Inhibition of Fumarase by S-2,3-Dicarboxyaziridine*

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S-2,3-Dicarboxyaziridine was found to be a potent competitive inhibitor \((K_i = 0.08 \mu M)\) of fumarase from pig heart. The aziridine did not inactivate the enzyme or exhibit any observable substrate activity. It is likely that it functions as a transition state analogue mimicking the carbaniom intermediate found in the normal catalytic reaction. The aziridine inhibited fumarate utilization in ruptured but not intact mitochondria.

Fumarase (fumarate hydratase, EC 4.2.1.2) catalyzes the reversible hydration of fumarate to form L-malate (Equation 1). The hydrogen and hydroxyl groups of L-malate are stereospecifically removed during catalysis. The overall stereochemical course of the reaction is a trans elimination of the elements of water from malate to form fumarate (1-3).

\[
\text{H} + \text{COO}^- + \text{H}_2\text{O} \rightleftharpoons \text{H} \text{COO}^- \text{OOC}^- \text{OH}
\]

Fumarase was originally thought to be highly specific for its substrates, fumarate and L-malate. In 1968, Nakamura and Ogata (4) reported that L-tartrate could be dehydrated by fumarase to form oxalacetate. Subsequently, a number of compounds were found which could serve as substrates for fumarase (5). All alternate substrates contain at least two negatively charged carboxyl groups, and their hydroxyl groups have the same configuration as the hydroxyl group of L-malate. In addition, malate derivatives require a hydrogen erythro to the hydroxyl group in order to be substrates of fumarase.

The chemical mechanism of the fumarase reaction has been the subject of considerable debate. In 1957, Albert and coworkers (6) proposed a carbonium ion mechanism based on isotope effect data. No primary deuterium isotope effect was found when erythro-3-monodeuteromalate was the substrate, suggesting that breaking of the C-H bond was not rate limiting. In addition, the rate of exchange of the erythro-hydrogen with solvent was consistent with hydration of fumarate being the sole route for incorporation of hydrogen at C-3. Further isotope exchange and isotope effect studies (7, 8) were interpreted in terms of a carbonium ion mechanism.

In 1980, Porter and Bright (9) reported the effect of 3-carbonionic substrate analogues on fumarase. Their results showed that the 3-carbanion of 3-nitro-2-hydroxypropionate bound 900 times tighter than L-malate to fumarase. Porter and Bright interpreted their findings in terms of the action of a transition state analogue. They concluded that fumarase recognized the nitronate valence bond structure of the 3-carbanion of 3-nitro-2-hydroxypropionate which is structurally analogous to the 3-carbanion of malate stabilized over the 3-carboxylate group. Additional evidence for a 3-carbanion intermediate mechanism was supplied by Blanchard and Cleland (10) from isotope effect studies at equilibrium and under steady state conditions. These data, coupled with the results of Porter and Bright, effectively establish that catalysis by fumarase proceeds via stabilization of a carbanionic intermediate in which C-3 is trigonal and C-2 is tetrahedral.

With these considerations in mind, we tested the effects of S-2,3-dicarboxyaziridine (DCAZ) (Fig. 1) on the fumarase-catalyzed reaction. DCAZ would seem to be an ideal choice for a transition state analogue of fumarase. The trigonal-like structure at C-3 (11) is analogous to the 3-carbanion of malate in the aci form, and the trans configuration of the carboxylate groups (which is fixed, since there is no free rotation about the C(2)-C(3) bond) imparts a resemblance to fumarate.

DCAZ was tested as a substrate of fumarase. Since some aziridines are very potent alkylating agents (12), control experiments were performed to determine if DCAZ was acting as a mechanism-based inactivator of fumarase. We also tested the effects of DCAZ on intact mitochondria. The data presented here support the conclusion that DCAZ is a competitive inhibitor of fumarase, binding 500 times tighter than fumarate.

MATERIALS AND METHODS

Fumaric acid and malonic acid were purchased from Fisher. S-2,3-Dicarboxyaziridine was obtained from a strain of Streptomyces found in Nara City, Japan, according to the method of Naganawa et al. (13). Pig heart fumarase was a product of Sigma. HEPES buffer was purchased from Calbiochem-Behring.

