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The inhibitory effect of various indolyl amino acid derivatives on arginase activity in macrophages

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Summary. Numerous indolyl amino acids and their derivatives inhibited arginase activity. The inhibition was found to be non-competitive, – at least partly – allosteric, and independent on manganese ions in the active site, and it cannot be explained by the dissociation of arginase homotrimers. Indole alone is weakly inhibitory; however, the presence of three-carbon side chains and their net charges is favorable for the inhibition. The binding of the inhibitory compounds caused only minor changes in the steric structure of arginase: a slight increase in α-helix content was detected by circular dichroism together with a decrease in parallel pleated sheet and β-turn sections. A slight alteration in the tertiary structure was also found using tryptophane fluorescence studies, but buried apolar side chains were not transposed to the protein surface. Computer studies that were performed did not provide additional structural information.

Keywords: Arginase – Indolyl amino acid derivatives – Inhibitors – Minor steric alterations – Nitric oxide synthase

Abbreviations: ANOVA, Analysis of Variances between groups; ANS, 8-anilino-1-naphthalenesulfonic acid; DMEM, Dulbecco Minimal Essential Medium; FBS, fetal bovine serum; NOS, nitric oxide synthase; SDS, sodium dodecyl sulfate

Introduction

L-Arginine is the substrate of two alternative metabolic pathways catalysed by arginase (E.C.3.5.3.1.) and nitric oxide (NO) synthase (E.C.1.14.13.39.) enzymes. The formed NO has various roles in different cell types; in macrophages it plays an important role in cytotoxic reactions against tumors, protozoan parasites, viruses and bacteria (MacMicking et al., 1997). In macrophages the Ca^{2+}-independent, cytokine-inducible NOS has been found and cloned (Xie et al., 1994). Macrophages also contain arginase (Currie, 1978), but its physiological function has not yet been clearly elucidated.

Arginase and NO synthase show characteristic differences in their affinities to L-arginine and in their sensitivity to various Arg-analogues as inhibitors (MacMicking et al., 1997). Arginase inhibitors, which are arginine analogues or various α-amino acids (Hrabák, 1994, 1996), were found to be more tightly fitted to the substrate (Hrabák, 1996). Several high affinity inhibitors discovered later (Custot et al., 1996, 1997; Hey, 1997) contain a hydroxyl group which can react with the double manganese cluster of the arginase active site (Kanyo et al., 1996). More recently, new, high affinity transition state analogue inhibitors belonging to ω-borono-α-amino acids were also discovered (Busnel et al., 2005). The description of human arginase I by fine resolution X-ray diffraction has also been performed by using these compounds (Di Costanzo et al., 2005). In another recent review, Christianson (2005) has stated that the best arginase inhibitors are those bearing N-hydroxyguanidinium or boronic acid ''warheads'' that can bridge the binuclear manganese cluster. By way of explanation, trigonal planar boronic acids undergo nucleophilic attack by hydroxide ions to form tetrahedral boronate anions mimicking the tetrahedral intermediate and its flanking transition states in the arginase mechanism. Amino acid aldehydes were also constructed on the basis of the same principle (Shin et al., 2004).

The two metabolic pathways are in reciprocal relationship with each other. This may be due to their different regulation by cytokines (Munder et al., 1998) and to the mutual inhibitory effect of their intermediates (Hrabák et al., 1996b).

In our previous studies (Hrabák et al., 1994, 1996a) we found that Arg-related inhibitors of NO synthases were selective for these enzymes: they were not inhibitors of arginase. The essential groups of the substrate and various

inhibitors in their binding to arginase were determined by using a computer-aided approximation. In these experiments we observed that amino acids of larger size could not be bound to NO synthase or arginase. Tryptophane was an exception which inhibited arginase in macrophages although its size was not suitable for binding to the active sites of the enzyme. On the basis of this observation, we carried out a systematic comparison of the effect of various aromatic amino acids and their derivatives on arginase, in order to find the explanation for their possible inhibitory effect. The results of these experiments are described in this paper.

Materials and methods

Chemicals

All chemicals were purchased from Sigma Hungary (Budapest). Bovine arginase was the product of Serva (Germany).

Isolation of peritoneal macrophages

Peritoneal macrophages were isolated from casein-elicited CFLP mice (30–35 g) purchased from Charles River Hungary (Gödöllö, Hungary) as described earlier (Hrabák, 1996a). Cells were cultured for 24 h at 37 °C in a 5% CO_2 atmosphere in RPMI containing 10% FBS and a mixture of antibiotics.

