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THE TWO HUMAN TRYPSINOGENS: CATALYTIC PROPERTIES OF THE CORRESPONDING TRYPSINS

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Summary

The catalytic properties of the two human trypsins obtained from purified trypsinogens have been studied. The catalytic rate constant k_{cat} and the pK of the ionisable residue implicated in the active site have been determined with Bz-Arg-OEt. The hydrolysis of Tos-Arg-OMe by human trypsins does not follow the simple Michaelis-Menten scheme and indicates a difference in the conformational flexibility of the active site-regions of the two enzymes. Both enzyme are readily autolyzed and calcium ion plays a fundamental role in stabilizing trypsin activity. However trypsin 2 self-digests more rapidly than trypsin 1. These results are a prerequisite to the elucidation of the fate of pancreatic enzymes in human digestive tract.

Introduction

Two trypsinogens have been described in the exocrine pancreatic secretion of a large number of species [1—6] including man. The two separate trypsins derived from these zymogens hydrolyze the alimentary proteins and catalyze the activation of all pancreatic zymogens, playing therefore an essential role in the digestive process. The understanding of the existence of two separate enzymes with similar specificities requires the knowledge of the functional as well as structural molecular properties of these enzymes. The characterization of two human pancreatic trypsins prepared from partially activated juice [7] or autolyzed pancreatic extracts has been reported by different authors [8—11]. Specific activities and amino acid compositions of those trypsins were quite different from those of trypsins prepared by controlled activation of purified trypsinogens (unpublished data). This may be explained by the sensitivity of trypsin to autolysis [12—14] leading to several active forms with different

structural and physico-chemical properties as demonstrated for bovine trypsin [15—17]. We have previously reported the interactions of trypsins 1 and 2 derived from their purified zymogens with proteinase inhibitors [18]. Based on the inhibition spectra we have demonstrated that the cationic trypsin purified by Travis and Roberts [9] the trypsin studied by Feeney [19] and the trypsin 2 prepared by Feinstein [11] derive from trypsinogen 1. The anionic trypsin prepared by Mallory and Travis [10] and the trypsin 1 purified by Feinstein derive from trypsinogen 2. No enzymatic study has been reported on any human trypsin. In this paper we describe some catalytic properties of the two human trypsins derived from their purified zymogens and we make on attempt to explain their function in vivo.

Materials and Methods

Materials

Human trypsins 1 and 2 were obtained after controlled activation of trypsinogens 1 and 2 isolated from pancreatic juice according to the method previously described [18]. The autocatalytic activation was performed in 20 mM $CaCl_2$, 40 mM NaCl, 5 mM Tris · HCl buffer (pH 8.0). Human trypsin concentration was 5 μg/ml and trypsinogen concentration was in the range of 50—500 μg/ml. In some cases, trypsins were purified by affinity chromatography on trypsin inhibitor-Sepharose [20]. Bovine chymotrypsinogen A was prepared according to the original technique of Kunitz [12]. The antiserum to human pancreatic proteins was prepared by immunization of rabbits with a mixture of activated pancreatic juices according to the method of Henry et al. [21].

Methods

Enzymatic activities were measured at 25°C, pH 7.9 in a pH stat (Radiometer) using 100 mM NaOH. Trypsin activity was assayed using α-*N*-benzoyl-L-arginine ethyl ester (Bz-Arg-OEt) in the concentration range of 1—400 mM or *p*-tosyl-L-arginine methyl ester (Tos-Arg-OMe) in the concentration range of 0.05—500 mM. The routine assay was performed with a 10 mM substrate concentration. All the assays were carried out in 5 mM Tris · HCl/20 mM $CaCl_2$/100 mM KCl. For the substrate concentrations below 2.5 mM, trypsin activity was determined using 20 mM NaOH. Chymotrypsin activity was assayed using *N*-acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt) 10 mM in 5 mM Tris · HCl/20 mM $CaCl_2$/100 mM KCl/3% CH_3OH.

Activations of bovine chymotrypsinogen A by human trypsins 1 and 2 were carried out at pH 8.0, 4°C in 5 mM Tris · HCl (pH 8.0)/40 mM NaCl/20 mM $CaCl_2$. The concentration of chymotrypsinogen was 0.6 mg/ml, of activating trypsin, 1.6 μg/ml. 0.1 ml aliquots were taken off periodically and measured for their Ac-Tyr-OEt-hydrolyzing activity.

