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STRUCTURE-ACTIVITY RELATIONSHIP OF NITRIC OXIDE SYNTHASE INHIBITORS: A THEORETICAL APPROACH

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Abstract - Nitric oxide (NO) has become an important intracellular and intercellular signal molecule and inhibition of the enzyme which produces NO (NOS, NO synthase) become a major goal for pharmacological researchers. We performed a complete search for the lowest-energy conformations at the AM1 calculation level, for zwitter-ionic species of NOS inhibitors, analogs to L-Arg. The lowest- energy conformations obtained were fully optimized at the *ab initio* theory levels: HF/3-21G and HF/6-31G*. L-NNA, L-NMA and L-CPA exhibited a conformational behavior quite comparable to that of L-Arg. L-NIL, L-NIO and L-NAME achieved to completely different conformations when compared to L-Arg. L-NIL, highly selective for the inducible isoform of NOS, exhibited conformational as well as charge distribution differences compared to L-Arg. L-NAME and L-NNA are highly selective compounds for the constitutive isoforms. Both compounds share the chain lengths of L-Arg and bear a nitro-substituent over the guanidinium group, which causes changes on the net atomic charges of the N-guanidinium atoms. Moreover, differences were observed on net atomic charges and density distribution analyzed by means of molecular electrostatic potentials (MEPs). These differences might be of great importance at determining the selectivity of the different inhibitors. On the basis of the conformational and molecular properties of the different NOS inhibitors and the selectivity of the analogs studied, we propose the requirements for the different NOS isoforms.

Keys words: NO synthase, substrate analogs, selective inhibitors, molecular properties

INTRODUCTION

Nitric oxide (NO) has become an important intracellular and intercellular signal molecule and inhibition of the enzyme which produces NO (NO synthase, NOS) become a major goal for pharmacological researchers. Nitric oxide synthase (NO synthase, NOS) is the enzyme that catalyses the five-electron oxidation of L-Arg to produce nitric oxide (NO) and citrulline (L-Cit) (1,3,4,9,10,13,14). L-Arg is the substrate of several NOS isoenzymes, which play a key role in many physiological cellular processes. A number of L-Arg analogs have been developed in the search of NOS inhibitors.

NO synthase constitutes a family of proteins: eNOS (endothelial NOS), nNOS (neuronal NOS) and iNOS (inducible) (5,7,16). All these enzymes share a common sequence with the cytocrome P450 reductase at the C-terminal region. The oxidation consists of two sequential, mechanistically distinct, heme-based oxidation, involving a five-electron oxidation of L-Arg to L-Cit and NO. Endogenous NO is synthesized from one of the two N-guanidinium atoms of L-Arg by the enzyme NO synthase (NOS) (16,20). One of the first experimental evidences for the reaction mechanism, provided by Stuher *et al*. (25), postulated that NO synthesis is initiated by N-hydroxylation of L-Arg. Radiolabelled analogs provided insights into the reaction mechanism. Consisting with this mechanism, the first mono-oxygenated product, N^G-hydroxy-L-Arg (L-NHA) has been shown to undergo oxidation exclusively at the hydroxy-bearing guanidino nitrogen (16,20,25).

Recently, the oxidant region of the enzyme (NOS_{ox}) has been crystallized, alone or in combination with the substrate, L-Arg, the product analog thiocitrulline or the intermediate L-NHA (5,6,7,8). The crystal data suggest that the active site

Abbreviations: **L-CPA**: N^G-cyclopropil-L-arginina; **L-NAME**: N^G-nitro-L-arginina metil ester; **L-NHA**: N^G-hydroxi-L-arginina; **L-NIL**: L-N^6-(1-iminoetil) lisina; **L-NIO**: N-(iminoetil)-L-ornitina; **L-NMA**: N^G-metiyl-L-arginina; **L-NNA**: N^G-nitro-L-arginina; **MEP**: molecular electrostatic potentials; **NO**: nitric oxide; **NOS**: nitric oxide synthase; **SAR**:

Fig. 1 Structure, atom numbering and dihedral angles studied for the species III of the different molecules investigated.

does not suffer any conformational change when interacting with L-Arg of L-NHA, meaning that the substrate should adopt the most adequate spatial disposition to interact at the enzyme active site.

