Studies of Hog Kidney Acylase I

II. SOME ASPECTS OF SUBSTRATE SPECIFICITY*

L. A. MOUNTER,† LIEN TIEN H. DIEN, AND FRED E. BELL‡

From the Department of Biochemistry, School of Medicine, University of Virginia, Charlottesville, Virginia and the Department of Biophysics, Medical College of Virginia, Richmond, Virginia

(Received for publication, April 10, 1958)

In a preceding paper (1), evidence has been presented that two enzymes of hog kidney, dialkylfluorophosphatase (2) and amino acid Acylase I (3) are separate and distinct enzymes (4). The DFPases are widely distributed (5, 6); naturally occurring substrates are unknown although these enzymes bear some resemblances to peptidases and esterases. It was also noted that there was some correlation between the distribution of DFPase and amino acid acylase in microorganisms, although the data on which comparison can be based are limited (7, 8).

The existence in a single tissue of several enzymes of overlapping specificity has been found to occur in many instances; some particularly well known examples are those of blood esterases (9, 10), cathepsins (11), and also other peptidases. Since specificity data for hog kidney DFPase and Acylase I had been based on the use of extracts containing a mixture of these enzymes, it was possible that previous conclusions might have to be modified when studies were made with more highly purified fractions. The present report describes investigations which were undertaken to determine (a) whether fractions obtained in the separation of DFPase and Acylase I would hydrolyze dialkyl phosphate esters and acetylated amino acids at the same rates as the parent preparations and (b) whether hog kidney DFPase might hydrolyze higher homologous acyl amino acids. The results obtained indicate that existing concepts of the specificity of DFPase and Acylase I are not altered using these new fractions. Additional information pertaining to the range of substrates hydrolyzed by Acylase I has been obtained.

MATERIALS AND METHODS

The enzymes used were obtained from hog kidney by the methods described in a preceding paper in which the methods of assay are also reported (1). The substrates used were DFP and other representative phosphorus triesters employed in earlier specificity studies (12). Most N-acetylated amino acids were obtained commercially, but acylated homologues were synthesized in the laboratory by the general procedure of Abbott (13) for the preparation of N-propionyl glycine. The method was modified with respect to reaction time and method of isolation of the product.

Typically, 0.085 mole of the amino acid and 0.098 mole of the appropriate acid anhydride were mixed in a glass-stoppered flask and heated on a water bath until solution was either complete or 90 per cent complete with the formation of a yellow to orange oil. The reaction mixture was then cooled overnight, taken up in approximately 100 ml. of ether, and filtered to remove any unreacted amino acid. The etherial filtrate was washed three times with 50 ml. portions of water, dried over anhydrous sodium sulfate, and the ether evaporated in a vacuum to a thick oil. The oil was then crystallized and recrystallized from an appropriate solvent or solvent pair.

N-Propionyl Glycine—The reaction mixture solidified on cooling after a reaction period of 0.5 hour. The final oily product was crystallized from ether and recrystallized from absolute ethanol: m.p., 125-127° (Abbott (13) 125-128°).

N-Butyryl Glycine—The reaction mixture solidified on cooling after a reaction period of 0.5 hour. The product was crystallized from benzene and recrystallized from ether-petroleum ether: m.p., 68.5-70° (Schachter and Taggart (14), m.p. 69°).

N-Propionyl Leucine—The product was crystallized and recrystallized from ethanol-water: m.p., 125-128°.

N-Butyryl Leucine—The impure solid was crystallized from ethanol-water and recrystallized from 95 per cent ethanol: m.p., 188-190° (evacuated tube).

N-Proplynyl Valine—Crystallized from ethanol-water and recrystallized from ether-petroleum ether: m.p., 199.5-201.5° (evacuated tube).

N-Butyryl Valine—Crystallized and recrystallized from chloroform: m.p., 164-166° (evacuated tube).

C₆H₁₂NO₃

Calculated: C 59.67, H 9.52, N 6.96
Found: C 59.53, H 9.38, N 6.87

N-Propionyl Valine—Crystallized from ethanol-water and recrystallized from ether-petroleum ether: 199.5-201.5° (evacuated tube).

C₆H₁₂NO₃

Calculated: C 55.47, H 8.73, N 8.09
Found: C 55.22, H 8.85, N 7.90

N-Butyryl-valine—Crystallized and recrystallized from chloroform: m.p., 164-166° (evacuated tube).

