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Heterologous expression and purification of kynurenine-3-monooxygenase from *Pseudomonas fluorescens* strain 17400

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Abstract

Kynurenine 3-monooxygenase (KMO) is an NADPH-dependent flavoprotein hydroxylase that catalyzes the conversion of L-Kynurenine (L-Kyn) to 3-hydroxykynurenine (3OHKyn). The reaction is central to the tryptophan degradative pathway and takes place within microglial cells defining cellular concentrations of the *N*-methyl-D-aspatate (NMDA) receptor agonist quinolinate and antagonist kynurenate. The influence over the cellular concentrations of these NMDA receptor effectors makes KMO an attractive target for the treatment of ischemic stroke. *Pseudomonas fluorescens* str 17400, expresses five activities of tryptophan catabolism including that of KMO. The KMO gene from *P. fluorescens* was cloned into the pET-17b plasmid using incorporated *Nde*I and *Xho*I restriction sites. This construct yielded *Pf*KMO to 20% of total cell protein after 12 h of expression at 22 °C without induction by isopropyl-β-thiogalactopyranoside (IPTG). The enzyme could be readily purified using ammonium sulfate fractionation and ion exchange chromatography, resulting in pure KMO with a turnover number of 5.0 s^{-1}. *Pf*KMO activity was dependent on the reduction state of the enzyme. Preparation and storage benefited from the presence of a reductant such as dithiothreitol or β-mercaptoethanol. The loss of activity was found to be directly related to the oxidation of thiols as measured by dinitrothiobenzoate assay. Steady-state assays monitoring the consumption of dioxygen were used to measure apparent kinetic parameters and ligand perturbation of flavin fluorescence was used to determine a K_d value for both L-Kyn and the inhibitor *m*-nitrobenzoylalanine. *Pf*KMO is offered as prototypical bacterial form of the enzyme to serve as a viable platform on which to base future KMO studies.

Keywords: Kynurenine; Stroke; Purification; Expression; Pseudomonas; Monooxygenase; Hydroxylase; Flavin; FAD; Oxygenase; Aromatic

Introduction

Kynurenine 3-monooxygenase (KMO)[1] catalyzes the irreversible conversion of L-Kynurenine (L-Kyn) to 3-hydroxy-kynurenine (3HOKyn) consuming one molecule of both NADPH and dioxygen per turnover (Scheme 1) [1]. This reaction is part of tryptophan catabolism by which higher organisms make nicotinamide adenine dinucleotide. The KMO reaction is particularly dominant within microglial cells where it controls the production of various metabolites that promote neurological function. There is considerable interest in KMO as it has a central enzymatic role within the tryptophan catabolism pathway, directly controlling the production of quinolinate, an *N*-methyl-D-aspartate (NMDA) receptor agonist and indirectly the production of kynurenate, an NMDA receptor antagonist with the reported capacity to suppress glutamate release [2]. The inhibition of KMO changes the balance of these molecules to diminish the cellular concentration of quinolinate and enhance that of kynurenate (Scheme 2) [2–4].

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[1] *Abbreviations used:* KMO, kynurenine-3-monooxygenase; L-Kyn, L-Kynurenine; 3OHKyn, 3-hydroxykynurenine; DTT, dithiothreitol; NMDA; *N*-methyl-D-aspartate; FAH, flavoprotein aromatic hydroxylase; *m*-NBA, *meta*-nitrobenzoylalanine; PCR, polymerase chain reaction; HEPES, (*N*-(2-hydroxyethyl)piperizine-*N*′-(2-ethane sulfonic acid)); BME, β-mercaptoethanol; MES, morpholinoethanesulfonic acid; CAPS, *N*-cyclohexyl-3-aminopropanesulfonic acid; FAD, flavin adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate, DMSO, dimethyl sulfoxide; IPTG, isopropyl-β-thiogalactopyranoside; DTNB, dithiobis-(2-nitrobenzoic acid).

