Biochemical Pharmacology, Vol. 33, No. 9, pp. 1575–1578, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

PRELIMINARY COMMUNICATION

MODE OF INTERACTION OF ORNITHINE DECARBOXYLASE WITH ANTIZYME AND α-DIFLUOROMETHYLORNITHINE

Dimitris A. Kyriakidis, Flavio Flamigni, Jan W. Pawlak and Evangelos S. Canellakis

Department of Pharmacology, Yale University School of Medicine,
New Haven, Connecticut 06510, U.S.A.

(Received 27 September 1983; accepted 20 January 1984)

α-Difluoromethylornithine (DFMO) is an enzyme-activated irreversible inhibitor of ornithine decarboxylase (EC 4.2.2.17) (ODC) of eukaryotic cells (1) that prevents the synthesis of putrescine (2) both *in vitro* and *in vivo*. The specificity of interaction between DFMO and ODC is such that DFMO has been used to determine the amount of ODC in cells (3,4). Based upon these properties, DFMO has also been used to determine the turnover rate of ODC (5,6) and the subunit size of ODC on sodium dodecyl sulfate (SDS) gels (6,7).

We have reported that putrescine elicits the appearance of a protein inhibitor of ODC, which was named antizyme to ODC (8-11). We have now found that DFMO reacts with the ODC-antizyme complex; therefore, treatment of tissues with (^{3}H)-DFMO would determine both free and antizyme-bound forms of ODC. It also appears that crude rat liver extracts may contain more than one form of ODC.

MATERIALS AND METHODS

α-(5-^{3}H)-Difluoromethylornithine (17.2 Ci/mmole) was purchased from the New England Nuclear Corp., Boston, MA, and (1-^{14}C)-D,L-ornithine (50 mCi/mmole) from Moravek Biochemicals, Brea, CA.

Preparation of rat liver ornithine decarboxylase. Ornithine decarboxylase was partially purified from livers of dexamethasone-induced female rats, approx. 125-150 g (10). Each liver was homogenized in 10 ml of Buffer A [50 mM Tris-HCl, pH 7.7, at 5°,0.1 mM EDTA, 0.1 mM NaN_3, 0.1 mM phenylmethane sulfonylfluoride and 1 mM dithiothreitol (DTT)] plus 50 mM KCl. The precipitate obtained between 20 and 45% ammonium sulfate saturation of the supernatant fraction (100,000 g - 90 min) was dissolved in Buffer A (1/2 of the original supernatant volume), dialyzed overnight against 40-50 vol. of Buffer A plus 50 mM KCl, clarified (10,000 g - 10 min), and applied on a DE52 cellulose column equilibrated with Buffer A plus 50 mM KCl. The column was eluted with a linear gradient of 100 to 250 mM KCl in Buffer A. The fractions with ODC activity (110-165 mM KCl) were concentrated with Amicon ultrafiltration (PM 30 filter) applied on a Sephadex G200 column equilibrated in Buffer A plus 50 mM KCl. The fractions with ODC activity were concentrated 8-fold (PM 30 filter); 77 units/mg protein; yield = 70%.

Preparation of rat liver antizyme. Female rats (200-250 g) fasted overnight were injected intraperitoneally at zero time and at 90 min with 2 mmoles putrescine dihydrochloride each time, per kg body weight. At 180 min, the livers were homogenized (see above), and the precipitate (30-50% ammonium sulfate saturation) was dialyzed (see above) and partially purified (12). It was applied to a DEAE Sephadex A25 column

(equilibrated with Buffer A plus 50 mM KCl) and eluted with 8 column volumes of a linear gradient from 100 to 450 mM KCl in Buffer A. The antizyme fractions (120-185 mM KCl) were concentrated (PM 10 filter), dialyzed, and applied on a Sephadex G150 column equilibrated with Buffer A plus 50 mM KCl. The active fractions in the 16,000 to 40,000 molecular weight region were concentrated (PM 10 filter); 56 units/mg protein; yield = 8%.

Assays, units and protein determination. These have been described (13-16).

