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Oxygen-Induced Seizures and Inhibition of Human Glutamate Decarboxylase and Porcine Cysteine Sulfinic Acid Decarboxylase by Oxygen and Nitric Oxide

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Key Words

γ-Aminobutyric acid · Glutamate decarboxylase · GAD65 · GAD67 · Nitric oxide · Oxygen · Radical mechanism · GABA

Abstract

The recombinant forms of the two human isozymes of glutamate decarboxylase, GAD65 and GAD67, are potently and reversibly inhibited by molecular oxygen (K_i = 0.46 and 0.29 m*M*, respectively). Inhibition of the vesicle-associated glutamate decarboxylase (GAD65) by molecular oxygen is likely to result in incomplete filling of synaptic vesicles with γ-aminobutyric acid (GABA) and may be a contributing factor in the genesis of oxygen-induced seizures. Under anaerobic conditions, nitric oxide inhibits both GAD65 and GAD67 with comparable potency to molecular oxygen (K_i = 0.5 m*M*). Two forms of porcine cysteine sulfinic acid decarboxylase (CSADI and CSADII) are also sensitive to inhibition by molecular oxygen (K_i = 0.30 and 0.22 m*M*, respectively) and nitric oxide (K_i = 0.3 and 0.2 m*M*, respectively). Similar inhibition of glutamate decarboxylase and cysteine sulfinic acid decarboxylase by two different radical-containing compounds (O_2 and NO) is consistent with the notion that these reactions proceed via radical mechanisms.

Introduction

Glutamate decarboxylase (GAD) was identified in the early literature on oxygen toxicity as a likely site of oxygen-induced seizures [23]. Of all the enzymes affected in the brains of animals exposed to hyperbaric oxygen, GAD is depressed to the greatest extent. However, the preeminence of GAD inhibition in oxygen-induced seizures was challenged when it was demonstrated that the *irreversible* losses of GAD in the brains of animals exposed to hyperbaric oxygen occur after the onset of seizures [9]. This effectively discredited the notion that inhibition of GAD by oxygen was responsible for triggering oxygen-induced seizures.

However, in 1991 it was reported that a bacterial form of GAD *(Escherichia coli)* had an oxygen-consuming side reaction [1]. This feature of GAD raised the possibility of direct interaction of the enzyme with molecular oxygen and direct, *reversible* inhibition of the enzyme by oxygen. *Reversible* inhibition of the enzyme under conditions of hyperbaric oxygen would not have been detected by the earlier experiments. Further, the irreversible losses of activity that were seen in vivo could possibly be due to self-inflicted oxidative inactivation by the enzyme, rather than by indirect, nonspecific oxidation as envisioned by earlier investigators.

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Table 1. Inhibition of human GAD (GAD65 and GAD67) by oxygen and nitric oxide

Assay environment (gas phase, 1 Atm)	GAD65 % activity	GAD67 % activity
Argon (anaerobic)[a]	100 ± 4	100 ± 5
Air (21% O_2)[a]	69 ± 5	52 ± 6
Oxygen (100% O_2)[a]	25 ± 3	24 ± 8
Nitric oxide (100% NO)[b]	19 ± 4	21 ± 3

[a] The average of 12 assays ± SD for each enzyme.
[b] The average of 9 assays ± SD for each enzyme.

In the present study we examine the sensitivity of the human forms of GAD, GAD65 and GAD67, and two fusion proteins containing the mechanistically related porcine cysteine sulfinic acid decarboxylase (CSAD) to inhibition by oxygen and nitric oxide. Both GAD and CSAD are inhibited by oxygen at physiological concentrations.

Materials and Methods

Recombinant Human GAD65 and GAD67

Both isozymes of human GAD were expressed as fusion proteins in *E. coli*, as previously described [5]. Culture of the bacteria at low temperature resulted in a majority of the expressed recombinant protein being in the soluble fraction. The fusion proteins were purified on a glutathione-affinity column, cleaved with factor Xa and then purified again by affinity chromatography. The specific activities of the purified, recombinant human GAD65 and GAD67 were 1.90 and 1.85 μmol of $^{14}CO_2$ produced min^{-1} mg^{-1}, respectively, when the enzymes were assayed under standard aerobic conditions.

