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NAD Biosynthesis: Identification of the Tryptophan to Quinolinate Pathway in Bacteria

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Summary

Previous studies have demonstrated two different biosynthetic pathways to quinolinate, the universal de novo precursor to the pyridine ring of NAD. In prokaryotes, quinolinate is formed from aspartate and dihydroxyacetone phosphate; in eukaryotes, it is formed from tryptophan. It has been generally believed that the tryptophan to quinolinic acid biosynthetic pathway is unique to eukaryotes; however, this paper describes the use of comparative genome analysis to identify likely candidates for all five genes involved in the tryptophan to quinolinic acid pathway in several bacteria. Representative examples of each of these genes were overexpressed, and the predicted functions are confirmed in each case using unambiguous biochemical assays.

Introduction

Nicotinamide adenine dinucleotide (NAD) and its phosphate (NADP) are essential redox cofactors in all living systems. In addition to its redox role as a hydride acceptor, this cofactor is a substrate for protein ADP-ribosylation, which occurs as a regulatory event in DNA repair [1] and in the activation of cholera and diphtheria toxins [2]. ADP-ribosylation also functions as part of the catalytic mechanism of NAD-dependent histone deacetylases [3].

Previous studies have demonstrated two different biosynthetic pathways to quinolinate, the universal de novo precursor to the pyridine ring of NAD [4, 5]. In prokaryotes, quinolinate is formed from aspartate and dihydroxyacetone phosphate; in eukaryotes, it is formed from tryptophan (Figure 1). In the eukaryotic pathway, tryptophan is cleaved by tryptophan-2,3-dioxygenase (TDO) to yield N-formylkynurenine [6]. Removal of the formyl group by N-formylkynurenine formamidase (KFA) results in kynurenine [7]. Hydroxylation, catalyzed by kynurenine-3-monooxygenase (KMO) gives 3-hydroxykynurenine [8]. Cleavage of the amino acid side chain by kynureninase (KYN) to give 3-hydroxyanthranilate, followed by the 3-hydroxyanthranilate-3,4-dioxygenase (HAD)-catalyzed complex oxidative rearrangement of 3-hydroxyanthranilate yields quinolinate [9–11].

In humans and in yeast the biosynthetic genes for all of the enzymes required for the conversion of tryptophan to quinolinate, with the exception of N-formylkynurenine formamidase, have been identified [12–19]. However, mechanistic studies on this pathway are still at an early stage because of the difficulty in overexpressing the enzymes.

While it is generally believed that the tryptophan to quinolinic acid biosynthetic pathway is unique to eukaryotes, labeling studies, using ^{14}C-tryptophan, demonstrate that *Streptomyces antibioticus*, *Cyanidium caldarium*, *Karlingia rosea*, and *Xanthomonas pruni* can utilize tryptophan to biosynthesize quinolinic acid [20, 21]. In addition, the biosynthesis of actinomycin by *Streptomyces antibioticus* involves the conversion of tryptophan to 3-hydroxyanthranilic acid, utilizing the first four of the five enzymes involved in quinolinic acid formation [22]. Furthermore, tryptophan dioxygenase, kynureninase, and anthranilate 1,2-dioxygenase activities have been detected in *Pseudomonas aureofaciens* [23], N-formylkynurenine formamidase has been purified from *Streptomyces parvulus* [24], and kynureninase has been purified from *Pseudomonas fluorescens* [9]. However, with the exception of the kynureninase, which has been thoroughly characterized, none of the other bacterial genes have been identified, and very little mechanistic enzymology has been reported on these important enzymes.

In addition to the biosynthesis of NAD, the tryptophan to quinolinic acid biosynthetic pathway plays an important role in several other biological processes [25]. For example, alterations in brain serotonin levels are known to result in mood disorders [26], and the availability of tryptophan for serotonin synthesis in the brain appears to be dependent on the rate of hepatic tryptophan-2,3-dioxygenase activity [15]. It has also been recently demonstrated that 3-hydroxykynurenine induces apoptosis of neurons prepared from rat striatum [27–31] and cross-linking reactions mediated by 3-hydroxykynurenine may play a role in cataract formation in the mammalian eye [32–35]. 3-hydroxykynurenine and quinolinate concentrations are elevated in patients with AIDS-related dementia, Huntington's disease, and hepatic encephalopathy. Furthermore, quinolinate acts on NMDA receptors in the brain causing neuronal injury and death. Mechanistic and structural studies on the quinolinate pathway biosynthetic enzymes will facilitate the identification of small molecule inhibitors to better understand and control these disease states.

In this paper, we describe the use of comparative genome analysis to identify likely candidates for all five genes involved in the tryptophan to quinolinic acid pathway in several bacteria. Representative examples of each of these genes are overexpressed, and unambiguous biochemical assays were used to confirm the predicted functions in each case.

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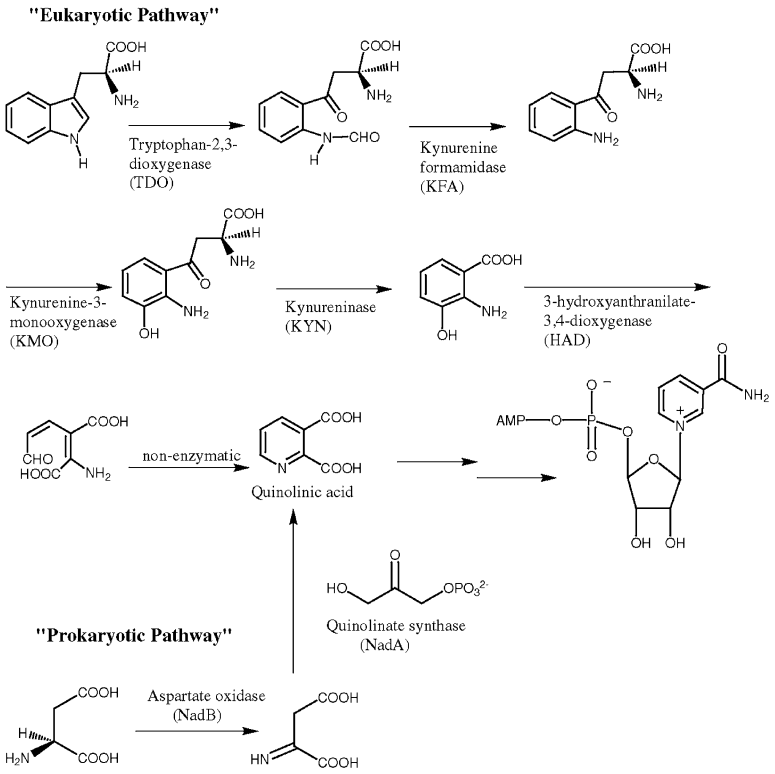

Figure 1. The Two Biosynthetic Routes to Quinolinic Acid

Results and Discussion

Identification of the Tryptophan to Quinolinate Biosynthetic Enzymes in Bacteria

While clear evidence from labeling studies exists for the occurrence of the tryptophan to quinolinate pathway in bacteria, until recently the kynureninase (KYN) gene from *Pseudomonas fluorescens* was the only characterized bacterial gene related to this pathway. By screening genomic archives, we detected the presence of eukaryotic tryptophan-2,3-dioxygenase (TDO), kynurenine-3-monooxygenase (KMO), and 3-hydroxyanthranilate-3,4-dioxygenase (HAD) homologs in a few recently sequenced bacterial genomes, including *Cytophaga hutchinsonii* and *Ralstonia metallidurans*. Identification of these genes along with the discovery of bacterial kynureninase homologs enabled us to identify a tryptophan to quinolinate pathway in bacterial species.