Arginase preparations

Arginase was partially purified from the supernatant of adhered mouse macrophages by heat treatment at 60 °C for 30 min in the presence of 1 mM Mn^{2+}-ions followed by ammonium sulfate precipitation (Schimke, 1970). The same purification steps were also used for commercial arginase (Serva, Germany). The purity of these preparations was tested by polyacrylamide gel electrophoresis without SDS, in 7% gels. After running, the gels were stained for 5 min with 1% coomassie brillant blue G 250 dissolved in 50% methanol-10% acetic acid and differentiated by washing thoroughly with 10% methanol-10% acetic acid. Gels were then dried and scanned for documentation.

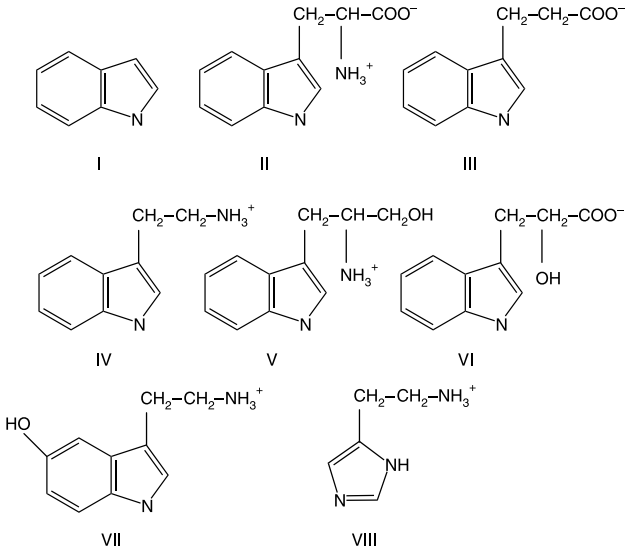
Chemical determinations and arginase measurements

Arginase activity was measured on the basis of urea release over the course of 30 min (Schimke, 1970). Briefly, 20 mM arginine substrate (pH set to 9.7), 0.5 mM $MnCl_2$ and the inhibitors were mixed in 250 µl and incubated at 37 °C. The reaction was stopped by adding 250 µl 1 N $HClO_4$; after centrifugation, an aliquot was tested for released urea according to Coulombe and Favreau (1963).

Studies on the possible dissociation of arginase into subunits

Arginase was gel filtrated on a Sephadex G 75 column (1.5 × 30 cm) in 50 mM Tris–HCl (pH = 8.5) containing 1 mM $MnCl_2$ both in the absence and in the presence of 10 mM tryptamine or L-tryptophane. Two millilitre fractions were collected and 50 µl aliquots diluted twofold were measured for protein at 590 nm by the coomassie blue method using an ELISA microplate reader (Bradford, 1976).

The dissociation of arginase has also been tested by Microcon centrifuge filter tubes (Millipore Inc., USA). The enzyme (0.5 mg/ml) was treated with 5 mM inhibitors (Scheme 1) for 30 min at 37 °C in the presence of 20 mM Arg substrate and 1 mM $MnCl_2$. Then the samples were filtered by centrifugation at 12,000 *g* for 12 min. Filtered solutions were then tested for protein content by the Coomassie blue method.



Scheme 1. Chemical structure of several indole and imidazole compounds tested for inhibition of arginase activity. I = Indole, II = L-tryptophane, III = indole-3-propionic acid, IV = tryptamine, V = tryptophol, VI = indole-3-L-lactic acid, VII = 5-hydroxy-tryptamine and VIII = histamine

Kinetic studies

Kinetic studies were performed on purified macrophage arginase and on bovine liver arginase (Serva, Heidelberg). The mechanism was tested in the presence of 0–100 mM Arg to determine K_M by double reciprocal plot, at various inhibitor concentrations. The determination of inhibitor constants (K_I) was carried out at 10 and 100 mM Arg using the Dixon plot.

Fluorescence analysis of arginase inhibition

Arginase contains 2 tryptophane residues per subunit; this makes it possible to observe their fluorescence as an indicator of conformational changes. Measurements were performed at 50 µg/ml arginase concentration, in the presence of 20 mM L-arginine substrate, 0.5 mM $MnCl_2$, at pH 9.7. Emission was measured between 300 and 500 nm; excitation wavelength was 285 nm. Indolyl compounds showed fluorescence themselves and a self-quenching effect of the indole backbone was also observed. The consideration of the latter effect is discussed in detail in the Results section.