Results

Hydrolysis of Bz-Arg-OEt

Fig. 1 shows trypsin activities on 10 mM Bz-Arg-OEt in relation to pH. The optimum activities of trypsins 1 and 2 occured between pH 7.5 and 8.5 and the

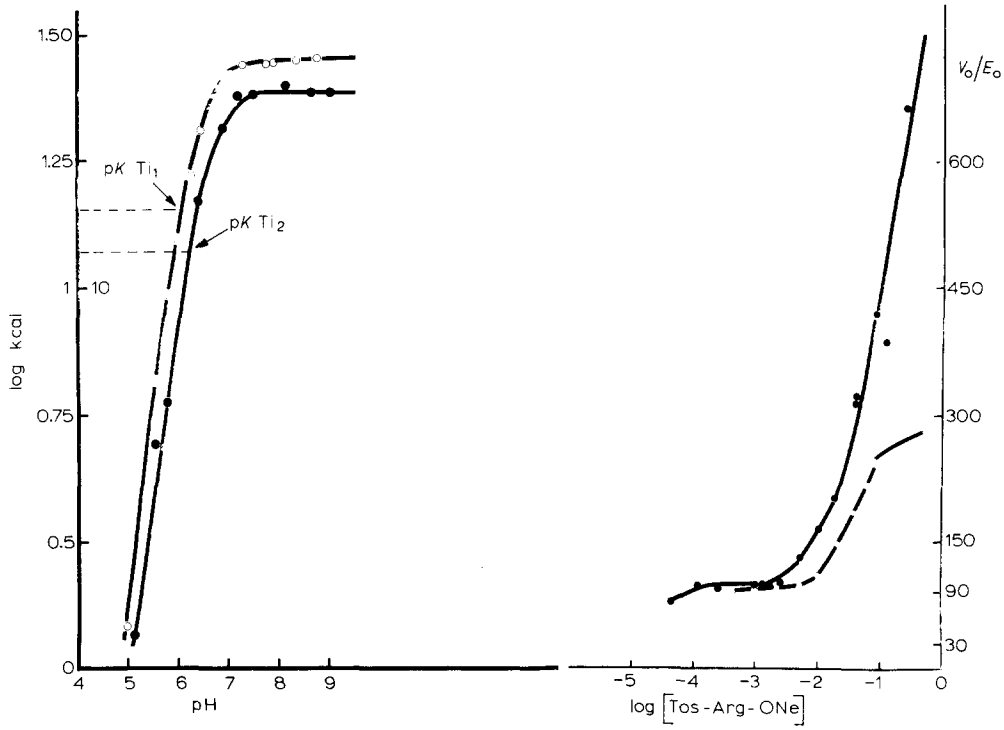

Fig. 1. Activities of human trypsins on Bz-Arg-OEt in relation with pH. Buffers used: pH 5.0—6.3: 5 mM sodium citrate/citric acid, 100 mM NaCl, 20 mM $CaCl_2$; pH 6.8—8.5: 5 mM Tris · HCl, 100 mM NaCl, 20 mM $CaCl_2$. The pK values are given by the pH values corresponding to log $k_{cat}/2$: ○———○, trypsin 1; ●———●, trypsin 2.

Fig. 2. Dependence of human trypsin activities on Tos-Arg-OMe upon substrate concentration. pH 7.9, 5 mM Tris · HCl, 20 mM $CaCl_2$, 100 mM NaCl ○———○, trypsin 1; ●———●, trypsin 2.

corresponding apparent k_{cat} values were 28 s^{-1} for trypsin 1 and 25 s^{-1} for trypsin 2. From this diagram the pK of the ionisable residue implicated in the active site of both trypsins were measured as 6.15 for trypsin 1 and 6.25 for trypsin 2, indicating the presence of an histidine residue at the active site of each trypsin, as previously suggested by the inhibition of both enzymes by TLCK [18].

