) M, for 15 minutes. Uniformly labeled L-phenylalanine-C\(^{14} \), 14.5 \( \mu \)moles containing 67,000 c.p.m., was then added and the incubation was continued for another 15 minutes.

<table>
<thead>
<tr>
<th>Protein of</th>
<th>Protein specific activity (c.p.m./mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without inhibitor</td>
</tr>
<tr>
<td>Coarse particulate fraction</td>
<td>80</td>
</tr>
<tr>
<td>Microsomes</td>
<td>313</td>
</tr>
<tr>
<td>Soluble fraction</td>
<td>379</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>178</td>
</tr>
</tbody>
</table>

Prevention and Relief of Inhibition of Lysine Incorporation—

The above pattern of inhibition was also observed when the amino acid was incorporated at a concentration adequate for saturating the protein synthetic system. This pattern, together with the prevention of inhibition by various concentrations of added valine, is shown in Fig. 2 with lysine-1-C\(^{14} \) as the incorporated amino acid.
protein at an earlier stage of hemoglobin synthesis than that associated with the microsomal particle. It may consist of accumulated material associated with the microsomes in the intact cell but solubilized during the isolation process.

The exchange reaction resembles a similar phenomenon observed in the absence of inhibitor, namely the decrease in specific activity of protein of the particulate fractions after addition of nonradioactive amino acid to cells previously incubated with the same labeled amino acid (Table II of (16), (19). In this case, however, the label in the particulate fraction may have been either transferred to soluble protein or released from protein altogether. In such uninhibited cells, the soluble protein soon accounts for the major portion of incorporated amino acid. Since in the uninhibited system there is no displacement of radioactive amino acid from the soluble protein (16), no measurable net loss of incorporated radioactive amino acid can be found. Thus, the magnitude and stability of the incorporated radioactivity in the soluble protein obscures the nature of the changes in the particulate proteins, and precludes the possibility of deciding whether the decrease in specific radioactivity of these fractions was the result of amino acid exchange or the release of newly formed protein molecules.

**Effect of α-Amino-β-Chlorobutyric Acid on Iron Incorporation**—Isolated reticulocyte microsomes, supported by components in the soluble fraction of the cell, participate in the incorporation of iron into hemoglobin, the sole acceptor in the soluble protein fraction (17). Also only in the presence of the soluble fraction of reticulocytes does radioactive iron become firmly bound to the microsomal protein. The kinetics of this reaction are shown in Fig. 5. The rapid attainment of a steady state in the Incorporation of iron into the microsomes is consistent with the assumption that these particles participate in the incorporation of iron into hemoglobin.

It was of interest to determine whether microsomes, isolated from reticulocytes in which the pathway of hemoglobin synthesis was blocked by α-amino-β-chlorobutyric acid, could participate in iron incorporation. As can be seen in Table III previous in-

**Table II**

<table>
<thead>
<tr>
<th>Protein of</th>
<th>Protein specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zero time</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole cells</td>
<td>140</td>
</tr>
<tr>
<td>Coarse particulate fraction</td>
<td>200</td>
</tr>
<tr>
<td>Microsomes</td>
<td>883</td>
</tr>
<tr>
<td>Soluble fraction</td>
<td>88</td>
</tr>
</tbody>
</table>

The participation of the various cell fractions in the exchange reaction is shown in Table II, and it is apparent that incorporated amino acid was released from protein of all fractions. However, 80 per cent of phenylalanine incorporated into the soluble protein was displaced, whereas less than 50 per cent displacement occurred in the particulate fractions.

The exchange reaction may be visualized as an equilibrium between free (and activated) amino acid and its incorporated counterpart in an early stage of protein synthesis. The higher displacement of incorporated amino acid from the soluble protein of inhibited cells may indicate that this fraction contains...
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**FIG. 5.** Kinetics of iron incorporation into microsomal and soluble protein in a cell-free reticulocyte preparation. Reticulocyte microsomes (5 mg. of protein) were incubated with the soluble extract of reticulocytes (30 mg. of protein) and iron (3.3 μg., 1.5 × 10⁶ c.p.m.) as ferrous ammonium sulfate.

