BBA 4027

THE ACID STRENGTH OF THE AMINO GROUP AS A FACTOR IN THE TRANSPORT OF AMINO ACIDS

HALVOR N. CHRISTENSEN AND DALE L. OXENDER

The Department of Biological Chemistry, The University of Michigan, Ann Arbor, Mich. (U.S.A.)

(Received November 23rd, 1962)

SUMMARY

1. Halogen atoms have been introduced on the β-carbons of amino acids in order to dissociate effects on pK_a of the amino group on transport, from effects of the hydrophilic or hydrophobic character of the side chain.

2. α-Monofluoromethyl- and α-trifluoromethylalanine have been synthesized by adaptations of the Strecker synthesis. The pK_a of the amino group in these 2 cases was lowered by 1.6 and 4.3 units, respectively, by the introduction of fluorine.

3. These fluorine-containing amino acids are resistant to metabolism, and apparently non-toxic. The first shows characteristic biological transport, but in all situations studied, whether *in vivo* or *in vitro*, its transport is weaker than for the unfluorinated methylalanine.

4. The anionic trifluoromethylalanine gives no evidence of specific mediation in its transport, either by reference to neutral amino acids or to glutamic acid.

5. α-Chloroalanine also has somewhat lower affinity for transport than alanine.

6. These results indicate that the charged rather than the uncharged form of the amino group probably participates in amino acid transport.

INTRODUCTION

Comparison of the extent to which various neutral amino acids are accumulated into the Ehrlich ascites tumor cell showed a tendency for greater steady-state accumulation to be reached whenever polar elements were present in their otherwise hydrocarbon sidechains[1]. This tendency might have any of several origins: (a) It might arise, as suggested previously[1], from a lowering of pK_a of the amino group, a behavior that would suggest that during transport a proton must be displaced from the charged amino group. (b) It might arise from the prevention or weakening by a hydrophilic group of apolar bonding to the sidechain during transport. (c) It might arise from the reactivity *per se* of the additional polar group (*e.g.* the hydroxyl group).

To examine this matter we have prepared the α-monofluoromethyl and trifluoromethyl derivatives of alanine by adaptations of the Strecker synthesis. Although the pK_a values of these two substances are successively lower than those of α-methylalanine (α-aminoisobutyric acid), their transport affinities also are successively lower, not higher. Similarly, a β-chloro atom lowers the pK_a of alanine by 1.6 units, and coincidentally lowers the transport affinity.

EXPERIMENTAL

DL-α-*Monofluoromethylalanine**

50 mmoles each of KCN and NH_4Cl were suspended in 10 ml of water. 50 mmoles (3.62 ml) of monofluoroacetone (K and K Laboratories) were added dropwise with shaking and cooling to keep the temperature below about 30°. The mixture was let stand 12 h at 37°. Three volumes of concentrated HCl ($\rho = 1.19$) were then added under a hood, and the mixture boiled under reflux for 3 h. The solution was then evaporated to complete dryness *in vacuo*, and the residue extracted with ether–commercial absolute alcohol (1:1, v/v), using a total of 150 ml in 4 portions. The residue remaining on evaporating this extract, was titrated with Amberlite IR4B resin (free amine form) to a pH of 5. Evaporation of the light-yellow filtrate (plus the washings) to about 4 ml, initiated crystallization. Two volumes of alcohol were added. The crystalline product was recrystallized from 70 % (v/v) aqueous alcohol as large needles of the cubic system. The yield was 64 % of theoretical. A preparation using a quantity of 2 mmoles of each reactant, including $K^{14}CN$ (1 mC) gave a similar result. On heating, the crystals began to sublime at 215°; they melted with decomposition at 226–228° (Fisher–Johns block). R_F on ascending chromatography on Whatman No. 1 paper, 0.52 (*tert.*-butanol–formic acid–water) (14:3:3, v/v). Found: C, 39.72; F, 15.5; H, 6.82. $C_4H_8O_2NF$ requires C, 39.65; F, 15.7; H, 6.66

