The competitive inhibition of tissue transglutaminase by α-difluoromethylornithine

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The transglutaminase-mediated insertion of putrescine into casein was inhibited competitively by α-difluoromethylornithine (α-DFMO), an enzyme-activated irreversible inhibitor of ornithine decarboxylase. Preincubation of the amine acceptor (casein) or the enzyme itself with the inhibitor did not affect enzyme activity. α-DFMO is a poorer substrate for transglutaminase (K_m = 2.10 mM) than putrescine (K_m = 0.17 mM). The inhibitory effect was also found with fibronectin as amine acceptor.

α-Difluoromethylornithine *Polyamine* *Transglutaminase* *Competitive inhibition*

1. INTRODUCTION

There are abundant reports in the literature showing that polyamines are essential for cell growth and proliferation [1,2]. This conclusion was established on the one hand from experiments in which polyamine levels were measured at various intervals after a growth stimulus [3] and on the other, from results obtained with α-DFMO, an enzyme-activated irreversible inhibitor of ornithine decarboxylase [4,5]. With this inhibitor, cell growth could be restored by the subsequent addition of putrescine [6,7].

The large number of intracellular enzymic reactions which are modulated by free polyamines (DNA, RNA, fatty acid and protein synthesis, oxidative phosphorylation) has made it difficult to ascribe to any one particular enzyme the role of the primary target of polyamine depletion. Further, there exist enzyme systems such as the transglutaminases which are capable of covalently inserting polyamines into the amide group of glutamine residues in proteins [8,9]. The latter fact raised the question whether the levels of protein-bound polyamines (such as putrescine) are affected by the addition of α-DFMO to cells either by the direct action of α-DFMO on transglutaminases or indirectly by reducing putrescine availability. If there is already evidence for the relationship between cadaverine availability and cadaverine binding (as its dansyl derivate) in cells in culture [10], to our knowledge, no information is available on the effect of α-DFMO on transglutaminase activity. To investigate this, the experiments described here were carried out.

Abbreviations: α-DFMO, α-difluoromethylornithine; PUT, putrescine; TPA, tetradecanoylphorbol acetate

2. MATERIALS AND METHODS

2.1. *Materials*

[^{14}C]Putrescine dihydrochloride {[1,4-^{14}C]tetramethylenediamine dihydrochloride, spec. act. 109 Ci/mol} and α-[^{14}C]DFMO {DL-α-difluoromethyl[5-^{14}C]ornithine, spec. act. 56 Ci/mol} were purchased from the Radiochemical Center (Amersham, Bucks.) and [^{14}C]ornithine monohydrochloride {DL-[1-^{14}C]ornithine monohydrochloride; spec. act. 50.3 Ci/mol} from New England Nuclear (Boston, MA). Putrescine dihy-

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drochloride and casein were obtained from Sigma (St Louis, MO), L-(+)-ornithine hydrochloride from Aldrich-Europe (Beerse, Belgium) and Pico-Fluor 30 from Packard Instrument Co. (Downers Grove, IL). α-DFMO was a kind gift from Centre de Recherche Merrel Int. (Strasbourg, France).

Transglutaminase was purified from guinea pig livers [11]. One unit of transglutaminase corresponds to the amount of enzyme capable of inserting 100 pmol putrescine into 20 μg casein for 30 min at 37°C.

2.2. *Methods*

To experimental tubes containing 20 μg casein in 30 μl buffer (see below) were added 25 μl [^{14}C]putrescine (5 μCi/ml) at levels ranging from 346 to 1046 μM (75–444 μM final concentration). To the mixture were added 20 μl α-DFMO in amounts ranging from 1 to 25 mM (0.17–4.35 mM final concentration) or 20 μl ornithine in amounts ranging from 25 to 100 mM (4.35–17.40 mM final concentration) and 20 μl transglutaminase (10 units). The reaction was initiated by the addition of calcium to a final concentration of 7 mM. All constituents of the reaction mixture were dissolved in 0.14 M NaCl/0.01 M Tris–HCl (pH 7.5) and the volume was adjusted to 115 μl with the same buffer.

Two types of control were performed: one contained all constituents except casein, the other all reagents except α-DFMO or ornithine, which were replaced by buffer.