Hydrolysis of Tos-Arg-OMe

Fig. 2 shows trypsin activity (pH 7.9) vs. Tos-Arg-OMe concentration. We have observed a substrate activation phenomenon with a first plateau at V_0/E_0 equal to 100 for both trypsins, where V_0 and E_0 are respectively the initial rate of hydrolysis (μmol · s^{-1}) and the enzyme concentration (μmol). When Tos-Arg-OMe concentration was above 5 mM, trypsins 1 and 2 had different behaviours. Trypsin 1 catalytic rate reached a second plateau corresponding to a V_0/E_0 close to 300 and trypsin 2 activity increased quickly until a V_0/E_0 value equal to 600 for a 500 mM Tos-Arg-OMe concentration. We have checked that there was no ionic strength dependence of human trypsin activity for a substrate concentration above 10 mM, as shown earlier for bovine trypsin in the same range of concentration [22].

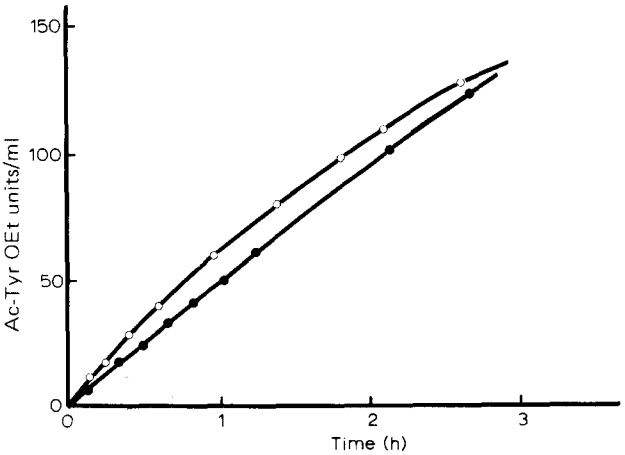

Fig. 3. Activation of bovine chymotrypsinogen A by human trypsins. Chymotrypsinogen concentration 0.6 mg/ml, activating trypsin concentration 1.6 μg/ml, pH 8.0, 4°C, 5 mM Tris · HCl, 20 mM $CaCl_2$, 40 mM NaCl, ○———○, trypsin 1; ●———●, trypsin 2.

Activation of bovine chymotrypsinogen A

The initial rates of activation of chymotrypsinogen A by each trypsin were similar; the average is equal to $1.3 \cdot 10^{-5}$ μmol chymotrypsinogen hydrolyzed per min, in the assay conditions (Fig. 3). This value is close to that obtained by Abita et al. for bovine trypsin in slightly different conditions [24].

Trypsin stability at acidic pH

The activities of trypsins 1 and 2 were constant at pH 3.0 and 4°C for 24 h. In strong acidic medium, trypsin 2 was less stable than trypsin 1. At pH 2.6, trypsin 2 lost 60% of activity after 24 h while trypsin 1 remained entirely active (Fig. 4). At pH 2.0 for 1 h, trypsin 2 was 50% inactive while trypsin 1 activity was unchanged.

Autocatalytic activities of human trypsins

Activities of both trypsins have been studied at pH 8.0 and 37°C using 10

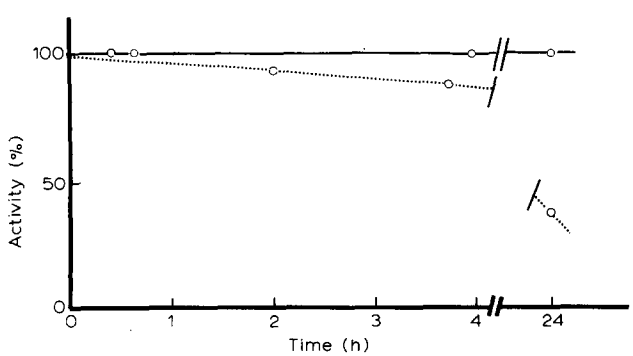

Fig. 4. Stability of human trypsins at pH 2.6. ———, trypsin 1; - - - - - -, trypsin 2.

