Requirement for Alanine in the Amino Acid Control of Deprivation-induced Protein Degradation in Liver

gluconeogenesis/autophagy/hepatocytes

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ABSTRACT Protein degradation in liver is actively controlled by a small group of inhibitory amino acids—leucine, tyrosine (or phenylalanine), glutamine, proline, histidine, tryptophan, and methionine. Other evidence, however, suggests that one or more of the remaining 12 noninhibitory amino acids is also required for suppression of proteolysis at normal concentrations. This question was investigated in livers of fed rats perfused in the single-pass mode. The deletion of alanine at normal (1×) but not at 4× or 10× normal, plasma amino acid concentrations evoked a near-maximal acceleration of protein degradation. No other noninhibitory amino acid was effective. Because alanine alone was not directly inhibitory and its omission was not associated with a decrease in inhibitory amino acid pools, alanine was presumed to act as a coregulator in the expression of inhibitory activity. When tested alone, the inhibitory group was as effective as the complete mixture at 0.5× and 4× levels, but it lost its suppressive ability within a narrow zone of concentration centered slightly above 1×. The addition of 1× (0.48 mM) alanine completely restored the inhibition. Pyruvate and lactate could be effectively substituted, but only at concentrations 10–20 times greater than that of alanine. These, together with earlier findings, indicate the existence of a regulatory complex that recognizes specific amino acids and transmits positive and negative signals to proteolytic sites. The results also suggest that alanine can provide an important regulatory link between energy demands and protein degradation.

A notable feature of liver is the exceptionally high rate of cellular protein loss that it exhibits early in starvation (1–3). At the systemic level, such a response is necessary to provide an adequate supply of amino acids for important metabolic processes such as gluconeogenesis as well as for protein renewal. There is now general agreement that overt or macroautophagy is largely if not solely responsible for the enhanced degradation (4–7) and that it is regulated primarily by an inhibitory action of amino acids (8–11). After amino acid removal in the perfused rat liver, for example, both macroautophagy and protein degradation accelerate dramatically (7). And because the autophagic response is rapid in onset (7), amino acid restriction must be linked to it by a discrete and potentially identifiable signal or set of signals.

Only a comparatively small number of amino acids appears to have demonstrable inhibitory activity (9–13). In a recent liver perfusion study designed to distinguish noninhibitory from inhibitory amino acids at the upper limit of physiological plasma concentration (4 times normal), we found that 12 amino acids were totally devoid of activity as a group whereas the remainder—leucine, tyrosine (or phenylalanine), glutamine, proline, methionine, tryptophan, and histidine—were as effective as the complete mixture (11).

EXPERIMENTAL PROCEDURES

Liver Perfusion. Male rats of the Lewis strain (M. A. Bio-products, Walkersville, MD), maintained on standard laboratory chow and water ad lib under controlled lighting (off 1900, on 0700), served as liver donors. They weighed from 120 to 140 g at the time of perfusion.

The technique of perfusion in situ has been described (7, 19, 20). In brief, livers were perfused in a single-pass or nonrecirculating mode for 40 min with perfusion media of various amino acid compositions. At the end of the single-pass phase, the flow of perfusate was switched to a second perfusion reservoir, containing cycloheximide, for the measurement of protein degradation.

Perfusion Medium. The composition of the basic medium was as follows: 3% bovine plasma albumin (fraction V, Pentex, Miles)/10 mM glucose/0.27% washed bovine erythrocytes (7, 19, 20) in Krebs–Ringer bicarbonate buffer. Prior to its addition to the medium, a concentrated solution of the bovine albumin was passed through a 0.3-μm Millipore filter. Additions of amino acids to the perfusion medium were made by using concentrated solutions of amino acids in 0.85% NaCl with pH adjusted to 7.4; additions of lactate, pyruvate, and octanoate were similarly prepared. The basic amino acid mixture, designed to simulate the composition of plasma, was the same as that used earlier (7, 11, 16, 17); an amino acid analysis is given in the legend to Fig. 1.
Measurement of Protein Degradation. Rates were determined as described (7, 17). Briefly, the procedure was as follows. After the 40-min single-pass perfusion, flow was switched to a reservoir containing the basic medium, 18 µM cycloheximide, and 0.35 mM valine. The medium was circulated through the liver for 15 min, and four perfusate samples were taken during the 5- to 15-min segment for valine analysis. Total free valine accumulation was computed at each point, and proteolytic rates were computed by least-squares regression. Values are expressed as µmol or nmol of valine/min per liver (100-g rat) after valve correction for valine released from short-lived components: the correction factor has been estimated to be 0.053 µmol/min (7). This procedure, which has been fully validated for the conditions used in this study (2, 7), provides a measure of the rate of long-lived or resident protein breakdown that exists at the moment the cycloheximide perfusion is started (2, 7). The corrected degradation rates are equivalent to those obtained by isotope release from previously labeled livers (7).