C₆H₁₂NO₃

Calculated: C 57.73, H 9.15, N 7.48
Found: C 57.48, H 8.87, N 7.29
Five additional N-acetylated amino acids were obtained from Drs. John Andrako and J. Doyle Smith of the Department of Pharmaceutical Chemistry, Medical College of Virginia, as follows: N-acetyl-dl-α-amino-3-methyl caproic acid; N-acetyl-dl-α-amino heptylic acid; N-acetyl-dl-α-amino caprylic acid; N-acetyl-dl-α amino pelargonic acid; and N acetyl dl-α-amino capric acid.

A full report of the synthesis of these and other related compounds will be published separately. The purity of the compounds was determined by (a) nitrogen analysis, and (b) failure to yield any positive ninhydrin reaction, indicating complete acetylation of the free amino group.

RESULTS

Kinetics of Hydrolysis of N-Acetyl Amino Acids—A preliminary investigation was made of the kinetics of hydrolysis of several N-acetyl amino acids to ensure the selection of optimal conditions for specificity studies. The effects of pH and substrate concentrations are shown in Figs. 1 and 2. At pH 7.4 and with 0.01 M substrate, the rate of hydrolysis was linear with respect to time and enzyme concentration.

Although Co++ is not essential for the enzymatic activity of DFPase and Acylase I, the presence of this metal ion has previously been shown to increase the rate of hydrolysis of certain substrates by those enzymes (2, 15). The effect of Co++ has been reported to vary considerably with different amino acid substrates; low rates of hydrolysis, such as that found with acetyl glycine, are increased by this metal ion whereas high rates of hydrolysis, such as that of acetyl methionine, are inhibited.

Fig. 2. Effect of substrate concentration on rate of hydrolysis of N-acetyl amino acids by Acylase I. The reaction was carried out at 37° in 0.05 M phosphate buffer, pH 7.2. Curves: 1, acetyl methionine; 2, acetyl alanine; 3, acetyl valine; 4, acetyl leucine; 5, acetyl glycine. The rate of hydrolysis of glycine, valine, and leucine was multiplied X5 for clarity of presentation.

Variations in the effect of Co++ on the hydrolysis of different acetylated amino acids were confirmed. The addition of 10^-4 M Co++ to the incubated mixture increased the rate of hydrolysis of N-acetyl glycine 120 per cent and N-acetyl alanine 80 per cent, but inhibited N-acetyl methionine approximately 50 per cent. Valine and leucine derivatives were little affected.

Comparison of Rates of Hydrolysis of Organophosphorus and N-Acetyl Amino Acids by Hog Kidney Fractions—The rates of hydrolysis of several organophosphorus compounds and N-acetyl amino acids were determined with hog kidney fractions including those in which a separation of DFPase and Acylase I activities had been obtained (1). The results (Table I) show that the subfractionation does not alter the relative rates of hydrolysis of the two types of substrates; the rates of hydrolysis of the organophosphorus compounds are identical with those previously reported (12), whereas the ratio of activities with different N-acetyl amino acids are in excellent agreement with the data of Birnbaum el al (3). The data support the belief in the separate identity of the two enzymes.

In view of the possibility of an overlapping specificity of the two enzymes, several homologous acetylated amino acids were tested as possible substrates for DFPase-rich fractions. Most of these compounds were found to be hydrolyzed by Acylase I, showing consistent relative rates of hydrolysis when compared to those of N-acetylated amino acids for which data were available. There was no evidence for hydrolysis by DFPase.
TABLE I

Relative rates of hydrolysis of organophosphorus compounds and acetylated amino acids by hog kidney fractions
Rates of hydrolysis = pmoles of substrate per mg. of N per 30 minutes.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Organophosphorus compounds*</th>
<th>N-acetyl amino acids</th>
<th>Ratios± acylase-DFPase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DFP</td>
<td>TEPP</td>
<td>DMCIVP</td>
</tr>
<tr>
<td>A-1</td>
<td>105 (100)‡</td>
<td>70 (65)</td>
<td>62 (60)</td>
</tr>
<tr>
<td>A-2</td>
<td>275 (100)</td>
<td>160 (70)</td>
<td>180 (58)</td>
</tr>
<tr>
<td>A-11</td>
<td>155 (100)</td>
<td>130 (67)</td>
<td>120 (60)</td>
</tr>
<tr>
<td>A-111</td>
<td>35 (100)</td>
<td>24 (68)</td>
<td>20 (58)</td>
</tr>
<tr>
<td>A-1111</td>
<td>5 (100)</td>
<td>9,500 (100)</td>
<td>2,300 (25)</td>
</tr>
<tr>
<td>A-11111</td>
<td>4 (100)</td>
<td>9,500 (100)</td>
<td>2,300 (25)</td>
</tr>
</tbody>
</table>

* DFP, diisopropyl fluorophosphate; TEPP, tetraisopropyl pyrophosphate; and DMCIVP, dimethyl chlorovinylphosphate.
† Rates calculated on basis of DFP and acetyl methionine hydrolysis.
‡ Figures in parentheses represent relative rates of hydrolysis. DFP and acetyl methionine = 100 per cent.