8-Anilino-1-naphthalenesulfonic acid (ANS) is also used to test conformational changes in proteins (Li et al., 1998; Chaffotte et al., 1992). Excitation wavelength was 360 nm and emission was registered from 400 to 600 nm. The fluorescence of ANS (both emission spectra and the intensity) may be changed when bound to proteins, indicating the partial unfolding of the tertiary structure. This property was used to analyse the possible effects of indole and imidazole compounds on the arginase conformation.

Fluorescence was measured in both cases by a Varian KS spectrofluorimeter, using Cary Eclipse software.

Circular dichroism studies

Purified bovine liver arginase was studied in these experiments. 5 mg/ml arginase was treated with the following inhibitors: 5 mM L-tryptophane,

tryptamine, 5-hydroxy-L-tryptophane, indole-3-propionic acid, indole-3-lactate, 10 mM imidazole-3-lactate, 10 mM tyramine.

A Jasco J700 instrument was used for the measurements and the obtained results were converted finally into dic files and evaluated using the Dicroprot software (created by CNRS IBCP, Lyon, France, http://dicroprot-pbil.ibcp.fr). The evaluation of the curves was performed by using the least square and Varselec methods.

Data analysis

The significance of differences was evaluated by ANOVA, and by Mann–Whitney *t*-test when only two values were compared.

Results

The effect of various aromatic amino acids and derivatives on arginase activity

Amino acids and their derivatives were tested for their effect on the activity of mouse macrophage and liver arginases at 0.5–20 mM concentrations, and the IC_{50} values (concentration of the compounds causing 50% inhibition) of each effective molecule were determined. The bovine enzyme was found to be pure, without any considerable other protein contamination, while the mouse preparation contained mainly (~80%) arginase, though a small amount of other proteins was also detected (Fig. 1). The indole derivatives did not change this electrophoretic pattern at all. Significant inhibition of arginase was found by indole derivatives only: L- and D-tryptophane, 5-hydroxy-L-tryptophane, tryptamine, 5-hydroxy-tryptamine, tryptophol, indole-3-lactic acid and indole-3-propionic acid (Table 1). The IC_{50} values of indole and N-acetyl-5-hydroxy-tryptamine were over 20 mM. Arginase activity was not affected at all by imidazolyl derivatives in the form of L-histidine, histamine, imidazole-L-lactate or L-histidinol. In addition, inhibition was not observed using L-phenylalanine, L-tyrosine or other derivatives containing only a benzene ring. The effects of the compounds were very similar, whether the arginase was derived from macrophage or from bovine liver (Serva, Heidelberg, Table 1).

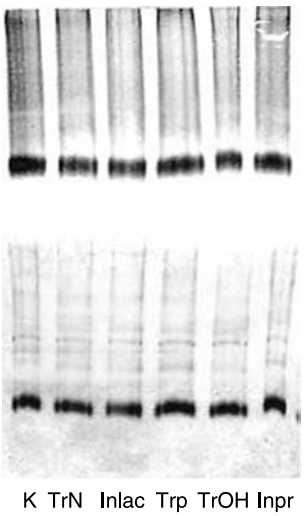


Fig. 1. Electrophoretic pattern of purified bovine and mouse macrophage arginase. Bovine arginase does not contain other detectable proteins (upper part), while mouse preparation contains mainly arginase, but a few other protein bands are also detected (below). Proteins were tested in 7% polyacrylamide gels without SDS and stained with 1% Coomassie brillant blue G 250, dissolved in methanol/acetic acid. *TrN* Tryptamine, *Trp* L-tryptophane, *Inlac* indole-3-lactic acid, *TrOH* L-tryptophol, *Inpr* indole-3-propionic acid

Table 1. Inhibitory effect of various aromatic amino acids on arginase

Compound added	IC_{50} mM for arginase	
	in bovine liver	in mouse macrophage
L-tryptophane	8.4	7.5
D-tryptophane	10.0	8.4
5-hydroxy-L-tryptophane	3.9	3.6
Tryptamine	3.2	3.4
5-hydroxy-tryptamine	3.3	3.6
Indole-propionic acid	2.8	2.8
N-acetyl-5-hydroxy-tryptamine	>20	N.D.
Indole-3-L-lactic acid	3.4	3.6
L-tryptophol	5.3	4.8
Indole	>20	>20
Imidazole-3-lactate	>20	>20
Tyramine	>20	>20