DL-α-*Trifluoromethylalanine*

5.5 g (0.1 mole) of KCN were suspended in 5 ml water in a Carius tube. 9.6 ml (0.1 mole) of 1,1,1-trifluoroacetone (Columbia Organic Chemicals) were added dropwise during 2 min with cooling. The KCN dissolved during the addition. The tube was stoppered firmly and shaken 15 min in a water bath at 70°. 25 ml of concentrated aqueous NH_3 ($\rho = 0.90$) were added with mixing, and the tube sealed with a glass-blower's torch. It was then held under boiling water for 5 h. After cooling, the tube was opened and the ammoniacal solution concentrated *in vacuo* to a thin syrup. (The aminonitrile could be obtained by sublimation from this syrup, but in poor yield.) An excess of concentrated hydrochloric acid was added under a hood, and the mixture boiled in an open flask for 15 min to steam-distill off what was presumably trifluoromethyllactic acid, and then for 4 h under a reflux condenser. The subsequent procedure followed that for the monofluoro compound, except that the titration with Amberlite IR4B was terminated at about pH 3.5. The combined filtrate and washings of the resin were treated with just enough charcoal (0.5 g acid-washed Nuchar) to eliminate almost all color. The combined filtrate and washings were taken to dryness *in vacuo*, the dry residue was taken up with heating in a minimal volume of commercial absolute alcohol, and the solution left several days at 5° to crystallize. It tended to remain supersaturated, and the initially formed crystals to be rather soluble; but the final crystalline form was rather difficultly soluble in alcohol. The mother liquors were concentrated and left at 5° for further crystallization. The product was recrystallized by dissolving in hot ethanol, then concentrating the solution to about one-tenth its volume, and leaving it at 5°. The total yield was 20 % of theoretical. A run at a level of 2 mmoles with $K^{14}CN$ (1 mC) gave a similar result. The crystals

* The name, α-methylalanine, is used in this paper for α-aminoisobutyric acid because of the greater ease of relating the structures of the fluoro derivatives to it.

sintered and melted incompletely at 198–205°. An R_F of 0.72 was obtained under the same conditions described for the monofluoro derivative. (Found: C, 30.7; F, 36.3; H, 3.99 %. $C_4H_6O_2NF_3$ requires C, 30.58; F, 36.3; H, 3.85 %.) The equivalent weight was found to be 157.

The amino acid was also obtained from a commercial preparation of the cyanohydrin of trifluoroacetone, but the yield was only 6 %, and adsorption on Amberlite IR4B followed by elution was needed to purify the product.

Several attempts to adapt the Strecker procedure to the preparation of monofluoroalanine and trifluoroalanine, using fluoroacetaldehyde and fluoral hydrate, respectively, were not productive.

RESULTS

The pK_a values were very effectively lowered by the presence of one or three fluorine atoms in the α-methylalanine molecule (Table I). The fluorinated derivatives, like α-methylalanine itself, are resistant to metabolic attack (Table II). In agreement, no obvious toxic action was observed in mice receiving single doses of 500 mg/kg of body weight. The fluorine-containing amino acids appeared more rapidly in the urine, a behavior that can not be attributed entirely to poorer renal transport, since they are also less strongly accumulated into the liver (Table II).

TABLE I

pK_a' VALUES FOR THE TWO FLUOROMETHYLALANINES AT 25°, COMPARED WITH PUBLISHED VALUES[2] FOR α-METHYLALANINE

The amino acids were titrated in 0.1 M solution at 25° with 0.5 N NaOH and 1 N or 5 N HCl, using a syringe microburette. The glass electrode was set at pH 4.00 with 0.05 M potassium acid phthalate, and at pH 7.00 with the commercial Beckman standard phosphate buffer.

Amino acid	$\Gamma/2$	pK_1'	$\Gamma/2$	pK_2'
α-Methylalanine	0.03	2.36*	0.03	10.21*
Monofluoromethylalanine	0.08	1.97	0.034	8.58
Trifluoromethylalanine	0.03	<0.9	0.03	5.94
	0.34	0.5		

* See ref. 2.

