1968

Fluorofumarate - a substrate for fumarate hydratase / Donald D. Clarke William J. Nicklas John Palumbo Department of Chemistry Fordham University Bronx, New York 10458

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Recommended Citation
Clarke, Donald Dudley PhD; Nicklas, William J.; and Palumbo, John, "Fluorofumarate - a substrate for fumarate hydratase / Donald D. Clarke William J. Nicklas John Palumbo Department of Chemistry Fordham University Bronx, New York 10458" (1968). Chemistry Faculty Publications. 35.
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Fluorofumarate—A Substrate for Fumarate Hydratase

Fumarate hydratase (EC 4.2.1.2) has been studied extensively and found to act only on the pair of substrates, L-malate in one direction and fumarate in the other (1, 2).

TABLE I
Loss of Fumarate Hydratase Activity on Heating Assayed with Fumarate and Fluorofumarate

<table>
<thead>
<tr>
<th>Data</th>
<th>Substrate</th>
<th>Fumarate</th>
<th>Fluorofumarate</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$, mm$^2$</td>
<td></td>
<td>5.0</td>
<td>44</td>
</tr>
<tr>
<td>$V_m$, umoles/ml/min/mg</td>
<td></td>
<td>443.</td>
<td>667</td>
</tr>
<tr>
<td>% Activity(^b) = 5 min</td>
<td></td>
<td>66.5</td>
<td>65.1</td>
</tr>
<tr>
<td>10 min</td>
<td></td>
<td>41.7</td>
<td>40.0</td>
</tr>
<tr>
<td>20 min</td>
<td></td>
<td>21.2</td>
<td>21.6</td>
</tr>
</tbody>
</table>

\(^a\) Measured in 0.033 M sodium phosphate buffer, pH 7.3, and 25°.

\(^b\) % activity remaining after enzyme was heated at 50° for various time periods.

The fluoro group is similar in size to the hydrogen atom, and this has led to the successful development of metabolic antagonists in which fluorine is substituted for hydrogen (3a, b, 4). The study of fluoro-analogs of citric acid cycle intermediates has shown that most of these are far less toxic in animals than is fluoroacetate. This led us to examine the action of fumarate hydratase from pig heart on fluorofumarate, and we report here that this compound is a substrate for the enzyme. Fluorofumaric acid was prepared from 2,2-di-fluorosuccinic acid by the method of Raasch et al. (5).

When fluorofumarate was incubated with a crystalline preparation of fumarate hydratase from pig heart (Boehringer Mannheim Corp.) under the assay conditions described by Racker for fumarate (6), and the reaction followed in a spectrophotometer at 300 mp, a rapid increase in optical density was observed instead of a decrease as with fumarate. This allowed the development of an assay for the enzyme in the presence of fluorofumarate as substrate. Addition of 2,4-dinitrophenylhydrazine solution to the incubation mixture gave a precipitate of a derivative, a reaction not observed with the control samples which contained no enzyme. Likewise, when enzyme denatured by prolonged heating was incubated with fluorofumarate, no reaction occurred. When the precipitated derivative was chromatographed on a thin layer of silica gel (Eastman Chromagram sheet) in a solvent system previously developed (7), the major product had the same $R_F$ as oxaloacetate-2,4-dinitrophenylhydrazone together with a minor component with the same $R_F$ as pyruvate-2,4-dinitrophenylhydrazone. The pyruvate apparently arose from the spontaneous decarboxylation of oxaloacetate. Hence, the reaction catalyzed by fumarate hydratase under these circumstances seemed to be the following (Eq. 1):

\[
\begin{array}{c}
\text{F} \quad \text{C} \quad \text{O} \quad \text{O} \\
\text{O} \quad \text{O} \quad \text{C} \quad \text{H} \\
\text{H} \quad \text{C} \quad \text{H} \quad \text{O} \\
\end{array}
\quad + \quad \text{HOH} \quad \rightarrow \quad
\begin{array}{c}
\text{C} \quad \text{O} \quad \text{O} \\
\text{C} \quad \text{H}_2 \quad \text{O} \\
\end{array}
\quad + \quad \text{H}^+ \quad + \quad \text{F}^-(\text{Eq 1})
\]

To demonstrate that this reaction was not catalyzed by some other enzymatic activity, which might have been present as an impurity in the enzyme preparation used, partial heat inactivation of the enzyme preparation was studied, and the inactivated preparations were separately incubated in the presence of fumarate or fluorofumarate. The results are presented in Table I.
It is evident from these results that both activities decreased in parallel over the range studied. The kinetic constants were similar for both substrates, although the apparent \( K_m \) for fluorofumarate was an order of magnitude higher than that for fumarate. Chloro- and bromofumarate, when tested under similar conditions, did not react with the enzyme.

When the enzymic reaction was examined by nmr spectroscopy, the decrease in the resonance due to fluorofumarate could be followed, but the product oxaloacetate could not be positively identified because of the strong water signal which swamped out the area where the signal due to oxaloacetate is known to occur (8).

The addition of water to the double bond of fluorofumarate follows the direction predicted by Markownikoff’s rule. Release of a proton by the hydroxyl group of the postulated 2-fluoromalic acid followed by elimination of fluoride ion should then be a very facile reaction. The free energy change of the overall process is thus large and of the order of magnitude as that for the normal fumarate hydratase reaction coupled with the action of malate dehydrogenase (EC 1.1.1.37). Therefore, the reaction is essentially irreversible. Such a reaction would also destroy the fluorofumarate, and this may explain the observation that this compound is relatively nontoxic to animals (4).

Anti-Markownikoff addition of water to the double bond of fluorofumarate would be expected to be a pathway of higher energy. This possibility, however, may be readily tested in the reverse direction by determining whether threo-3-fluoromalic acid followed by elimination of fluoride ion should be a very facile reaction. The free energy change of the overall process is thus large and of the order of magnitude as that for the normal fumarate hydratase reaction coupled with the action of malate dehydrogenase (EC 1.1.1.37). Therefore, the reaction is essentially irreversible. Such a reaction would also destroy the fluorofumarate, and this may explain the observation that this compound is relatively nontoxic to animals (4).

It is reported in the literature that this compound is not acted on by the bacterial enzyme (9a, b), but was a competitive inhibitor. However, we plan to test this compound with the enzyme from pig heart.

While this work was in progress another preliminary report appeared in the literature (10) in which it was observed that L(-)-tartrate is also a substrate for fumarate hydratase and that the product of the reaction is oxaloacetate. Pyruvate, which probably results from the spontaneous decarboxylation of oxaloacetate, was also present in the incubation mixture.

Fumarate hydratase, while still an enzyme exhibiting very high specificity, can no longer be considered an example of an enzyme with absolute specificity. To quote Reiner (11), “Some enzymes are more discriminating than others, but it seems fair to say that any enzyme can be fooled if one goes to enough trouble.”

ACKNOWLEDGMENTS

We wish to thank Dr. M. S. Raasch of E. I. DuPont Co. for a generous gift of 2,2-difluorosuccinic acid. This work was supported by a grant from the U.S. Public Health Service, Institute of Neurological Diseases and Blindness, 9-R01-NB-07890-04. One of authors (WJN) is a predoctoral National Aeronautics and Space Administration Trainee (1965–68).

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Received November 16, 1967

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