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Carbonic anhydrase activators: Activation of the human tumor-associated isozymes IX and XII with amino acids and amines

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Abstract—The first activation study of the human carbonic anhydrase (hCA, EC 4.2.1.1) isoforms associated to tumors, hCA IX and XII, with a small library of natural and non-natural amino acids as well as aromatic/heterocyclic amines is reported. hCA IX was activated efficiently by dopamine, adrenaline and heterocyclic amines possessing aminoethyl-/aminomethyl-moieties (K_As of 9 nM–1.07 μM), whereas the best hCA XII activators were serotonin, L-adrenaline, 4-(2-aminoethyl)-morpholine and D-Phe (K_A of 0.24–0.41 μM). Precise steric and electronic requirements are needed to be present in the molecules of effective hCA IX/hCA XII activators, in order to assure an adequate fit within the enzyme active site cavity for the formation of the enzyme–activator complex, and for an efficient proton transfer process within this complex, leading to the release of a proton and formation of the catalytically active, zinc-hydroxide species of the enzyme. Selective activation of these CA isoforms might be useful to develop pharmacologic tools or to understand whether some of these biogenic amines/amino acids may influence the progression of tumors over-expressing CA IX and/or CA XII.

1. Introduction

In previous work from this group we reported that many of the 13 catalytically active carbonic anhydrase (CA, EC 4.2.1.1) isozymes described so far in mammals, are subject to activation processes by several classes of compounds, among which the amines and amino acid derivatives are the most investigated ones.[1–7] Such phenomena started to be understood in detail only after our report of X-ray crystal structures of adducts of the ubiquitous human isoforms hCA I and hCA II with histamine, L- and D-histidine, L- and D-phenylalanine or L-adrenaline, among such investigated activators.[1,4,5] Thus, insights of the activation mechanisms of many mammalian isoforms at the molecular level are now available, being proven that CA activators (CAAs),[2,3] similarly with the pharmacologically relevant CA inhibitors (CAIs)[8–10] intervene in the catalytic cycle of the enzyme, at different stages of the catalytic turnover. Whereas inhibitors generally bind to the catalytic Zn(II) ion displacing the fourth zinc ligand, a hydroxide ion/water molecule acting as nucleophile in the catalytic cycle, activators bind at the entrance of the active site cavity, in a region in which the proton shuttle residue His64 is placed, facilitating the rate-determining step of the CA catalytic cycle.[1–5] In most CAs, this step is the proton transfer reaction between the zinc-coordinated water molecule and the reaction medium.[1–3] CAAs thus supplement the proton-shuttling capacity of His64 (or other amino acid residues playing this role in isoforms different from CA II, IV, VI, VII, IX, etc.) and lead to a supplementary formation of the nucleophilic (zinc hydroxide) species of the enzyme.[1–7] Indeed, many typical CAAs are able to increase k_{cat} (without having an effect on K_M) for the physiologic reaction (CO_2 hydration to bicarbonate and protons) even ten-fold as compared to the rate of the enzyme in the absence of endogenous activators,[1–6,10] leading thus to a drastic enhancement of the catalytic power of these enzymes,

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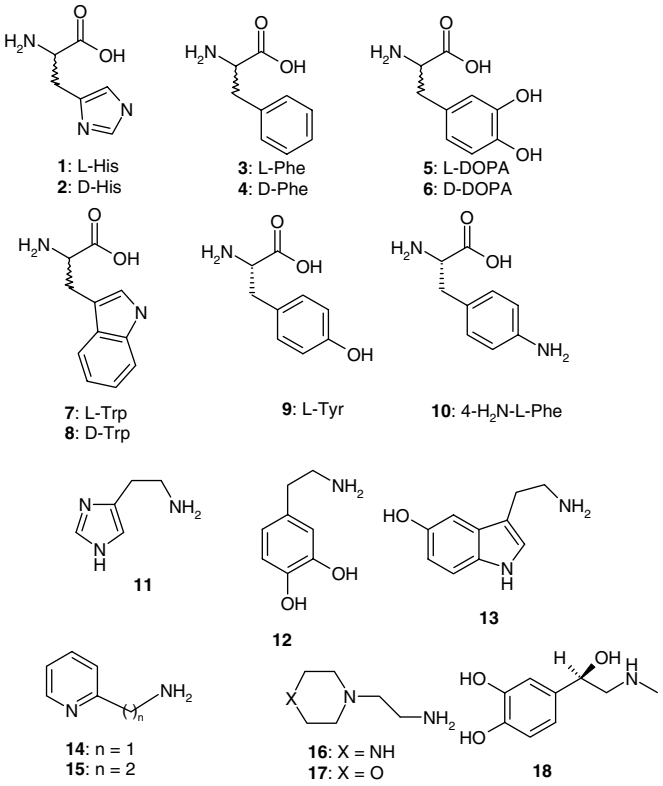
some of which are already among the most efficient catalysts that nature has 'designed' (hCA II is one of the fastest enzymes known until now).[1,3,8]