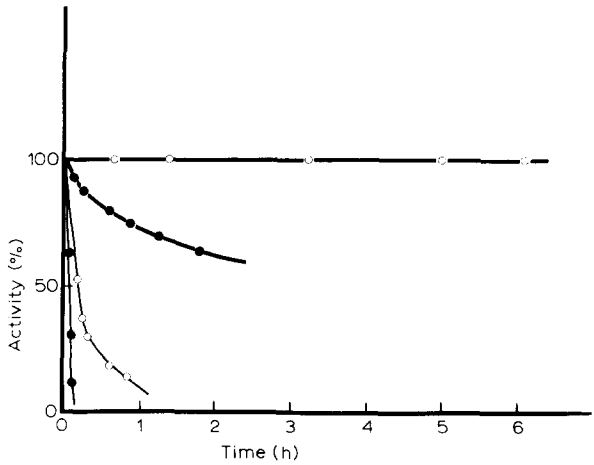

Fig. 5. Autolysis of human trypsins in presence of calcium and in absence of calcium, with 1 mM EDTA. Trypsin concentration 12 μg/ml; substrate: Tos-Arg-OMe, 10 mM Tris · HCl, 100 mM NaCl, pH 8.0, 37°C. ○——○, trypsin 1; ●——●, trypsin 2. Bold-faced lines indicate samples with 20 mM $CaCl_2$.

mM Tos-Arg-OMe in the presence of 20 mM calcium and in the absence of calcium with 1 mM EDTA (Fig. 5). In the presence of calcium, trypsin 1 activity was constant for 6 h while trypsin 2 lost 40% of activity in 2 h. In the absence of calcium and in the presence of EDTA, both trypsins were rapidly autolyzed. After 10 min, trypsin 1 was 50% inactive whereas trypsin 2 was completely inactive.

Immunological characterization

Fig. 6 shows the results obtained by immunodiffusion of trypsin 1 and 2 against antiserum to activated pancreatic juice which contains anti-trypsin 1 and anti-trypsin 2. Precipitation lines indicate a partial identity between the

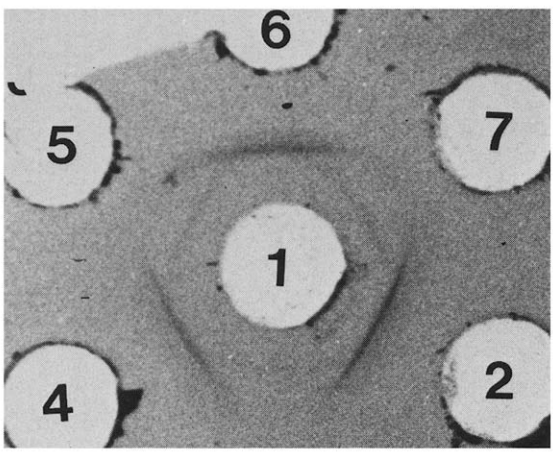

Fig. 6. Immunological characterization of human trypsins 1 and 2. Double diffusion test. 1% agarose in 50 mM veronal acetate pH 8.6. The wells contain respectively antiserum anti-proteins of pancreatic juice [1], 0.4 μg of trypsin 1 [2,4,6] and 0.5 μg of trypsin 2 [5,7].

two human trypsins with some additional antigenic determinants for trypsin 1. These observations are in agreement with partial results previously reported with antiserum containing only anti-trypsin 1 [10,11].

Discussion

The presence of two trypsins derived from two distinct trypsinogens in human pancreatic secretion has led us to study the catalytic power of the two enzymes in vitro and in some physiological conditions. This work is a prerequisite to understand the catalytic process of these enzymes in the digestive tract. The apparent catalytic rate constants (k_{cat}) on Bz-Arg-OEt and the histidine residue pK of human trypsins summarized in Table I are compared to the values reported for other trypsins. The k_{cat} values of both human trypsins are very similar to those of bovine cationic α- and β- and porcine cationic trypsins but very different from the k_{cat} value of rat trypsin 2 which is much higher. The pK values of the histidine residues of human trypsins 1 (6.15) and 2 (6.25) are respectively identical to those of bovine trypsins β and α. This result demonstrates that the environment of the histidine at the active site is the same in the four trypsins.

The kinetics of Tos-Arg-OMe hydrolysis by human trypsins does not follow the simple Michaelis-Menten scheme. This could be explained by the presence of two substrate binding sites on each human trypsin. The ternary complex enzyme · substrate would decompose faster than the binary enzyme · substrate complex, following the same pattern as bovine [23,25] and rat [26] pancreatic trypsins. Yon et al. [25] have suggested that the binding of substrate on a second site would induce a conformational change in the trypsin molecule leading to a more functional active center. The conformational flexibility in the active site regions has been mentioned as a determining factor in the catalytic properties [16,27—29]. Our results suggest that this is also the case for both human trypsins and that there is a difference between the two enzymes. When the Tos-Arg-OMe concentration is sufficient to saturate the first site (catalytic site) V_0/E_0 is the same for the two human trypsins. At higher substrate concentration (corresponding to the ternary complex) the rate of hydrolysis of Tos-Arg-OMe by trypsin 2 is twice higher than the rate of hydrolysis of Tos-Arg-OMe by trypsin 1. This fact points out a different flexibility degree in the active site regions of trypsins 1 and 2.

