Published in final edited form as:

Biochem Biophys Res Commun. 2009 April 24; 382(1): 21–25. doi:10.1016/j.bbrc.2009.02.084.

Role of substrate functional groups in binding to nitric oxide synthase

Baochen Fan^{a,1}, Dennis J. Stuehr^b, and Denis L. Rousseau^{a,*}

^aDepartment of Physiology and Biophysics, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461, USA

^bDepartment of Immunology, NN-I, The Cleveland Clinic, Cleveland, OH 44195, USA

Abstract

The interactions between the heme CO ligand in the oxygenase domain of nitric oxide synthase and a set of substrate analogues were determined by measuring the resonance Raman spectra of the Fe–C–O vibrational modes. Substrates were selected that have variations in all the functional units: the guanidino group, the amino acid site and the number of methylene units connecting the two ends. In comparison to the substrate free form of the enzyme, Interactions of the analogues with the CO moiety caused the Fe–CO stretching and the Fe–C–O bending modes to shift in frequency due to the electrostatic environment. An unmodified guanidino group interacted with the CO in a similar fashion despite changes in the amino acid end. However, an unmodified amino acid end is required for catalysis owing to the H-bonding network involving the substrate, the heme and the pterin cofactor.

Keywords

Raman spectroscopy; Carbon monoxide; Nitric oxide synthase; Substrate analogues

Nitric oxide synthase (NOS) catalyzes the synthesis of NO in a two-step mechanism in which the reaction of L-Arg with oxygen forms N-hydroxy-L-Arginine (NOHA) which in turn reacts with another molecule of oxygen to yield NO and citrulline. In addition to L-Arg and NOHA, only two other L-Arg derivatives (NMA and homoarginine) have been shown to be substrates for NOS [1–3]. Three major mammalian isoforms of the enzyme have been identified, two constitutive forms from brain (nNOS) and endothelium cells (eNOS), and one inducible form (iNOS), from macrophage [4, 5]. The functional form of all three isoforms are homodimeric with a heme-containing oxygenase domain incorporating a tetrahydrobiopterin (H4B) cofactor binding site, and a reductase domain that has the site for the electron donor, NADPH. The electron transfer to the oxygenase domain is enabled by the binding of Ca-calmodulin.

The NOS substrates possess multiple points for interaction with the enzyme. Both the positively charged guanidino group and the amino acid end interact directly with either amino acid residues or prosthetic groups in the active site as revealed by crystal structure studies [6, 7]. The length of the intervening methylene groups also affects the binding and activity [8]. The similarity of the substrate binding sites in each of the isoforms has made the development of isoform-specific inhibitors a challenging task. A major advance in selective

inhibitor design was made recently by constructing inhibitors that can bind in the substrate site but also have bulky tails that can bind in remote sites [9]. These remote sites differ among the isoforms so outstanding selectivity can be achieved. As these inhibitors need structures to interact both with the normal substrate site as well as the remote site a clear understanding of the interactions in the substrate binding site are of crucial importance for efficient inhibitor design.

To identify substrate-enzyme interactions in NOS, resonance Raman spectroscopy coupled with small molecular probes has proven to be a powerful tool as it reflects the position of the substrate or substrate analogue with the bound ligand [10, 11]. Well before the determination of NOS structures, it was established that substrates strongly interact with heme bound ligands and the interactions are dependent on the nature of bound substrate [10]. Also, structural differences among the three NOS isoforms were identified in which the interaction of the substrate with the ligand was the same in iNOS and eNOS and distinct from that in nNOS [12]. This work reports the studies of the interaction of a bound-CO ligand of iNOS_{oxy} with substrates and analogues with variations in all the functional units: the guanidino group, the number of methylene groups and the amino acid site (Fig. 1).

Materials and methods

L-thiocitrulline and S-methyl-thiocitrulline were obtained from Calbiochem (La Jolla, CA) and all of the others substrates and analogues were purchased from Sigma (St. Louis, MO). Carbon monoxide was purchased from Matheson (CP grade, 99.5%).

