SPECIFICITY OF PAPAIN-CATALYZED
TRANSAMIDATION REACTIONS*

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An earlier report from this laboratory (1) has described the application
of column chromatography on ion exchange resins (2) to the quantitative
study of papain-catalyzed transamidation reactions of the type:

\[
\text{Cbzo-NH(CHR)CO-NH}_2 + \text{NH}_2(\text{CHR'})\text{CO-NH(CHR")COO}^- + \text{H}^+ \rightleftharpoons \\
\text{Cbzo-NH(CHR)CO-NH(CHR')CO-NH(CHR")COO}^- + \text{NH}_3^+
\]

In the experiments reported in the present communication, these studies
have been extended with special reference to the specificity of the enzyme
toward the dipeptide used as replacement agent. It had been suggested
(1) that the value of pK_a' of a dipeptide is a determining factor in the
efficiency with which it can participate in a transamidation reaction at a
given pH. This suggestion has been examined by the determination of
pK_a' for several dipeptides under the conditions of the enzymic tests and
the comparison of the relative magnitude of these values with the observed
efficiency of transamidation. Earlier data (1) had also indicated that, in
addition to the pK_a' values, other factors that are important in determining
the efficiency of a dipeptide as a replacement agent are the chemical nature
of the R' group and the configuration of the N-terminal amino acid residue.
This aspect of the problem was investigated further by the use of a variety
of dipeptides.

Methods

The chromatographic procedures were essentially the same as those de-
scribed previously (1) except that Dowex 50-X2 resin (3) was employed
instead of the Dowex 50-X4 resin. The chromatographic behavior of the
peptides involved in the present studies is summarized in Table I. The
color values for these peptides were determined in the same manner as
reported previously (1) and are also listed in Table I.

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for the degree of Doctor of Philosophy.

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New York.

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The conditions of the enzymic incubations were similar to those described earlier (1). Ammonia liberation was followed by the Conway microdiffusion method (1) and was used to measure the rate of the overall reaction, i.e. both hydrolysis and transamidation.

The samples were prepared for chromatography in the manner described previously (1), except that the hydrogenolysis of the carbobenzyoxy compounds was performed in a semimicrohydrogenation assembly and the

Table I

Chromatographic Behavior of Peptides on Dowex 50-X2

0.5 ml. samples containing 1.0 to 2.0 μmoles of each compound were used. Column dimensions, 0.9 X 30 cm.; flow rate, 1.5 to 2.0 ml. per hour; temperature, 31°. A stated volume of citrate buffer, pH 4.0, was passed through the column, and the eluent was then changed to citrate buffer, pH 5.0. The composition of the buffers was reported previously (1). The color values of the peptides were determined at pH 5.0, after heating with ninhydrin at 100° for 30 minutes (1).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Volume of buffer at pH 4</th>
<th>Effluent peak</th>
<th>Color value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycylglycine</td>
<td>31</td>
<td>36</td>
<td>0.86</td>
</tr>
<tr>
<td>Glycyl-L-leucine</td>
<td>31</td>
<td>47</td>
<td>0.94</td>
</tr>
<tr>
<td>L-Leucyl-L-leucine</td>
<td>36</td>
<td>64</td>
<td>1.13</td>
</tr>
<tr>
<td>L-Leucyl-L-phenylalanine</td>
<td>35</td>
<td>87</td>
<td>0.78</td>
</tr>
<tr>
<td>L-Leucyl-L-tyrosine</td>
<td>32</td>
<td>79</td>
<td>1.19</td>
</tr>
<tr>
<td>L-Prolylglycine</td>
<td>36</td>
<td>45</td>
<td>0.12†</td>
</tr>
<tr>
<td>Glycylglycylglycine</td>
<td>33</td>
<td>49</td>
<td>0.81</td>
</tr>
<tr>
<td>Glycylglycyl-L-leucine</td>
<td>31</td>
<td>50</td>
<td>0.83</td>
</tr>
<tr>
<td>Glycyl-L-leucylglycine</td>
<td>34</td>
<td>82</td>
<td>0.89</td>
</tr>
<tr>
<td>Glycyl-L-leucyl-L-leucine</td>
<td>36</td>
<td>70</td>
<td>0.93</td>
</tr>
<tr>
<td>Glycyl-L-leucyl-L-phenylalanine</td>
<td>35</td>
<td>96</td>
<td>0.88</td>
</tr>
<tr>
<td>Glycyl-L-leucyl-L-tyrosine</td>
<td>32</td>
<td>87</td>
<td>1.05</td>
</tr>
<tr>
<td>Glycyl-L-prolylglycine</td>
<td>36</td>
<td>53</td>
<td>0.78</td>
</tr>
</tbody>
</table>