Cytophaga hutchinsonii was the first bacterial genome available to us containing orthologs of both the KMO and HAD enzymes encoded within an operon-like genomic cluster with the KYN encoding gene (see Figure 2). A putative ortholog of the TDO gene was also identified outside of the KMO-KYN-HAD cluster. In addition, the presence of the *nadC* gene and the absence of the *nadB-nadA* genes (see Table 1) provided strong evidence for the prediction of a eukaryotic-like quinolinate biosynthesis from L-tryptophan in this bacterium. An intriguing feature of *C. hutchinsonii*, as well as of the recently sequenced *Xanthomonas axonopodis* and *Xanthomonas campestris* genomes, is the absence of bacterial KFA homologs (see Table 1). Furthermore, these genomes do not contain any homologs of the recently identified eukaryotic KFA [36]. These observations indicate the possible existence of a nonorthologus form of KFA, characteristic of *Cytophaga* and *Xanthomonas* ssp.

Potential *Ralstonia metallidurans* genes for TDO, KYN, and HAD were readily identified using homology searches of public sequence archives and the ERGO database. The *R. metallidurans* KFA gene could not be identified in the same way because a reference sequence had not yet been determined. Instead, we made a functional prediction for a potential KFA gene by using the genome context analysis approach [37]. We identified a previously uncharacterized gene, possessing a metal-dependent hydrolase sequence signature, as a putative bacterial KFA-encoding gene due to its strong tendency to cluster on the chromosome with the TDO and KYN genes. This functional prediction was experimentally verified by overexpression and preliminary enzymatic characterization of the protein and is fully described by us elsewhere [38].

The predicted gene functions from *R. metallidurans* and *C. hutchinsonii* were validated as part of the tryptophan to quinolinate pathway by coexpressing the KYN-KFA-TDO gene cluster from *R. metallidurans* with the HAD-KYN-KMO gene cluster from *C. hutchinsonii* in an *E. coli* mutant incapable of biosynthesizing quinolinate. Further confirmation of the predicted functions was accomplished by the biochemical characterization of a gene product from either organism for each unique functional assignment.

While this work was in progress, the recently sequenced bacterial genomes *Gemmata sp*. and *Polaribacter filamentus* became available. Since neither organism contains the genes required for de novo biosynthesis or niacin salvage, the tryptophan to quinolinate pathway

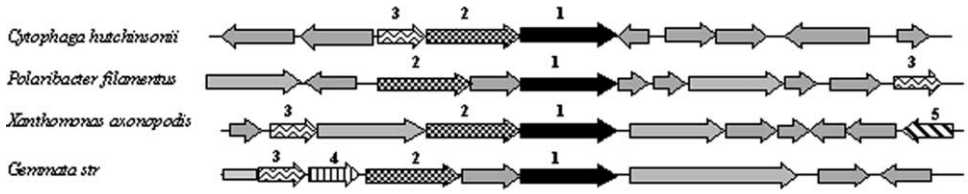

Figure 2. Chromosomal Clustering of Genes Involved with the L-Tryptophan to Quinolinate Pathway

Contigs of four microbial genomes are aligned ("pinned") around orthologs of KMO gene (1). Relevant homologous genes conserved in this genomic neighborhood are labeled with matching patterns and numbers: 2, KYN; 3, HAD; 4, KFA. Nonconserved genes are colored gray. The presence of NAD kinase (5) in the same chromosomal neighborhood was detected only for *Xanthomonas* ssp.

is the only conceivable route for NAD biosynthesis. These two genomes do in fact contain all five genes required for biosynthesis of quinolinate from tryptophan. Remarkably, these genomes also revealed a characteristic chromosomal clustering pattern. In *P. filamentus*, the KMO, KYN, and HAD genes are clustered on the chromosome, and *Gemmata sp*. provides the first example of bacterial KFA coclustered with these genes (see Figure 2). The presence of a complete tryptophan to quinolinate pathway in *Gemmata sp*. and *P. filamentus* is further validation of the functional assignments made to the *C. hutchinsonii* and *R. metallidurans* genome sequences.

Verification of the Tryptophan to Quinolinate Pathway by In Vivo Complementation

E. coli MG1655:Δ(*pnuC-nadA*), which is unable to biosynthesize quinolinic acid, was cotransformed with plasmids containing the *R. metallidurans*, KFA-TDO-KYN putative operon [pPRO-*rm*Locus(RT)], and the *C. hutchinsonii* HAD-KYN-KMO putative operon (pBAD-*ch*Locus) to verify the possibility of in vivo conversion of tryptophan to quinolinate. Stable cotransformants containing both plasmids grew normally in rich medium and in minimal medium supplemented with niacin. In the absence of niacin but in the presence of 100 μM tryptophan, they grew slowly, requiring approximately 72 hr to reach an OD_{600} ~2 in liquid minimal medium and to form colonies on minimal agar plates. Control strains cotransformed by both "empty" vectors (pPROLar and pBAD) or by one vector plasmid and one construct [either pPRO-*rm*Locus(RT) or pBAD-*ch*Locus] grew normally in the minimal medium supplemented by niacin but did not grow in the presence of tryptophan. No growth of the *E. coli* MG1655:Δ(*pnuC-nadA*) [pPRO-*rm*Locus(RT); pBAD-*ch*Locus] was observed over 96 hr in the absence of added tryptophan. This was unanticipated; while the *E. coli* strain used is not a tryptophan auxotroph, apparently the endogenous tryptophan concentrations are not sufficient to support quinolinate biosynthesis using the installed tryptophan pathway, possibly because of the competing nonproductive degradation of tryptophan to anthranilic acid. The observed growth of the doubly transformed *nadA* mutant provided strong support for the putative functional assignments.

Overexpression and Characterization of Tryptophan-2,3-Dioxygenase

The putative TDO gene from *R. metallidurans* was overexpressed as a soluble 34 kDa protein and readily purified by Ni-NTA affinity chromatography (Figure 3, lanes 2 and 3). The enzyme is a tetramer in its active form. The enzyme catalyzes the oxidation of tryptophan to N-formylkynurenine, and the identity of the reaction product was confirmed by HPLC and NMR analysis. The enzyme was assayed by monitoring the appearance of N-formylkynurenine at 321 nm. Kinetic parameters are shown in Table 2. The enzyme demonstrated no activity increase with the addition of external heme to the reaction mixture, establishing that the cofactor was not limiting.

The oxidative ring opening of aromatic compounds is typically catalyzed by nonheme iron-dependent enzymes, and the use of the heme cofactor for the opening of the indole ring is unusual. The mechanism of this reaction is still poorly understood, and there is no structure available for any member of this family.