Among the 16 CA isoforms characterized so far in mammals (13 are catalytically active and 3 are devoid of activity as they miss one or two histidines acting as Zn(II) ligands),[11] CA I, CA II, CA IV, CA VA, CA VB, CA VI, CA VII, CA XIII and CA XIV were investigated for their activation by many classes of compounds, most of which belong to the aromatic/heterocyclic amines, amino acids and small oligopeptide classes.[1–7] Furthermore, X-ray crystal structures are available at high resolutions for complexes of hCA I and hCA II with activators such as histamine, D-/L-histidine, D-/L-phenylalanine and L-noradrenaline[1–7] which may be helpful for designing such isozyme-specific and tighter-binding derivatives, some of which may have pharmacological applications for the treatment of Alzheimer's disease, for enhancing synaptic efficiency or for the memory therapy.[12,13] However, although the in vitro CA activation of many CA isoforms is understood in detail, there are very few in vivo studies[12,13] investigating these phenomena. On the other hand, no CA activation data of the tumor-associated,[14,15] highly overexpressed by hypoxia isoforms CA IX and XII are available at this moment, and this is the first study reporting the activation of these two isozymes with a series of amines and amino acid derivatives.

2. Results and discussion

The discovery that isoform CA IX, a transmembrane tumor-associated protein,[16] was prevalently expressed in several human cancer cells and not in their normal counterparts[17–21] suggested a role for CA in oncogenesis.[14,15] Several studies showed then a clear-cut relationship between high CA IX levels in tumors and a poor prognosis of patients harbouring this genotype.[14–21] CA IX was also shown to act on cells adhesion and differentiation by its N-terminal proteoglycan related-region which is absent in other transmembrane CA isozymes, such as CA XII (which is also present in tumors)[14,15] and CA XIV (which is not associated with tumors).[8–10] The level of CA IX, which efficiently catalyzes CO_2 hydration to bicarbonate with release of a proton,[14,15] is strongly increased by tumor hypoxia via a direct transcriptional activation of the *CA9* gene by the hypoxia-inducible factor type 1 (HIF-1).[22,23] Furthermore, CA IX is negatively regulated by von Hippel Lindau (VHL) tumor suppressor protein, and its expression in renal cell carcinomas is related to inactivating mutation of the VHL gene.[23,24] CA IX was also shown to serve as a marker of tumor hypoxia and its predictive and prognostic potential has been demonstrated in a number of clinical studies.[14–25]

The high catalytic activity of CA IX and CA XII isozymes leading to formation of protons by the hydration of CO_2 was then demonstrated to be critical for the tumor microenvironment acidification processes, by maintaining the extracellular acidic pHe, and thus contributing to tumor propagation and malignant progression.[26] Indeed, Svastova et al.[26] showed that the acidic extracellular pHe of tumor microenvironment, is mainly generated by the activity of the tumor-associated isozymes, that is, CA IX and probably also CA XII, and that this acidification can be perturbed by deletion of the enzyme active site and inhibited by CA-selective inhibitors of the sulfonamide type, which bind only to hypoxic cells containing the active enzyme.[26] As a consequence, targeting the tumor microenvironment via CA IX inhibition constitutes an attractive new approach for the management of hypoxic tumors.[26–28] The potential use of CA inhibitors as antitumor agents opens thus a new important research direction,[26–28] but no investigations regarding the possible activation processes of these enzymes by many biogenic amines or amino acids present in high enough concentrations in the human body are available in the literature up to now. In fact, considering the important role of CA IX (and presumably also CA XII) in the acidification of tumors, an enhancement of their activity by CAAs may lead to a supplementary production of H^+ ions, which may contribute to the progression of the tumor.[15] For better understanding whether CA IX and XII, similarly to other CA isoforms are activated by such compounds, we investigated here the in vitro activating effects of L-/D-amino acids and amines **1**–**18**on the purified, recombinant catalytic domain of CA IX and XII.