The expression and reconstitution of iNOS_{oxy} was described previously [13]. Samples were buffered (pH 7.6, 40 mM Bis-Tris with 1 mM of dithiothreitol) and mixed with substrates or inhibitors. The final heme and substrate analog concentrations were 30 μ M and 3 mM, respectively. Ferrous CO-adducts of the enzyme were prepared by reducing the sample in a sealed Raman cell with 5-fold excess of sodium dithionite, followed by exposure to the CO gas and monitored by optical absorption. To avoid any spectral complications from H4B [14], the pterin was not introduced in any of the samples.

Resonance Raman measurements were performed with a 1.25 m polychromator (Spex) equipped with a CCD detector (Princeton Instruments). The signal was collected in a right angle geometry with a 100 micron entrance slit. Excitation at 441.6 nm, provided by a He-Cd laser (Liconix), was used to selectively enhance the active form of the CO-bound enzyme which has an absorption maximum at ~445 nm [14]. Spectra were calibrated with lines from toluene and an aqueous solution of sodium ferrocyanide. Data processing and analysis were performed with routines provided by Grams386 (Galactic Industries Corp.).

Results

Arginine analogs were introduced into the CO-bound complex of iNOS_{oxy} and the resonance Raman spectra were measured. The orientation of the substrate with respect to the CO, and also the bound oxygen during catalytic activity, is determined by the H-bonding interactions between the substrate, or analogue, and the residues in the catalytic site. The binding of native substrates, such as L-Arg, involves H-bonding interactions to both the guanidino end and the amino acid end of the molecule as shown in Fig. 2 for the oxygenase domain of nNOS. The guanidino end is held in place by Glu-592 (the nNOS sequence numbering is used throughout) and by an H-bond to the polypeptide carbonyl group of Trp-587. The carboxylate group of the amino acid end of L-Arg is H-bonded to Tyr-588 and the amino group is H-bonded to Glu-592 and to one of the carboxylate oxygen atoms of a heme propionate group. The other oxygen atom of the propionate group is H-bonded to the

pterin cofactor. As shown in Fig. 2A, when CO is present there is an H-bonding interaction to it from the NH group of the L-Arg and from a water molecule coordinated in the distal pocket. The distance of each of these groups to the oxygen atom of the CO is $\sim 3 \text{ \AA}$.

To determine the affect of these two ends of the Arg and its analogues on their juxtaposition with respect to the heme ligand, the selected analogues are divided into two groups: those in which the native guanidino group is present and those in which the amino acid end of the molecule has its native substituents (Fig. 1). By this organization, the influence of each end of the analogues on the interactions at the heme catalytic site can be evaluated. For the native guanidino group, HomoArg and AGPA represent cases in which the methylene spacing between the two ends is either longer or shorter, respectively, than that in L-Arg . Argmt and ArgA have modified amino acid ends. For those cases in which the amino acid terminal is intact some of the molecules have derivatized NH groups in the guanidino end, such as NOHA, a native substrate, and NMA. All of the others, including the final product, Cit have one of the terminal NH groups replaced by a different atom or atoms.

To measure the interactions between each of the analogues and the catalytic site, the vibrational modes of heme-bound CO, an oxygen surrogate, were examined. The Fe–CO vibrational modes are very sensitive to the electrostatic environment owing to changes in the back-bonding between the heme and the CO as reflected in the relationship below relating the limiting case structures [15].

A positively charged distal environment shifts the equilibrium toward structure II, thereby increasing the Fe–CO bond strength and hence its stretching mode frequency and concomitantly decreasing the C–O bond strength and its associated stretching mode frequency as compared to structure I.

Several prior studies of the effect of substrates and their analogues on the FeCO modes in the NOS isoforms have been reported and have revealed important properties of the heme active site [10, 11, 14]. In the absence of substrates and the pterin cofactor the Fe–CO stretching mode is broad and can be deconvoluted into two or three different components reflecting alternate conformations in the distal pocket accessible in the absence of the substrates [16]. Although multiple components were also detected in the presence of substrates, a single component tended to dominate resulting in a sharper and better-defined line making the central component a useful indicator of the distal interactions between the substrate and the bound CO. In iNOS_{oxy} and eNOS_{oxy} the multicomponent line in the $480\text{--}490 \text{ cm}^{-1}$ region in the absence of substrate shifted to a well defined line at $\sim 512 \text{ cm}^{-1}$ in the presence of L-Arg , whereas in nNOS_{oxy} the line is located at 503 cm^{-1} in the presence of L-Arg [12].