Overexpression and Characterization of N-Formylkynurenine Formamidase

The putative KFA gene from *R. metallidurans* contained a stop codon in the middle of the coding region, and the corresponding truncated protein had no enzymatic activity. This mutation was corrected, and the resulting KFA gene was overexpressed as a soluble 23.3 kDa protein and readily purified by Ni-NTA affinity chromatography (Figure 3, lanes 4 and 5). The product of the enzymatic reaction was identified as kynurenine by HPLC and NMR analysis. The enzyme was assayed by monitoring the disappearance of N-formylkynurenine at 321 nm. Kinetic parameters are shown in Table 2 and are corrected for the slow nonenzymatic hydrolysis of the substrate. The enzyme displays a sharp pH optimum at pH 8.0 and is inhibited by EDTA but not PMSF. In

Table 1. Genes and Pathways Related to Aerobic L-Tryptophan Degradation and NAD Biosynthesis

		BACTERIA										EUKARYA		
PATHWAYS/ENZYMES	Example in human (Genbank ID)	*Escherichia coli*	*Bacillus subtilis*	*Pseudomonas aeruginosa*	*Bacillus cereus*	*Ralstonia solanacearum*	*Ralstonia metallidurans*	*Cytophaga hutchinsonii*	*Xanthomonas axonopodis*	*Gemmata str*	*Polaribacter filamentus*	*Saccharomyces cerevisiae*	*Homo sapiens*	*Arabidopsis thaliana*
								quinolinate pathway						
(aerobic Tryptophan degradation pathways in bacteria)		N/A		anthranilate pathway				incomplete			complete			N/A
DE NOVO PATHWAYS TO NaMN														
TRYPTOPHAN TO QUINOLINATE (mostly eukaryotes)		*no*	*no*	*no*	*no*	*no*	*?*	*yes*	*yes*	*yes*	*yes*	*yes*	*yes*	*no*
Tryptophan 2,3-dioxygenase														
Tryptophan 2,3-dioxygenase (EC 1.13.11.42)	gi|993046	−	−	+	+	+	+	+	+	+	+	+	+	−
or Indoleamine 2,3-dioxygenase (EC 3.7.1.3)	gi|306956	−	−	−	−	−	−	−	−	−	−	−	+	−
N-formylkynurenine (aryl-) formamidase (EC 3.5.1.9)								?	?					−
form I (bacteria)	(PA2081)[1]	−	−	+	+	+	+	−	−	+	+	−	−	−
form II (eukaryotes)	gi|17481546	−	−	−	−	−	−	−	−	−	−	−	+	−
Kynurenine-3-monooxygenase (EC 1.14.13.9)	gi|3695027	−	−	−	−	−	−	+	+	+	+	+	+	−
3-hydroxy-kynureninase (EC 3.7.1.3)	gi|1323715	−	−	+	+	+	+	+	+	+	+	+	+	−
3-hydroxyanthranilate 3,4-dioxygenase (1.13.11.6)	gi|443919	−	−	−	−	−	+	+	+	+	+	+	+	−
or ASPARTATE TO QUINOLINATE (mostly prokaryotes)		*yes*	*yes*	*yes*	*yes*	*yes*	*yes*	*no*	*no*	*no*	*no*	*no*	*no*	*yes*
Aspartate Oxidase (*nadB*; EC 1.4.3.16)	(PA0761)[1]	+	+	+	+	+	+	−	−	−	−	−	−	+
Quinolinate Synthase (*nadA*; EC 4.1.99.-)	(PA1004)[1]	+	+	+	+	+	+	−	−	−	−	−	−	+
and QUINOLINATE TO NaMN (universal)		yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
Quinolinate Phosphoribosyl Transferase (*nadC*; EC 2.4.2.19)	gi|1060907	+	+	+	+	+	+	+	+	+	+	+	+	+
SALVAGE/RECYCLING TO NaMN/NMN														
NICOTINAMIDE TO NaMN (via nicotinate phosphoribosyltransferase)		*yes*	*yes*	*yes*	*yes*	*no*	*yes*	*no*	*yes*	*no*	*no*	*yes*	*yes*	*yes*
or NICOTINAMIDE TO NMN (via nicotinamide phosphoribosyltransferase)		*no*	*no*	*no*	*no*	*yes*	*yes*	*yes*	*yes*	*no*	*no*	*no*	*yes*	*no*
or NICOTINAMIDE RIBOSE TO NMN (via ribosylnicotinamide kinase)		*yes*	*no*	*yes*	*no*	*no*	*no*	*no*	*no*	*no*	*no*	*no*	*yes*	*no*
UNIVERSAL PATHWAYS: NaMN/NMN TO NAD and NADP		*yes*	*yes*	*yes*	*yes*	*yes*	*yes*	*yes*	*yes*	*yes*	*yes*	*yes*	*yes*	*yes*

Genes marked with a "1" are not present in human; representatives from *P. aeruginosa* are shown using original genome ID numbers. Gene PA2081 was originally annotated as "hypothetical protein".

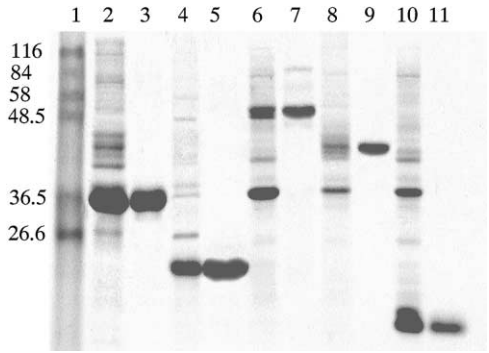

Figure 3. SDS PAGE Analysis of the Overexpression of the Enzymes Involved in the Conversion of Tryptophan to Quinolinic Acid

Lane 1: molecular weight standards. Lanes 2 and 3: TDO-soluble cell-free extract and purified protein. Lanes 4 and 5: KFA-soluble cell-free extract and purified protein. Lanes 6 and 7: KMO-soluble cell-free extract and purified protein. Lanes 8 and 9: KYN-soluble cell-free extract and purified protein. Lanes 10 and 11: HAD-soluble cell-free extract and purified protein.

addition, a conserved metal binding site can be identified in the sequence. This suggests that bacterial KFA may be a zinc ion-dependent metalloprotease and not a serine protease as has been found for the mammalian and the avian enzyme.

Overexpression and Characterization of Kynurenine-3-Monooxygenase

The predicted KMO gene from *C. hutchinsonii* was overexpressed as an insoluble 54 kDa protein. However, soluble preparations could be obtained by adding 0.2% Triton X-100 to the buffers used for cell lysis and enzyme purification. Under these conditions, the enzyme was readily purified by Ni-NTA affinity chromatography (Figure 3, lanes 6 and 7). KMO contains a tightly bound flavin cofactor (FAD). The enzyme was assayed by monitoring the disappearance of NADPH at 340 nm. Kinetic parameters are shown in Table 2. The enzyme will also utilize NADH with a 50% reduction in V_{max}.

Kynurenine-3-monooxygenase is a member of a well-studied enzyme family that utilizes a flavin hydroperoxide to hydroxylate electron-rich aromatic compounds. The availability of this enzyme in large quantities will facilitate the design of inhibitors.