Kinetic experiments for the physiological reaction (carbon dioxide hydration to bicarbonate and a proton)[29] (Table 1) showed that as for hCA I and II,[1–7] activators of the amino acid or amine type enhance k_{cat} of the enzyme, with no effect on K_M. Data of Table 1 show that

Table 1. Kinetic parameters for the activation of hCA isozymes I, II, IX and XII with L- and D-Phe, at 25 °C and pH 7.5, for the CO_2 hydration reaction[29]

Isozyme	$(k_{cat})^a$ (s^{-1})	$(k_{cat})_{L\text{-Phe}}{}^b$ (s^{-1})	$(k_{cat})_{D\text{-Phe}}{}^b$ (s^{-1})	$K_A{}^c$ (μM)	
				L-Phe	D-Phe
hCA I[d]	2.0×10^5	19.8×10^5	2.3×10^5	0.07	86
hCA II[d]	1.4×10^6	5.7×10^6	5.2×10^6	0.013	0.035
hCA IX[e]	3.8×10^5	4.7×10^5	6.1×10^5	16.3	9.30
hCA XII[e]	4.2×10^5	12.4×10^5	16.7×10^5	1.38	0.37

[a] Observed catalytic rate without activator. K_M values in the presence and the absence of activators were the same for the various CA isozymes (data not shown).

[b] Observed catalytic rate in the presence of 10 μM activator.

[c] The activation constant (K_A) for each isozyme was obtained as described earlier,[7] and represents the mean from at least three determinations by a stopped-flow CO_2 hydrase assay method.[7] Standard errors were in the range of 5–10% of the reported values.

[d] Human recombinant isozymes.

[e] Human catalytic domain recombinant isozyme.[26,27]

L- and D-Phe activate all investigated isoforms for the CO_2 hydration, but in a very different manner. It must be stressed that the activators had no influence on the K_M values (data not shown), as the Michaelis–Menten constants were identical with or without activator, but a very strong influence has been observed on k_{cat} (Table 1). This parameter is generally greatly enhanced in the presence of activators, proving that it is just the rate-determining step, that is, the proton transfer reactions between the active site and the reaction medium, which is favored by CAAs, as already proved in our first CA activation study on histamine.[1–7] For example, in the case of CA IX, the k_{cat} in the absence of activator (of 3.8×10^5 s^{-1}) becomes 6.1×10^5 s^{-1} in the presence of 10 μM of D-Phe, whereas the L-enantiomer has a weaker activating effect (k_{cat} of 4.7×10^5 s^{-1}). CA XII is on the other hand much more activatable by the same concentration of L- and D-Phe, with a k_{cat} which is enhanced from 4.2×10^5 s^{-1} (in the absence of CAAs) to 12.4–16.7×10^5 s^{-1} (Table 1). Thus, the activation mechanism of the newly investigated isoforms hCA IX and XII seems to be identical to that of the better-studied isoforms hCA I and II, for which detailed X-ray crystallographic studies of enzyme–activator adducts are available.[1–7] This is after all not so surprising, since, as shown in Figure 1, where an alignment of the amino acid residues of isoforms I, II, IX and XII is presented, many active site residues critical for the catalytic cycle (represented by a combination of z, + and * signs) are identical for these 4 isoforms. These include among others: (i) the zinc(II) ion-coordinating residues His94, 96 and 119 (hCA I numbering system); (ii) the proton shuttle (His64); (iii) the gate-keeping residues Thr199 and Glu106 which orient the substrate molecule (CO_2) prop-