Analytical Procedures. Valine in perfusate plasma was determined chromatographically by the method of Mortimore and Mondon (19). Complete amino acid analyses of liver and plasma were carried out as before (7, 17); liver values were corrected for extracellular amino acids, and the results are expressed as µmol/ml of intracellular water. Liver ATP was measured by the method of Williamson and Corkey (21). The significance of differences between means was determined by Student’s t test.

RESULTS

Effects of Deletion of Noninhibitory Amino Acids on Protein Degradation. The central observation from which this study evolved (16) is shown in Fig. 1 (column C). It is evident that deletion from a complete 1× mixture of the four glucogenic amino acids (glutamate, glutamine, alanine, and glycine) whose intracellular pools are decreased by glucagon (17, 18) accelerates protein degradation to near maximum. The effect could not be duplicated by removal of glutamine alone or of glutamine/proline but was obtained when glutamate/alanine/glycine as a group (column F) or alanine alone (column G) was deleted. Omission of glutamate or glycine evoked no responses nor did deletion of the remaining nine noninhibitory amino acids (column J). Although none of the latter were tested individually, the findings generally indicate that alanine is the only noninhibitory amino acid whose omission is capable of inducing a response. As shown in Fig. 2, the degree of proteolytic stimulation induced by the omission of alanine was strongly modulated by the concentration of plasma amino acids; no effects were obtained at 4× and 10× normal levels but strong responses were observed at 1× and 2×. This pattern of response corresponded closely to those previously observed after glucagon administration (17) and deletion of the four glucogenic amino acids (16).

Mechanism of the Proteolytic Response to Alanine Deprivation. In general terms, the proteolytic acceleration observed after alanine omission at the 1× level can be explained by either (i) the loss of direct inhibitory activity or (ii) removal of an indirect coregulatory function. The first would be demonstrable as proteolytic suppression following the administration of alanine alone or of mixtures of noninhibitory amino acids containing alanine; the second would be manifest only in the presence of the inhibitory amino acid group, whose effectiveness would diminish in the absence of alanine. With regard to i, it should be noted that noninhibitory amino acid mixtures have consistently failed to suppress protein degradation (refs. 11, 16; see Table 2), findings that appear to exclude the possibility that alanine is directly inhibitory. Results of Parrilla and Goodman (22), though, have indicated that alanine loading may suppress net protein ca-

FIG. 1. Effects on protein degradation of various deletions from a complete 1× mixture of plasma amino acids. Livers from normal fed rats were perfused in the single-pass mode for 40 min. Protein degradation was determined from the release of valine in a second-stage cyclic perfusion containing cycloheximide; results were corrected for short-lived components. Amino acid analyses of 15 samples of perfusate plasma after the addition of amino acids at normal (1×) concentrations gave the following mean µmol values: Ala, 475; Arg, 220; Asp, 53; Cys, 34; Glu, 158; Gly, 370; His, 92; Ile, 114; Leu, 204; Lys, 408; Met, 60; Phe, 96; Pro, 437; Ser, 657; Thr, 329; Tyr, 98; Val, 250; Asn, 101; Gln, 716. The average deviation from values reported earlier after similar additions (7) was 6%. Results shown are increases above the mean basal rate of degradation at 4× (131 nmol of valine/min per 100-g rat) expressed as percentages ± 1 SEM of the maximal depression response. The term “balance” refers to all remaining noninhibitory (Ala, Glu, Gly, Asp, Asn, Cys, Ser, Thr, Arg, Lys, Val, Ile) or inhibitory (Leu, Tyr/Phe, Gln, Pro, Met, Trp, His) amino acids (total minus amino acids indicated separately). The horizontal broken line represents the response to a normal complete 1× plasma mixture. Three to 28 experiments comprise each group.

tabolism in liver, and for this reason we tested alanine alone. In three experiments, the mean rate of protein degradation with 0.48 mM (1×) alanine was 316 ± 17 nmol of valine/min per 100-g rat compared with 339 ± 11 for 0× controls. We concluded that, if alanine had any direct inhibitory activity, its contribution to the deprivation response would be very small.