Assay of GAD

Routine assay of the enzyme was carried out by capture of $^{14}CO_2$ produced from L-[1-^{14}C]glutamate, as previously described [5]. Assays of enzymes under defined atmospheres were conducted in septum-sealed tubes. With the exception of assays conducted under air (21% oxygen), all tubes were first purged with argon by introducing the gas through the septum via a syringe needle and allowing evacuation of the headspace through a second needle. It was important to first eliminate oxygen from the headspace in those assays conducted under nitric oxide, since nitric oxide reacts rapidly with molecular oxygen [4]. All assays were conducted at 25 °C, pH 7.3, with other details of the assay equivalent to those used in the routine assay [5].

Recombinant Porcine CSADI and CSADII

CSAD was cloned from a porcine *(Sus scrofa)* genomic library as recently described [11]. Two forms of porcine CSAD, CSADI and CSADII, were expressed as fusion proteins with β-galactosidase in *E. coli* and used without cleavage. The specific activity of CSAD I and CSAD II fusion proteins under aerobic conditions was 0.31 and 0.26 μmol of $^{14}CO_2$ produced from L-[1-^{14}C]cysteic acid min^{-1} mg^{-1}.

Assay of CSAD

Routine assay of the enzyme was carried out by capture of $^{14}CO_2$ produced from L-[1-^{14}C]cysteic acid, under similar conditions to GAD assays [5], with the exception that radiolabeled cysteic acid was substituted for glutamate [25]. Glutathione (1 m*M*) was included in all assays in addition to other assay components normally present in GAD assays [*S*-(2-aminoethyl)isothiuronium bromide and pyridoxal phosphate]. A final concentration of 20 m*M* glutamate was included in all assays to inhibit any residual GAD activity *(E. coli)* that may be present.

Results

The relative activities of GAD65 and GAD67 when assayed under various atmospheres are summarized in table 1. Even under physiologic conditions (air, 21% oxygen) GAD65 and GAD67 are substantially inhibited (31 and 48%, respectively) relative to anaerobic conditions (argon). This inhibition is reversible, as is true for the bacterial enzyme [1], since assay time courses are linear (i.e. the inhibition is not time dependent, data not shown) and the inhibition is concentration dependent. A combination of time-independent and concentration-dependent inhibition is only consistent with a reversible process, although either characteristic alone would not provide evidence for reversibility. Assays in the presence of nitric oxide were also linear. However, since only one concentration of nitric oxide was examined in this study, these data do not define whether the inhibition by nitric oxide is reversible or irreversible.

Making the assumption that oxygen and nitric oxide are linear, noncompetitive inhibitors of GAD65 and GAD67, inhibition constants can be estimated from these data. When the equation for linear, noncompetitive inhibition [% activity = (100%)/(1 + I/K_i), where I is the concentration of oxygen or nitric oxide and K_i is the inhibition constant] was applied to these data, inhibition constants (K_i) of 0.46 ± 0.05 and 0.29 ± 0.03 m*M* were obtained for inhibition of GAD65 and GAD67 by O_2 and 0.5 m*M* for inhibition of both enzymes by NO. The inhibition constants for oxygen are apparent values that apply to our assay conditions. However, independent studies of the GAD from *E. coli* have demonstrated that the K_i for oxygen is independent of glutamate concentration (i.e. noncompetitive).

The relative activities of CSADI and CSADII when assayed under various atmospheres are summarized in

table 2. Both of these enzymes display very similar sensitivity to inhibition by oxygen and nitric oxide (table 2) to that seen for human GAD (table 1). The data presented in table 2 were used to calculate apparent inhibition constants (K_i) for CSADI and CSADII, as was done for GAD. Values of 0.30 ± 0.03 and 0.22 ± 0.04 m*M*, respectively, were obtained for oxygen. Comparable values of 0.3 and 0.2 m*M*, respectively, were obtained for nitric oxide.

Table 2. Inhibition of porcine cysteinesulfinic acid decarboxylase (CSAD) by oxygen and nitric oxide

Assay environment (gas phase, 1 Atm)	CSAD I % activity	CSAD II % activity
Argon (anaerobic)[a]	100±5	100±4
Air (21% O_2)[a]	53±5	45±6
Oxygen (100% O_2)[a]	24±3	22±6
Nitric oxide (100% NO)[b]	13±9	11±7

[a] The average of 6 assays ± SE for each enzyme.
[b] The average of 3 assays ± SE for each enzyme.