To assess the occupancy of the substrate analogues in the substrate binding site, the shape and frequency of the Fe–CO stretching mode was examined along with the frequency and intensity of the Fe–C–O bending mode. Sharpening of the stretching mode indicates restriction of the conformational freedom and therefore occupancy of the substrate binding site. The higher the frequency of the mode is, the greater the positive electrostatic interaction. Similarly, a higher frequency of the bending mode tends to correlate with a stronger positive electrostatic interaction although such a correlation has not been as thoroughly substantiated as that of the stretching mode. Stronger interactions also give more intense bending modes.

Based on the data in Fig. 3, for the compounds with an intact guanidino group, all but AGPA show evidence of coordination in the substrate binding site with variable interaction

strengths. However, the variation is relatively small demonstrating that the position of the guanidine group is relatively insensitive to the changes that occur at the amino acid end of the molecules. The three compounds shown in Fig. 4A, which have modified guanidino groups, all bind to the enzyme but the interactions with the CO are very different. As reported and discussed previously [16], NOHA, a physiological substrate, results in a weaker interaction with the CO than that of L-Arg. NMA has a strong but heterogeneous interaction as indicated by the broad Fe–CO stretching mode. L-NIO has a low Fe–CO stretching frequency but the line is narrow indicating that the conformation is relatively homogeneous. Of the three compounds in Fig. 4B, SMTC shows evidence for binding but Cit and ThioCit show no indication of any modification to the distal pocket.

Discussion

The data reported here reveal clear differences between the effect of structural changes in the guanidino end versus the amino acid end of iNOS_{oxy} substrate analogues on the heme binding site. When the guanidino group is near the CO ligand it exerts a positive interaction that shifts the Fe–CO stretching mode to higher frequency. In addition, the mode narrows when the conformational heterogeneity is restricted by the presence of this moiety. Associated with these changes the bending mode also shifts to higher frequency and gains intensity.

Of the five compounds with intact guanidino groups, L-Arg, HomoArg, Agmt, and ArgA all show the characteristic changes of an increased $\nu_{\text{Fe-CO}}$ frequency, a sharpening of the mode and a significant enhancement of the bending mode, $\delta_{\text{Fe-C-O}}$. This indicates that despite structural changes in the amino acid end, the H-bonding interactions between the guanidino end with the heme-pocket residues can position the compounds in similar positions with respect to the heme. The observation that AGPA, with its shortened methylenic section results in a similar spectrum to that obtained in the absence of substrate indicates that either it does not bind to the protein or if it does it is too far from the CO to interact with it. The former possibility is excluded by a back titration experiment. A sample was prepared with L-Arg to generate a spectrum characteristic of arginine binding. Then AGPA was added and the spectrum reverted to that of the substrate free form, showing that AGPA competes with L-Arg for binding to the active site even with CO-bound to the heme. However, its bound orientation does not allow it to be close enough to the heme to influence the CO ligand. As may be seen in Table 1, its IC₅₀ reveals that its binding affinity is relatively high. This suggests that the amino acid end binds with its normal interactions placing the guanidino end too far away from the CO to be detected owing to the short methylenic group.

The fact that the large differences in the amino acid tail of the analogues have a relatively small effect on the Fe–CO modes has implications with regard to the differences in the mode frequencies in the presence of L-Arg between nNOS (502 cm⁻¹) and those in iNOS and eNOS (512 cm⁻¹) [12]. Li et al obtained the crystal structure of the CO-bound complexes of nNOS_{oxy} and eNOS_{oxy} [7]. The H-bonding distance between the L-Arg and the CO was 2.9 Å in nNOS_{oxy} but a very short 2.4 Å in eNOS_{oxy}. Based on the fact that none of the changes in the amino acid end of the analogues examined in this study resulted in a frequency as low as 502 cm⁻¹, it is the differences in direct H-bonding to the guanidino end of the molecule that must be the origin of the isoform differences. Thus, the difference between nNOS and the other two isoforms are in the positions of the groups forming the H-bonds to the guanidino end of the molecule. These differences need not be very large to cause a slight shift or re-orientation of the analogue thereby changing the local electrostatic potential on the CO.