Arginase was directly measured in the presence of indolyl or other aromatic derivatives. IC_{50} was determined in experiments using a concentration range of 0.5–20 mM of effectors. *N.D.* Not determined

Kinetic experiments on arginase

The mechanism of the inhibition of arginase was tested by using effective representatives of indolyl derivatives. Figure 2 shows the effect of tryptamine, L-tryptophane and L-tryptophol. The inhibitory effect was tested at various arginine and constant inhibitor concentrations (5 mM) as well as at various inhibitor and constant arginine concentrations. Tryptamine caused a non-competitive inhibition (Fig. 2A) of arginase, similarly to L-tryptophane, tryptophol, indole-3-lactate and indole-3-propionic acid (the last two compounds are not shown). K_I values of the inhibitors were 3.1 mM for tryptamine (Fig. 2B) and 8.2 mM for L-tryptophane (not shown), respectively.

The independence of the inhibition on manganese

The possible chelating effect of indole derivatives on the essential Mn^{2+}-ions of arginase was also considered. To test this, 1–10 mM $MnCl_2$ was added prior to the addition

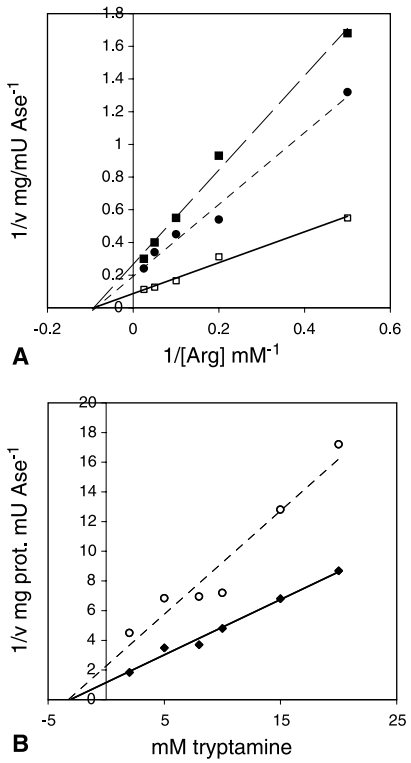


Fig. 2. Kinetic properties of the inhibitory effect of tryptamine and L-tryptophane on arginase. Tryptamine caused a noncompetitive inhibition as evidenced by double reciprocal plot (**A**), □ non-inhibited reaction, ■ reaction inhibited with 5 mM tryptamine, ◆ 10 mM L-tryptophane. According to the Dixon plot (**B**), the K_I of tryptamine is 3.1 mM. The Dixon plot supports the inhibitory mechanisms proposed by double reciprocal plots. ○, 10 mM L-arginine, ◆ 100 mM L-arginine used as substrate for arginase determination

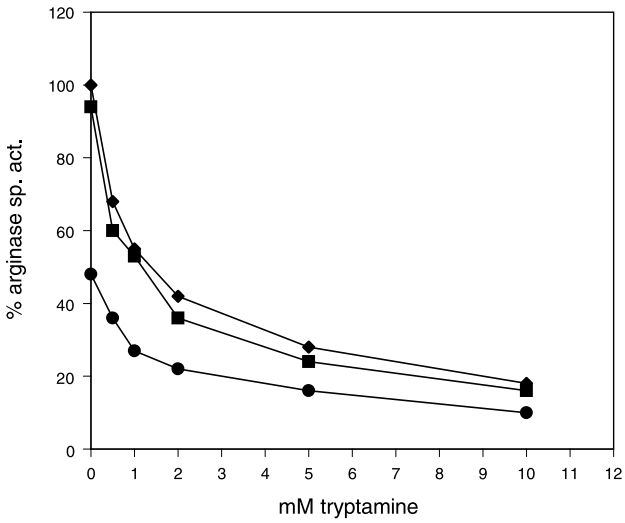


Fig. 3. The effect of tryptamine on arginase activity cannot be reversed by an increase of Mn^{2+}-concentration. 5 μg/ml final arginase and 20 mM L-arginine were used, tryptamine-concentration varied. ◆ Control (0.5 mM Mn^{2+} final concentration), ■ 2 mM Mn^{2+} final concentration, ● 5 mM Mn^{2+} final concentration

of the inhibitory compounds. Arginase activity could not be preserved by adding manganese; on the contrary, excessively added Mn^{2+}-ions caused a further decrease in enzyme activity (Fig. 3).