After incubation at 37°C for 30 min with gentle shaking, 20 μl incubation mixture were spotted onto a Whatman 3MM filter paper disc pre-soaked with 100 μl EDTA (100 mM). The filters were immediately immersed in 10% ice-cold trichloroacetic acid. Free [^{14}C]putrescine was removed by 3 repeated washings (20 min each at 4°C with shaking) with 10% trichloroacetic acid containing 0.01 M putrescine and finally with 95% ethanol (5 min). The filters were then dried, and the remaining covalently bound radioactivity was measured in a Packard Liquid Scintillation Spectrometer BPLD (efficiency for ^{14}C of 85%) with 5 ml Pico-Fluor 30. The number of nmol putrescine bound/30 min per 20 μg casein was determined from the specific radioactivity of putrescine as follows:

(Transglutaminase + [^{14}C]putrescine + casein + Ca^{2+}) − (transglutaminase + [^{14}C]putrescine + buffer + Ca^{2+})

When [^{14}C]ornithine or α-[^{14}C]DFMO was used as substrate, the experimental procedure was the same as that described above for [^{14}C]putrescine with the following modifications:

(i) [^{14}C]Putrescine was replaced by 25 μl [^{14}C]ornithine (5 μCi/ml) at levels ranging from 400 to 2100 μM (87–456 μM final concentration) or by 25 μl α-[^{14}C]DFMO (20 μCi/ml) at levels ranging from 1860 to 4360 μM (400–950 μM final concentration).

(ii) Unlabelled α-DFMO or ornithine was replaced by buffer.

(iii) Filters were washed, respectively, in 0.01 M ornithine/10% trichloroacetic acid and 0.01 M α-DFMO/10% trichloroacetic acid.

All experiments were carried out in duplicate, and duplicates agreed within 5%.

3. RESULTS

3.1. *Effect of α-DFMO on transglutaminase-mediated insertion of putrescine into casein*

This was investigated as described in section 2 with concentrations of α-DFMO ranging from 2 to 30 mM in the presence of 23 mM putrescine. Ornithine over a similar concentration range was used as control. It can be seen from fig.1 that 50% inhibition was obtained with 2.6 mM α-DFMO, whereas with ornithine an almost 5-fold greater concentration (14.2 mM) is needed.

3.2. *α-DFMO, a competitive inhibitor of transglutaminase*

This was assessed at 3 different concentrations of α-DFMO: 3.3, 1.7 and 0.8 mM, and concentrations of putrescine ranging from 0.07 to 0.44 mM. It is apparent (fig.2) that α-DFMO is a competitive inhibitor of transglutaminase which shows a K_m for putrescine of 0.17 mM and a V_{max} of 1.14 nmol putrescine bound/20 μg casein per 30 min. This competitive inhibition can be annulled by increasing the amount of putrescine relative to that of α-DFMO. It decreases from 70% at a putrescine/α-DFMO ratio of 0.02 to 4% at a ratio of 0.51.

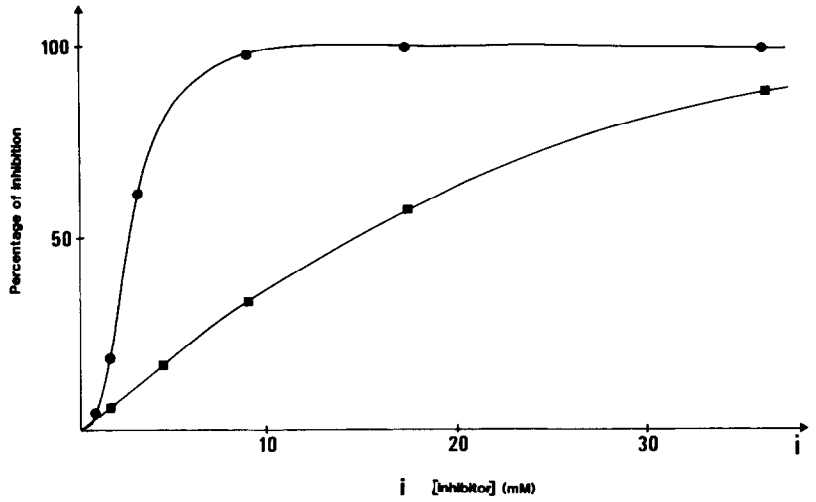

Fig.1. Inhibition of the transglutaminase-mediated insertion of putrescine (23 mM) into casein by α-DFMO (●) or ornithine (■). Each point represents the average of duplicate determinations carried out as described in section 2, in two different experiments.