Fig. 3. Influence of carbon chain length on rates of hydrolysis of N-acetyl amino acids. The abscissa represents the number of carbon atoms in amino acid. The maximal rate of hydrolysis is observed at C6, norleucine. The branched chain acids are: C6, valine; C6, leucine; C7, α-amino heptylic acid.

The studies of Acylase I specificity showed that numerous N-acetyl amino acids are hydrolyzed by Acylase I. Data presented in Fig. 3 show that straight chain derivatives of amino acids have an optimal length of 6 carbons. Branching at the end of the carbon chain produces a noticeable decrease in the rate of hydrolysis. The results are similar to those observed with the esterases (9) and lipases (16).

When higher acyl homologues of the amino acids were tested as substrates, some variations of rates of hydrolysis were observed with different kidney fractions in preliminary experiments. It was found however that this was due to marked autoinhibition as substrate concentration was increased. Representative data are shown in Fig. 4. It was also apparent that the rates of hydrolysis were very dependent on the over-all...
Relative rates of hydrolysis of N-acyl amino acids by hog kidney Acylase I

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Acyl group*</th>
<th>N-propionyl</th>
<th>N-butyryl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>100</td>
<td>100</td>
<td>680</td>
</tr>
<tr>
<td>Alanine</td>
<td>100</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>100</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Leucine</td>
<td>100</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

* Rate of hydrolysis of N-acetyl derivative = 100 per cent.

The relative rates of hydrolysis of N-propionyl and N-butyryl valine and leucine indicate that the hydrolysis of both series of compounds is due to a single enzyme. Studies of the effect of pH on the rates of hydrolysis of N-propionyl and N-butyryl valine and leucine indicate pH optima in the region of pH 7.5, and the same value was obtained with the N-acetyl derivatives. These experiments were carried out with the same methods used to obtain the data presented in Table II have been calculated on the basis of maximal observed velocities. The relative rates were independent of the enzyme preparation used, indicating that the hydrolysis is due to a single enzyme. Studies of the effect of pH on the rates of hydrolysis of N-propionyl and N-butyryl valine and leucine indicated pH optima in the region of pH 7.5, and the same value was obtained with the N-acetyl derivatives. These experiments were carried out with the same methods used to obtain the data presented in Fig. 1, except for the higher enzyme concentration required to produce adequate liberation of free amino acid for measurement.

The heat stability of Acylase I samples, measured with higher acyl amino acid homologues as substrates, was identical to that obtained for N-acetyl amino acids. This also supports the belief that a single enzyme is responsible for the hydrolysis of both series of compounds.

**DISCUSSION**

The experiments reported show no evidence for an overlapping specificity of hog kidney DFPase and Acylase I. Relative rates of hydrolysis of organophosphorus compounds and N-acyl amino acids with various kidney fractions show that a separation of DFPase and Acylase I activities does not affect the specificity within the classification of the two types of substrate. Like many other hydrolytic enzymes, Acylase I seems to be relatively nonspecific for an homologous series of substrates. Amino acid derivatives with a straight carbon chain are hydrolyzed more rapidly than those in which there is chain branching. The optimal chain length is at 6 carbons, corresponding to norleucine. It may be noted that the most rapidly hydrolyzed acetylated amino acid is the methionine derivative (3); this is almost isosteric with norleucine. Unlike the cholinesterases and lipases (9), there seems to be a change in the optimal size of the acyl group with change of size in the remainder of the molecule. If atomic models of the various Acylase I substrates are constructed, it can be seen that higher homologues show appreciable steric hindrance between the large acyl group and amino acid moiety; the configurations suggest that the carbonyl portion of the acyl group, together with the amino nitrogen and carboxyl group, must be able to attain a planar configuration before significant hydrolysis can be effected by the enzyme.

**SUMMARY**

Further evidence is presented indicating that hog kidney dialkylfluorophosphatase and Acylase I are separate and distinct enzymes; there is no evidence for an overlapping specificity within the range of phosphate esters and N-acyl amino acids tested as substrates for these enzymes.

The rates of hydrolysis of several new acyl amino acids by Acylase I have been determined. The specificity of Acylase I is discussed.

**Acknowledgment**—The authors are grateful to Dr. C. E. Griffin for the preparation of some of the N-acyl amino acids.

**REFERENCES**

Studies of Hog Kidney Acylase I: II. SOME ASPECTS OF SUBSTRATE SPECIFICITY

L. A. Mounter, Lien Tien H. Dien and Fred E. Bell