Two possibilities for indirect inhibition (ii) were considered. The first is that the lack of alanine had in some way decreased pools of certain critical inhibitory amino acids. An extensive analysis of intracellular amino acids, however, failed to show any diminution of inhibitory amino acids (Table 1). Leucine, histidine, and the noninhibitory amino acid, threonine (data not shown), were increased, alterations that could be attributed to enhanced intracellular proteolysis (17, 20). The only other effects of note were small, but significant, decreases in alanine and glutamate. Our failure to observe more than a 16% reduction in alanine is in agreement with Sips et al. (23), who reported negligible effects of alanine omission on intracellular pool size in perfused hepatocytes. With regard to glutamate, the statistical significance of the decrease could be established only indirectly. We found that glutamate and glutamine correlate positively over a rather wide (3-fold) range of control values (r = 0.78). Because the slope regressed close to the zero intercept, we were able to express changes in glutamate in relation to glu-
Fig. 2. Effect on protein degradation of the deletion of alanine from various concentrations of a complete mixture of plasma amino acids. Experimental procedures were identical to those in Fig. 1. Values are means ± SEM expressed as percentages of the average maximal rate of degradation (339 nmol of valine/min per 100-g rat). Each group consists of 3–28 observations. □, Complete amino acid mixture; □, alanine-lacking mixture.

The ratio, glutamate/glutamine, averaged 1.048 ± 0.049 for the 15 control livers in Table 1 and 0.883 ± 0.044 for the alanine-deleted group (P < 0.025). The percentage change, 16, was the same as that for alanine.

Concerning the second possibility, we reported earlier that the inhibitory amino acid, leucine, lost its effectiveness with-

Table 1. Effects of alanine deletion on intracellular amino acid pools in rat livers perfused in the single-pass mode with 1× plasma amino acids

<table>
<thead>
<tr>
<th>Amino acid pool</th>
<th>Complete mixture</th>
<th>Ala-lacking mixture</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitory</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>0.356 ± 0.009</td>
<td>0.393 ± 0.008</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.106 ± 0.004</td>
<td>0.100 ± 0.006</td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>0.114 ± 0.007</td>
<td>0.118 ± 0.009</td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>0.836 ± 0.045</td>
<td>0.994 ± 0.013</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Proline</td>
<td>0.161 ± 0.011</td>
<td>0.177 ± 0.014</td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>9.180 ± 0.369</td>
<td>9.190 ± 0.230</td>
<td></td>
</tr>
<tr>
<td>Noninhibitory</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td>9.190 ± 0.425</td>
<td>8.070 ± 0.339</td>
<td>&lt;0.025†</td>
</tr>
<tr>
<td>Alanine</td>
<td>2.190 ± 0.108</td>
<td>1.840 ± 0.090</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Glycine</td>
<td>5.090 ± 0.173</td>
<td>5.070 ± 0.218</td>
<td></td>
</tr>
<tr>
<td>Aspartate</td>
<td>4.970 ± 0.179</td>
<td>4.570 ± 0.212</td>
<td></td>
</tr>
</tbody>
</table>

Livers from normal fed rats were perfused in the single-pass mode for 40 min with either a complete mixture of normal (1×) plasma amino acids or the same minus alanine (Fig. 1). Phenylalanine is listed even though its inhibition is believed to be mediated via tyrosine (see text). Trp and Arg were not determined; values for Ser, Thr, Asn, Cys, Lys, Ile, and Val are not shown. P values not indicated are >0.05. Values are means ± SEM.

*P < 0.05.
†P < 0.025.
‡From comparison of the glutamine/glutamate ratio.