Overexpression and Characterization of 3-Hydroxy-Kynureninase

The putative KYN gene from *R. metallidurans* was overexpressed as a soluble 44.8 kDa protein and purified by Ni-NTA affinity chromatography (Figure 3, lanes 8 and 9). The enzyme contains tightly bound pyridoxal phosphate as a cofactor. KYN catalyzes the cleavage of the side chain from both 3-hydroxykynurenine and from kynurenine to give 3-hydroxyanthranilic acid and anthranilic acid, respectively. In each case, the products of the enzymatic reaction were confirmed by HPLC and NMR analysis. The enzyme was assayed by monitoring the disappearance of L-3-hydroxykynurenine at 371 nm or L-kynurenine at 360 nm. Kinetic parameters are shown in Table 2.

Overexpression and Characterization of 3-Hydroxyanthranilate-3,4-Dioxygenase

The predicted HAD gene from *R. metallidurans* was overexpressed as a soluble 22.5 kDa protein. While the enzyme was readily purified by Ni-NTA affinity chromatography, the purified protein lost most of its catalytic activity (Figure 3, lanes 10 and 11). Functional characterization was therefore carried out using gel-filtered cell-free extract from the overexpression strain. HAD requires iron (II) as a cofactor. HPLC and NMR analysis of the enzymatic reaction mixture demonstrated the formation of α-amino-β-carboxymuconic semialdehyde, which underwent a nonenzymatic conversion to quinolinate. The enzyme was assayed by monitoring the appearance of α-amino-β-carboxymuconic semialdehyde at 360 nm. The reaction shows substrate inhibition (K_i, 1.35 mM), and the kinetic parameters are shown in Table 2.

The mechanism of 3-hydroxyanthranilate-3,4-dioxygenase is likely to be similar to the mechanism of the well-studied catechol dioxygenases [39]. However, the mechanism of its inhibition by such molecules as 4-chloro-3-hydroxyanthranilate, as well as the mechanism by which α-amino-β-carboxymuconic semialdehyde cyclizes to quinolinic acid, remain to be elucidated.

Significance

Quinolinate is the precursor to NAD in both prokaryotic and eukaryotic organisms. The biosynthesis of quinolinate from tryptophan, previously identified only in eukaryotic organisms, is still poorly understood due to the experimental intractability of the eukaryotic en-

Table 2. Kinetic Parameters for the Enzymes of the Tryptophan to Quinolinate Pathway

Enzyme	Subunit Mass (kDa)	Km (mM)	kcat (s^{-1})	kcat/ Km($M^{-1}s^{-1}$)	Cofactor
Tryptophan-2,3-dioxygenase[a]	34	0.35 ± 0.3	22.6	6.4×10^4	heme/Fe^{++}
N-formylkynurenine formamidase[a]	23.3	0.075 ± 0.009	0.77 ± 0.03	1.03×10^4	ND
Kynurenine-3-monooxygenase[b]	54	0.076 ± 0.004 (Kyn) 0.089 ± 0.006 (NADPH)	2.15	2.8×10^4	FAD
3-hydroxy-kynureninase[a]	44.8	0.07 ± 0.06 (Kyn) 0.040 ± 0.002 (hydroxyKyn)	4.52 (Kyn) 2.9 (hydroxyKyn)	6.4×10^4 7.4×10^4	PLP
3-hydroxyanthranilate-3,4-dioxygenase[a]	22.5	1.22 ± 0.07	8.65	7.1×10^3	Fe^{++}

[a] From *Ralstonia metallidurans*.
[b] From *Cytophaga hutchinsonii*.

zymes. In this study, comparative genome analysis and metabolic reconstruction of the tryptophan to quinolinate pathway in several bacterial species was performed using the ERGO genomic database and a set of tools implemented therein. Implementation of these techniques culminated with the discovery of the tryptophan to quinolinate pathway in several bacterial species. The five bacterial enzymes required to convert tryptophan to quinolinate were cloned, overexpressed, and unambiguously characterized. The stability of the bacterial proteins allows for mechanistic and structural studies that are not possible with the eukaryotic enzymes, and the neurotoxicity of the pathway metabolites makes detailed study of their enzymatic formation of great interest. This study demonstrates the enormous potential within the extensive bacterial genomic archives available for exploration. There are many examples of important enzymes, considered unique to eukaryotic biosynthetic pathways, that are experimentally intractable due to instability, inclusion body formation, or failure to overexpress. As demonstrated here, the use of comparative genomics to identify bacterial orthologs for all of the five enzymes involved in the conversion of tryptophan to quinolinate enabled us to overexpress each of these enzymes as soluble proteins, opening up this important "eukaryotic" biosynthetic pathway to mechanistic and structural studies. With the availability of more than 400 bacterial genome sequences, the search for bacterial orthologs of experimentally intractable eukaryotic proteins is likely to be a productive one.

Experimental Procedures

Bacterial Strains, Media Components, Plasmids, Reagents, and General Procedures

Escherichia coli strains DH5α (Life Technologies, Rockville, MD), TOP10, BL21(DE3), and Tuner(DE3) (Stratagene, La Jolla, CA) were used for cloning and protein overexpression. *E. coli* K-12 strain MG1655 was used for the complementation experiments. Chromosomal DNA from *Cytophaga hutchinsonii* ATCC 33406 was obtained from M.J. McBride (University of Wisconsin, Milwaukee). *E. coli* strains were grown on LB media or M9 minimal media (Sigma) under standard laboratory conditions. A pProEX HTa vector (Life Technologies) containing a *Trc* promoter, N-terminal His_6tag, and TEV-protease cleavage site, or a pET16b vector (Novagen) containing a T7 promoter, N-terminal His_{10}tag, and a Factor Xa cleavage site were used for cloning and protein expression. Engineering of the expression plasmid pPRO-*rm*Locus(RT) containing a modified operon-like segment from *R. metallidurans* encoding KFA*-KYN-TDO in the pPROLar.A122 vector (Clontech) under control of the IPTG-inducible $P_{lac/ara-1}$ hybrid promoter used in this study is described by us elsewhere [38]. The vector pBAD-TOPO (Stratagene) with the L-arabinose-inducible *ara*BAD promoter (P_{BAD}) was used for cloning of the *C. hutchinsonii* ATCC 33406 operon-like locus encoding HAD-KYN-KMO. Oligonucleotide primers were made by the Oligonucleotide Synthesis Facility at the Cornell University Bioresource Center, or were obtained from Sigma-Genosys (Woodlands, TX), or from MWG Biotech Inc (High Point, NC). PCR reagents and enzymes for DNA manipulations were from New England Biolabs (Beverly, MA) and Fermentas (Vilnius, Lithuania). Plasmid purification kits and Ni-NTA resin were purchased from Qiagen (Valencia, CA). Reagents for DNA sequencing were from PE Biosystems (Foster City, CA). Antibiotics, buffer components, and all reagents for enzymatic assays and chemical synthesis were from Sigma-Aldrich. The basic molecular biology procedures for bacterial growth, plasmid preparation, restriction analysis, and transformation of competent cells were performed as described by Maniatis et al. [40].