The addition of compounds with a modified guanidino group brings about a wide range of changes. L-NIO and SMTC both show evidence of binding by a narrowing of the Fe–CO

stretching mode and an increase in intensity of the bending mode. However, the frequency of the stretching mode is relatively low, indicating that there a strong positive polar interaction is not present. This may be interpreted as an interaction of the methyl group for these two compounds with the CO. Both of these compounds are relatively good inhibitors (IC_{50} s of 1.8 and 34 μ M, respectively, as listed in Table 1). This suggests that both of these compounds bind with the same interaction as does L-Arg but with the methyl substituent pointing toward the CO thus accounting for the Raman data.

NMA also shows evidence for binding with a strengthened bending mode and a shifted stretching mode to higher frequency. However, both of these modes are relatively broad indicating heterogeneity in the binding. This is likely a result of the methyl amine end of the guanidino group pointing toward the CO ligand resulting in an interaction of the CO with either the methyl group or the proton of the $HNCH_3$ moiety. The placement of this group over the iron atom is consistent with the formation of L- N_G -hydroxy- N_G -methylarginine as shown by Feldman et al. [2] Thus, NMA can bind with the same interactions as L-Arg accounting for its strong binding (Table 1).

The binding of NOHA differs from that of L-Arg and other analogues as the proton on the hydroxide group is H-bonded to a backbone peptide nitrogen [17]. This may displace the substrate slightly away from the heme ligand, accounting for a weaker electrostatic interaction [16]. Furthermore, in the crystal structures of L-Arg and NOHA adducts of bsNOS [17], a water molecule was found to be in H-bonding distance of the terminal oxygen on the NO in the presence of L-Arg just as shown in Fig 2A for the CO derivative reported by Li et al. [7] However, in the bsNOS study the water was not in H-bonding distance of the terminal oxygen of the NO when NOHA was present. The combination of these differences accounts for the lowered positive electrostatic potential near the CO ligand.

Cit and ThioCit have spectra of the CO derivative identical to that of the substrate free form. We conclude that these do not bind in the presence of CO. Indeed, for the enzymatic product, Cit, even in the ferric form the binding has been shown to be very weak [18]. However, for ThioCit it has been shown that it binds in the ligand-free form in different conformations, one of which involves apparent coordination to the heme by the sulfur atom [19, 20]. When CO is present both of the molecules plausibly exert too great an electrostatic repulsion to the CO to bind in the catalytic site.

In summary, the data reported here clarify the effect of the two ends of the substrate analogues on binding in the catalytic site of $iNOS_{oxy}$. Both ends of the molecules contribute to the stability and the guanidino end very precisely positions the substrate over the heme to enable catalysis to proceed. However, the amino acid end is also important for catalysis as indicated by argininic acid and agmatine which bind to the CO-bound active site in a very similar manner to that of the native substrates. Thus both of these analogues appear to be positioned appropriately for catalysis but they are inactive owing to the structure of the amino acid end. These observations indicate that proper static poising of the substrate in the active site is not the sole requirement for catalysis. This suggests that the amino acid end is needed to stabilize the H-bonding interactions between the heme and the pterin cofactor presumably to facilitate the electron transfer from the pterin to the heme catalytic site.

Acknowledgments

This work was supported by NIH Grants GM54806 to D.L.R. and CA53914, GM51491 and HL76491 to D.J.S. We thank Dr. T. Poulos for sending us the 2G6M data file and Dr. Syun-Ru Yeh for helpful discussions.

Abbreviations

HomoArg	L-homoarginine
NOHA	N ^ω -hydroxy-L-arginine
NMA	N ^ω -methyl-L-arginine
ArgA	argininic acid
Agmt	agmatine
AGPA	L-2-amino-3-guanidino-propionic acid
L-NIO	L-N5-(L-iminoethyl)-ornithine
Cit	L-citrulline
ThioCit	L-thiocitrulline
SMTC	S-methyl-thiocitrulline
iNOS_{oxy}, nNOS_{oxy} and eNOS_{oxy}	oxygenase domains of the inducible, neuronal and endothelial isoforms of nitric oxide synthase
BH4	tetrahydrobiopterin
bsNOS	Bacillus subtilis nitric oxide synthase

