Hydrolysis of carnosine and related compounds by mammalian carnosinases

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Abstract

Comparative study of hydrolysis of carnosine and a number of its natural derivatives by human serum and rat kidney carnosinase was carried out. The rate of carnosine hydrolysis was 3–4-fold higher than for anserine and ophidine. The rate of homocarnosine, N-acetylcarnosine and carcinine hydrolysis was negligible by either of the enzymes used. Our data show that methylation, decarboxylation or acetylation of carnosine increases resistance of the molecule toward enzymatic hydrolysis. Thus, metabolic modification of carnosine may increase its half-life in the tissues. © 2000 Elsevier Science Inc. All rights reserved.

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1. Introduction

Carnosine (β-alanyl-L-histidine) and related compounds (CRC) are natural constituents of excitable tissues possessing diverse biological activities (Severin, 1964; Boldyrev and Severin, 1990). Synthesis of carnosine, the compound most studied, is provided by a specific ligase (carnosine synthase, EC 6.3.2.11).

The level of carnosine in tissues is controlled by a number of enzymes transforming carnosine into other CRCs, such as carcinine, N-acetylcarnosine, anserine or ophidine (by decarboxylation, acetylation or methylation, respectively) or its cleavage into the amino acids, histidine and β-alanine.

Hydrolysis is mainly due to tissue carnosinase (EC 3.4.13.3) which is widely distributed among different subjects (Lenny, 1976; Jackson et al., 1991) or serum carnosinase (EC 3.4.13.20), obtained in brain and blood plasma of primates and humans (Kunze et al., 1986; Lenny, 1990). Both carnosinases are characterized by higher activity toward carnosine compared with anserine or homocarnosine (Murphey et al., 1972; Lenny et al., 1982), while their affinity towards other natural CRCs has not been investigated.

Because of specific tissue distribution of different CRCs, a question arises whether these compounds may be enzymatically hydrolyzed. In this study, the comparative efficiency of hydrolysis of carnosine and a number of CRCs by carnosinase isolated from rat kidney or human serum is determined.
2. Materials and methods

Carnosine was obtained from St. Petersburg Biomedical Factory (Russia) with a purity of 97% (with histidine as contaminant). Anserine and ophidine were prepared from natural sources (99.5% purity); N-acetylcarnosine (99.2% purity) was synthesized by Victor Shavratsky (Institute of Neurology, Russian Academy of Medical Sciences) and carcinine (99% purity) was a gift of Dr Alexey Khomutov (Institute of Molecular Biology, Russian Academy of Sciences); in all these cases, small contaminations of carnosine were present. Homocarnosine was purchased from Sigma (St. Louis, MO). The purity of the compounds used was tested by HPLC.

Human blood serum was obtained from non-hemolyzed blood of eight donors and stored at −20°C before use. Rat kidney homogenate partially purified by dialysis (Margolis et al., 1979) was used in kidney carnosinase assays. Kidneys of five rats were separately homogenized with two volumes of distilled water, centrifuged for 30 min at 24,000 × g and subjected to dialysis against 40 mM Tris–HCl, pH 7.5 (two times for 2 h). The final preparation was frozen and stored at −20°C.

Blood carnosinase activity was measured as described earlier (Lenny et al., 1985) with Tris–HCl (pH 8.5) substituting ammonia buffer which interferes with NH₂-determining reagents. A 10 μl volume of serum sample was pre-incubated for 30 min with 40 μl of 125 mM Tris–HCl (pH 8.5), 15 μl of 5 mM CdCl₂ (prepared in 30 mM sodium citrate), and 15 μl of H₂O (36°C). The reaction was started by addition of 20 μl of substrate (final concentration is noted in the tables and figure). The incubation time was from 5 to 45 min for substrates undergoing fast enzymatic hydrolysis (carnosine, anserine, ophidine); when poorly hydrolyzed substrates were used, the reaction time was prolonged up to 6 h. In all cases, the reactions were monitored for linearity. The reaction was stopped by 0.3 ml of 96% ethyl alcohol, protein was removed by centrifugation and the supernatant was used to determine the reaction products. For this procedure, aliquots of the supernatant were treated with 4-chloro-7-nitrobenzo-2-oxo-1,3-diazole (NBD-chloride) as described earlier (Tkachuk and Malinovskaya, 1977) and thin-layer chromatography procedure was used to identify the products on the Silufol-UV plates (Kavalier, Czech Republic). The yellow-colored spots were scanned on a ‘Mustek 600 CP’ color scanner, and analyzed using the ‘Chromo’ program, developed by Dr A.B. Merkov (Institute for Systemic Analysis, Russian Academy of Sciences).

Kidney carnosinase activity in dialyzed homogenates was measured as described earlier (Wolos et al., 1982). Samples of 0.1 ml, containing 40 mM Tris–HCl buffer (pH 8.0) with or without 1 mM MnCl₂, and 20 μl of enzyme preparation were pre-incubated for 1 h. The reaction was initiated by substrate addition (1 or 10 mM). The incubation time was of 1 h (for carnosine) or 1.5 h (anserine and ophidine) and for other substrates as long as 3 h. The reaction was stopped by 96% ethyl alcohol and all other procedures were performed as described above. All data were obtained from three to four measurements and presented as mean ± S.D.