Replotting the data of fig.2 in the form of a Dixon plot (fig.3) determined the K_i to be 2.28 mM which is in good agreement with the calculated value of 2.31 mM. In the case of ornithine (fig.4), the K_i value was found to be 12.10 mM, a 5-fold increase.

Transglutaminase is a two-substrate enzyme and is capable of interacting with the second substrate (the amine donor) only after the formation of an acyl enzyme complex with the first substrate (the amine acceptor) [8]. It was therefore possible that α-DFMO could inhibit the overall reaction by interacting with the amine acceptor or the enzyme itself. To investigate this, 20 μg casein and 10 units transglutaminase were preincubated individually for 30 min at 37°C with α-DFMO at 8 and 10 mM,

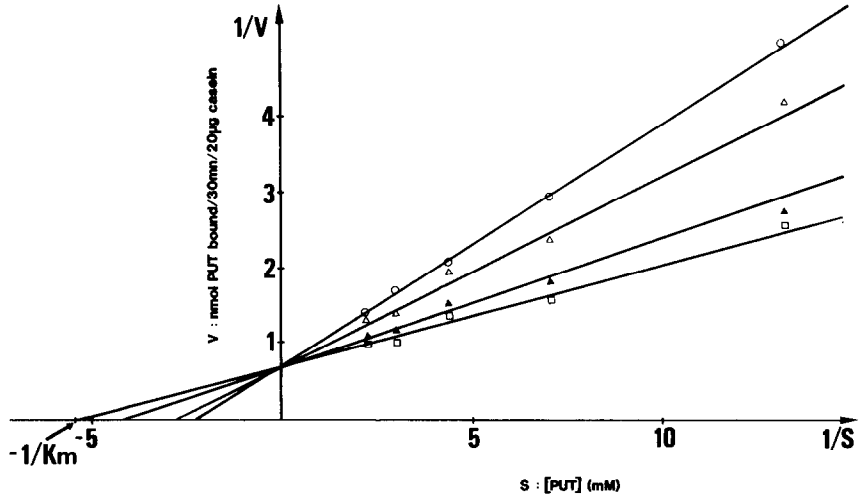

Fig.2. Lineweaver-Burk representation of the results of the transglutaminase (10 units)-mediated incorporation of putrescine (PUT) into casein (20 μg) in the absence of α-DFMO (□) or in the presence of α-DFMO at different concentrations: (▲) 0.87 mM; (△) 1.74 mM; (○) 3.50 mM. Each point represents the average of duplicate determinations carried out as described in section 2, in two different experiments.