* On a molar basis relative to leucine.
† Determined at 440 μμ.

reaction was followed by noting the hydrogen uptake; the hydrogenation vessel was equipped with a side arm which contained soda lime to absorb the carbon dioxide released. The reaction was allowed to proceed until the uptake of hydrogen had ceased. It has been assumed that any losses incurred in the course of preparing the sample for chromatography were the same for all the compounds in the incubation mixture. In the calculation of the per cent of the reacted substrate that had undergone transamidation, therefore, the amount of transamidation product found was divided by the sum of the amounts of hydrolysis product and transamidation product, and multiplied by 100.
The determination of the values of $pK_z'$ for several dipeptides was carried out under the same conditions as the enzymic reactions. A Radiometer pH meter was employed. The saturated potassium chloride of the calomel electrode was replaced by 30 per cent methanol saturated with potassium chloride. Both the calomel and glass electrodes were allowed to stand in 30 per cent methanol for 1 week prior to the titrations. The hydrochloric acid and sodium hydroxide used as titrants were prepared in 30 per cent methanol. The titrations were performed at $37^\circ \pm 0.1^\circ$ in 0.1 M NaCl ($\Gamma/2 = 0.1$), and moist nitrogen was bubbled through the solution. Acetate buffer (sodium acetate 0.1 M, acetic acid 0.1 M) in 30 per cent methanol was selected as the standard; its $pK$ is 5.17 in 30 per cent methanol at 25$^\circ$ (5), and it was assumed to have the same $pK$ at 37$^\circ$ since the $pK$ of the aqueous acetate buffer of the same composition varies only by 0.005 from 25–38$^\circ$ (6).

**Results**

The data obtained in the transamidation experiments with carbobenzoxyglycinamide as the substrate bearing the sensitive CO—NH group are summarized in Tables II and III. It will be noted that the glycyl peptides tested as replacement agents were approximately equally effective in this regard. It is of interest that the extent of replacement with glycyl-$L$-leucine and glycyl-$D$-leucine is nearly the same, indicating that the configuration of the C-terminal amino acid of the dipeptide has little influence

### Table II

*Chromatographic Analysis of Papain-Catalyzed Transamidation Reactions*

<table>
<thead>
<tr>
<th>Replacement agent (0.05 M)</th>
<th>pH of incubation mixture</th>
<th>Ammonia liberation* (µmoles per ml)</th>
<th>Transamination product (µmoles per ml)</th>
<th>Hydrolysis product (µmoles per ml)</th>
<th>$(a) \times 100 \div (a + b)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycyl-$L$-leucine</td>
<td>7.4</td>
<td>19.4</td>
<td>3.8</td>
<td>9.0</td>
<td>30</td>
</tr>
<tr>
<td>Glycyl-$D$-leucine</td>
<td>7.5</td>
<td>18.5</td>
<td>2.7</td>
<td>10.1</td>
<td>21</td>
</tr>
<tr>
<td>L-Leucyl-$L$-phenylalanine</td>
<td>7.5</td>
<td>16.4</td>
<td>8.9</td>
<td>4.7</td>
<td>65</td>
</tr>
<tr>
<td>L-Leucyl-$L$-tyrosine</td>
<td>7.5</td>
<td>14.9</td>
<td>7.0</td>
<td>3.2</td>
<td>69</td>
</tr>
<tr>
<td>L-Leucyl-$D$-leucine</td>
<td>7.2</td>
<td>17.4</td>
<td>3.9</td>
<td>11.0</td>
<td>26</td>
</tr>
<tr>
<td>L-Prolylglycine</td>
<td>7.5</td>
<td>16.7</td>
<td>0.3</td>
<td>12.6</td>
<td>2.5</td>
</tr>
</tbody>
</table>