For the determination of kinetic parameters, initial rates were plotted versus the substrate concentration and fit to the Michaelis Menten equation using Origin 6.0 [41]. For HAD, which shows substrate inhibition, initial rates were plotted versus the substrate concentration and fit to $\upsilon = V_{max}S/(S+K_m+(S^2/K_i))$.

Identification of the Tryptophan to Quinolinate Pathway in Bacteria

Comparative genome analysis and metabolic reconstruction of quinolinate/NAD biosynthesis in various species was performed using the ERGO genomic database and a set of tools implemented therein [42]. The genes coding for the enzymes involved in the tryptophan to quinolinate pathway in bacterial genomes were tentatively identified by homology with known sequences from *H. sapiens* and *S. cerevisiae*, except for bacterial KFA. The gene encoding for the latter enzyme was identified and experimentally verified by us in representative gram-negative and gram-positive bacteria [38]. Orthologs of bacterial KFA are not present in eukaryotes but they are found by homology searches in a number of bacteria, including those containing other putative genes involved with the tryptophan to quinolinate pathway. Additional public bioinformatics resources used were GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html), PSI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), and PFAM (http://pfam.wustl.edu/). Genomic sequences used in this study were *E. coli* K12 MG1655 (GenBank U00096), *Pseudomonas aeruginosa* PA01 (GenBank AE004091), *Ralstonia solanacearum* (GenBank AL646052), *Ralstonia metallidurans* CH34 (formerly *Ralstonia eutropha* or *Alcaligenes eutrophus*; http://www.jgi.doe.gov/JGI_microbial/html/ralstonia/ralston_homepage.html), *Cytophaga hutchinsonii* ATCC 33406 (GenBank AE016877), *Bacillus cereus* ATCC 14579 (GenBank AE016877), *Bacillus subtilis* subsp. *subtilis* str. 168 (GenBank AL009126), *Xanthomonas axonopodis* pv. citri str. 306 (GenBank AE008923), *Saccharomyces cerevisiae* (http://genome-www.stanford.edu/*Saccharomyces*/), *Homo sapiens* (http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9606), and *Arabadopsis thaliana* (http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=3702). *Polaribacter filamentus* and *Gemmata sp*. genomes were sequenced and analyzed by Integrated Genomics. Sequences of the genes related to quinolinate/NAD biosynthesis referred to in this study are deposited in GenBank.

Cloning of the Quinolinate Biosynthetic Gene Clusters

The KFA-KYN-TDO gene cluster from *R. metallidurans* CH34 was PCR amplified and cloned into a pPro-LAR vector, and the naturally occurring stop codon disrupting the KFA encoding was corrected (TAA→TAC; Stop82Tyr) by site-directed mutagenesis [38]. The resultant plasmid pPRO-*rm*Locus(RT) was used by us in this study for (a) in vivo quinolinate pathway verification and (b) amplification, cloning, overexpression, purification, and in vitro characterization of *R. metallidurans* KFA* (with corrected stop codon), *R. metallidurans* KYN, and *R. metallidurans* TDO.

The PCR amplification of the HAD-KYN-KMO gene cluster from *C. hutchinsonii* ATCC 33406 was achieved using 5′-ATGCTCAAGC CATTTAACTTCAAAGTCTG-3′ and 5′-TTATATATATTTTTTTGTCAC TGTAATGACTTTATTTTTTAATTCTTCTG-3′ as the forward and reverse primers, respectively. The PCR-amplified fragment was purified and directly cloned into pBAD-TOPO via the "TOPO TA" cloning strategy. *E. coli* strain TOP10 was used for subsequent transformation. The resultant plasmid pBAD-*ch*Locus was verified by DNA sequence analysis.

Engineering the *E. coli* MG1655 *pnuC-nadA* Deletion Mutant

The *E. coli* nadA-pnuC operon was deleted using the PCR product-mediated one-step gene inactivation procedure as previously described [43]. A pair of long composite primers each containing a 20 bp long priming sequence (shown below in lower case) and a 40 bp extension homologous to the region immediately upstream of the *nadA* or downstream of the *pnuC* coding sequence (upper case) were designed as follows. Forward primer: 5′-TGTCGGTTTTAACG TTAAGCCTGTAAAACGAGATGGTAAGgtgtaggctggagctgcttc-3′; reverse primer: 5′-ACCTGAATGAAGGGGTGTCTGGCCATTCACATC ATCACCAcatatgaatatcctccttagt-3′. Using this set of primers and the pKD3 plasmid [43] as a template, the chloramphenicol-resistance

cassette flanked by regions of homology with the *nadA-pnuC* locus was amplified. The PCR product was gel purified and electroporated into MG1655 cells expressing the lambda red recombination proteins from the pKD46 expression plasmid [43]. Recombinants were isolated by plating on LB supplemented with 25 μg/ml chloramphenicol, colony purified by passage at nonpermissive temperature (37°C), and then tested for ampicillin sensitivity to achieve the loss of the helper plasmid pKD46. Introduction of the desired deletion was confirmed by PCR and also phenotypically: Cm-resistant recombinant colonies were unable to grow on M9 media and growth could be restored by addition of 50 μM niacin.

In Vivo Verification of the Assembled Tryptophan to Quinolinate Pathway

E. coli MG1655: Δ(*pnuC-nadA*) was transformed with the compatible plasmids pPRO-*rm*Locus(RT) and pBAD-*ch*Locus. As a control, this strain was also transformed with the parent plasmids. These strains were grown in liquid LB media in the presence of the appropriate antibiotics (20 μg/ml kanamycin and 20 μg/ml ampicillin) to OD_{600} ~0.8. After induction with 0.2% arabinose and 2 mM IPTG, the cells were grown for 3 hr, harvested by centrifugation, washed with M9 media, and diluted 1:100 in M9 media supplemented with 0.4% glycerol or glucose, 0.2% arabinose, and 2 mM IPTG in the presence or absence of 500 μM L-tryptophan or 50 μM niacin. Cell growth was monitored by OD_{600} over 5 days. Alternatively, overnight cultures washed with M9 media were streaked onto M9 plates supplemented with 0.4% glycerol, 0.2% arabinose, and 0.8 mM IPTG in the presence or absence of 500 μM L-tryptophan or 50 μM niacin and grown for ~72 hr.

Overexpression and Purification of Tryptophan-2,3-Dioxygenase

The TDO gene from *R. metallidurans* was PCR amplified using primers 5′-GGGGA*TCATGA*GCGAATTCAAGGGATGC-3′ and 5′-GGG *GTCGAC*TACAGGTCTGTCCGCAGTTTCCACA-3′ as the forward and reverse primers, respectively, and plasmid pPRO-*rm*Locus(RT) as a template. The PCR fragment was purified, digested with BspHI and SalI, cloned into appropriately digested pProEX Hta, and the resulting DNA used to transform *E. coli* BL21(DE3). The nucleotide sequence of the insert was confirmed by DNA sequencing.