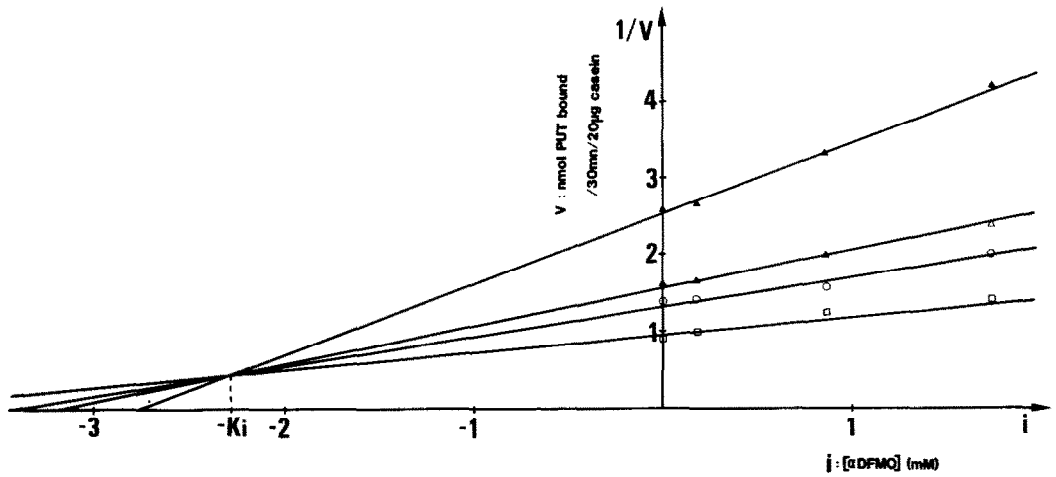

Fig.3. Dixon plot of the results obtained on adding increasing concentrations of α-DFMO to a mixture of transglutaminase (10 units), casein (20 μg) and calcium (7 mM) in the presence of different concentrations of putrescine: (▲) 75 μM, (△) 140 μM, (○) 227 μM, (□) 444 μM. Experimental details as in text. Each point represents the average of duplicate determinations carried out in two different experiments.

respectively. Control incubation mixtures contained the same amounts of casein and transglutaminase but without α-DFMO. At the end of the preincubation, the contents of each preincubation mixture were dialysed against 0.14 M NaCl/0.01 M Tris–HCl (pH 7.5). Then a 50-μl aliquot of casein preincubation mixture was added to the normal reaction mixture containing [^{14}C]-putrescine (12.5 Ci/8.5 nmol), calcium (7 mM final concentration) and 10 units transglutaminase

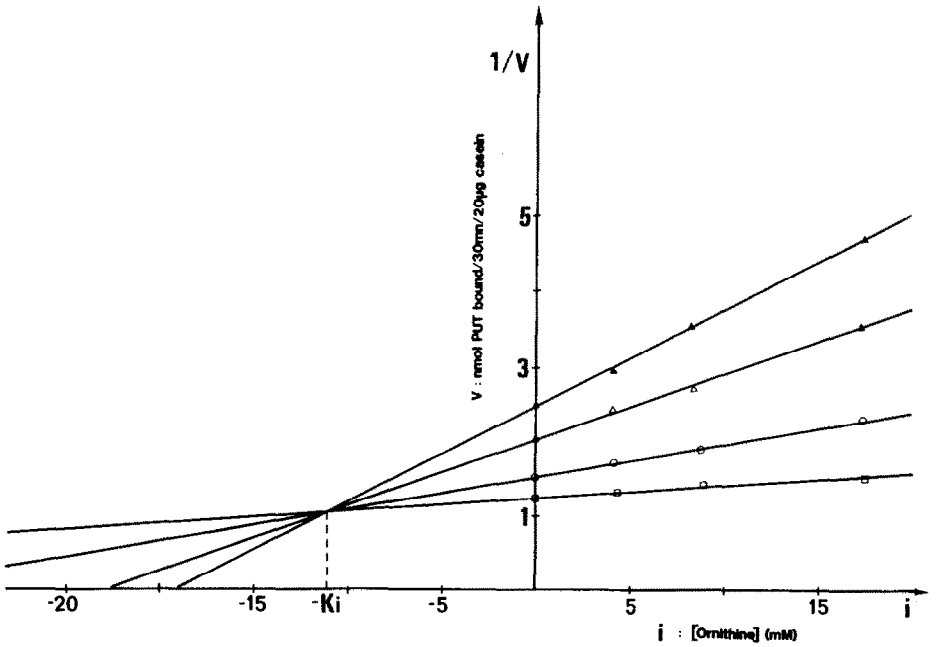

Fig.4. Same as for fig.3, except that α-DFMO was replaced by ornithine.

in a final volume of 115 μl. In the case of the transglutaminase preincubation mixture, a 40-μl aliquot was added to an incubation mixture containing [^{14}C]putrescine and calcium as described above, and casein at 20 μg. It is apparent (table 1) that α-DFMO has no influence on the transglutaminase-mediated insertion of putrescine when preincubated alone with either casein or transglutaminase.

Since transglutaminase has an absolute requirement of calcium, a further control for transglutaminase was carried out by preincubating the enzyme in the presence of calcium with or without α-DFMO. There was no significant difference in the residual activity of transglutaminase incubated in the presence or absence of calcium and in the presence or absence of α-DFMO.

It would therefore seem that inhibition of transglutaminase by α-DFMO required the simultaneous presence of casein, transglutaminase, calcium, α-DFMO and putrescine.

In view of this, attempts were made to determine whether α-DFMO is a substrate of transglutaminase.

