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Butylmalonate is a transition state analogue for aminocylase I

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Butylmalonate (butyl propanedioic acid) is a slow-binding inhibitor of porcine renal aminoacylase I (EC 3.5.1.14), causing transients of activity with half-times of more than 10 min. At 25°C and pH 7.0, the dissociation rate of the complex is approximately 6×10^{-4} s^{-1}, while the rate constant of complex formation is in the order of 20 $M^{-1} \cdot s^{-1}$. In good agreement with these data, steady-state kinetics yield an estimated inhibition constant around 100 μM. Molecular mechanics calculations showed that conformation and charge distribution of butylmalonate are strikingly similar to those of the putative transition state of aminoacylase catalysis.

Aminoacylase; Reaction mechanism; Inhibitor; Transition state; (Hog kidney)

1. INTRODUCTION

Aminoacylase I from porcine kidney (EC 3.5.1.14) is inhibited by a variety of N-substituted amino acids, including *p*-toluenesulfonyl-, *t*-butyloxycarbonyl-, or trichloroacetyl derivatives [1,2]. The inhibition constants for all of these compounds are in the millimolar range; in general, they are similar to the K_m values of structurally related substrates. Free amino acids are poor inhibitors, except at high pH, when their amino groups are uncharged [3]. Highest aminoacylase activities or, in the case of substrate analogues, highest degrees of inhibition are observed with substituted straight-chain aliphatic amino acids [4]. So *N*-acetyl norleucine (*N*-acetyl α-aminohexanoic acid) is among the best aminoacylase substrates.

When screening a series of potential aminoacylase inhibitors, we noticed that butylmalonic acid (butylpropanedioic acid) inhibits the enzyme in a time-dependent fashion. The results of a more detailed study on the mechanism of butylmalonate inhibition are described in the present paper.

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2. EXPERIMENTAL

2.1. *Materials*

Aminoacylase I was purified from hog kidney as described elsewhere [5]. Butylmalonic acid and benzylmalonic acid are commercially available.

2.2. *Methods*

Aminoacylase activities were measured by flow injection analysis according to a published procedure [5]. This method is more reliable than the conventional spectrophotometric assay, especially when the reaction has to be followed for some time. All assays were performed at 25°C in 50 mM Mops/NaOH, pH 7.0, with acetyl-L-Met as the substrate. Kinetic parameters were estimated by non-linear regression analysis with our KINFIT program [6].

Molecular mechanics calculations were performed using the MMX force field (Serena Software, Bloomington, IN, USA), which is an enhanced version of Allinger's MMP2 [7]. In contrast to former programs, MMX also handles transition states.

3. RESULTS

Under the conditions of our experiments, butylmalonate inhibition of aminoacylase-catalyzed acetyl-L-Met hydrolysis was a surprisingly slow process. When the reaction was started by adding enzyme to substrate and inhibitor (fig.1, curve 2) the rate of product release gradually decreased for more than half an hour before reaching a new steady-state level. On the other hand, when the enzyme was preincubated with in-

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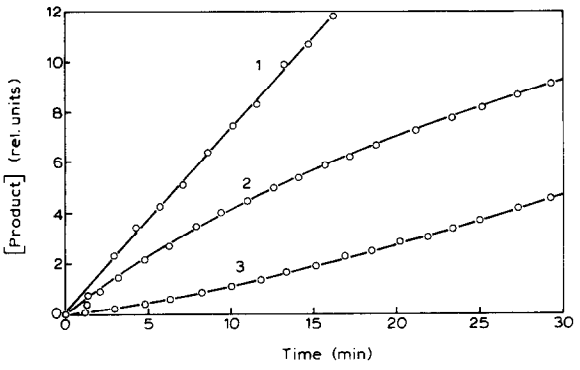

Fig.1. Time course of inhibition by butylmalonate at 25°C. The samples contained 50 mM Mops/NaOH, pH 7.0, 8.3 mM acetyl-L-Met and 0.4 mM butylmalonate (curves 2 and 3 only). The reaction was started with enzyme (curve 2) or with substrate after a 30 min preincubation of enzyme with butylmalonate (curve 3). Curve 1 shows the uninhibited reaction. Product concentrations were measured by flow injection analysis with fluorimetric detection [5]. Solid lines (curves 2 and 3) are fits of eqn I (see text) with the following parameters: curve 2, $-v_i$ = 0.51 ± 0.02 units·min^{-1}, v_f = 0.18 ± 0.04 units·min^{-1}, k = 0.071 ± 0.018 min^{-1}; curve 3, $-v_i$ = 0.072 ± 0.008, v_f = 0.218 ± 0.027, k = 0.056 ± 0.02; curve 1 is a linear fit with v_o = 0.71 ± 0.01.