Combined effect of arginase inhibitors

In order to test the independence of the target points of the inhibitors, we tested their combined effect on arginase. In this experiment we used 5 mM L-valine as a previously known inhibitor and 5–10 mM of tryptamine, L-tryptophane, indole-3-propionic acid and histamine, respectively. The concentrations of indolyl inhibitors were chosen on the basis of their IC_{50} values, and histamine was used as an inefficient compound. The effect of the mixed inhibitors was more marked than the effect of the inhibitors alone, but they did not reach the sum of their individual effects (Fig 4). In addition, when both tryptamine (TrN) and L-tryptophane (Trp) were added together to arginase inhibited by valine (Val), their additional effect was not significantly different from the combination of Val + TrN or Val + Trp. These results suggested that although the indole compounds may be bound partially at the substrate binding site of the enzyme, an independent, probably allosteric site may also exist.

Indole derivatives did not cause the dissociation of arginase into subunits

Purified bovine liver arginase, which was affected in the same way by indole derivatives as macrophage arginase,

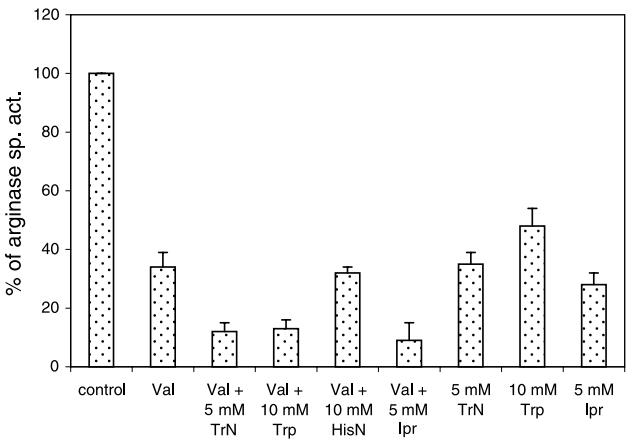


Fig. 4. The combined inhibitory effect of L-valine and some indoles on arginase activity. TN, 5 mM tryptamine, V, 5 mM L-valine, W, 5 mM L-tryptophane, HN, 10 mM histamine. Val and indole derivatives caused a summarized inhibition, suggesting the existence of distinct binding sites. Data ± S.E.M. are shown

was used to study the effect of tryptamine on the quaternary structure of arginase. Arginase was gel filtrated in 50 mM Tris–HCl (pH = 8.5) containing 1 mM $MnCl_2$ both in the absence and in the presence of 5 mM tryptamine and L-tryptophane. The presence of the two indole derivatives did not influence the gel filtration pattern (not shown).

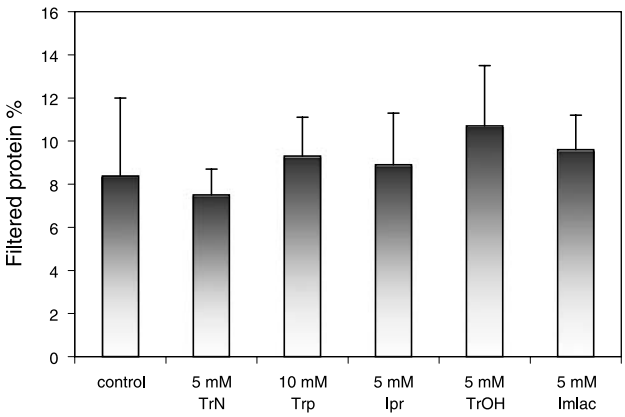


Fig. 5. The proportion of the filtered arginase protein. The amount of filtered arginase was measured by the coomassie blue method, after centrifuging arginase in the presence and absence of indole compounds, at 12,000 rpm for 12 min on YM-100 Microcon tubes. No significant differences were found. Data ± S.E.M. are shown. *TrN* Tryptamine, *Trp* L-tryptophane, *Ipr* indole-3-propionic acid, *TrOH* L-tryptophol, *Imlac* imidazole-3-lactic acid

The dissociation of arginase was also tested by Microcon centrifuge filter tubes. The percentage of filtered protein content is shown in Fig. 5. Protein retention is dependent on membrane type: YM-100 causes the exclusion of the proteins of 100 kD or higher molar mass from the filtrate. A small portion may penetrate, and for this reason, an inhibitor-free control sample was also filtered. None of the tested compounds caused the dissociation of arginase into subunits, supporting the gel filtration results (Fig. 5).