Discussion

Physiological concentrations of oxygen substantially inhibit GAD65 and GAD67. Under hyperbaric conditions, the extent of inhibition of GAD could be a contributing factor in the genesis of oxygen-induced seizures. In particular the inhibition of the vesicle-associated GAD, GAD65, is likely to contribute to triggering an oxygen-induced seizure. GAD (GAD65) appears to be coupled to the V-type ATPase [15]. The V-type ATPase in turn provides the required proton gradient for the transport of GABA into vesicles for release at GABAergic synapses. Coupling of GAD65, the V-type ATPase, and GABA transporter would render vesicle-filling sensitive to inhibition by molecular oxygen. As such, seizures could potentially be triggered without the need for a global imbalance in the relative pool sizes of glutamate and GABA. This view of GAD65 as the critical form of human GAD responsible for triggering oxygen-induced seizures is consistent with the results of gene knockout experiments in mice. Mice that are deficient in GAD65 are prone to seizure, while those that are deficient in GAD67 die shortly after birth [16]. Such phenotypes would be consistent with a primary role for GAD67 in maintaining the intracellular pool of GABA (about 1 m*M*), required to establish the thermodynamically determined levels of GABA in the synaptic vesicles (about 100 m*M*) and extracellularly (about 1 μ*M*). The intracellular concentration of GABA would establish the levels in the synaptic vesicles and outside of the cell through the actions of the vesicle-associated GABA transporter (H^+ dependent) and membrane-associated GABA transporter (Na^+ dependent). Coupling of the vesicle-associated GABA transporter with GAD65 and the V-type ATPase would yield primarily a kinetic effect on vesicle filling (steady state) and GABA release, rather than a thermodynamic one. As such it is easy to rationalize why loss of GAD67 would be lethal (house keeping), while loss of GAD65 would render an animal prone to seizure (rate of GABA release). Similarly, inhibition of GAD65 by oxygen under hyperbaric conditions would likely lead to seizures, especially if the animals were stressed.

The physiological significance of the inhibition of CSAD by oxygen is less clear. However, deficiencies in CSAD have been linked to seizure disorders. Cats are deficient in CSAD [24]. CSAD is considered to be the rate-limiting step in the biosynthesis of the inhibitory amino acid taurine [6]. This deficiency in CSAD is the metabolic basis for the recognized dietary requirement for taurine in cats [7]. Dietary taurine has been demonstrated to reverse the occurrence of epileptic seizures in cats [21]. The effects of taurine and homotaurine in seizure disorders are complex, in that both prevent epilepsy in animal models for focal epilepsy, but potentiate the occurrence of seizures in corticoreticular epilepsy [10]. Taurine and other anticonvulsants prevent cortical spiking activity induced by cortical freezing [14]. Taurine has also been reported to prevent kainate-induced seizures, although these observations have recently been challenged [8]. The absence of CSAD in the optic nerve is thought to be responsible for the occurrence of macular degeneration in cats fed a taurine-deficient diet [20]. Although the role of CSAD inhibition in oxygen-induced seizures cannot be easily assessed, it has the potential to be a contributing factor.

We believe that the reversible inhibition of GAD and CSAD by oxygen is an indication that these reactions proceed via radical mechanisms. Similarly, the oxygen-consuming side reaction [1] that produces succinate semialdehyde, ammonia, carbon dioxide and hydrogen peroxide from glutamate and oxygen is inconsistent with the currently held view of the mechanism for GAD [17]. The rate at which molecular oxygen reacts with molecules in their singlet state (as they would be in the currently accepted mechanism for GAD) is simply too slow to account for the oxygenase reaction of GAD and other sim-