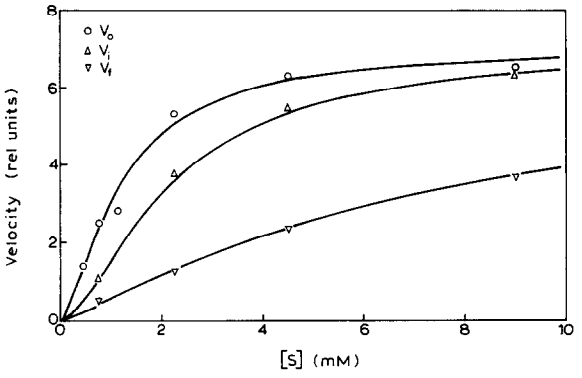

Fig.2. Dependence of butylmalonate inhibition on substrate concentration. Experimental conditions were as in fig.1 except that 0.09 mM butylmalonate was employed. Velocities of the uninhibited reaction (v_o, ○) as well as initial (v_i, Δ) and steady-state rates (v_f, ▽) in the presence of butylmalonate are plotted vs the concentration of acetyl-L-Met. Solid lines are fits of the Hill equation with the following parameters: $v_o - V$ = 7.0 ± 0.6 units, $[S]_{0.5}$ = 1.20 ± 0.21 mM, Hill coefficient n = 1.55 ± 0.25; $v_i - V$ = 7.0 ± 0.5, $[S]_{0.5}$ = 2.1 ± 0.3, n = 1.6 ± 0.3; $v_f - V$ = 6.6 ± 0.8, $[S]_{0.5}$ = 7.3 ± 1.5, n = 1.2 ± 0.1.

hibitor for 20–30 min before adding substrate (curve 3), activity increased for about 15 min to attain the same final value. In the absence of inhibitor the time course of product release was strictly linear (curve 1).

The solid lines in fig.1 are fits of eqn I (cf. [8])

$$[P]_t = v_f \cdot t + (v_i - v_f) \cdot \{1 - e^{-kt}\}/k \qquad (I)$$

where $[P]_t$ is the product concentration at time t, v_i is the initial rate after starting the reaction, v_f is the final velocity, and k is the first-order rate constant of the transient. For curves 2 and 3 first-order rate constants around 1×10^{-3} s^{-1} were obtained which corresponds to a half-time of about 12 min.

The dependence of v_i and v_f on substrate concentration is shown in fig.2. Note that the saturation curve for the uninhibited reaction is distinctly sigmoidal (see also fig.3, upper curve). Statistical tests [6] applied to the respective parameter estimates confirmed that the deviation from hyperbolic behaviour is highly significant at the 95% level. So far, sigmoidal kinetics were only found with substrates bearing extended unbranched alkyl side chains. With acetyl-L-Ala or chloroacetyl-L-Ala, both routinely used in our laboratory, non-hyperbolic kinetics are not observed.

The v_f values in fig.2 do not allow a clear-cut distinction between hyperbolic and sigmoidal kinetics. However, from the results of curve fitting, it is obvious that the substrate concentration at half-maximal velocity $[S]_{0.5}$ for v_f was much

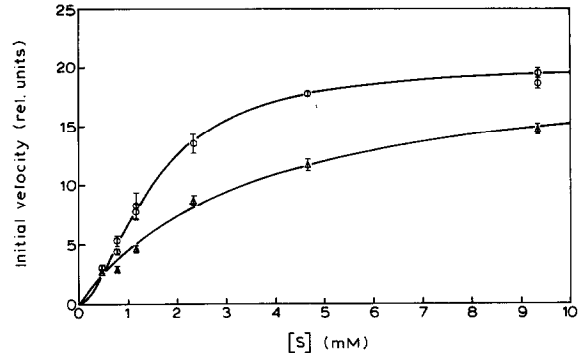

Fig.3. Inhibition of aminoacylase by benzylmalonate. Assay conditions were as in fig.1. Velocities in the absence of inhibitor (○) and in the presence of 9.3 mM benzylmalonate (Δ) are plotted vs substrate concentration. Bars indicate standard deviations. Solid lines are fits of the Hill equation with V = 20.3 ± 0.8, $[S]_{0.5}$ = 1.53 ± 0.13 mM, n = 1.74 ± 0.15 (○) or of the Michaelis-Menten equation with V = 20.8 ± 2.2, $[S]_{0.5}$ = 3.7 ± 0.7 mM (Δ).

higher than the $[S]_{0.5}$ values for v_o or v_i, while the maximal velocities were the same within experimental error. Applying the competitive model of inhibition to fig.2 and using $[S]_{0.5}$ for comparison, we estimate an inhibition constant K_i of about 100 μM. This value is markedly lower than any published value for aminoacylase inhibitors.

In contrast to butylmalonate, benzylmalonate did not exhibit slow-binding effects. Inhibition by this analogue was competitive (that is, V was unchanged) with an inhibition constant of 6.2 mM (fig.3). The Michaelis constant for acetyl-L-Phe is 6.0 mM under the same conditions [5].

4. DISCUSSION

Morrison [8] has defined 'slow-binding' inhibitors as compounds that exert their effects on the target enzyme in a reversible manner but at rates low enough to be measured by ordinary kinetic techniques. Many of these inhibitors are also 'tight-binding', i.e., effective at exceptionally low concentrations.