TABLE I

VALUES OF k_{cat} ON Bz-Arg-OEt AND pK OF IMIDAZOLE HISTINE RESIDUE OF HUMAN TRYPSINS AND SOME OTHER TRYPSINS

	Human trypsin		Bovine trypsin [16,17]		Cationic porcine trypsin [30]	Rat trypsin [26]	
	1	2	α	β		1	2
k_{cat} (s^{-1})	28	24	23.3 23.4	27.8 27.8	24.8	27.3	78.9
pK	6.15	6.25	6.24	6.15	6.40		

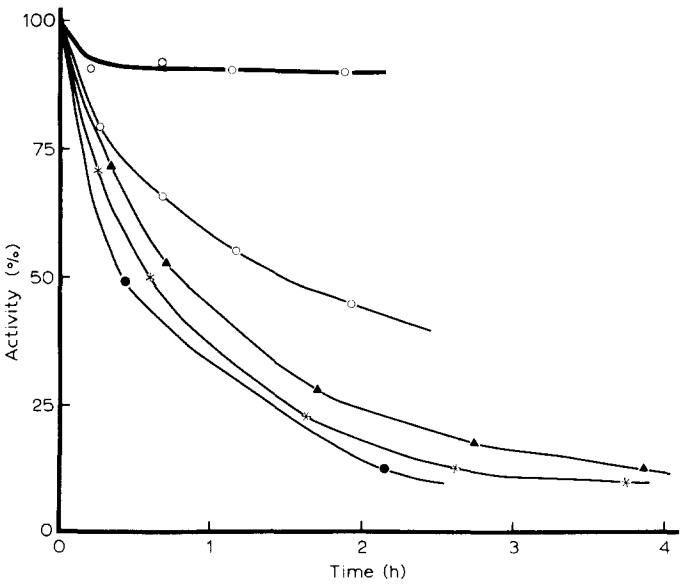

Fig. 7. Kinetics of trypsin degradation in four human duodenal juices at 37°C. Initial trypsin concentrations: 87 Tos-Arg-OMe units/ml for sample 1 (▲———▲); 121 units/ml for sample 2 (X———X); 50 units/ml for sample 3 (○———○) and 38 units/ml for sample 4 (●———●). Bold-faced line indicates the sample with 20 mM $CaCl_2$ added.

The proteolytic activities of the two human trypsins have been found identical. The initial rates of activation of bovine chymotrypsinogen A are similar and as reported before [18] the hydrolysis of casein by the two human trypsins is identical. As described for other trypsins the autolysis of human trypsins is markedly important. However trypsin 2 self-digests more rapidly than trypsin 1. We have shown the importance of calcium to stabilize both human trypsins. Calcium prevents inactivation and degradation of both enzymes, which does not confirm the results of Travis et al. [9] who found that the addition of 0.5 M calcium had no effect on trypsin 2 activity. This calcium dependent stability of human trypsins in vitro has been questioned in vivo. We have studied the life of trypsins in duodenal juices. As shown in the kinetics in Fig. 7, trypsin activity decreases rapidly at pH 8.0 and 37°C in the physiological concentration of calcium (1 mM). However the addition of 20 mM calcium to the duodenal juice stabilizes trypsins which inactivate more slowly, as observed with the purified enzymes. Therefore we can postulate that in vivo trypsin inactivation is mainly due to autolysis in the presence of a low concentration of calcium and that trypsin 2 is the first one to inactivate. The 30—60% of trypsin activity present in duodenal juice after 1 h of incubation must be attributed to trypsin 1 since this trypsin is more stable and twice more concentrated than trypsin 2 in pancreatic secretion [2]. It is difficult, at this time, to explain the difference between the two trypsins in terms of molecular mechanism but 2 possibilities can be suggested: (1) the same peptide bonds are cleaved but the affinity of active trypsin for inactive trypsin is increased in the trypsin 2 system; (2) the affinity for inactive trypsin is the same for the two human

trypsins but trypsin 2 possesses a peculiar peptide bond not protected by calcium ion and playing a role in the functional conformation of the enzyme.