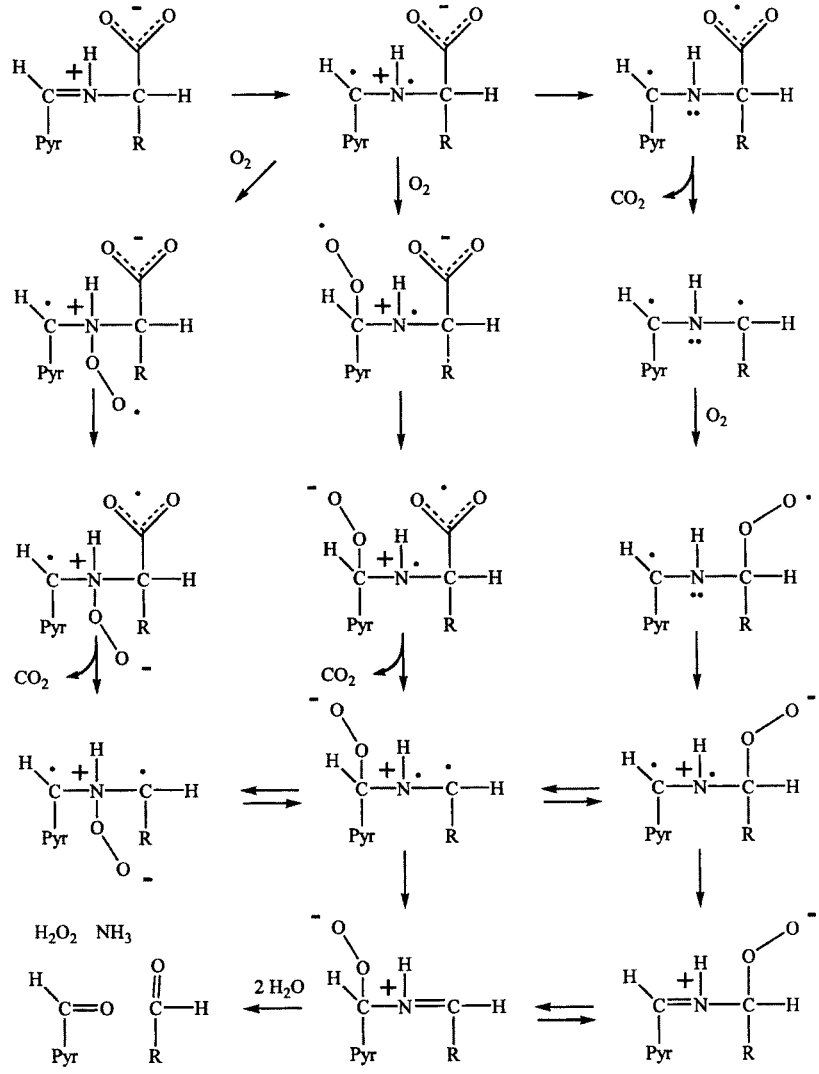

Fig. 1. Possible mechanisms for the oxygenase activity of an amino acid decarboxylase.

ilar oxygen-dependent side reactions of 'carbanion-forming' enzymes [19]. In our opinion, GAD and other amino acid decarboxylases achieve a triplet state (biradical) by unpairing electrons in the iminium formed from enzyme and glutamate. Electron transfer from the carboxylate of glutamate to the nitrogen-centered radical cation would allow for a radical-assisted decarboxylation, as illustrated in figure 1. By contrast to oxygen, formation of an adduct with nitric oxide would be expected to be a dead end complex (i.e. would not turnover to form products) due to the fact that a biradical is required for the production of singlet products (fig. 2). If this is the mechanism for the reaction of GAD, then all amino acid decarboxylases should be sensitive to inhibition by oxygen and other radical species. Consistent with this expectation, an oxygen-consuming side reaction has also been reported for DOPA decarboxylase [3]. Data presented here demonstrate that CSAD exhibits similar sensitivity to GAD for both oxygen and nitric oxide. Inhibition of GAD65, GAD67, CSADI and CSADII by nitric oxide under anaerobic conditions is easy to rationalize if they have radical mechanisms.