LB media (5 ml) containing 100 μg/ml of ampicillin was inoculated with a single colony of transformed *E. coli* BL21(DE3). The culture was incubated overnight at 37°C with agitation. On the next day, 1 liter of LB medium containing 100 μg/ml ampicillin was inoculated with 100 μl of the starter culture. The cells were grown with shaking until the culture reached an A_{590} of 0.6. IPTG was added to a final concentration of 0.6 mM, and the culture was incubated for an additional 3 hr at 37°C. The cells were then harvested by centrifugation at 5000 × *g* for 15 min at 4°C and stored at −80°C. The frozen cells were resuspended in potassium phosphate buffer (0.1 M, pH 7.0, 15 ml/g cell paste). Protease inhibitors (1 mM PMSF, 2 μM leupeptin, and 0.02 mg/ml aprotinin) were added, and the cells were sonicated on ice. The cells debris was removed by centrifugation at 20,000 × *g* for 15 min at 4°C, and the supernatant containing TDO was further fractionated by Ni-NTA-affinity chromatography (Ni-NTA superflow resin). Before application to the Ni-NTA column, the imidazole concentration of the supernatant solution was adjusted to 10 mM. The Ni-NTA column was subsequently washed with 20 ml of 0.05 M potassium phosphate buffer (pH 8.0, 20 mM imidazole, 300 mM NaCl). TDO was eluted from the column with 0.05 M potassium phosphate, 200 mM imidazole, 300 mM NaCl (pH 8.0). The fractions containing TDO were combined and exchanged into 0.1 M potassium phosphate (pH 7.0) to remove imidazole and NaCl from the solution.

Identification of the TDO Reaction Product by HPLC Analysis

A typical reaction mixture contained 30 μl of L-tryptophan (10 mM), 25 μl of ascorbic acid (2 mM), 10 μl of enzyme sample (10 μM), and 435 μl of 0.1 M potassium phosphate buffer (pH 7.0). The enzymatic reaction was stopped after various times by the addition of 10% v/v trifluoroacetic acid (2 M), and the precipitated protein was removed by centrifugation at 9200 × *g* for 2 min. A 50 μl sample was analyzed by HPLC (reverse-phase LiCrospher RP18, 5 μm; flow rate, 1ml/min; solvent 9:1 0.1% aqueous trifluoroacetic acid:acetonitrile) with detection of N-formylkynurenine by absorbance at 321 nm or the fluorescence of tryptophan at 366 nm (excitation at 286 nm). N-formylkynurenine and tryptophan eluted after 5 and 8 min, respectively.

Characterization of the TDO Reaction Product by NMR

TDO (290 μg) was added to a 0.8 ml solution of L-tryptophan (11.6 mM) and ascorbic acid (1 mM) in D_2O. After 15 min at room temperature, the NMR spectrum of the reaction mixture demonstrated 100% conversion of L-tryptophan to N-formyl-L-kynurenine. ^1H NMR (N-formyl-L-kynurenine), D_2O: 3.75 (m, 2H), 4.14 (m, 1H), 7.34 (t, 1H, J = 7.8), 7.66 (t, 1H, J = 7.8), 7.99 (d, 1H, J = 7.8), 8.15 (d, 1H, J = 7.8), 8.34 (s, 1H).

Kinetics Studies on TDO

For kinetics studies, the activity of TDO was determined by monitoring the absorbance increase of the reaction mixture at 321 nm (ϵ = 3152 $M^{-1}cm^{-1}$ for N-formyl-L-kynurenine). A typical reaction mixture consisted of 0.4 mM L-tryptophan, 0.1 mM l-ascorbate, and the 0.1 μM TDO in potassium phosphate buffer (0.1 M; pH 7.0) saturated with O_2 at room temperature. The reaction was initiated by the addition of the enzyme.

Overexpression and Purification of N-Formylkynurenine Formamidase

For characterization of KFA, the expression construct containing the KFA gene from *Ralstonia metallidurans* (with the stop codon at position 82 replaced by Tyr) in the pProEX Hta expression vector was used [38]. The overexpression of this construct was performed in *E. coli* BL21(DE3).

E. coli BL21(DE3) transformed with the KFA-containing plasmid were grown as described above for TDO. After addition of IPTG, the culture was incubated for an additional 15 hr at 27°C. The frozen cells were resuspended in 0.1 M potassium phosphate buffer (pH 7.4; 15 ml/g cell paste), and the imidazole concentration adjusted to 10 mM. Ni-NTA chromatography was performed as described above for TDO. KFA eluted from the column was exchanged into 0.1 M potassium phosphate (pH 7.4) to remove imidazole and NaCl.

Characterization of the KFA Reaction Product by HPLC Analysis and by NMR

A typical reaction mixture contained 30 μl of L-tryptophan (10 mM), 25 μl of ascorbic acid (2 mM), 10 μl of TDO sample (15 μM), and 435 μl of potassium phosphate buffer (0.1 M; pH 7.0). The formation of N-formylkynurenine was monitored spectrophotometrically. After the L-tryptophan was quantitatively converted to N-formylkynurenine, 10 μl of KFA (50 μM) was added to the reaction mixture. The enzymatic reaction was stopped after various times by the addition of 10% v/v trifluoroacetic acid (2 M), and precipitated protein was removed by centrifugation at 9200 × *g* for 2 min. A 50 μl sample was analyzed by HPLC (reverse-phase LiCrospher RP18, 5 μm; flow rate, 1ml/min, with mobile phase of 50% MeOH in 0.1 M aqueous acetic acid, 0.1 M NH_4OAc, pH 4.6). N-formyl-kynurenine and kynurenine were detected at 321 nm and 360 nm, respectively, and eluted after 4.6 and 6 min. For the NMR analysis, the reaction was scaled up 5-fold and the peak eluting after 6 min was collected. ^1H NMR (L-kynurenine) DMSO d-6: 3.25 (dd, 1H, J = 18 Hz, J = 8.78 Hz), 3.52 (dd, 1H, J = 18.06 Hz, J = 3.41 Hz), 3.61 (dd, 1H, J = 8.78 Hz, J = 2.93 Hz), 6.54 (t, 1H, J = 7.5 Hz), 6.76 (dd, 1H, J = 8.78 Hz, J = 0.97 Hz), 7.26 (t, 1H, J = 7.5 Hz), 7.7 (dd, 1H, J = 8.3 Hz, J = 1.46 Hz).

Kinetics Studies on KFA

For kinetics studies, the activity of KFA was determined by monitoring the absorbance increase of the reaction mixture at 365 nm resulting from the conversion of N-formyl-L-kynurenine to L-kynurenine (molar extinction coefficients of N-formyl-L-kynurenine and kynurenine at 365 nm are ~200 and 4530 $M^{-1}cm^{-1}$, respectively). The reaction mixture contained 100 mM Tris-HCl buffer (pH 7.4), 1 mM N-formyl-L-kynurenine, 20 μM $ZnCl_2$, and KFA in a final volume of 0.5 ml. The reaction was initiated by addition of enzyme after equilibration for 5 min at 37°C.