Fluorescence analysis of arginase inhibition

The effect on the position of tryptophane side chains

Going by the supposition that these compounds not related to arginine acted as allosteric effectors on arginase, a fluorescence analysis was carried out to study

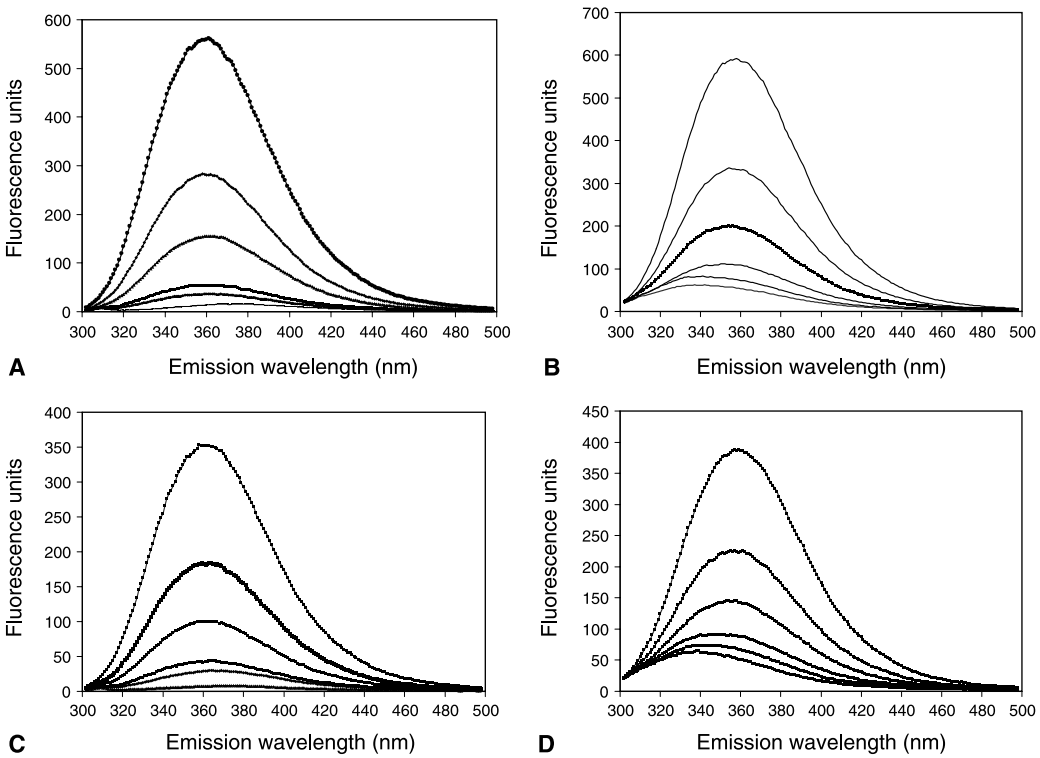


Fig. 6. Fluorescence analysis of the effect of tryptamine on arginase structure. The fluorescence of tryptamine at various concentrations between 100 nM and 2 μM, in the absence of arginase (**A**); when arginase is added, the fluorescence of tryptamine is added to that of arginase and a wavelength shift towards higher values was observed, indicating an interaction between the tryptophane side chains of arginase and tryptamine (**B**); the previous experiment using L-tryptophane as a ligand (**C**, **D**). Curves symbolize the indole concentrations of 0–100–200–500 nM–1–2 μM tryptamine (**A**, **B**) or L-tryptophane (**C**, **D**) upwards