Structural analogs of L-Arg are inhibitors of the different NO synthase isoforms. For a selective treatment of some pathophysiologies, a selective inhibition of the inducible isoform is absolutely necessary. Other pathologies, such as severe hypertension could be treated by inhibition of the constitutive endothelial isoform. We selected for the presents study a number of inhibitors with selectivity for different NOS isoforms (2,12,15,16,17,19). L-NIL is a highly selective inhibitor of the inducible isoform, L-NNA and L-NAME are selective and with high activity for the constitutive isoforms. L-NIO is a non-selective inhibitor while L-NMA, L-CPA selective inhibitors with low activity for the constitutive isoform.

In previous papers (21,22) we modeled and performed a conformational and thermodynamic study of the reaction of formation of NO starting from L-Arg. In order to model this reaction, we used N-methyl guanidine as a model compound for L-Arg to study the mechanism of oxidative release of NO by means of *ab initio* molecular computations (21,22). Intermediate species (A to F) were proposed and the thermodynamic feasibility of the reaction was studied at different theory levels.

The conformational behavior and molecular parameters of L-Arg and L-NHA were fully explored by computational methods (17) and we found a good agreement with crystal experimental data (5,8). This observation also suggests that *in vacuo* calculations might be a good representation of the crystal conformation.

On the present study, we undertook a complete conformational and molecular analysis of NOS inhibitors and compared their properties with those of L-Arg in the search of a structure-activity relationship for NOS inhibitors as well as the requirements of the enzyme at the active site.

MATERIALS AND METHODS

We performed a complete comparative analysis of the conformational and molecular properties of L-Arg and several NOS inhibitors, at the two zwitter-ionic species named as species III and IV.

L-Arg can exhibit ionization not only at the amino-carboxylic groups, but also at the guanidinium portion. Thus, four different species could be available for L-Arg. Species I is a completely neutral one; species II bears a charge on the guanidinium group; species III is the zwitter-ion with a charge on the guanidinium group, fully charged and species IV is a zwitter-ionic species with no charge on the guanidinium group. Species III is the thermodynamically more stable species and thus, has the highest probability to be present in aqueous solution under physiological conditions and in the environment of the enzyme active site (7). For all the compounds studied, species III and IV were analyzed. Fig. 1 shows the molecules under study, atom numbering and rotations studied.

For each species, more than 200 conformations were analyzed by using the AM1 version 6.0 of the MOPAC package (24), resulting from the combination of the allowed position for the different dihedral angles with the configurational possibilities of the guanidinium group. In all cases L isomers were studied, since it is the isomer recognized by the enzyme.

To obtain good structural parameters, we performed full optimizations at *ab initio* levels HF/3-21G and HF/6-31G* of the lowest-energy conformations obtained with AM1. *Ab initio* quantum-mechanical calculations were performed by using the Gaussian 98 program system (11). Charge densities were represented by molecular electrostatic potentials (MEPs) obtained by using SPARTAN package (23).

RESULTS

Conformational flexibility

A complete conformational search was conducted for

Fig. 1 Continuation

Table 1 Lowest-energy conformations for the *species III* of L-Arg and inhibitors obtained at the different calculation levels: AM1 version 6.0, HF/3-21G y HF/6-31G*

Molecules	Conformation	χ_1	χ_2	χ_3	χ_4	χ_5	χ_6
			AM1				
L-ARG	a g- g+ g+	174.5	-109.3	65.8	72.8	-	-
L-NMA	a g- g+ g+ a	174.3	-109.4	66.5	72.4	-165.6	-
L-NNA	a g- g+ g+ a	174.6	-108.3	67.5	73.4	-165.2	-
L-CPA	a g- g+ g+ a	174.2	-109.5	65.7	74.8	-167.1	-
L-NIO	g+ g+ g- g- g-	70.8	107.2	-64.4	-86.3	-25.2	-
L-NIL	g+ g+ a g+ g+ g+	81.3	98.4	-142.6	70.3	84.5	21.9
L-NAME	a a g+ a g-	-157.3	-145.8	78.2	170.4	-8.2	-
			HF/3-21G				
L-ARG	a g- g+ g+	174.5	-109.3	65.8	72.8	-	-
L-NMA	a g- g+ g+ a	160.5	-110.7	60.1	107.4	-155.6	
L-NNA	a g- g+ g+ a	161.6	-109.4	59.3	106.3	-158.1	
L-CPA	a g- g+ g+ a	174.3	-109.4	65.9	74.3	-167.2	
L-NIO	g+ g+ g- g- g-	63.6	117.9	-64.8	-129.4	-14.4	
L-NIL	g+ g+ a g+ g+ g+	81.5	98.3	-142.2	70.5	84.7	21.8
L-NAME	a a g+ a g+	-161.8	-138.5	71.4	174.2	3.7	
			HF/6-31G*				
L-ARG	a g- g+ g+	167.8	-107.2	62.2	91.1	-	-
L-NMA	a g- g+ g+ a	168.2	-107.6	62.8	86.1	-161.0	
L-NNA	a g- g+ g+ a	167.7	-105.2	62.7	88.4	-162.1	
L-CPA	a g- g+ g+ a	167.8	-108.6	63.5	91.1	-156.3	
L-NIO	g+ g+ g- g- g-	66.5	107.6	-63.4	-103.5	-19.7	
L-NIL	g+ g+ a g+ g+ g+	82	98.3	-142.2	70.5	84.7	21.8
L-NAME	a a g+ a g+	-158.6	-141.6	72.6	174.5	4.6	