References

1. Stuehr DJ, Griffith OW. Mammalian nitric oxide synthases. *Adv. Enzymol. Relat. Areas Mol. Biol.* 1992; 65:287–346. [PubMed: 1373932]
2. Feldman PL, Griffith OW, Hong H, Stuehr DJ. Irreversible inactivation of macrophage and brain nitric oxide synthase by L-NG-methylarginine requires NADPH-dependent hydroxylation. *J. Med. Chem.* 1993; 36:491–496. [PubMed: 7682617]
3. Sennequier N, Stuehr DJ. Analysis of substrate-induced electronic, Catalytic, and structural changes in inducible NO synthase. *Biochemistry.* 1996; 35:5883–5892. [PubMed: 8639550]
4. Stuehr DJ. Mammalian nitric oxide synthases. *Biochim. Biophys. Acta.* 1999; 1411:217–230. [PubMed: 10320659]
5. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. *Biochem. J.* 2001; . 357:593–615. [PubMed: 11463332]
6. Crane BR, Arvai AS, Ghosh DK, Wu C, Getzoff ED, Stuehr DJ, Tainer JA. Structure of nitric oxide synthase oxygenase dimer with pterin and substrate. *Science.* 1998; 279:2121–2126. [PubMed: 9516116]
7. Li H, Igarashi J, Jamal J, Yang W, Poulos TL. Structural studies of constitutive nitric oxide synthases with diatomic ligands bound. *J. Biol. Inorg. Chem.* 2006; 11:753–768. [PubMed: 16804678]
8. Grant SK, Green BG, Stiffey-Wilusz J, Durette PL, Shah SK, Kozarich JW. Structural requirements for human inducible nitric oxide synthase substrates and substrate analogue inhibitors. *Biochemistry.* 1998; 37:4174–4180. [PubMed: 9521739]
9. Garcin ED, Arvai AS, Rosenfeld RJ, Kroeger MD, Crane BR, Andersson G, Andrews G, Hamley PJ, Mallinder PR, Nicholls DJ, St-Gallay SA, Tinker AC, Gensmantel NP, Mete A, Cheshire DR, Connolly S, Stuehr DJ, Aberg A, Wallace AV, Tainer JA, Getzoff ED. Anchored plasticity opens doors for selective inhibitor design in nitric oxide synthase. *Nat. Chem. Biol.* 2008; 4:700–707. [PubMed: 18849972]
10. Wang J, Stuehr DJ, Rousseau DL. Interactions between substrate analogues and heme ligands in nitric oxide synthase. *Biochemistry.* 1997; 36:4595–4606. [PubMed: 9109669]
11. Rousseau DL, Li D, Couture M, Yeh SR. Ligand-protein interactions in nitric oxide synthase. *J. Inorg. Biochem.* 2005; 99:306–323. [PubMed: 15598509]