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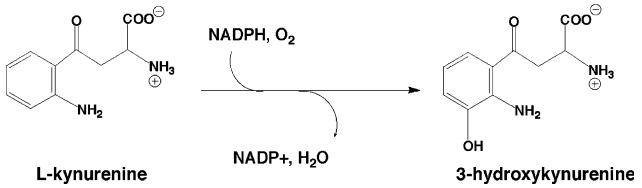
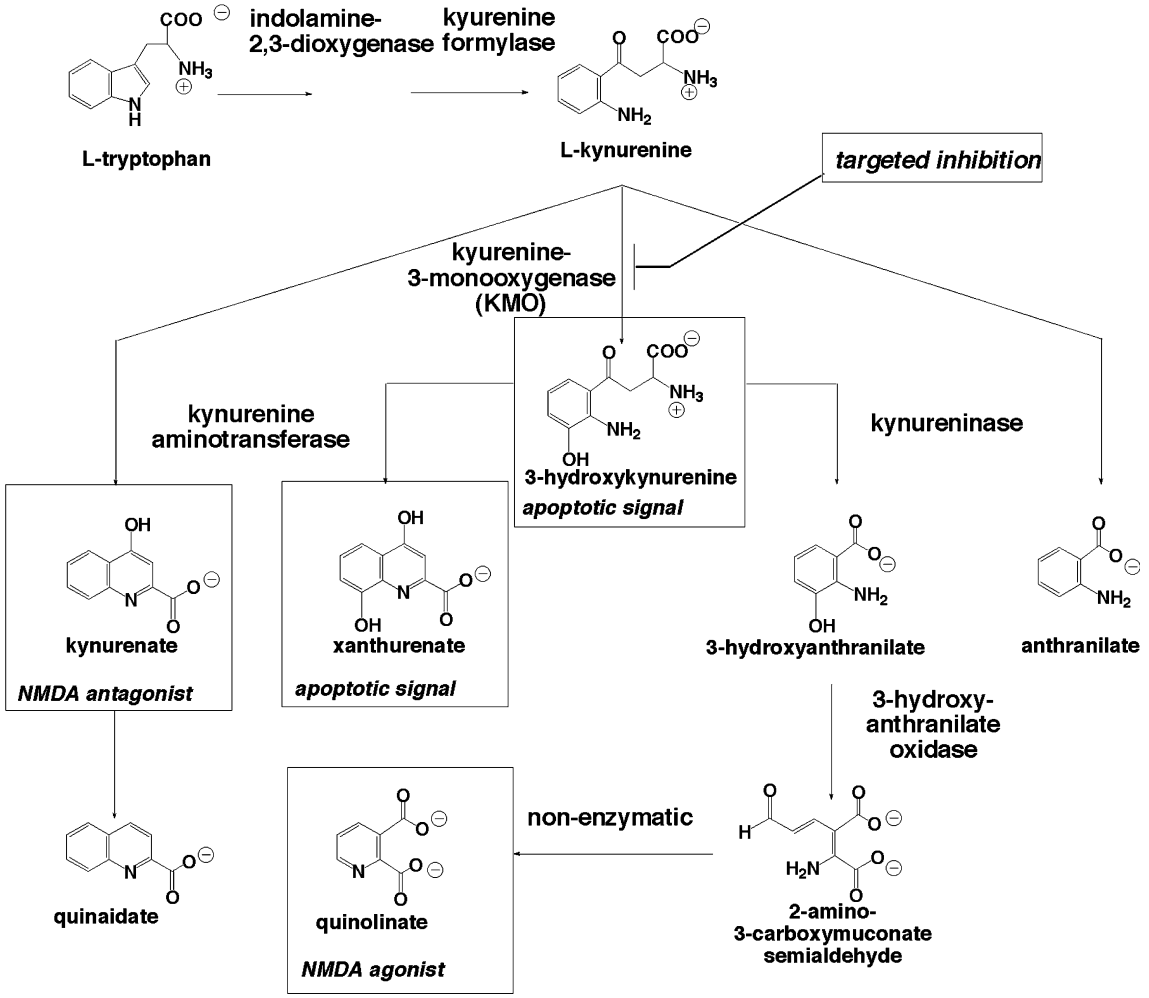


Scheme 1.

Moreover, this inhibition directly prevents the production of the product, 3OHKyn, and xanthurenate both of which have been implicated as apoptotic signals [5,6]. It has also been reported that auto-oxidation of 3HOKyn and the resulting radical species promote neurological cell death [7].

It has generally been regarded that molecules designed to block NMDA receptor interaction may be important in the treatment of ischemic stroke. However, the ubiquitous nature of the NMDA receptor has meant that the side effects of such drugs often outweigh the benefit [8]. The local targeting of KMO inhibition is an alternate method that uses endogenous metabolites to suppress the cascade of glutamate release that occurs during and after an ischemic event [4,9,10].

KMO is a member of the external flavoprotein aromatic hydroxylase (FAH) family of enzymes. However, KMO is distinct from other FAHs having only ~10% amino acid identity with other members of the family. Mammalian KMO is associated with the mitochondrial outer membrane [11–14]. These forms of KMO have proven to be generally quite unstable and difficult to express or purify in significant quantity [11,15]. A small number of bacteria express a number of the activities of tryptophan catabolism [16] including *Pseudomonas fluorescens* strain 17400 that carries the gene for KMO. This form of KMO has 36% identity and 54% homology with human KMO. We have cloned the *P. fluorescens* gene into the pET17b expression vector and defined conditions under which it is possible to successfully express and purify native KMO in *Escherichia coli* in high yield. This has involved assessment of basic stability requirements for the protein during expression, preparation and storage. In addition we have defined the dissociation constant for L-Kyn and the inhibitor *m*-nitrobenzolylalanine (*m*-NBA) and measured apparent



Scheme 2.