analogs of L-Arg, species III and IV, at the semi-empirical calculation level AM1. In all cases, 8-12 low-energy conformations were obtained within a range of 3 kcal/mol above the global minimum, thus indicating conformational flexibility for all the molecules under consideration. We performed *in vacuo* calculations, which have demonstrated to provide good account of the crystal structure of related molecules (17) in agreement with the requirements at the active site of the enzyme (7). Solvent effect was not included in the calculations because of the small size of the molecules under study. It is well-known from experimental data that peptides in solution present a high degree of conformational freedom.

Using as the starting points the lowest-energy conformations obtained at the AM1-calculation level, full optimization was performed at the *ab initio* HF/3-21G and HF/6-31G* theory levels.

Species III

Table 1 shows the lowest-energy conformations obtained at the AM1 calculation level compared with the fully optimized, final conformations obtained at the *ab initio* HF/3-21G and HF/6-31G* theory levels, for species III of all the molecules studied.

As it can be seen on this table, L-Arg, L-NNA, L-NMA and L-CPA, arrive to identical final conformations at the different calculation levels for species III. The present results are indicative of a similar conformational behavior for these molecules, in spite of the different substituents over the guanidinium group (see Fig. 1 and Fig. 2). All these compounds have in common with L-Arg the side chain length, which can be an important feature for the recognition at the enzyme active site. All these inhibitors are selective ones for the constitutive isoforms, with different relative affinities (2,12,15,16,17,19).

L-NIO and L-NIL adopt conformations quite different as compared to those of L-Arg. Similar data were obtained at the different calculation levels (Table 1). L-NIL and L-NIO both recognize the inducible isoform and both compounds have in common the substituent which replace the guanidinium group (see Fig. 1), but a different side chain length. The side chain of L-NIL, a derivative of L-lysine is longer than that of L-Arg and this explains its different conformational behavior. L-NIO, a derivative of the aminoacid L-ornitine, with similar length to that of L-Arg, recognizes both enzyme isoforms, the constitutive as well as the inducible.

L-NAME is the only species, which carries a methyl ester on the C-terminal group (Fig. 1), a feature that modifies its possibility of being a zwitter-ionic species. Thus, the double charged species, with charge over the guanidinium and over the amino-terminal groups was compared with species III. This special feature accounts for the different conformational behavior of this compound (Fig. 3).

Fig. 2 Spatial view of the lowest-energy conformations and MEPs obtained for species III and IV of L-Arg, L-NNA, L-NMA and L-CPA at the HF/6-31G* calculation level.

Species IV

Species IV is a zwitter-ionic species with no charge over the guanidinium group. Thus, it is interesting to note that species IV seems to be more sensitive to the substituent effect on the conformational behavior of the molecules under study. Table 2 shows the final conformations obtained at the three theory levels.

With very few exceptions, the final conformations obtained were comparable at the different calculation levels. For L-NIL different dihedral angles χ_1 and χ_2 were obtained at the *ab initio* calculation levels.

Molecular properties

Ionization potential (IP) and net atomic charges were analyzed at the different calculation levels and a similar tendency was observed for the different molecules under study. Thus, we are reporting only values obtained at the HF/6-31G*, a theory level which could provide better estimations of these values (Table 3). Quite similar IP values were obtained for the different compounds, with exception of L-NAME, molecule with no charges over the carboxyl-group due to the methyl substituent.

Interatomic distances and spatial disposition of potentially active groups for the final conformations obtained agrees with the possibility of internal H-bonds. However, on physiological media, as well as at the active site of the enzyme, all potentially active groups will be interacting with residues of the enzyme. Thus, possible internal H-bonds were considered as informative of the potential capacity of the different groups.