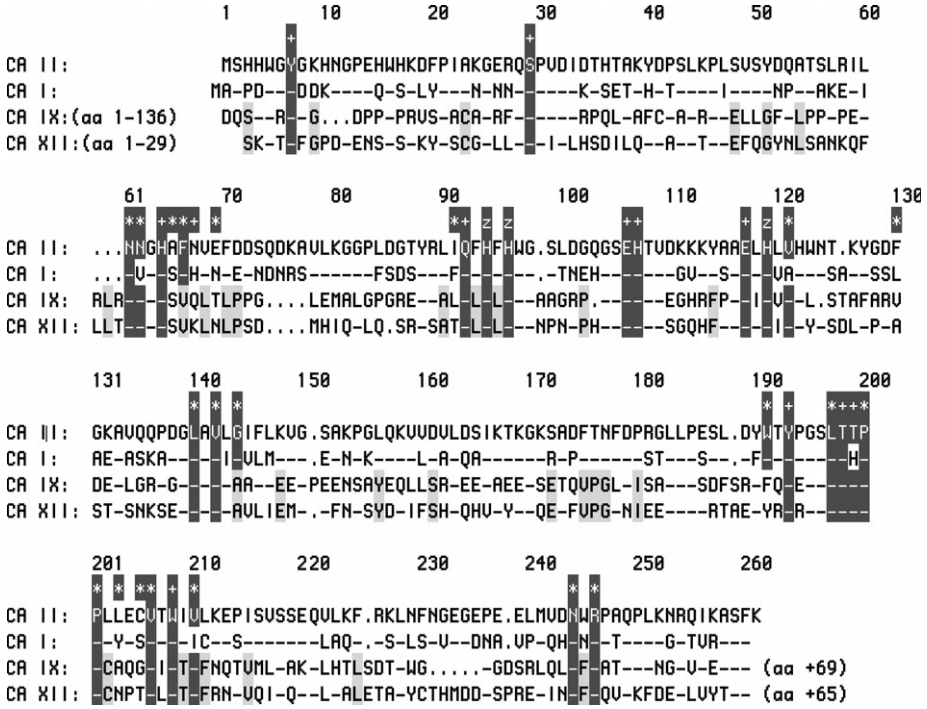


Figure 1. Alignment of the human CA II, I (cytosolic isoforms) with the CA IX and XII (transmembrane isoforms) sequences. Residues involved in the active site architecture are represented by a combination of z, + and * signs (hCA II numbering). Dark gray bars represent conserved amino acid residues participating to the active site architecture. Light gray bars show amino acid residues commonly used in CA IX and XII, but not in CA I and II (indicating amino acid residues, relatively specific to the transmembrane isoforms).

Table 2. Activation constants of hCA I/hCA II (cytosolic isozymes), as well as hCA IX/hCA XII (transmembrane, tumor-associated isoforms) with amino acids and amines **1**–**18**

No.	Compound	K_A[a] (μM)			
		hCA I[b]	hCA II[b]	hCA IX[c]	hCA XII[c]
1	L-His	0.03	10.9	9.71	37.5
2	D-His	0.09	43	12.5	24.7
3	L-Phe	0.07	0.013	16.3	1.38
4	D-Phe	86	0.035	9.30	0.37
5	L-DOPA	3.1	11.4	51.3	1.67
6	D-DOPA	4.9	7.8	54.7	0.89
7	L-Trp	44	27	37.5	26.0
8	D-Trp	41	12	43.6	28.1
9	L-Tyr	0.02	0.011	25.3	25.8
10	4-H_2N-L-Phe	0.24	0.15	48.7	1.09
11	Histamine	2.1	125	35.1	27.9
12	Dopamine	13.5	9.2	0.92	0.67
13	Serotonin	45	50	33.1	0.30
14	2-Pyridyl-methylamine	26	34	1.07	41.5
15	2-(2-Aminoethyl)pyridine	13	15	0.013	0.69
16	1-(2-Aminoethyl)-piperazine	7.4	2.3	0.009	48.3
17	4-(2-Aminoethyl)-morpholine	0.14	0.19	0.43	0.24
18	L-Adrenaline	0.09	96	0.87	0.41

Data for hCA I and II activation with these compounds are from Ref. 7.
[a] Mean from three determinations by a stopped-flow, CO_2 hydrase method.[29] Standard errors were in the range of 5–10% of the reported values.
[b] Human recombinant isozymes, stopped-flow CO_2 hydrase assay method.[29]
[c] Catalytic domain, human recombinant enzyme, stopped-flow CO_2 hydrase assay method.[29]

erly for the nucleophilic attack,[1,8–10] etc. Obviously, there are also many differences in the active site architecture and amino acid composition of these isoforms, which clearly explain their different catalytic activity, affinity for inhibitors and activators (see later in the text) (Fig. 1).