Binding of (5-^{3}H)-DFMO to ODC. The binding of (5-^{3}H)-DFMO to ODC was performed at 30^0 in assay buffer (50 mM Tris-HCl, pH 7.2, at 20^0, 0.1 mM EDTA, 50 µM pyridoxal phosphate and 5 mM DTT). The extent of incorporation was then determined (5). Samples were removed and added to 100 µl of non-radioactive DFMO (58 µM) and 100 µl of bovine serum albumin (2 mg/ml), precipitated with 1 ml of 0.5 M perchloric acid. After 20 min at 0^0, the pellet (8,000 g - 5 min) was washed once with 0.5 M perchloric acid, twice with ethanol, once with ethanol-ether (1:1, v/v), twice with ether, dissolved in 0.1 ml of 0.1 N NaOH, heated at 86^0 for 2 min, and reprecipitated with 0.5 M perchloric acid. The final pellet was dissolved in 0.2 N NaOH and counted in 5 ml of Liquiscint in a liquid scintillation spectrometer.

RESULTS AND DISCUSSION

Absence of interaction between DFMO and antizyme. Antizyme retained 95-97% of its activity after incubation for 4 hr with non-radioactive DFMO (0.5 mg/ml)at 30^0 (Fig. 1). This experiment was repeated with (^{3}H)-DFMO (1 µCi), and the mixture was applied on a Biogel P100 column. No radioactivity was found in the antizyme fractions (inset of Fig. 1), indicating that the (^{3}H)-DFMO does not bind to antizyme protein(s) and that this extract is free of ODC protein that may bind DFMO.

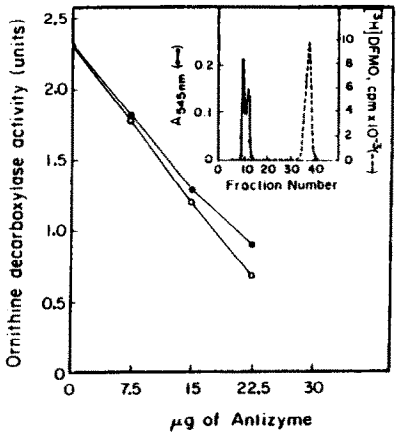

Fig. 1. Treatment of rat liver antizyme with DFMO. The rat liver antizyme was incubated at 30^0 for 4 hr in 0.6 ml of assay buffer in the presence and absence of DFMO (0.5 mg/ml). The two solutions were dialyzed for 16 hr against 800 ml [50 mM Tris-HCl, pH 7.2, at 20^0, 0.1 mM EDTA, 50 mM pyridoxal phosphate (PLP), 0.1 mM DTT, 50 mM KCl] and for 4 hr against 800 ml of the same buffer without the KCl before being assayed (9,12). Key: control antizyme (o—o); DFMO-treated antizyme (●—●). Inset: Antizyme (0.29 mg) in 0.1 ml assay buffer was incubated for 4 hr at 30^0 with (^{3}H)-DFMO (1 µCi). The solution was applied on a Biogel P100 column (60 x 1.4 cm), equilibrated, and eluted with 50 mM Tris-HCl, pH 7.2, at 20^0, 0.1 mM EDTA, 0.1 mM DTT. Key: protein (———); radioactivity (– – –).

Kinetics of inhibition of ODC by DFMO in the presence and absence of antizyme. Preparations containing a constant amount of ODC and various amounts of antizyme were incubated to form solutions containing different amounts of "ODC-antizyme" complexes. The ODC:antizyme activity ratios of these preparations were 1:1.06, 1:0.53, 1:0.25 and 1:0. Regardless of the degree of inactivation of ODC activity by the antizymes, the total incorporation of (^{3}H)-DFMO into these preparations remained constant and characteristic of the amount of ODC that was originally added; it did not vary with the degree of inactivation of ODC by the different amounts of antizyme added (Fig. 2A).

Since each preparation contained a different amount of free, non-antizyme bound ODC, we also determined whether this free ODC was inactivated by the added (^{3}H)-DFMO. It can be seen that in all these preparations the (^{3}H)-DFMO progressively inhibited the residual free, non-antizyme bound ODC (Fig. 2B). Nevertheless, some ODC activity, which accounts

for 5-8% of the initial activity, was still detectable even after 4 hr, although plateau inhibition had occurred at 3 hr.