Rate Determinations—Kinetic data were recorded on a Cary 118 spectrophotometer with a water-jacketed cell chamber at 22 °C. Reaction rates were followed by monitoring the decrease in absorbance at 240 nm due to the conversion of fumarate to malate. A 1-ml assay contained 0.1 mM EDTA, 5 mM MgCl₂ in 0.1 M HEPES, pH 7.0, plus fumarase varied from 25 to 200 μM. Assay solutions for inhibition studies were the same as above with the addition of a fixed concentration of inhibitor. All reactions were initiated by the addition of 1 μl of fumarase which had been diluted 1:200 (0.04 mg/ml) in 0.1 M HEPES, pH 7.0. In order to be sure that the diluted fumarase was not losing activity over the course of the experiment, kinetic data with no inhibitor present were collected at the beginning and end of the experiment. All reaction rates were linear up to 5 min.

Control Experiments—The following incubations were performed at room temperature: enzyme + aziridine (equal volumes of fumarase

*The abbreviations used are: DCAZ, S-2,3-dicarboxyaziridine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

†Recipient of a predoctoral fellowship from the Robert A. Welch Foundation.

‡Supported by Grant CA 14030 from the National Cancer Institute and Grant C-582 from the Robert A. Welch Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Inhibition of Fumarase

RESULTS AND DISCUSSION

Compounds containing aziridine rings are often powerful alkylating agents (12). Such molecules are of biological interest due to their antibiotic and antitumor activities and as potential insect chemosterilants. It has been reported that aziridine-2-carboxylate is an active site-directed reagent for proline racemase (15). A possible mechanism of inactivation of proline racemase by aziridine-2-carboxylate involves alkylation of an amino acid side chain.

In 1975, Umezawa and co-workers (13) described a metabolite from a strain of Streptomyces which exhibited antibacterial activity against Aeromonas salmonicida. These workers identified the structure of this metabolite to be S-2,3-dicarboxyaziridine. Specific sites of inhibition by DCAZ were not described.

As a part of a study of aspartate-fumarate analogues, we investigated the effect of DCAZ on the reaction catalyzed by fumarase from swine heart. When the concentration of fumarase was varied at different fixed levels of DCAZ, the family of lines in a double reciprocal plot intersected on the l/x axis (Fig. 2A), indicative of competitive inhibition. The K_m for fumarate was 40 \mu M and the K_i for DCAZ was 0.08 \mu M, as determined from the slope repot (Fig. 2B). Since all reactions were initiated by the addition of enzyme to a reaction mixture containing DCAZ, we ran control experiments to determine if the apparent competition was real or simply the result of slow inactivation of fumarase by DCAZ. Enzyme and DCAZ were incubated together for 60 min as described under "Materials and Methods." The reaction was inhibited 55% for each time point relative to enzyme which had been incubated under the same conditions without DCAZ. Interestingly, when enzyme was incubated with a volume of 0.1 m HEPES, pH 7.0, equal to the volume of the DCAZ solution, a loss in activity was observed over the 60-min interval (at 60 min, 34% of enzyme activity was lost). It has been reported that fumarase is inactivated in dilute solution and that substrates or competitive inhibitors will protect from inactivation (16). From our data, we conclude that the binding of DCAZ to fumarase protects the enzyme from inactivation.
the enzyme. The electron-donating capacity of the hetero atom of aziridines is considerably greater than that of cyclic ethers (11, 19). This difference in basicity may be reflected in the difference in the nature of the interactions of DCAZ and \( \text{L-trans-2,3-epoxysuccinate} \) with fumarase.

Attempts were made to inhibit fumarase in intact mitochondria. The isolated mitochondria were capable of supporting respiration with either succinate or fumarate as substrate. As a control, malonate was found to inhibit succinate-associated respiration, having no effect when fumarate was the active substrate. Concentrations of DCAZ up to 70 \( \mu \text{M} \) had no effect on respiration. When ruptured mitochondria (either by sonication or Triton X-100 treatment) were tested, we were able to completely inhibit fumarate-supported respiration with DCAZ. These results would suggest that DCAZ is unable to cross the mitochondrial membrane.