* Theory for 100 per cent deamination, 50 µmoles per ml.
† Per cent transamidation.
in the transamidation reaction. This result is in decided contrast to the earlier observation (1) that the papain-catalyzed reaction exhibits specificity in respect to the configuration of the N-terminal amino acid of a dipeptide used as the replacement agent; thus, L-leucylglycine was found to be much more efficient than D-leucylglycine.

Most of the L-leucyl peptides tested appear to be more effective as replacement agents than the glycyl peptides examined. The extent of replacement observed with L-leucyl-L-phenylalanine and L-leucyl-L-tyrosine is especially noteworthy; as will be seen from Table II, with equimolar (0.05 M) concentrations of one of these dipeptides and of the substrate, the extent of replacement was about twice that of hydrolysis, despite the much greater concentration of water. L-Leucylglycine gave about 50 per

Table III

<table>
<thead>
<tr>
<th>Replacement agent</th>
<th>Glycine</th>
<th>Glycylglycine</th>
<th>Glycyl-L-leucylglycine</th>
<th>Transamidation per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>24.5</td>
<td>6.6</td>
<td>8.7</td>
<td>GG 21</td>
</tr>
<tr>
<td>LG</td>
<td>9.6</td>
<td>1.6</td>
<td>8.8</td>
<td>LG 48</td>
</tr>
<tr>
<td>GG + LG</td>
<td>11.8</td>
<td>1.6</td>
<td>8.8</td>
<td>GG 12</td>
</tr>
</tbody>
</table>

cent transamidation (Table III), a value slightly lower than that found previously (1). However, L-leucyl-L-leucine appears to be no more effective than is glycyl-L-leucine.

The greater effectiveness of some L-leucyl peptides as replacement agents is illustrated by an experiment in which carboxbenzoxycglycinamide (0.05 M) was incubated with both L-leucylglycine (0.05 M) and glycylglycine (0.05 M) in the presence of papain. The data in Table III show that the reaction with glycylglycine had been markedly inhibited, when compared to the transamidation reaction in the presence of this peptide as the sole replacement agent. On the other hand, the extent of reaction with L-leucylglycine was essentially the same in both the presence and absence of glycylglycine. It is of interest that the extent of hydrolysis in the presence of L-leucylglycine is much lower than in the presence of glycylglycine.

A comparison of the relative efficiency of various glycyl and L-leucyl peptides with their respective pK\textsubscript{a}' values (Table IV) shows that, although
the dissociation of the ammonium group of these compounds is an important factor, it is by no means the only determinant of the extent of transamidation. It appears that, at least in the papain-catalyzed reactions of carbobenzyloxyglycinamide, the enzyme system exhibits relative specificity in regard to the structure of the replacement agent. The difference between L-leucylglycine and D-leucylglycine, mentioned above, is further evidence on this point. It remains to be determined whether similar specificity relations apply to the action of papain on other substrates and in transamidation reactions catalyzed by other enzymes.