the possible conformational changes. Arginase contains 2 tryptophane residues per subunit; this makes it possible to observe their fluorescence as an indicator of conformational changes. In the absence of indoles, arginase showed a maximum emission at 336 nm. However, in the case of indole compounds, the evaluation of this analysis is complicated by the fact that tryptamine and other indoles have their own fluorescence. In addition, during the measurement, a self-quenching effect of the indole backbone was observed, even in the absence of arginase; i.e. the addition of tryptamine over 10 μM concentration caused a decrease of fluorescence instead of an increase. In order to overcome this difficulty, we applied a method in which the relationship between fluorescence and tryptamine concentration was registered in the absence (Fig. 6A) and presence (Fig. 6B) of arginase. In Fig. 6A the fluorescence curves of 100 nM–2 μM tryptamine range are shown in the presence of 20 mM L-arginine substrate and 0.5 mM $MnCl_2$ at pH 9.7 (the optimal pH for arginase measurement). In Fig. 6B the same curves are shown after the addition of 50 μg/ml arginase. The figures show clearly that in the presence of the enzyme, an dose-dependent increase of fluorescence intensity by tryptamine was observed. In addition, tryptamine also caused a wavelength shift from the 341-nm maximum emission towards higher wavelengths, suggesting alterations in the tertiary structure. A similar tendency was observed with L-tryptophane, although lower fluorescence intensities were obtained. Although the self-quenching effect made it impossible to measure the direct effect of higher doses of tryptamine and other indoles on the tryptophane fluorescence of arginase, it can be supposed that higher tryptamine and indole concentrations may act similarly, even more markedly.

No effect on ANS fluorescence was observed

Indole compounds did not change the fluorescence intensity of protein-bound ANS. The binding of arginase caused a marked emission shift itself, while the effect of certain compounds was followed by the change of the fluorescence intensity. These results suggest that the inhibitory effect of indole derivatives on arginase activity may not be due to an ANS-detectable conformational change.

Minor changes were detected by circular dichroism studies

Circular dichroism is an established method to test the secondary structure of proteins. The effect of potent arginase inhibitors was tested at 10 mM concentrations and the results were evaluated by Dicroprot software. Least-square and Varselec methods were used for evaluation; the latter seems to be more realistic. The α-helix, β-pleated sheet and β-turn contents are summarized in Table 2. Two compounds, tryptamine and indole-3-lactate, markedly altered the distribution of secondary structure elements. The proportion of pleated sheet structures, mainly their antiparallel forms and β-turns, was markedly decreased, together with the increase in α-helical and other (random coil) structures. This result was obtained by both evaluation methods; nevertheless, the Varselec method seems to be more informative.

Table 2. The proportion of secondary structure elements in arginase as detected by circular dichroism experiments

Inhibitor 5 mM	α-helix	β-strand*	β-turn	random coil
Control	43	17	0	29
	40	12 + 8	18	29
Tryptamine	59	6	0	23
	50	1 + 8	9	33
L-tryptophane	43	14	0	32
	41	13 + 7	18	27
Indole-3-lactate	60	8	0	20
	55	1 + 8	10	27
Histamine	42	12	0	40
	39	11 + 7	18	25

The upper rows show the results of the least square method, lower rows that of Varselec method. * In Varselec method: antiparallel + parallel pleated sheets (may be distinguished)
All values are given in percentage (total values may slightly differ from 100%, due to the inherent insufficiency of evaluation methods of Dicroprot)

Discussion

In our previous papers we have described the effect of various amino acids and their derivatives on the activity of NO synthase and arginase enzymes specifically for the same substrate, L-arginine (Hrabák et al., 1994, 1996). In those studies, the structural features needed for binding to arginase were well approximated by computer programs. Numerous other studies proved that various hydroxy derivatives of arginine and other amino acids are more potent arginase inhibitors (Custot et al., 1996, 1997). The effect of these hydroxy compounds is based on the binding of hydroxy groups to the double manganese cluster of arginase involved in the active site. Similar effects were observed in a comparative study with boron-substituted amino acid analogues (Cama et al., 2004).

Although aromatic amino acids containing an abundant ring structure are generally not inhibitory to arginase, we found that L-tryptophane inhibits this enzyme. On the

basis of this observation, a series of experiments was carried out to clarify the requirements of this inhibition. Arginase is inhibited by various indolyl amino acid-related compounds without strong stereospecificity (both L- and D-tryptophane are inhibitory, Table 1). Free indole was also inhibitory to a lesser extent, suggesting its partial involvement in the inhibitory effect of D-tryptophane.

The mechanism of this inhibition of arginase was studied by kinetic methods. Because of the great number of possible inhibitory compounds, only some representatives of indole derivatives were tested in detail. Both tryptamine and L-tryptophane were non-competitive inhibitors of arginase, with a K_I of 3.1 and 8.2 mM, respectively (Fig. 2). Since the structures of these inhibitors are not related to arginine, and their size is not compatible with the binding properties previously determined by computer-aided methods (Hrabák et al., 1996), they were considered as possible allosteric effectors.