**TABLE III**

<table>
<thead>
<tr>
<th>Protein of</th>
<th>Iron incorporation mediated by microsomes from cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Preincubated in buffer</td>
</tr>
<tr>
<td></td>
<td>c.p.m./μg protein</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>Microsomes</td>
</tr>
<tr>
<td></td>
<td>Soluble fraction</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>Microsomes</td>
</tr>
<tr>
<td></td>
<td>Soluble fraction</td>
</tr>
</tbody>
</table>

* Deviation from the mean of duplicate incubations.

**DISCUSSION**

**Locus of Inhibition**—The block in hemoglobin synthesis and the concomitant accumulation of incorporated radioactive amino acid in the microsomal protein indicate that the inhibition may occur at a site after the incorporation reaction but before the release of a completed protein molecule. In view of the general ability of amino acid antagonists to be incorporated, the results suggest that α-amino-β-chlorobutyric acid enters into a precursor protein associated with the ribonucleoprotein particles, which then is unable to assume the proper configuration of globin (Fig. 6). The failure of the synthetic site to release completed protein thus might cause an accumulation of earlier protein intermediates along the pathway.

Neither α-fluorophenylalanine (20) nor α-amino-β-chlorobutyric acid interfered with the protein synthetic process of the Ehrlich ascites tumor cell. The ability of the valine analogue to interrupt protein synthesis is therefore not only determined by its structure, but is also a function of the specific protein synthetic system. The structure of the protein synthesized may also be a factor in determining whether an analogue could be accepted within its framework. In this regard, it is of interest that valine is the sole N-terminal residue of rabbit hemoglobin (21), and its role in this location could play a part in the inability of the analogue to substitute for it during the terminal phase of protein synthesis.

**Incorporation as Protein Synthesis**—The observation by Borisok et al. (8) that an amino acid mixture and iron stimulated the incorporation of a single radioactive amino acid into hemoglobin implied the participation of other components in the incorporation process and thus supplied evidence that the major fraction of amino acid incorporation was due to synthesis of protein from free amino acids. The results reported in this communication support and extend this observation. Neither phenylalanine nor lysine was incorporated into hemoglobin when the incorporation of valine was inhibited. The obligatory participation of component amino acids indicates that all incorporation of amino acids into hemoglobin is by protein synthesis, and

* M. Rabinovitz and M. E. Olson, unpublished observations.
that there is no independent mechanism for exchange of amino acids with corresponding residues in the complete hemoglobin molecule.

Exchange at Synthetic Sites—Gale and Folkes (22) reported that labeled amino acids incorporated into the total protein of disrupted Staphylococcus aureus preparations were displaced on incubation with the unlabeled amino acid and an energy source. Such exchange was not found in incorporation studies with rat liver microsomes (25) or calf thymus nuclei (26).

The demonstration of exchange between free and incorporated amino acid in the intact rabbit reticulocyte suggests that such a phenomenon may influence the magnitude of unequal labeling (21) of an amino acid within the hemoglobin molecule (28) as well as the apparent time for synthesis of a molecule of the protein (29). Indeed, any estimation dependent upon precise determinations of initial incorporation rates would be subject to errors introduced by such equilibration. This phenomenon, however, may occur only when the synthetic pathway is inhibited or functioning at a suboptimal rate, and may not be a major factor in the normal protein synthetic system.

Preformed Protein Precursors—The ability of microsomes with a blocked pathway of globin synthesis to participate in the incorporation of iron into hemoglobin demonstrates that these two aspects of hemoglobin synthesis are easily dissociated. Although this dissociation indicates the availability of globin (16) which can act as an acceptor for heme, the possibility exists that this protein is hemoglobin, and that the reaction observed is heme or iron exchange at the synthetic site.

London et al. (30, 31) have described a similar dissociation in intact duck erythrocytes. They produced a larger inhibition of glycine incorporation into globin as compared to incorporation of lysine in intact duck erythrocytes. They produced a larger inhibition of glycine incorporation into globin as compared to incorporation of lysine in intact rabbit reticulocytes, and to Dr. M. Rothstein for a gift of lysine-l-14C.

Acknowledgments—We are grateful to Professor D. M. Greenberg for his continued interest and active support of this work, and to Dr. M. Rothstein for a gift of lysine-l-14C.

REFERENCES


3 This exchange may in part be due to release of amino acid from the hexosamine-peptide of Staphylococcus aureus cell wall (25, 24).