12. Fan B, Wang J, Stuehr DJ, Rousseau DL. NO synthase isozymes have distinct substrate binding sites. *Biochemistry*. 1997; 36:12660–12665. [PubMed: 9376373]
13. Ghosh DK, Stuehr DJ. Macrophage NO synthase: characterization of isolated oxygenase and reductase domains reveals a head-to-head subunit interaction. *Biochemistry*. 1995; 34:801–807. [PubMed: 7530045]
14. Li D, Stuehr DJ, Yeh SR, Rousseau DL. Heme distortion modulated by ligand-protein interactions in inducible nitric-oxide synthase. *J. Biol. Chem.* 2004; 279:26489–26499. [PubMed: 15066989]
15. Egawa T, Yeh SR. Structural and functional properties of hemoglobins from unicellular organisms as revealed by resonance Raman spectroscopy. *J. Inorg. Biochem.* 2005; 99:72–96. [PubMed: 15598493]
16. Li D, Kabir M, Stuehr DJ, Rousseau DL, Yeh SR. Substrate- and isoform-specific dioxygen complexes of nitric oxide synthase. *J. Am. Chem. Soc.* 2007; 129:6943–6951. [PubMed: 17488012]
17. Pant K, Crane BR. Nitrosyl-heme structures of *Bacillus subtilis* nitric oxide synthase have implications for understanding substrate oxidation. *Biochemistry*. 2006; 45:2537–2544. [PubMed: 16489746]
18. Santolini J, Adak S, Curran CM, Stuehr DJ. A kinetic simulation model that describes catalysis and regulation in nitric-oxide synthase. *J. Biol. Chem.* 2001; 276:1233–1243. [PubMed: 11038356]
19. Frey C, Narayanan K, McMillan K, Spack L, Gross SS, Masters BS, Griffith OW. L-thiocitrulline. A stereospecific heme-binding inhibitor of nitric oxide synthases. *J. Biol. Chem.* 1994; 269:26083–26091. [PubMed: 7523401]
20. Salerno JC, Frey C, McMillan K, Williams RF, Masters BS, Griffith OW. Characterization by electron paramagnetic resonance of the interactions of L-arginine and L-thiocitrulline with the heme cofactor region of nitric oxide synthase. *J. Biol. Chem.* 1995; 270:27423–27428. [PubMed: 7499198]
21. Young RJ, Beams RM, Carter K, Clark HA, Coe DM, Chambers CL, Davies PI, Dawson J, Drysdale MJ, Franzman KW, French C, Hodgson ST, Hodson HF, Kleanthous S, Rider P, Sanders D, Sawyer DA, Scott KJ, Shearer BG, Stocker R, Smith S, Tackley MC, Knowles RG. Inhibition of inducible nitric oxide synthase by acetamidine derivatives of heterosubstituted lysine and homolysine. *Bioorg. Med. Chem. Lett.* 2000; 10:597–600. [PubMed: 10741561]
22. Bretscher LE, Li H, Poulos TL, Griffith OW. Structural characterization and kinetics of nitric-oxide synthase inhibition by novel *N*5-(iminoalkyl)- and *N*5-(iminoalkenyl)-ornithines. *J. Biol. Chem.* 2003; 278:46789–46797. [PubMed: 12960153]
23. Narayanan K, Spack L, McMillan K, Kilbourn RG, Hayward MA, Masters BS, Griffith OW. *S*-alkyl-L-thiocitrullines. Potent stereoselective inhibitors of nitric oxide synthase with strong pressor activity in vivo. *J. Biol. Chem.* 1995; 270:11103–11110. [PubMed: 7538112]

Fig. 1.
Structures of the NOS substrate analogs used in this study.

Fig. 2.

The structure of the L-arginine-bound nNOS_{oxy} catalytic site. The models were depicted with PyMOL from the structure (2G6M) reported by Li et al. [7]. (A) The interactions between the CO heme-ligand and the L-Arg and water molecule in the distal pocket. (B) The H-bonding interaction that stabilize the L-Arg substrate in the catalytic site.

Fig. 3.

The low frequency resonance Raman spectrum of CO-bound ferrous iNOS_{oxy} in the substrate free (SF) form (trace a) compared to its spectra in the presence of various substrate analogues. All of the analogues have an unmodified guanidino end. Their structures are depicted in Fig. 1. The sample conditions are given in Material and methods section. Spectra are averages of 10 one minute accumulations at room temperature. The Fe–CO stretching mode and the Fe–C–O bending modes are designated by $\nu_{\text{Fe-CO}}$ and $\delta_{\text{Fe-C-O}}$, respectively.

Fig. 4.

The low frequency Raman spectra of the CO-bound ferrous of the iNOSox in the presence of substrate analogues with an unmodified amino acid end. The experimental conditions and labeling are the same as described in Fig. 3.

Table 1

The Fe–CO stretching ($\nu_{\text{Fe-CO}}$) and the Fe–C–O bending ($\delta_{\text{Fe-C-O}}$) modes compared to the activities of iNOS_{oxy} substrate analogues.

Compound	$\nu_{\text{Fe-CO}}$ (cm ⁻¹)	$\delta_{\text{Fe-C-O}}$ (cm ⁻¹)	NO synthesis	Inhibitor IC ₅₀ (μM)
Substrate-free	486 (broad)	560	NA	NA
L-Arg	512	570	Y	NA
Agmatine	509	568	N	98.5 ^a
NMA	509 (broad)	573 (broad)	Y	6.6 ^b
HomoArg	508	568	Y	19.4(Ki) ^c
Argininic acid	507	562	N	388 ^a
NOHA	499	564	Y	NA
L-NIO	494	567	N	1.8 ^a
SMTc	486 (sharp)	561 (strong)	N	2.2 ^d
Citrulline	486	561	NA	NA
ThioCitrulline	486	561	N	3.6(Ki) ^e
AGPA	486	561	N	23 ^a

IC₅₀ data references

^a[8];

^b[21];

^c[22];

^d[23];

^e[19].