In conclusion proteolytic activities of human trypsins are the same. Both enzymes are sensitive to autolysis in vitro as well as in vivo and the inactivation rates of trypsins 1 and 2 are different. This process could be the first step of trypsin degradation in the digestive tract.

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References

1 Marchis-Mouren, G. Pasero, L. and Desnuelle, P. (1963) Biochem. Biophys. Res. Commun. 13, 262—266
2 Figarella, C., Clemente, F. and Guy, O. (1969) FEBS Lett. 3, 351—353
3 Puigserver, A. and Desnuelle, P. (1971) Biochim. Biophys. Acta 236, 499—502
4 Ohlsson, K. and Tegner, H. (1973) Biochim. Biophys. Acta 317, 328—337
5 Voytek, P. and Gjessing, E.C. (1971) J. Biol. Chem. 246, 508—516
6 Clemente, F., De Caro, A. and Figarella, C. (1972) Eur. J. Biochem. 31, 186—193
7 Keller, P.J. and Allan, B.J. (1967) J. Biol. Chem. 242, 281—287
8 Robinson, L.A., Churchill, C.L. and White, T.T. (1970) Biochim. Biophys. Acta 222, 390—395
9 Travis, J. and Roberts, R.C. (1969) Biochemistry 8, 2884—2889
10 Mallory, P.A. and Travis, J. (1973) Biochemistry 12, 2847—2851
11 Feinstein, G., Hofstein, R., Koifmann, J. and Sokolowsky, N. (1974) Eur. J. Biochem. 43, 569—581
12 Northrop, J.H., Kunitz, M. and Merriot, R.M. (1948) Crystalline Enzymes, 2nd edn., Columbia University Press, New York
13 Bier, M. and Nord, F.F. (1951) Arch. Biochem. 33, 320—333
14 Maroux, S., Rovery, M. and Desnuelle, P. (1967) Biochim. Biophys. Acta 140, 377—380
15 Maroux, S. and Desnuelle, P. (1969) Biochim. Biophys. Acta 181, 59—72
16 Roberts, D.V. and Elmore, D.T. (1974) Biochem. J. 141, 545—554
17 Green, G.D.J. and Tomalin, G. (1976) Eur. J. Biochem. 68, 131—137
18 Figarella, C., Negri, G.A. and Guy, O. (1975) Eur. J. Biochem. 53, 457—463
19 Feeney, R.E., Means, G.E. and Bigler, J.C. (1969) J. Biol. Chem. 244, 1957—1960
20 Guy, O., Bartelt, D.C., Amic, J., Colomb, E. and Figarella, C. (1976) FEBS Lett. 62, 150—153
21 Henry, C., Bernard, D. and Depieds, R. (1968) Ann. Inst. Pasteur, Paris 114, 395—416
22 Bechet, J.J., Chevallier, J., Labouesse, J., Arnould, B., Aldin-Cicarelli, S. and Yon, J. (1968) Biochim. Biophys. Acta 151, 165—177
23 Trowbridge, C.G., Krehbiel, A. and Laskowski, Jr., M. (1963) Biochemistry 2, 843—850
24 Abita, J.P., Delaage, M. and Luzdunski, M. (1969) Eur. J. Biochem. 8, 314—324
25 Bechet, J.J. and Yon, J. (1964) Biochim. Biophys. Acta 89, 117—126
26 Vandermeers, A., Vandermeers-Piret, M.C., Rathe, J. and Christophe, J. (1973) Arch. Biochem. Biophys. 159, 492—496
27 Berezin, I.V., Savin Yu, V. and Martinek, K. (1971) FEBS Lett. 14, 178—180
28 Hinman, L.M., Coan, C.R. and Deranleau, D.A. (1976) Biochemistry 15, 2212—2219
29 Koshland, Jr., D.E., Yankeelov, Jr., J.A. and Thoma, J.A. (1962) Fed. Proc. 21, 1031—1038
30 Lazdunksi, M. (1964) Thèse de Doctorat Es-Sciences Physiques, Université Aix Marseille, France