An alternative assay protocol involved measuring the production of formate by monitoring the formate dehydrogenase-catalyzed conversion of NAD to NADH. A typical reaction mixture contained 100 mM Tris-HCl (pH 7.5), 2 mM NAD, 2 units of formate dehydrogenase (Sigma), and an appropriate amount of KFA in 500 μl. The reaction was started by adding N-formylkynurenine (or other formylated substrate analogs) to 100–200 μM and monitored at 340 nm over a 20 min period.

Overexpression and Purification of Kynurenine-3-Monooxygenase

The KMO gene from *Cytophaga hutchinsonii* was PCR amplified using 5′-TAGTTCG*CATATG*AAAGAACAGATTACCATTTGCGGCG-3′ and 5′-CTACTA*CTCGAG*TTATATATATTTTTTTGTCACTGTAATGAC-3′ as the forward and reverse primers and *C. hutchinsonii* genomic DNA as a template. The purified PCR product was digested with NdeI and XhoI, cloned into the appropriately digested pCR4Blunt vector with the Zero Blunt TOPO PCR cloning kit, and the resulting DNA (pChuKO.28) used to transform *E. coli* BL21(DE3). The nucleotide sequence of the insert was confirmed by DNA sequencing.

E. coli BL21(DE3) transformed with pChuKO.28 were grown as described above for TDO, but in LB media containing 20 μg/ml of kanamycin. After addition of IPTG, the culture was incubated for an additional 12 hr at 30°C. Frozen cells were resuspended in a solution of 1 mM DTT, 0.2 M mannitol, 0.2% Triton X-100, and 5 μM FAD in 0.05 M Tris-acetate buffer (pH 8.0; 15 ml/g cell paste) for sonication. Ni-NTA chromatography was performed as described for TDO but with the following buffers. For equilibration, 0.05 M Tris-acetate buffer (pH 8.0, 0.2 M mannitol, 0.2% Triton X-100, 5 μM FAD, 10 mM imidazole); for washing, 0.05 M Tris-acetate buffer (pH 8.0, 0.2 M mannitol, 0.2% Triton X-100, 5 μM FAD, 0.5 mM DTT, 20 mM imidazole); and for elution, 0.05 M Tris-acetate buffer (0.2 M mannitol, 0.2% Triton X-100, 5 μM FAD, 1 mM DTT, 200 mM imidazole, pH 8.0). KMO eluted from the column was exchanged into 0.05 M Tris-acetate buffer (pH 8.0, 0.2 M mannitol, 0.2% Triton X-100, 5 μM FAD) to remove imidazole from the enzyme-containing solution.

Cofactor Identification

The flavin cofactor content was determined by ESI mass spectrometry.

The flavin cofactor was released by boiling KMO 100 μl (21.6 mM) in 200 μl of 1 mM ammonium acetate buffer (pH 6.0) for 5 min and then cooling rapidly on ice. Denatured protein was removed by centrifugation at 13,300 × *g* for 5 min. The supernatant was analyzed by ESI-MS in a positive mode. The flavin cofactor was identified as FAD (m/z = 786.2; MS/MS analysis m/z = 438, 348, 250, 136).

KMO can use both NADH and NADPH as cofactors. In the case of NADH, the specific activity was half that obtained with NADPH.

Identification of the KMO Reaction Product by HPLC and NMR Analysis

A typical reaction mixture contained L-kynurenine (0.1 mM), NADPH (0.2 mM), and 6 μg of KMO in Tris acetate buffer (0.05 M; pH 8.0). The enzymatic reaction was stopped after various times by the addition of 1/10 volume of ice-cold 2.4 M $HClO_4$, and the precipitated protein was removed by centrifugation at 9200 × *g* for 2 min. A 50 μl sample was analyzed by HPLC (reverse-phase LiCrospher RP18, 5 μm; flow rate, 1ml/min; solvent A: 0.1 M aqueous acetic acid, 0.1 M NH_4Oac, pH 4.65, 2% acetonitrile; solvent B: methanol; gradient: 100% A to 50% A–50% B over 15 min). The column eluent was monitored by absorbance at 365 nm. Kynurenine and 3-hydroxykynurenine eluted after 10.3 and 7 min, respectively. The reaction was scaled up 5-fold, and the HPLC-purified reaction product was analyzed by NMR. ^1H NMR (3-hydroxy-kynureinine) DMSO d-6: 3.25 (m, 1H, J = 8.78 Hz), 3.52 (dd, 1H, J = 18.6, J = 2.93), 3.61 (dd, 1H, J = 8.78 Hz, J = 2.93 Hz), 6.41 (t, 1H, J = 7.81 Hz), 6.73 (br s, 2H), 6.83 (d, 1H, J = 6.83 Hz), 7.20 (d, 2H, J = 7.81 Hz).

Kinetics Studies on Kynurenine-3-Monooxygenase

The activity of kynurenine-3-monooxygenase was determined by monitoring the absorbance decrease of the reaction mixture at 340 nm resulting from the oxidation of NADPH (molar extinction coefficient of NADPH = 2.7 ± 0.1 mM^{-1}). A typical assay mixture contained 530 μl of buffer (0.05 M Tris-acetate, pH 8.0, 1 mM DTT, 0.2 M mannitol, 0.2% Triton X-100, 5 μM FAD), 6 μl 10 mM kynurenine, 10 μl 12 mM NADPH, 4 μl 1.5 M KCl. Reaction was initiated by the addition of 50 μl of enzyme sample (21.6 μM). Kinetic parameters were determined by initial-velocity measurements at a fixed concentration of NADPH (0.5 mM) and varying concentrations of kynurenine.

Overexpression and Purification of 3-Hydroxy-Kynureninase

The KYN gene from *R. metallidurans* was PCR amplified using 5′-GGGGA*TCATGA*CAACGCTGACCCGAGC-3′ and 5′-GGG*GTCGAC* TCAGGTCACCGCCGTCCG-3′ as the forward and reverse primers, respectively, and plasmid pPRO-*rm*Locus(RT) as a template. The PCR fragment was purified, digested with BspHI and SalI, cloned into appropriately digested pProEX Hta, and the resulting DNA used to transform *E. coli* BL21(DE3). The nucleotide sequence of the insert was confirmed by DNA sequencing.

E. coli BL21(DE3) transformed with the KYN-containing plasmid was grown as described above for TDO. After addition of IPTG, the culture was incubated for an additional 10 hr at 30°C. Frozen cells were resuspended in a solution of 1 mM DTT, 0.25 M sucrose, 10 μM PLP in 0.1 M Tris-HCl buffer (pH 8.0; 15 ml/g cell paste) for sonication. Ni-NTA chromatography was performed as described above for TDO but with the following buffers. For equilibration, 0.1 M Tris-HCl buffer (pH 8.0, 0.25 M sucrose, 10 μM PLP, 10 mM imidazole); for washing, 0.1 M Tris-HCl buffer (pH 8.0, 0.25 M sucrose, 10 μM PLP, 0.5 mM DTT, 20 mM imidazole); and for elution, 0.1 M Tris-HCl buffer (pH 8.0, 0.25 M sucrose, 10 μM PLP, 1 mM DTT, 200 mM imidazole). KYN eluted from the column was exchanged into 0.1 M Tris-HCl buffer (pH 8.0, 0.25 M sucrose, 7 μM PLP) to remove imidazole from the enzyme-containing solution.