**Table IV**

*Relation of pKₐ of Dipeptides and Their Effectiveness As Replacement Agents*

<table>
<thead>
<tr>
<th>Dipeptide</th>
<th>pKₐ*</th>
<th>pKₐ**</th>
<th>Transamidation†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycylglycine</td>
<td>8.17 (7)</td>
<td>8.17 (7)</td>
<td>21</td>
</tr>
<tr>
<td>Glycyl-L-leucine</td>
<td>8.29 (7)</td>
<td>8.29 (7)</td>
<td>30</td>
</tr>
<tr>
<td>Glycyl-D-leucine</td>
<td>8.29 (7)</td>
<td>8.29 (7)</td>
<td>21</td>
</tr>
<tr>
<td>L-Leucylglycine</td>
<td>7.82 (8)</td>
<td>7.82 (8)</td>
<td>48</td>
</tr>
<tr>
<td>L-Leucyl-L-leucine</td>
<td>7.62</td>
<td>7.62 (8)</td>
<td>26</td>
</tr>
<tr>
<td>L-Leucyl-L-tyrosine</td>
<td>7.47</td>
<td>7.47 (8)</td>
<td>69</td>
</tr>
<tr>
<td>L-Prolylglycine</td>
<td>8.62</td>
<td>8.62 (8)</td>
<td>2</td>
</tr>
</tbody>
</table>

* The figures in parentheses denote bibliographic references.
† Papain-catalyzed reaction with carbobenzyloxyglycinamide under the conditions given in Tables II and III.

It will be seen from Table II that, under the conditions of these experiments, L-prolylglycine did not participate in a transamidation reaction to a significant extent. A similar failure of L-prolyl compounds to serve as replacement agents was noted previously with cathepsin C (9); at pH 7.5, L-prolyl-L-phenylalaninamide underwent only hydrolysis and no polymerization, in contrast to the behavior of glycyl-L-phenylalaninamide. The pKₐ value of L-prolylglycine, and presumably of other L-prolyl compounds (10), is higher than those of the glycyl and L-leucyl peptides examined, and it appears likely that the failure of L-prolylglycine to act as a replacement agent in the papain-catalyzed reaction at pH 7.5 is primarily a consequence of the low concentration of the unprotonated imino group.

In several of the experiments discussed above, the effluent curves obtained on chromatography showed the appearance of ninhydrin-positive components other than those derived from the starting materials or those expected from the hydrolysis of the substrate and from the transamidation
reaction leading to the formation of the carboxymethyltripeptide. For example, in the papain-catalyzed reaction between carboxymethylglycinamide and the mixture of glycylglycine and L-leucylglycine, incubated for 10 hours, a peak emerged at 11 ml in the effluent (buffer at pH 4) upon chromatography of the hydrogenated incubation mixture. Examination of this fast moving component by paper chromatography on Whatman No. 1 paper, and with butanol-acetic acid-water (6:1:1) as the solvent, gave an $R_F$ of 0.06. Upon hydrolysis with 6 N HCl at 120° for 20 hours, this material gave two spots in paper chromatograms, with $R_F$ values of 0.56 and 0.12, identical to those given by authentic samples of leucine and glycine. The chromatographic behavior of the fast moving component present in the 10 hour incubation mixture suggests that it is a polypeptide (or a mixture of peptides) composed of glycine and leucine. It is apparently formed during the initial stage of the reaction, and decomposes as the reaction proceeds, since it was not noted in the 25 hour incubation mixture. The nature of this material and the mode of its formation require further investigation.

**DISCUSSION**

The data reported here further document the conclusion that the efficiency of a proteinase-catalyzed transamidation reaction depends not only on the structure of the sensitive substrate, but also on the structure of the replacement agent. It may be suggested, therefore, that in transamidation reactions two enzymic binding sites are operative, one for the substrate and another for the replacement agent. The possibility exists that in the reaction catalyzed by papain, as well as by other proteinases (11), the substrate (amide or ester) bearing the sensitive carbonyl group interacts with the enzyme to form an "acyl-enzyme" which can react with a replacement agent or water specifically bound at an adjacent enzyme site (12).

The authors wish to acknowledge with thanks the assistance of Dr. Frederic M. Richards in this investigation.

**SUMMARY**

The catalysis by papain of transamidation reactions between carboxymethylglycinamide and several dipeptides has been studied further, and additional evidence has been presented for the view that the enzyme exhibits specificity not only toward the substrate bearing the sensitive CO—NH bond, but also toward the dipeptide used as the replacement agent.

**BIBLIOGRAPHY**

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