Most cases of slow-binding inhibition are due to a very slow rate of dissociation of the enzyme-inhibitor complex. A recent review [9] cites cases where reversible competitive inhibitors are released from the enzyme with half-times of years. The simplest model to describe slow-binding interactions is the classical scheme for competitive inhibition

$$\begin{array}{l} \quad\quad +S \rightleftharpoons ES \longrightarrow \ldots\ldots \text{Products} \\ E \\ \quad\quad +I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} EI \end{array} \qquad \text{(II)}$$

When both the time course of inhibition after starting with enzyme and the time course of partial reactivation after preincubation with inhibitor can be followed, the rate constants of association and dissociation, k_{on} and k_{off} may be estimated [8,10,11]. Applying the method of Baici and Gyger-Marazzi [10] to the data of fig.1, we find $k_{off} = 6 \pm 2 \times 10^{-4}\ s^{-1}$ and an association constant k_{on} between 10 and 40 $M^{-1} \cdot s^{-1}$. These results are in reasonable agreement with the estimated steady-state inhibition constant of about 100 μM (fig.2), since model II predicts that $K_i = k_{off}/k_{on}$. On the other hand, the competitive scheme is not strictly applicable to cooperative systems, which renders the above comparison somewhat questionable. In any case, the data show that butylmalonate is not an exceptionally tight-binding inhibitor because its rate of association is slow, too.

In recent years evidence has accumulated indicating that a large fraction of slow-binding inhibitors mimic intermediates in enzyme catalysis [9]. For instance, there is good evidence that peptidase inhibitors like bestatin [11] or leupeptin [10] are analogues of the tetrahedral transition state formed by nucleophilic attack of a water molecule or a hydroxyl ion at the carbonyl group of amide substrates. In order to examine whether butylmalonate may act by a similar mechanism, we employed molecular mechanics calculations to study the presumed transition state for SN2-hydrolysis of *N*-acetyl norleucine. A conformation obtained by global energy minimization of such a structure is shown in fig.4. It is a 'late' transition state with a fractional bond order of 0.8. Clearly, the oxygen atoms at the transition state carbon (marked by #) and the α-carboxylate oxygens form a structure resembling dianionic

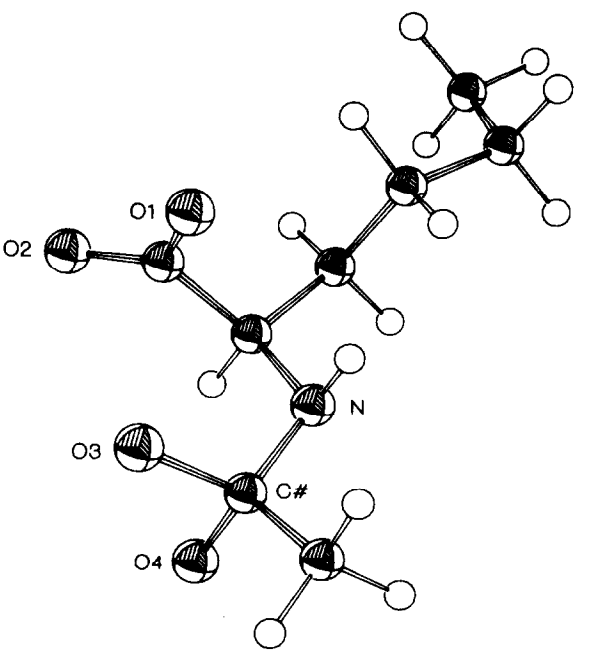

Fig.4. Conformation of the transition state of acetyl norleucine hydrolysis (see text).

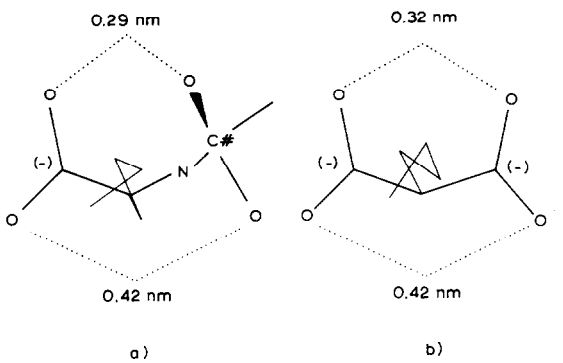

Fig.5. A comparison of the transition state? of acetyl norleucine hydrolysis (a) with butylmalonate (b), see text.

malonate. This similarity becomes quite obvious by inspection of fig.5, where the same transition state (a) and butylmalonate (b) are viewed along the direction of the respective butyl side chains. It can be seen that the inter-oxygen distances in both conformations are almost identical. The same holds for the distribution of negative charges, since one of the transition state oxygens bears a fractional charge close to unity.

Our observation that benzylmalonate acts as a normal competitive inhibitor indicates that the transition states for the aminoacylase-catalyzed hydrolysis of aromatic substrates may be different. As compared to aliphatic compounds, the turnover numbers of *N*-acyl derivatives of aromatic amino acids are lower by 1–2 orders of magnitude, although the stabilities of the scissile amide bonds are comparable [4,5]. We assume that the bulky side chains of such substrates impede catalysis.

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