3. Results

Comparison of the enzymatic hydrolysis of carnosine and related compounds was made at two concentrations — close to physiological level of CRCs (1 mM) and higher (10 mM). The rate of hydrolysis of carnosine, anserine and ophidine by serum carnosinase is compared in the Fig. 1 showing double-reciprocal plots for carnosine, anserine and ophidine hydrolysis. The initial rate of hydrolysis is highest with carnosine and lowest with ophidine. When carcinine, homocarnosine or N-acetylcarnosine were used, no hydrolytic activity was detectable (Table 1).

From a Lineweaver — Burk plot for carnosine, anserine and ophidine hydrolyses the kinetic parameters were calculated. The \( K_m \) value for all the three substrates is the same (8.0 ± 0.6 mM), while the \( V_{max} \) values are 8.3 ± 0.6, 13.3 ± 1.0 and 33.0 ± 2.5 μmoles per ml serum per h for ophidine, anserine and carnosine, respectively (the data obtained on the plasma samples from eight donors).

Rat kidney carnosinase demonstrated similar activity with the substrates tested. The same order of affinity for carnosine, anserine, ophidine and homocarnosine was found (Table 2). Lenny (1976) reported that the rate of carnosine hydrolysis by rat kidney carnosinase was 17.6 ± 6.3 μmoles per g tissue per h, and the small dis-
crepancy between our data and his may be related to different levels of enzyme purification. For other CRCs, the rate of anserine hydrolysis was 8–30% of that of carnosine whilst that of homocarnosine was less than 1% (Margolis et al., 1979; Wolos et al., 1982). This is consistent with the data presented in Table 2.

It is known that manganese ions activate tissue carnosinase at high and inhibit it at low concentrations of the substrate (Margolis et al., 1979). Thus, we compared hydrolytic activity of rat kidney carnosinase in the presence or absence of 1 mM manganese ions. The order of hydrolytic rates of carnosine, anserine and ophidine (all in 10 mM concentration) was the same as for human serum carnosinase, other substrates being not hydrolyzed at all (Table 2). When concentration of the substrates was decreased 10-fold, manganese ions decreased the activity with no change in the order of the affinity for the substrates used. At the same time, relatively low, but detectable homocarnosine hydrolysis was noted. Hydrolysis of carcinine and N-acetylcarnosine was not detectable.

### Table 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>10 mM %</th>
<th>1 mM %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carnosine</td>
<td>21.4 ± 0.3</td>
<td>100</td>
</tr>
<tr>
<td>Anserine</td>
<td>7.0 ± 0.5</td>
<td>32.7</td>
</tr>
<tr>
<td>Ophidine</td>
<td>4.8 ± 0.3</td>
<td>22.4</td>
</tr>
<tr>
<td>Homocarnosine</td>
<td>0.10</td>
<td>0.20</td>
</tr>
<tr>
<td>Carcinine</td>
<td>0.05</td>
<td>0</td>
</tr>
<tr>
<td>N-acetylcarnosine</td>
<td>0.10</td>
<td>0</td>
</tr>
</tbody>
</table>

*The data presented are mean from 24 observations (three observations for each of eight donors) ± S.D.*

### Table 2

<table>
<thead>
<tr>
<th>Substrate</th>
<th>10 mM + MnCl2 %</th>
<th>1 mM + MnCl2 %</th>
<th>1 mM − MnCl2 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carnosine</td>
<td>31.6 ± 3.2</td>
<td>3.7 ± 0.5</td>
<td>6.3 ± 0.5</td>
</tr>
<tr>
<td>Anserine</td>
<td>8.16 ± 2.5</td>
<td>0.8 ± 0.15</td>
<td>1.3 ± 0.3</td>
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<tr>
<td>Ophidine</td>
<td>5.32 ± 2.0</td>
<td>0.8 ± 0.30</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>Homocarnosine</td>
<td>0.05</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Carcinine</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N-acetylcarnosine</td>
<td>0.01</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*The data presented are mean from 15 ophidine observations (three observations for each of five rats) ± S.D.*

### 4. Discussion

The substrate specificity of serum carnosinase was studied by several authors but hydrolysis of carnosine was compared only with two natural CRCs — anserine and homocarnosine (Lenny, 1976). In the last 30 years, a number of other CRCs were described such as carcinine and acetylated dipeptides (Boldyrev, 1998). The ability of carnosinase to hydrolyze these substrates was not studied, although these compounds are known to possess specific biological action. For example, carcinine has been demonstrated to regulate the heart beat (Brotman et al., 1990) and N-acetylcarnosine to protect heart against ischemic injury.
(Alabovsky et al., 1997). Moreover, there are some examples of the use of different CRCs therapeutically (Boldyrev, 1998; Babizhaev et al., 1998). Thus, efficiency of the tissue carnosinase toward these derivatives of carnosine is of special interest.

With respect to recent data, modification of carnosine by methylation, acetylation or decarboxylation strongly modifies its biological activity (Boldyrev and Abe, 1999). We show here that such modification decreases the rate of hydrolysis of CRCs by both forms of carnosinase. This means that the modified molecule may be characterized by a longer half-life in the blood stream. Because the carnosine derivatives show different levels of biological activity (Boldyrev and Abe, 1999), one may conclude that carnosine turnover in different tissues may reflect accommodation of their metabolism to different environmental conditions. Finally, the natural derivatives of carnosine with increased resistance to carnosinase may have potential as carnosine substitutes in clinical trials.

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References