The presence of different substituents over the guanidinium group or the carboxylic portion of the molecule (i.e. L-NAME) will certainly reduce the interactions at the active site and thus the progress of the oxidation reaction. Moreover, substituents of N^{11} on the guanidinium group clearly modify the net atomic charge over this atom and therefore, interactions at the active site (Table 3). Similar charges were observed for molecules with the same replacement over the guanidinium group, such as L-NNA and L-NAME or L-NIO and L-NIL.

Molecular electrostatic potentials (MEPs)

Charge density distribution over the molecules studied is better represented by MEPs, as a descriptor of the electronic structure. MEPS were obtained by using the SPARTAN package for the final conformations obtained at the HF/6-31G* theory level. Fig. 2 and 3 show the final conformations obtained at this theory level and its corresponding MEPs for the different molecules studied as compared to L-Arg, species III.

The negative charges (red areas) over $-COO^-$ or $-NO_2$ groups are evident, as well as the positive areas (blue) on the

Table 2 Lowest-energy conformations for the *species IV* of L-Arg and inhibitors obtained at the different calculation levels AM1 version 6.0, HF/3-21G y HF/6-31G*

Molecules	Conformation	χ_1	χ_2	χ_3	χ_4	χ_5	χ_6
			AM1				
L-ARG	g+ g+ a g+ g+	45.7	69.2	-150.3	66.6	22.3	-
L-NMA	g+ g+ g- g- g+	46.9	57.7	-92.5	-51.4	110.4	-
L-NNA	g- g+ a g+ g+	-68.3	110.2	-154.4	70.4	4.7	-
L-CPA	g+ g+ g- g- g+	47.5	58.3	-91.2	-51.6	109.4	-
L-NIO	g- g+ a g+ a	-60.3	104.6	-154.3	78.1	-174.2	-
L-NIL	g- g+ g+ a g+ a	-86.7	63.4	63.8	-177.2	71.4	-174.5
L-NAME	g- g- a g- g+	-65.3	-67.2	146.5	-69.3	4.8	-
			HF/3-21G				
L-ARG	g+ g+ a g+ g+	45.7	69.2	-150.3	66.6	22.3	-
L-NMA	g+ g+ g- g- g+	51.6	49.6	-82.4	-55.3	102.8	
L-NNA	g- g- a g- g-	-59.3	-60.8	153.8	-68.2	-2.1	
L-CPA	g+ g+ g- g- g+	47.1	58.1	-91.9	-51.5	109.6	
L-NIO	g- g+ a g+ a	-54.7	105.6	-152.6	79.5	176.3	
L-NIL	g+ g- g+ a g+ a	82.3	-73.8	91.4	-167.0	70.6	-179.2
L-NAME	g- g- a g- g+	-65.5	-67.2	146.6	-69.6	4.8	
			HF/6-31G*				
L-ARG	g+ g+ a g+ g+	48.1	64.7	-151.3	65.1	21.6	
L-NMA	g+ g+ g- g- g+	54.5	50.2	-83.3	-54.9	111.4	
L-NNA	g- g+ a g+ g+	-63.4	92.5	-166.6	79.3	2.3	
L-CPA	g+ g+ g- g- g+	54.8	50.3	-83.3	-54.6	113.1	
L-NIO	g- g+ a g+ a	-55.8	99.1	-154.6	78.8	178.2	
L-NIL	g+ g- g+ a g+ a	82.3	-73.8	91.4	-167.0	70.6	-179.0
L-NAME	g- g- a g- g+	-65.5	-67.2	146.6	-69.6	4.8	

Fig. 3 Spatial view of the lowest-energy conformations and MEPs obtained for species III and IV of L-NIL and L-NIO and doubled charged species of L-NAME compared to that of L-Arg at the HF/6-31G* calculation level.

Table 3 Net atomic charges of potentially active atoms for the species III of L-Arg and inhibitors obtained on fully optimized conformations on *ab initio* calculations at the HF/6-31G* level of theory

Molecules	O^5	O^6	N^9	N^{11}	N^{12}
L-Arg	-0.655	-0.787	-0.802	-0.925	-0.904
L-OHArg	-0.662	-0.784	-0.809	-0.453	-0.917
L-NMA	-0.663	-0.785	-0.812	-0.814	-0.914
L-NNA	-0.656	-0.792	-0.820	-0.684	-0.919
L-CPA	-0.658	-0.786	-0.832	-0.807	-0.939
L-NIO	-0.732	-0.765	-0.784	-	-0.887
L-NAME	-0.512	-0.602	-0.821	-0.639	-0.928
L-NIL	-0.642	-0.810	-0.783	-	-0.885

amino and guanidinium portions. Since similar conformations were obtained for L-Arg, L-NNA, L-CPA and L-NMA compounds, quite similar spatial MEPs were obtained (Fig. 2).