Inhibition of GAD and CSAD by nitric oxide under anaerobic conditions is distinct from the mechanism by which *S*-nitrosoglutathione inhibits ornithine decarboxylase [2]. Anaerobically and under one atmosphere of nitric oxide, glutathione has a half-life of approximately 200 h

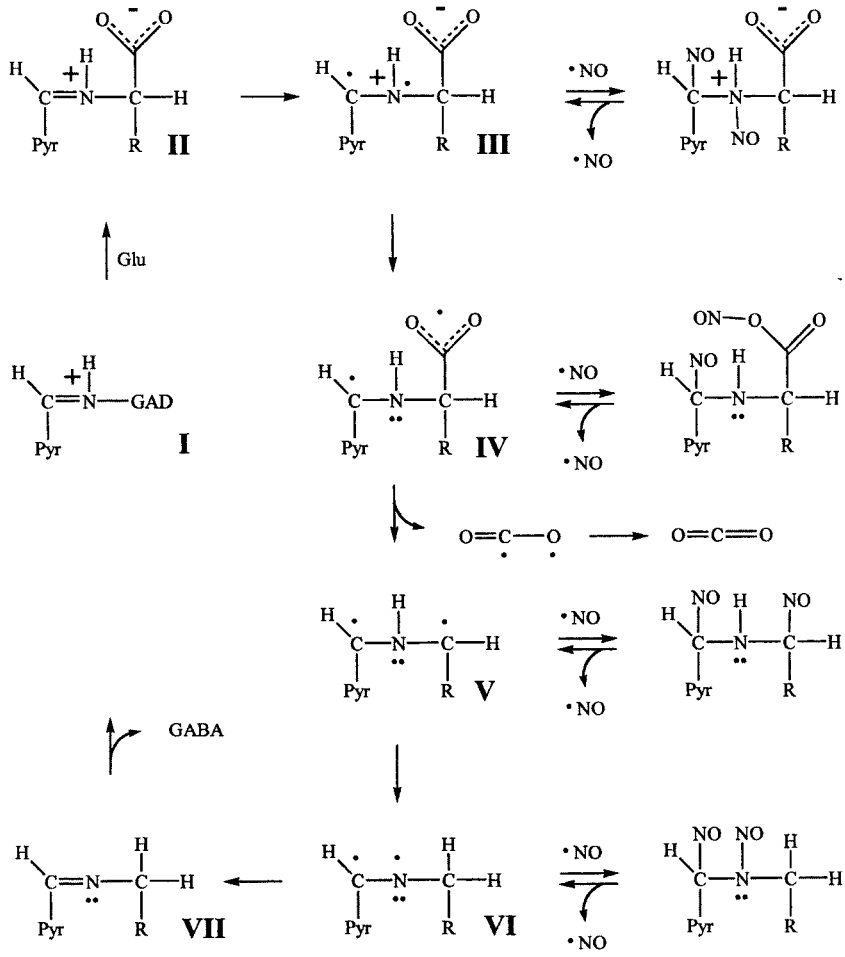

Fig. 2. Reversible inhibition of the GABA-producing reaction of GAD by nitric oxide.

(8 days) [13]. The rate at which nitric oxide reacts with thiols under anaerobic conditions is too slow for this to account for the mechanism by which nitric oxide inhibits GAD. By contrast, aerobically nitric oxide forms NO_2, N_2O_4, and N_2O_3 [4]. Dinitrogen trioxide (N_2O_3), which is formed from nitrogen dioxide and nitric oxide, is generally considered to be the nitrosylating agent that forms *S*-nitrosoglutathione in vivo [22]. *S*-Nitrosoglutathione inactivates a number of thiol-containing proteins, such as ornithine decarboxylase [2] and the V-type ATPase [12], by either *S*-nitrosylation or *S*-glutathionylation. It is likely that *S*-nitrosoglutathione also inactivates GAD, although we have not examined that possibility here. However, under the conditions employed in these experiments (anaerobic), we believe the mechanism by which nitric oxide inhibits GAD65 and GAD67 is by forming reversible adducts with enzymatic radical reaction intermediates (fig. 2). We do not attach any physiological significance to these interactions. Physiologically, the effect of oxygen on GAD65 may be further augmented by the inhibition by *S*-nitrosoglutathione of GAD65 and the V-type ATPase. In vivo nitric oxide has been reported to inhibit GABA transaminase [18]. The effect of nitric oxide on GABA transaminase may partially offset or balance the effects of oxygen and nitric oxide on GAD65 under some conditions. Clearly, a complete understanding of the relative importance of these interactions and their ultimate role in triggering an oxygen-induced seizure will require further investigation.

Acknowledgments

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