TABLE II

DISTRIBUTION IN THE MOUSE OF ^{14}C ADMINISTERED AS α-METHYLALANINE AND ITS FLUORO DERIVATIVES

1 μC (0.1–0.3 mg) of the 1-^{14}C-labeled amino acid in 0.5 ml of 0.9 % NaCl was injected subcutaneously in the scapular region into white Swiss mice weighing 25–32 g. For the next 6 h the air around the animals was passed through methanol solutions of Hyamine hydroxide, as described previously[3]. Blood was then collected by heart puncture, and the thigh muscles removed for analysis. The methods of extraction and radioactivity determination have been described already[3]. The distribution ratio represents counts/ml of cell water, divided by counts/ml of plasma.

Amino acid	*Percent excreted as CO_2*	*Percent excreted in the urine*	*Distribution ratio*	
			Liver/plasma	*Muscle/plasma*
α-Methylalanine	0.04	2	6.5	0.97
Monofluoromethylalanine	0.07	39	2.6	1.06
Trifluoromethylalanine	0.27	60	1.5	0.92

The monofluoro derivative is concentrated by the Ehrlich cell, although less strongly than α-methylalanine (Table III). The inhibitory interactions of these 2 amino acids show that the same transport system is involved in their entry, and confirm the order of their transport affinities (Table III). The trifluoro derivative enters the Ehrlich cell much more slowly, however, and no evidence could be obtained that the transport was mediated, since it did not inhibit the uptake of other neutral amino acids, nor was its uptake inhibited by their presence (Table III). Because trifluoromethylalanine is an anion at neutral pH, it might possibly use the separate transport mediation serving for the anionic amino acids[1]. Glutamic acid fails, however, to inhibit its entry into the Ehrlich cell (Table III).

Table IV shows that trifluoromethylalanine enters the red blood cell more rapidly than do the other two amino acids. This rapid entry does not arise from its affinity

TABLE III

COMPARATIVE UPTAKE OF FLUORO DERIVATIVES OF α-METHYLALANINE BY EHRLICH ASCITES-TUMOR CELLS

The cells were incubated at 37° in 25 volumes of Krebs–Ringer bicarbonate medium. The separation and extraction of the cells and the counting of radioactive disintegrations have been described previously[3,4]. The amino acid named at the left was present at a concentration of 1 mM, labeled with ^{14}C in the carboxyl group. The distribution ratio represents counts/ml cell water, divided by counts/ml suspending medium.

Amino acid whose uptake was observed	*Amino acid present as inhibitor at 10 mM*	*Distribution ratio*	
		1 min	*30 min*
α-Methylalanine	None	0.85	27.7
α-Methylalanine	Fluoromethylalanine	0.69	9.2
Fluoromethylalanine	None	0.95	13.0
Fluoromethylalanine	α-Methylalanine	0.43	5.5
Fluoromethylalanine	L-Methionine	0.24	1.6
Trifluoromethylalanine	None	0.27	0.74
Trifluoromethylalanine	L-Glutamic acid	0.45	1.0

TABLE IV

UPTAKE OF α-METHYLALANINE AND ITS FLUORO DERIVATIVES BY HUMAN-RED-BLOOD CELLS

Fresh human erythrocytes were incubated in 5 volumes of the medium of RAKER *et al.*[5] at 37°. The amino acid named at the left was present in 1-^{14}C-labeled form at a level of 1 mM in the suspending medium. The distribution ratio represents counts/min per gram cells, divided by counts/min/ml medium. Typical experiment by C. G. WINTER.

Amino acid whose uptake was observed	*Amino acid present as inhibitor at 5 mM*	*Distribution ratio after*		
		30 min	*90 min*	*180 min*
α-Methylalanine	None		0.23	0.32
α-Methylalanine	Trifluoromethylalanine		0.22	0.32
Fluoromethylalanine	None	0.19	0.32	
Fluoromethylalanine	Leucine	0.18	0.28	
Trifluoromethylalanine	None	0.40	0.47	
Trifluoromethylalanine	Leucine	0.37	0.50	

for the transport mediators serving for neutral amino acids, however, since no competition could be observed between this and other neutral amino acids (Table IV). The unusual permeability of the red blood cell to anions probably accounts for the observed inversion of the order of the rates of entry of this cell.