From the results of their inhibition studies, Porter and Bright (9) concluded that fumarase recognized the nitronate valence bond structure of the 3-carbanion of 3-nitro-2-hydroxyaspartate. This structure is analogous to the 3-carbanion of malate stabilized over the -carboxylate, having an \( \text{sp}^2 \) hybridized carbon at position 3 and an \( \text{sp}^3 \) hybridized carbon at position 2.

Even though carbons 2 and 3 of DCAZ are each formally bonded to four atoms, the strained conformation of the aziridine ring imparts \( \pi \) character to their bonds. The hybridization of carbons 2 and 3 is intermediate between \( \text{sp}^2 \) and \( \text{sp}^3 \). Since DCAZ and the nitronate 3-carbanion show enhanced binding to fumarase of the same order of magnitude, the configuration at C-2 must not contribute substantially to the attainment of the transition state.

The data presented here, along with the structural features of DCAZ discussed above, support the conclusion that DCAZ is acting as a transition state analogue (20) by binding to fumarase in a configuration similar to that found during the catalytic reaction.

REFERENCES

1. Fisher, H. F., Frieden, C., McKee, J. S. M., and Alberty, R. A. (1955) \( \text{J. Am. Chem. Soc.} \) 77, 4436
2. Englard, S., and Colowick, S. P. (1966) \( \text{J. Biol. Chem.} \) 221, 1019–1035
3. Gawron, O., and Fondy, T. P. (1959) \( \text{J. Am. Chem. Soc.} \) 81, 6333–6334
4. Nakamura, S., and Ogata, H. (1968) \( \text{J. Biol. Chem.} \) 243, 528–532
5. Hill, R. L., and Teipel, J. W. (1971) in \textit{The Enzymes} (Boyer, P. D., ed) p. 556, 3rd Ed., Academic Press, New York
6. Alberty, R. A., Miller, W. G., and Fisher, H. F. (1957) \( \text{J. Am. Chem. Soc.} \) 79, 3973–3977
7. Hansen, J. N., Dinovo, E. C., and Boyer, P. D. (1969) \( \text{J. Biol. Chem.} \) 244, 6270–6279
8. Schmidt, D. E., Jr., Nigh, W. G., Tanzer, G., and Richards, J. H. (1969) \( \text{J. Am. Chem. Soc.} \) 91, 5849–5854
9. Porter, D. J. T., and Bright, H. J. (1980) \( \text{J. Biol. Chem.} \) 255, 4772–4780
10. Blanchard, J. S., and Cleland, W. W. (1980) \textit{Biochemistry} 19, 4506–4513
11. Deyrup, J. A. (1983) in \textit{Small Ring Heterocycles} (Hassner, A., ed) Part 1, p. 8, John Wiley & Sons, New York
12. Dermer, O. C., and Ham, G. E. (1969) Ethyleneimine and Other Aziridines, pp. 394–443, Academic Press, New York
13. Naganawa, H., Uratani, N., Takita, T., Hamada, M., and Umezawa, H. (1975) \textit{J. Antibiot.} (Tokyo) 28, 828–829
14. Johnson, D., and Lardy, H. (1967) \textit{Methods Enzymol.} 10, 94–96
15. Walsh, C. T. (1979) \textit{Enzymatic Reaction Mechanisms}, p. 574, W. H. Freeman and Co., San Francisco
16. Teipel, J. W., and Hill, R. L. (1971) \( \text{J. Biol. Chem.} \) 246, 4859–4865
17. Albright, F., and Schroepfer, G. J., Jr. (1971) \( \text{J. Biol. Chem.} \) 246, 1350–1357
18. Teipel, J. W., Hass, G. M., and Hill, R. L. (1968) \( \text{J. Biol. Chem.} \) 243, 5684–5694
19. Barrett, E. M., and Wu, C. Y. (1962) \( \text{J. Am. Chem. Soc.} \) 84, 1684–1688
20. Wolfenden, R. (1977) \textit{Methods Enzymol.} 46, 15–28