The data of Table 2 show that as for the ubiquitous cytosolic isozymes hCA I and II investigated earlier,[1–7] hCA IX and XII are also activated by amino acids and amines **1**–**18** in a quite distinct manner, leading thus to activation profiles typical for these two isozymes and distinct of those observed for other cytosolic, mitochondrial or membrane-associated CAs investigated earlier.[1–7] Thus, for hCA IX, the following SAR was observed for the activation with this small series of amino acids and amines: (i) a first group of predominantly amino acids, such as **2**, **3**, **5**–**10**, but also the amines **11** and **13**, showed quite weak CA IX inhibitory properties, with activation constants in the range of 12.5–48.7 μM. It seems that the enantiomeric form of the amino acid or the type of derivative (amine or amino acid, respectively) does not greatly influence the CA IX activating properties of these compounds. For example, both the L- and D-enantiomers of DOPA (or the corresponding enantiomers of L-Trp) showed a rather similar activity. Also, the amine serotonin or the amino acids L-/D-Trp possessing the structurally related indole ring (with slightly different substitution patterns, however), showed rather comparable activity; (ii) two other derivatives, for example, L-His **1** and D-Phe **4**, acted as medium potency CAAs for CA IX, with activation constants in the range of 9.30–9.71 μM. These data clearly demonstrate that both the L- as well as the D-enantiomer of some aromatic amino acids can act as efficient CAAs, a situation we also proved for CA I and II, cases in which the X-ray crystal structures of adducts of such enantiomeric pairs of amino acids showed a very different binding within the enzyme active site;[1–7] (iii) the best CA IX activators were the amines **12** and **14**–**18**, which showed activation constants in the range of 9 nM–1.07 μM (Table 2). Several comments must be added here: compound **14** and the two catecholamines **12** and **18** showed quite similar CA IX activating properties (K_As around 1 μM), whereas the heterocyclic amines possessing an aminoethyl moiety of type **15**–**17** presented much better such properties, with activation constants in the low-nanomolar range for **15** and **16** (K_As of 9–13 nM). The difference of CA IX activating properties between dopamine **12** and the structurally related amino acids from which it can be formed by decarboxylation, L-/D-DOPA **5** and **6** are very high, with the amine being a 55.7–59.4 times better activator as compared to the amino acids.

CA XII was also prone to be activated by these compounds, with an activation profile totally different from that of CA IX. Thus, a first group of compounds, including **1**, **2**, **7**–**9**, **11**, **14** and **16**, showed weak CA XII activating properties, with activation constants in the range of 24.7–48.3 μM. It should be observed that again the stereochemistry of enantiomeric pairs of amino acids, such as **1** and **2** or **7** and **8**, respectively, is irrelevant to the activating power of these compounds. Both amino acids as well as amines are found among these weak CAAs, similarly to the case for the CA IX (as well as CA I and II) activation phenomena discussed earlier. As for the CA IX activators, again L-/D-His (**1** and **2**) and the corresponding decarboxylated compound, histamine **11**, showed similar, weak activities. However,

dopamine **12** and its carboxylated precursors, L-/D-DOPA (**5** and **6**) showed potent CA XII activating properties, similarly with several other such compounds **3**, **4**, **10**, **13**, **15**, **17** and **18**. These compounds possessed activation constants in the range of 0.24–1.67 μM (Table 2). What is striking as SAR in this small library of compounds is the net variation of activity even for rather small structural changes for congeneric compounds. For example, L-Phe **3** is a good CA XII activator (K_A of 1.38 μM) whereas the incorporation of a 4-hydroxy moiety in its phenyl ring, such as in L-Tyr **9**, leads to a drastic diminution of the CA XII activating properties (K_A of 25.8 μM). However, a second hydroxyl group in the same ring, such as in L-DOPA **5**, restores the good CA activating properties (K_A of 1.67 μM). The differences between the congeneric amines **14** and **15**, differing just by one methylene group, are also dramatic, with **15** being a 60-times better CA XII activator as compared to **14**. This is obviously due to different favorable or unfavorable interactions with amino acid residues in which these compounds participate when bound within the enzyme cavity in order to enhance the proton transfer processes between the active site and the environment, as shown by X-ray crystallography for adducts of CA I and II with various such CAAs.[1–7]