AKEDO[6] has made parallel observations showing successively weaker transport for the mono- and trifluoro derivatives across the isolated small intestine of the rat (unpublished results from this laboratory). Similarly, the fluorine-containing amino acids are less strongly concentrated across the placenta of the guinea pig[7].

β-Chloro-L-alanine[8] shows an initial rate of uptake by Ehrlich cells, and also a competitive action, somewhat less than that of L-alanine (Table V). Introduction of the β-chloro atom lowers the pK_2' of alanine from 9.8 (see ref. 2) to 8.2 (see ref. 9), which should increase the concentration of the species with an uncharged amino group at pH 7.4, by 220 times. Unless we suppose that the modest metabolic lability of β-chloroalanine already interferes with its transport behavior in the first minute of contact with the cells, this comparison offers no support to the view that the amino group needs to be deprotonated for transport. Over longer term intervals, β-chloroalanine interferes more strongly with transport.

TABLE V

COMPARATIVE UPTAKE OF β-CHLOROALANINE

Same conditions as for Table III, except that the β-chloroalanine was uniformly labeled, prepared from L-[$^{14}C_3$]serine[8].

Amino acid whose uptake was observed	*Amino acid present as inhibitor at 5 mM*	*Distribution ratio*	
		1 min	*10 min*
α-Methylalanine	None	1.86	15.50
α-Methylalanine	β-Chloroalanine	0.71	2.86
α-Methylalanine	Alanine	0.32	2.43
β-Chloroalanine	None	1.20	1.66
β-Chloroalanine	β-Chloroalanine	0.84	1.06
β-Chloroalanine	Alanine	0.57	1.05
Alanine	None	2.12	9.24
Alanine	β-Chloroalanine	1.49	3.34
Alanine	Alanine	0.79	—

We conclude from these experiments that the lowering of the pK_a of the amino group does not, *per se*, improve the affinity for transport. The greater steady-state accumulation observed earlier for the amino acids with hydrophilic groups in their sidechains appears therefore not to arise from a more rapid initial uptake; but it may arise from a slower escape from the cells. It appears likely that the effect of hydrophilic groups may be to handicap exodus rather than to intensify uptake[10–12].

ACKNOWLEDGEMENT

This work was supported in part by a grant (C2645(C6)) from the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

REFERENCES

[1] H. N. CHRISTENSEN, in W. D. MCELROY AND B. GLASS, *Amino Acid Metabolism*, Johns Hopkins Press, Baltimore, 1955, p. 91.
[2] P. K. SMITH, A. C. TAYLOR AND E. R. B. SMITH, *J. Biol. Chem.*, 122 (1937) 109.
[3] H. N. CHRISTENSEN AND J. C. JONES, *J. Biol. Chem.*, 237 (1962) 1203.
[4] H. N. CHRISTENSEN AND T. R. RIGGS, *J. Biol. Chem.*, 194 (1952) 57.
[5] J. W. RAKER, I. M. TAYLOR, J. M. WELLER AND A. B. HASTINGS, *J. Gen. Physiol.*, 33 (1950) 691.
[6] H. AKEDO, unpublished results.
[7] B. H. FELDMAN AND H. N. CHRISTENSEN, *Proc. Soc. Exptl. Biol. Med.*, 109 (1962) 700.
[8] E. FISCHER AND K. RASKE, *Ber.*, 40 (1907) 3717.
[9] T. R. RIGGS, B. A. COYNE AND H. N. CHRISTENSEN, *J. Biol. Chem.*, 209 (1954) 395.
[10] H. N. CHRISTENSEN, H. AKEDO, D. L. OXENDER AND C. G. WINTER, in J. F. HOLDEN, *Free Amino Acid Pools*, Elsevier, Amsterdam, 1962, p. 527.
[11] D. L. OXENDER AND H. N. CHRISTENSEN, *Nature*, 197 (1963) 765.
[12] H. N. CHRISTENSEN, *Biological Transport*, Benjamin, New York, 1962.