L-NNA and L-NAME, both having a $-NO_2$ substituent at the guanidinium group, exhibited a clear negative area localized over this group. The MEPs for L-NIO and L-NIL, with a different residue replacing the guanidinium group showed a different charge density on this area (Fig. 3).

DISCUSSION

NOS are highly regulated enzymes responsible for the synthesis of the potent cytotoxin and signal molecule NO. NOS are composed of two domains: the catalytic oxygenase domain (NOS_{ox}) that binds the substrate L-Arg and the electron supplying reductase domain (NOS_{red}). To be active, the NOS_{ox} domain requires dimerization as stated by crystal resolution (5,6,7,8). Recently, crystal structures of dimeric NOS oxygenase domain for different isoenzymes have been solved and detailed information was provided about the active site.

On the present paper, we performed a complete theoretical conformational and structural study of L-Arg analogs, inhibitors of NOS and compared to L-Arg, in the search of a structure-activity relationship. Selective inhibitors were studied in order to provide structural requirements for the interaction between these molecules and the active site of different enzyme isoforms.

Apparently, a partially folded conformation is required to fit at the enzyme active site, conformation that can be easily achieved by both L-Arg as well as the inhibitors. Also, it has been pointed out that the most probable species to be found on the enzyme media is the charged species III (7). The fact that no conformational changes have been observed for the active site when complex the substrate L-Arg or the intermediate L-NHA, indicates that the active site is well defined and that the molecules should accommodate at the site. The present studies do suggest that L-Arg and its analogs are highly flexible molecules, which can easily adopt the conformation required for the interaction at the active enzyme site.

Hydrogen bonds seems to be crucial for the interaction at the active site, since a number of residues are located at short distance of the potentially active atoms of the substrate or the intermediate L-NHA. The capacity to form hydrogen bonds is indicated by the atomic charges over potentially active sites. Most of the inhibitors showed a number of potentially active groups, which could easily interact at the active site. This might be an important feature in order to produce the interaction with the enzyme. Since calculations have been made *in vacuo*, distances and orientation for H-bonds are indicative of the possibility of formation of internal H-bonds. Taking into account that the molecule will interact with a number of residues at the active site, we consider this effect as indicative of the potential capacity of the studied species to form H-bonds.

On the crystal, L-Arg has close contacts with the heme prosthetic group through the guanidinium portion of the molecules. Guanidinium deslocalization allows us to justify the multiple interactions at the active site, such as the interaction with Glu^{371} while interacts with the peptide bond of the Gly^{365}-Trp^{366}.

Moreover, for the species III, which bears a protonated guanidinium group, the crystal data do suggest that some of the nitrogen atoms could suffer a pyramidalization in order to fit the active site. Our present results do agree with these observations, since N^{12} for L-Arg III or N^{11} for L-NHA exhibited this pattern.

Substituents over the guanidinium group would interfere with this interaction and prevent the reaction progress. The different substituents not only provide steric hindrance but also cause modification of charge distribution. L-NNA and L-NAME suffer an important change on charge distribution, which could explain the selectivity of these compounds on constitutive NOS. Most of the inhibitors that are active on constitutive isoforms, have a side chain of the same length than L-Arg. This pattern appears to be an important feature on constitutive enzymes.

L-NIL, with a longer side chain is selective for inducible isoforms and exhibited a different conformational behavior compared to L-Arg. Replacement of the guanidinium for an iminoethyl substituent over an amino group (L-NIL and L-NIO) strongly affects delocalization of the guanidinium group. Both, L-NIO and L-NIL have such a modification and both compounds are recognized by the inducible isoform. This fact suggests that these isozymes accept such a modification. However, the selectivity of L-NIL indicates that the longer chain of this compound compared to L-NIO would strongly favor preference for the inducible isoform. L-NIO,

shares the side chain length with L-Arg and the guanidinium substituent with L-NIL and thus would account for its lack of selectivity.

In summary, on the basis of the conformational and molecular properties of the different NOS inhibitors in combination with the selectivity for certain isoforms of the analogs studied, we propose the requirements for the different NOS isoforms. The present data obtained from theoretical calculations are in good agreement with experimental data (5,7,8) and strongly support the recognition of both L-Arg and L-NHA at the same spatial ordering of the active site, thus indicating that theoretical studies could be a good help to interpret the enzyme-substrate interaction.

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I don't find the word "deslocalization" in any dictionary .
Ref 18: is not in the text. Please add.