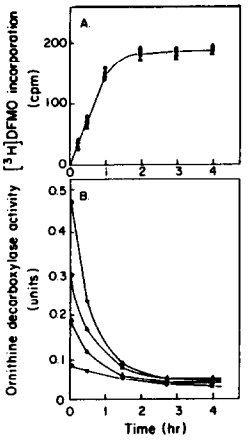

Fig. 2. Interaction of (^{3}H)-DFMO with rat liver ODC and with the preformed ODC-antizyme complexes. ODC (17 units), in 500 µl assay buffer, was allowed to form a complex with 18 (o—o), 9 (Δ—Δ), 4.5 (▲—▲) and 0 (●—●) units of antizyme at 0° for 30 min. (^{3}H)-DFMO (5 µCi) was then added (time zero), incubation was continued at 30°, and samples were removed at the indicated times. (A) Protein-bound (^{3}H)-DFMO. (B) ODC activity (per 5 µl, diluted to 100 µl); at this dilution and in the presence of the (1-^{14}C) ornithine substrate used during the assay, the residual (^{3}H)-DFMO did not inhibit ODC activity.

Reaction of antizyme with DFMO-inactivated ornithine decarboxylase. When an excess of ODC was added to antizyme, no antizyme activity was detected although excess ODC could be determined (Table 1, conditions 1-3). When a similar excess of "DFMO-inactivated" ODC was added to antizyme, no ODC activity was detectable. The antizyme activity was titratable (Table 1, condition 4) and recovered off a Sephadex column (Fig. 3B). These results indicate that the affinity between antizyme and the "DFMO-inactivated ODC" molecule was less than that between antizyme and native ODC.

In a separate experiment, when an excess ODC was again added to antizyme but the mixture was now treated with DFMO (to bind both the free and the antizyme-inactivated ODC as per Fig. 2), only about one-half of the antizyme activity (Table 1, condition 5, 40%) could be recovered. Since we have already established that antizyme activity can be completely recovered in the presence of DFMO or in the presence of the DFMO treated "ODC-antizyme" complex, this result indicates that approximately one-half of the antizyme was free and the other one-half remained bound to ODC. One plausible interpretation of these results is that the ODC was bound to two antizyme molecules and that the configurational change imposed upon ODC by treatment with DFMO permitted only one of the two antizyme molecules to be free of the complex.

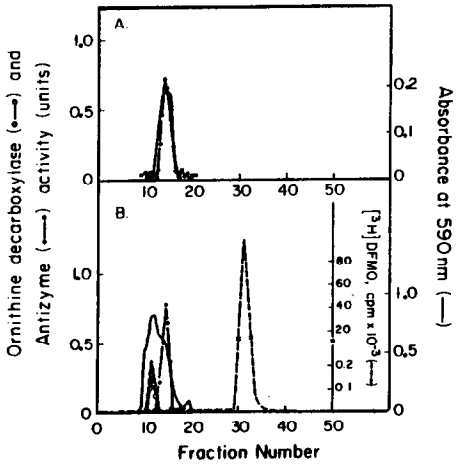

Fig. 3. Lack of interaction of antizyme with (^{3}H)-DFMO-inactivated ODC. (A) Control: Rat liver antizyme (step Sephadex G150, 17 units) was applied on a Sephadex G75 superfine (20 x 0.6 cm) column. Fractions of 0.18 ml were collected and assayed for antizyme activity (●—●) and protein (——) to localize the position of the antizyme and to determine percent recovery. (B) Experimental: (^{3}H)-DFMO-inactivated ODC (17 units - 100 µl) preincubated with 1 µCi (^{3}H)-DFMO for 4 hr at 30° was mixed with antizyme (10 units) and left at 0° for 30 min. The mixture was applied on a Sephadex G75 superfine column (20 x 0.6 cm). Fractions of 0.18 ml were collected, and ODC activity (o—o), antizyme activity (●—●), protein (——) and radioactivity [(^{3}H)-DFMO] (- - - -) were determined in each fraction.

For these experiments, we used minimally, but equivalently, purified preparations of ODC and of antizyme, in case more than one form of ODC (13, 17) or of antizyme may exist (12). Our earlier results (13) indicated that antizyme does not inactivate ODC completely.