3.3. *α-DFMO: an amine donor for transglutaminase*

This was checked experimentally using α-[^{14}C]DFMO as the amine donor and casein as the amine acceptor under the same experimental conditions as those used for [^{14}C]putrescine. For comparative purposes [^{14}C]ornithine was also tested as an amine donor.

Table 1

Transglutaminase-mediated insertion of putrescine into casein after preincubation of casein (20 μg) alone or transglutaminase (10 units) alone for 30 min in the presence or absence of α-DFMO

Composition of preincubation mixture		Putrescine insertion (nmol bound/30 min per 20 μg casein)
	α-DFMO	
Casein	+	0.16
	−	0.17
Transglutaminase	+	0.09
	−	0.10
Transglutaminase + calcium (7 mM)	+	0.07
	−	0.06

Results are the means of 2 experiments in duplicate

Table 2

Apparent K_m and V_{max} of the transglutaminase-mediated insertion of putrescine, ornithine and α-DFMO into casein

Amine donor	K_m (mM)	V_{max} (nmol bound/30 min per 20 μg casein)
Putrescine	0.17	1.14
Ornithine	1.35	0.53
α-DFMO	2.10	0.04

Results are the means of 2 experiments in duplicate

The results (table 2) show that ornithine and α-DFMO are relatively poor substrates for transglutaminase compared to putrescine and this order of affinity was substantiated by V_{max} values of 0.04, 0.53 and 1.14 nmol bound/30 min per 20 μg casein, respectively, for α-DFMO, ornithine and putrescine.

4. DISCUSSION

The only well documented effect of α-DFMO is the specific 'suicide' inhibition of ornithine decarboxylase. Our results provide evidence that α-DFMO is also a good competitive inhibitor of transglutaminase (fig.2).

This inhibition was observed with putrescine as amine donor for an artificial amine acceptor, casein (K_m = 0.17 mM).

The inhibition of transglutaminase by α-DFMO was not limited to casein as amine acceptor but was also found when fibronectin, one of its natural substrates [12], was used. With this glycoprotein as amine acceptor, the K_i of α-DFMO for transglutaminase was found to be 4.09 mM whereas in its absence the K_m of putrescine for transglutaminase was 0.06 mM with a V_{max} of 1.38 nmol bound/30 min per 50 μg fibronectin (not shown).

The K_i of α-DFMO for ornithine decarboxylase is 39 μM [13], and for transglutaminase with casein as substrate 2.31 mM. Therefore, inhibition of ornithine carboxylase requires a 6-fold smaller dose of α-DFMO than inhibition of transglutaminase. However, in studies using cells in culture, concen-

trations of 5 mM α-DFMO are commonly used [6,14], and for some cell types such as 9L rat brain tumor cells even higher concentrations (20 mM) are employed [15]. The possibility that cellular transglutaminase may be inhibited at such concentrations cannot be ignored.

Since α-DFMO (K_i = 2.31 mM) is a better competitive inhibitor of transglutaminase than ornithine (K_i = 12.10 mM), it would appear that the difluoromethyl moiety might contribute to the increased inhibition. However, when α-[^{14}C]DFMO and [^{14}C]ornithine were used as substrates, K_m values of 2.1 and 1.35 mM were found, respectively. This apparent similarity in K_m was not paralleled by V_{max} values which were 0.04 nmol bound/30 min per 20 μg casein in the case of α-DFMO and 0.53 nmol bound/30 min per 20 μg casein in that of ornithine: i.e., 13-fold increase. Bound α-DFMO may cause more structural hindrance to the subsequent binding of [^{14}C]putrescine than bound ornithine, but this is purely hypothetical for the moment.

Regardless of the mechanism by which α-DFMO inhibits transglutaminase, its effects on this enzyme may be relevant to two findings.

(i) Animals treated with α-DFMO show an increase in clotting time [7]. One interpretation of this result may be a direct effect of α-DFMO on the plasma transglutaminase–fibrinogen system.

(ii) α-DFMO could dissociate between the ornithine decarboxylase increase linked to the differentiation and that linked to the proliferation of HL60 cells induced by TPA and retinoic acid [16]. Since differentiation of these cells is accompanied by an increase in transglutaminase activity [17], the above system is being re-evaluated in the light of the results presented here.

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