Table 2. Effects of inhibitory and noninhibitory amino acid mixtures on hepatic protein degradation; coregulatory effect of alanine

<table>
<thead>
<tr>
<th>Amino acid mixture</th>
<th>Protein degradation, nmol of valine/min per 100-g rat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>None</td>
<td>339 ± 11</td>
</tr>
<tr>
<td>Noninhibitory</td>
<td>339 ± 17</td>
</tr>
<tr>
<td>Ala, Glu, Gly</td>
<td>340 ± 20</td>
</tr>
<tr>
<td>Inhibitory</td>
<td>290 ± 14</td>
</tr>
<tr>
<td>Inhibitory + noninhibitory</td>
<td>165 ± 6</td>
</tr>
<tr>
<td>Inhibitory + alanine</td>
<td>164 ± 16</td>
</tr>
</tbody>
</table>

Livers from normal fed rats were perfused for 40 min in the single-pass mode with the indicated amino acid additions at their normal (1×) plasma concentrations; resident (long-lived) protein degradation was determined as in Fig. 1. The mean basal rate with 4× complete plasma amino acids was 131 ± 6 nmol of valine/min per 100-g rat. Total values are means ± SEM; numbers of experiments are in parentheses.

*Equivalent to normal plasma amino acid mixture.

in a sharply localized zone between 0.5× and 2× whereas the full inhibitory mixture appeared to mimic responses of total plasma amino acids (11). The effect of this mixture, though, was not tested at the 1× level (11). If suppressive activity of the inhibitory group is actually diminished at normal plasma concentrations, then one would have to consider the hypothesis that alanine is necessary for expression of inhibitory activity. Results in Table 2 in fact show that most of the suppression is lost. The decrease in inhibitory effectiveness at 1×, which was 125 nmol of valine/min per 100-g rat, explained 83% of the total alanine deprivation effect at this level; effectiveness was completely restored by the addition of alanine.

Fig. 3 displays responses over the full concentration range for the inhibitory and complete amino acid mixtures. Results with the complete mixture were comparable with those previously reported from our laboratory (7, 11, 17), but responses of inhibitory amino acids deviated dramatically in showing a narrow zonal loss of suppressive activity, peaking at 1.25× (Fig. 3 Inset). Except for differences in magnitude, the 0–4× segment of the response curve in Fig. 3, including the sharp peak at ≈1×, corresponded exactly to the curve for leucine reported earlier (11). The downward deviation in proteolytic rates between 4× and 10× has been discussed previously and was attributed to an inhibition of lysosomal proteolysis from ammonia generated at unphysiologically high concentrations of glutamine (11, 16).

Effects of Pyruvate, Lactate, and Octanoate on the Alanine-Deprivation Response. Both pyruvate and lactate decreased the proteolytic response to alanine deprivation, but the amounts needed greatly exceeded the normal (1×) concentration of plasma alanine (0.48 mM). As shown in Table 3, 5 mM additions of these substrates inhibited the effect by ≈40%; nearly complete suppression was achieved with 10 and 20 mM pyruvate. Because pyruvate and lactate were comparable in effectiveness, the deprivation response was probably not the result of oxidoreductive alterations in the cytosol.

The addition of 1 mM octanoate also inhibited the effect of alanine omission (Table 3). This was surprising because earlier studies had shown that neither octanoate nor acetooce-
felt it important to determine whether the stringent removal of alanine might for some reason limit energy production. We found that ATP was unaffected by the loss. In four pairs of 40-min perfusions, values were 11.8 ± 0.31 μmol/g of dry weight in the presence of 1× plasma amino acids and 11.7 ± 0.19 after the deletion of alanine.

The striking inhibition by octanoate suggests that fatty acids play a permissive role similar to that of alanine. Should future experiments show that plasma long-chain fatty acids are as effective as octanoate, the finding would be of major importance in interpreting proteolytic regulation in vivo as well as in isolated liver cell preparations. A question of immediate interest, however, is the extent to which fatty acids bound to perfusate albumin might have affected the present results. Fig. 3 indicates that proteolysis would accelerate by ~180 nmol/min with complete loss of inhibitory activity at 1.25× plasma amino acids. An approximation of the maximal effect of alanine removal at this level (Fig. 3 Inset) is 175 nmol/min. Thus the probability that albumin-bound fatty acids inhibited the alanine effect appreciably appears to be small. The question, though, should be reexamined in the future with the use of defatted albumin.

**DISCUSSION**

The finding that alanine is required for inhibitory amino acids to express their suppressive activity at normal plasma concentrations adds a new dimension to our rudimentary knowledge of how protein degradation in liver is regulated. Apart from the possibility that the response to alanine deprivation could be abolished by fatty acids in vivo, the fact that the removal of alanine from a normal mixture of plasma amino acids during perfusion evokes consistent increases in proteolytic activity clearly means that alanine or its products are important components of the regulatory mechanism.