A last aspect deals with the activation profile of the two transmembrane, tumor-associated isozymes CA IX and XII as compared to that of the cytosolic ones CA I and II (Table 2). It may be observed that every one of these 4 isoforms shows a rather distinct activation pattern by each investigated activator. However, CA IX seems to be less prone to be activated by amino acid derivatives and more activatable by amines, whereas CA XII shows a rather mixed behavior, with some amines and amino acids both acting as efficient CAAs. Thus, L- and D-His, for example, may be considered as quite selective CA I activators, whereas their affinities for CA II, IX and XII is two orders of magnitude lower. A CA II—selective activator may be considered to be D-DOPA, with an affinity of 35 nM for CA II and 9.30 μM for CA IX, 86 μM for CA I and 0.37 μM for CA XII (there is, however, not much discrimination between CA II and XII). The most selective CA IX activators in the library of investigated derivatives are **15** and **16**, which possess a low nanomolar affinity for this isozyme, and much higher activation constants for the other isoforms (Table 2). Finally, the most CA XII selective compound was serotonin, with a good affinity for CA XII (K_A of 0.30 μM) whereas its affinity for the other isoforms was in the range of 33.1–50 μM (Table 2).

Many of the investigated compounds such as the amino acids L-His, L-Phe, L-DOPA, L-Trp, L-Tyr, or the amines histamine, dopamine, serotonin or L-adrenaline are present in high enough concentration in various human tissues,[30,31] and might thus act as endogenous ligands for some of the CA isozymes investigated there, leading thus to a notable enhancement of their catalytic properties. Undoubtedly, this probably has important yet unravelled physiologic consequences but might be also useful to develop pharmacological agents based on CAAs, a field not much investigated up to now.[12,13]

3. Conclusion

In conclusion, we report here the first hCA IX and XII activation study with a small library of natural and non-natural amino acids as well as aromatic/heterocyclic amines. hCA IX was activated efficiently by dopamine, adrenaline and heterocyclic amines possessing aminoethyl-/aminomethyl moieties (K_As of 9 nM–1.07 μM), whereas the best hCA XII activators were serotonin, L-adrenaline, 4-(2-aminoethyl)-morpholine and D-Phe (K_A of 0.24–0.41 μM). Precise steric and electronic requirements are needed to be present in the molecule of effective hCA IX/hCA XII activators, in order to assure an adequate fit within the enzyme active site cavity for the formation of the enzyme–activator complex, and for an efficient proton transfer process within this complex, leading to the release of a proton and formation of the catalytically active, zinc-hydroxide species of the enzyme. Selective activation of these brain CA isoforms may be useful to develop pharmacologic tools or to understand whether some of these biogenic amines/amino acids may influence the progression of tumors overexpressing CA IX and/or CA XII.

4. Experimental

4.1. Chemistry

L-/D-Amino acids and amines **1**–**18** investigated as hCA IX/XII activators were commercially available from Sigma–Aldrich (Milan, Italy) and were used without further purification. The recombinant CA isozymes (hCA I, II, IX and XII) have been obtained as described earlier.

4.2. CA assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyszd CO_2 hydration activity.[29] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and activation constants. For each activator at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of activators **1**–**18** (10 mM) were prepared in distilled-deionized water and dilutions up to 0.001 μM were done thereafter with distilled-deionized water. Activator and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–A complex. The activation constant (K_A), defined similarly with the inhibition constant K_I,[1–3,7] may be obtained by considering the classical Michaelis–Menten equa-

tion (Eq. 1), which has been fitted by non-linear least squares by using PRISM 3:

$$v = v_{max}/\{1 + K_M/[S](1 + [A]_f/K_A)\} \quad (1)$$

where $[A]_f$ is the free concentration of activator.

Working at substrate concentrations considerably lower than K_M ($[S] \ll K_M$), and considering that $[A]_f$ can be represented in the form of the total concentration of the enzyme ($[E]_t$) and activator ($[A]_t$), the obtained competitive steady-state equation for determining the activation constant is given by Eq. 2[7]:

$$v = v_0 \cdot K_A/\{K_A + ([A]_t - 0.5\{([A]_t + [E]_t + K_A) - ([A]_t + [E]_t + K_A)^2 - 4[A]_t \cdot [E]_t)^{1/2}\}\} \quad (2)$$

where v_0 represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator.[7]

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