The titration curves of Fig. 2B verify that 5-8% of the ODC did not react with DFMO nor was it inactivated by antizyme.

Table 1. Recovery of antizyme activity in the presence of ODC and DFMO*

Additions	Recovery of antizyme activity (%)	Recovery of ODC activity (%)
1. Antizyme	100	
2. Antizyme plus DFMO	100	
3. ODC plus antizyme	0	50
4. ODC inactivated by DFMO, then antizyme added	94.7	5
5. ODC plus antizyme, then DFMO added	40.0	5

*ODC (5.0 units) and DFMO (0.23 nmole) were either incubated for 4 hr at 30° and then antizyme (1.9 units) was added, or ODC was preincubated with antizyme (1.9 units) for 30 min at 0°, DFMO (0.23 nmole) was added and the mixture incubated at 30° for 4 hr.

In summary, we found that difluoromethylornithine reacted in an almost identical manner with the "ornithine decarboxylase-antizyme" complex as it does with ornithine decarboxylase. Consequently, treatment of tissues with (^{3}H)-difluoromethylornithine measures not only the free ornithine decarboxylase but also any intracellular antizyme-bound, cryptic or inactive forms of ornithine decarboxylase that may exist. It also appears that native ODC may bind with two molecules of antizyme.

Acknowledgements - This investigation was supported by a USPH Service Grant CA-26546, by a Research Career Award to E.S. Canellakis, and by a gift from Mr. and Mrs. J. Seward Johnson, The Johnson Seward Johnson 1961 and 1963 Charitable Trusts and by Johnson and Johnson.

REFERENCES

1. B.W. Metcalf, P. Bey, C. Danzin, M.J. Jung, P. Casara and J.P. Verert, J. Am. chem. Soc. 100, 2551 (1978).
2. N. Seiler, C. Danzin, N.J. Prakash and J. Koch-Weser, in Enzyme-Activated Irreversible Inhibitors (Eds. N. Seiler, M.J. Jung and J. Koch-Weser), pp. 55-71. Elsevier North-Holland Biochemical Press, Amsterdam (1978).
3. M.L. Pritchard, J.E. Seely, H. Poso, L.S. Jefferson and A.E. Pegg, Biochem. biophys. Res. Commun. 100, 1597 (1981).
4. A.E. Pegg, J.E. Seely and I.S. Zagon, Science 217, 68 (1982).
5. J.E. Seely, H. Poso and A.E. Pegg, J. biol. Chem. 257, 7549 (1982).
6. J.E. Seely, H. Poso and A.E. Pegg, Biochemistry 21, 3394 (1982).
7. T. Kameji and S.I. Hayashi, Biochim. biophys. Acta 705, 405 (1982).
8. W.F. Fong, J.S. Heller and E.S. Canellakis, Biochim. biophys. Acta 428, 456 (1976).
9. J.S. Heller, W.F. Fong and E.S. Canellakis, Proc. natl. Acad. Sci. 73, 1858 (1976).
10. E.S. Canellakis, J.S. Heller and D.A. Kyriakidis, in Advances in Polyamine Research (Eds. C.M. Caldarera, V. Zappia and U. Bachrach), Vol. 3, pp. 1-13, Raven Press, New York (1981).
11. E.S. Canellakis, D.A. Kyriakidis, J.S. Heller and J.W. Pawlak, Med. Biol. 59, 279 (1981).
12. E.S. Canellakis, D. Viceps-Madore, D.A. Kyriakidis and J.S. Heller, in Current Topics in Cellular Regulation (Eds. B. Horecker and E. Stadtman), Vol. 15, pp. 155-202, Academic Press, New York (1979).
13. J.S. Heller and E.S. Canellakis, J. cell. Physiol. 107, 209 (1981).
14. K.Y. Chen, D.A. Kyriakidis and E.S. Canellakis, Biochim. biophys. Acta 716, 72 (1982).
15. M. Bradford, Analyt. Biochem. 72, 248 (1976).
16. M.K. Haddox and D.H. Russel, Biochemistry 20, 6721 (1981).
17. L. Alhonen-Hongisto, P. Seppayen, E. Holtta and J. Janne, Biochem. biophys. Res. Commun. 106, 291 (1982).