Virtually nothing is known of the way or ways in which regulatory agents are recognized or where the recognition occurs. However, we can conclude from our findings that, if the signal from alanine lack is initiated at the cell surface, then alanine rather than its transamination product, pyruvate, would be the probable agent. But because there is little relevant information on the question of location, it is more appropriate to ask whether alanine itself or its metabolic products is the active regulator, independent of locus. Lac- tate, pyruvate, and alanine all produce glucose as their major end product (26–28). The first two, though, are more effective substrates even at physiological concentrations (28). On the other hand, only a very small fraction of lactate (and probably pyruvate also) is converted to alanine; the ratio of glucose to alanine produced is ~30:1 (28). It seems likely therefore that at the 1× plasma level equimolar quantities of lactate or pyruvate can replace alanine as a source of carbon for gluconeogenesis and oxidative pathways without appreciably altering the alanine deficiency. These considerations support the hypothesis that physiological alterations of alanine rather than pyruvate or other intermediates generate the signal or signals that regulate proteolysis. Presumably, very high concentrations of pyruvate or lactate would provide enough alanine to offset the deficit and return proteolytic rates to control values (Table 3).

Of no less importance to proteolytic regulation is the suppression that is achieved by the inhibitory amino acids at their lowest concentration, 0.5×. The same effect probably occurs at higher levels and is very likely the one that is defeated when alanine is omitted from the complete mixture. Because alanine is not required for inhibitory expression at 0.5× as it is at 1×, the suppression at 0.5× may be considered a direct response to the inhibitory group (compare 0.5× amino acid effects in Fig. 3). Responses to all the individual inhibitory amino acids have not been established at 0.5×.
However, past studies indicate that leucine is the dominant component of the group and that its suppressive activity can explain more than 60% of the total inhibition at 0.5 × as well as at 4 × (10, 11). It is likely that effects of the remaining amino acids would parallel those previously established at 4 × (11).

Although the mechanism of this primary inhibition is obscure, it is possible that aminoacylation is involved. From considerations given earlier (11) we have calculated that, as a maximum, 8% of the leucine entering the hepatocyte is transamminated. Thus the suppression must be achieved either through free leucine or leucine bound to tRNA (11). The latter would generally agree with the proposal of Scornik and co-workers (29, 30), who have suggested that protein degradation in histidine-dependent mutants of Chinese hamster ovary cells is controlled by tRNA or aminoacylated tRNA.

Because the response to alanine deletion is an indirect, coregulatory phenomenon, it will become fully understood only in the context of the underlying inhibitory activity that it complements. The main question, therefore, is one of explaining the intrinsic loss of effectiveness of the inhibitory amino acids between 0.5 × and 1 × in the absence of alanine (Fig. 3). Why is the primary inhibition abruptly switched off at amino acid concentrations above 0.5 ×? Although we have no definitive answer to this question, the findings do point to the existence of a second action of inhibitory amino acids that nulls the inhibitory effectiveness of the first; it is the latter that is prevented by alanine. The striking difference in the concentration–response characteristics of these opposing activities clearly establish their independence. However, we have no way of knowing whether they ultimately arise from one or two sites of amino acid recognition.

While the aforementioned second regulatory action is presumed to be responsible for the intrinsic loss of effectiveness of the specific inhibitory amino acids at plasma concentrations greater than 0.5 × (demonstrable in the absence of alanine), a third action of the same amino acids would be required to achieve the proteolytic suppression observed between 1.25 × and 4 × (Fig. 3). One possible explanation for this suppression is illustrated in our previous study of leucine (11). We showed that protein degradation in perfused livers was not inhibited by octanoate or acetooacetate in the absence of added amino acids although it was suppressed by the ketoacid of leucine. The response curve for α-ketoisocaproate paralleled the descending limb of the leucine curve from 1 × to 4 × but differed from it in failing to show an initial inhibitory effect at a concentration equivalent to 0.5 × leucine. Obviously these responses were not physiological since they were obtained at concentrations higher than branched chain ketoacids are normally found in plasma (31). But they do suggest the existence of a third site capable of recognizing structural features common to leucine and its ketoacid. By these criteria, the regulation would not involve aminoacylation. Although proteolytic suppression by this mechanism would probably not be expressed under normal conditions, it might be of importance in metabolic states characterized by low plasma alanine.

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