An experiment to exclude a possible chelating effect on the double manganese cluster was also carried out. Inhibitory compounds do not act on arginase by the chelation of Mn^{2+}-ions: the addition of an equivalent or higher dose of $MnCl_2$ did not prevent the inhibition of arginase by indole derivatives. On the contrary, higher Mn^{2+}-concentrations rather caused a decrease in arginase activity. Similar effects of other transient metals (e.g. cadmium) were described (Tormanen, 2006). The possible dissociating effect of indole derivatives was also excluded, either by gel filtration or by ultrafiltration experiments (Fig. 5). It is to be noted that arginase monomers containing certain mutations may also be more active at a lower level than the trimeric form (Lavulo et al., 2001).

The possible structural background of this allosteric effect of indoles on arginase was studied thoroughly. Tryptamine was studied by fluorescence methods. The wavelength shift caused by indoles suggests that these compounds may cause the change of the position of several tryptophane side chains. This effect was detected even at concentrations lower than those which were effective on the enzyme activity. Nevertheless, it is likely that this effect is similar, even more marked, at higher concentrations of indole derivatives, at which levels the fluorescence changes cannot be detected because of the complete self-quenching effect of tryptophane and other indolyl compounds above 10 μM concentration (Fig. 6).

In the case of tryptamine and L-tryptophane (and, likely, other indoles) only the tertiary/secondary structures are distorted by the inhibitor. The availability of indolyl side chains is modified (shown by fluorescence change), but the enzyme is not dissociated into subunits (see above). Therefore, the change in the tertiary structure is not accompanied by a similar change in the quaternary structure. The second fluorescence study, based on the measurement by ANS, did not show significant changes. This result suggests that the position of apolar side chains is not altered markedly and they are not available at the protein surface.

Finally, a circular dichroism study was also performed to approximate the steric changes: tryptamine, L-tryptophane, indole-3-lactic acid and indole-3-propionic acid were investigated and histamine and tyramine, as other non-indole derivatives, were also used for comparison. Minor, but significant, alterations in secondary structure were observed with indole derivatives, while no changes were found using others (Table 2). The very weak effect of L-tryptophane compared with other indoles may have been due to its 5 mM concentration, which was below its IC_{50} value. The α-helix content of arginase was similar to that based on X-ray diffraction data (i.e. near to 40%), according to the protein data bank (SwissProt, http://ca.expasy.org/cgi-bin/sprot-search-de?arginase). In addition, this proportion is not significantly different either in arginase I proteins of different mammalian species or between arginase I and II of the same species (e.g. 85% homology can be detected between bovine and mouse arginase I sequences and more marked similarities can be observed in their helix content). According to these minor changes, the inhibitor binding decreases the proportion of antiparallel pleated sheet and β-turn elements with an increase of α-helix and random coil contents. These changes may induce the minor changes in the tertiary structures detected by tryptophane fluorescence (Fig. 6) which may be responsible – at least partly – for the inhibitory effect.

We also investigated the possible common target of indole derivatives and other previously described arginase inhibitors (e.g. N-hydroxy-L-arginine and L-valine). The effect of the mixed inhibitors was more marked than that of the inhibitors alone, but they did not reach the sum of their individual effects (Fig. 3). In addition, when both tryptamine and L-tryptophane were added together to arginase inhibited by valine, their additional effect (Val + TrN + Trp) was not significantly different from the combination of Val + TrN or Val + Trp. These results suggest the existence of an independent, probably allosteric, site for indole derivatives.

Summarizing these results, indole derivatives of amino acids are non-competitive, at least partly allosteric inhibitors of arginase. The inhibitory effect of these compounds may be due to minor, but detectable, alterations

in the secondary and tertiary structures of arginase, without causing the dissociation of homotrimers into subunits. The inhibitors do not influence the position of Mn^{2+}-ions bound strongly to arginase. The requirements of the inhibition cannot be defined clearly. However, although the indole ring alone is weakly inhibitory, a side chain at position 3 increases its effect. L-Alanine alone is not inhibitory (Hrabák, 1996), but the involvement of a charged side chain is also favorable for the inhibition (see higher IC_{50} value of tryptophane, which has no net charge). Although a computer-aided approximation was also performed for the identification of the important characteristics, this was not successful: significant requirements of the indole binding could not be defined (data not shown). Other non-indolyl cyclic amino acids, such as histidine, tyrosine, phenylalanine and their analogous derivatives, did not cause inhibition on arginase at all.

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