Identification of the 3-Hydroxy-Kynureninase Reaction Product by HPLC and by NMR

A typical reaction mixture consisted of 772 μl of 100 mM Tris HCl buffer (pH 8.0), 7 μM PLP, 500 μM of 3-hydroxykynurenine, and 50 μl of KYN (0.21 mg/ml). The reaction was stopped after various times by the addition of 10% v/v of 2.4 M $HClO_4$. The precipitated protein was removed by centrifugation, and a 50 μl sample was injected into the HPLC column (reverse-phase LiCrospher RP18, 5 μm; flow rate, 1ml/min; solvent A: 0.1 M aqueous acetic acid, 0.1 M NH_4Oac, pH 4.65, 2% acetonitrile; solvent B: methanol; gradient: 100% A to 50% A-50% B over 15 min). The column eluent was monitored by absorbance at 365 nm (3-hydroxykynurenine) and by fluorescence at 410 nm (excitation at 330 nm). 3-hydroxyanthranilic acid eluted with a retention time of 11 min. The reaction was scaled up 5-fold for NMR analysis. ^1H NMR (3-hydroxyanthranilic acid) DMSO d-6: 6.36 (t, 1H, J = 7.8), 6.79 (dd, 1H, J = 1.46, J = 7.32), 7.2 (dd, 1H, J = 1.46, J = 8.3), 9.6 (s, 1H).

Kinetic Studies on 3-Hydroxy-Kynureninase

The activity of kynureninase was determined by monitoring the decrease in absorbance of L-3-hydroxykynurenine at 371nm ($\Delta\epsilon$ = 3775 $M^{-1}cm^{-1}$). A typical assay mixture contained 2.8 μl of 1 mM PLP in 100 mM Tris-HCl buffer at pH 8, 0.5–20 μl of 3-hydroxykynurenine (6.2 mM) or kynurenine (10 mM) in a final reaction volume of 0.5 ml. The reaction was initiated by addition of 15 μl of the enzyme (0.2 mg/ml).

Overexpression and Purification of 3-Hydroxyanthranilate-3,4-Dioxygenase

The HAD gene from *Cytophaga hutchinsonii* was PCR amplified using 5′-TAG*CATATGC*TCAAGCCATTTAACTTCAAAGTC-3′ and 5′-GCTAC*TCGAG*TTATCGTTTAACAGGTGC-3′ as the forward and reverse primers and pBAD-*ch*Locus as a template. The PCR product was purified (with the QIAquick PCR purification kit), digested with NdeI and XhoI, cloned into the appropriately digested pET16b, and the resulting DNA (pKLC101) used to transform *E. coli* Tuner(DE3). The nucleotide sequence of the insert was confirmed by DNA sequencing. *C. hutchinsonii* HAD was insoluble upon overexpression; therefore, the HAD gene from *Ralstonia metallidurans* was used for biochemical characterization.

The HAD-KYN genes from *Ralstonia metallidurans* were PCR am-

plified using 5′-ATGCTGACCTACGGAGCGCCCT-3′ and 5′-TCAGG TCACTGACTTGCGCGTG-3′ as the forward and reverse primers, respectively. The PCR-amplified fragment was directly cloned into pBAD-TOPO with the "TOPO TA" cloning strategy. The *E. coli* strain TOP10 was used for subsequent transformation. The resulting plasmid pBAD-*rm*Locus was verified by DNA sequence analysis.

The HAD gene from *Ralstonia metallidurans* was PCR amplified using 5′-TAGC*CATATG*CTGACCTACGGAGCGCC-3′ and 5′-GCT A*GGATCC*TCAGGCCGCGCGTCCGG-3′ as the forward and reverse primers and pBAD_1029-28.4 plasmid as a template. The PCR product was purified (with the QIAquick PCR purification kit), digested with NdeI and BamHI, cloned into the appropriately digested pET16b, and the resulting DNA (pKLC100) used to transform *E. coli* Tuner(DE3). The nucleotide sequence of the insert was confirmed by DNA sequencing.

LB medium (5 ml) containing ampicillin (200 μg/ml) was inoculated with a single colony *E. coli* Tuner(DE3) cells transformed with pKLC100. The culture was incubated overnight at 37°C with agitation. On the next day, 1 liter of LB media containing 200 μg/ml ampicillin was inoculated with 1.0 ml of the starter culture. The cells were grown with shaking at 37°C until the culture reached an A_{595} of 0.4. The temperature was then reduced to 25°C, and the cells were allowed to grow to an A_{595} of 0.6. IPTG was added to a final concentration of 0.1 mM and the culture was incubated for an additional 5 hr at 25°C. The cells were then harvested by centrifugation at 8300 × *g* for 15 min at 4°C and stored at −20°C. The frozen cells were resuspended in potassium phosphate buffer (50 mM, pH 8) containing sodium chloride (300 mM) and imidazole (10 mM). Lysozyme (0.1 mg/mL) was added, and the cells were sonicated on ice. The cell debris was removed by centrifugation at 27,000 × *g* for 15 min at 4°C. The supernatant containing HAD was purified by Ni-NTA affinity chromatography. Ni-NTA superflow resin (1 mL) was equilibrated with 50 mM potassium phosphate buffer (pH 8, 300 mM NaCl, 10 mM imidazole). HAD was bound to the resin (200 mg protein/ml of resin) and washed with 50 mM potassium phosphate buffer (pH 8, 300 mM NaCl, 20 mM imidazole). HAD was eluted in 1 ml fractions (50 mM potassium phosphate buffer, pH 8, 300 mM NaCl, 250 mM imidazole). HAD eluted over fractions 2–5. The yield of activity in the purified HAD was 1.1%. The recombinant enzyme was stable (half-life, 13 days at 4°C) in gel-filtered cell-free extracts containing 20 mM DTT; however, the purified HAD was quite unstable, with a half-life of only 1 hr at 20°C in the absence of DTT. In the presence of DTT, the activity could be increased 8- to 9-fold from freshly purified protein and maintained for 12–24 hr at 4°C; however, after 72 hr at 4°C the activity was completely lost. Therefore, the enzymatic assays to confirm function were carried out using gel-filtered crude cell extracts from the overexpression strain maintained in 20 mM DTT at 4°C.

Identification of the HAD Reaction Product by HPLC Analysis

3-hydroxyanthranilate (500 μM) was added with stirring to a solution of 120 μg of cell-free extract (5% HAD) in 2 ml total volume of 10 mM phosphate buffer (pH 7.2). After 2, 5, 10, 15, 30, 45, 60, 75, and 120 min, 100 μl of the reaction was withdrawn and shaken with an equal volume of ice-cold chloroform to precipitate the protein. After centrifugation for 3 min, the aqueous layer was removed and immediately frozen until just prior to analysis. The sample (75 μl) was analyzed by HPLC (reverse-phase LiCrospher RP18, 5 μm; 1.0 ml/min; solvent A: 20 mM aqueous phosphoric acid containing 20 mM acetic acid, pH 4.5; solvent B: acetonitrile; gradient: 100% A to 20% A–80% B over 20 min). Quinolinate, α-amino-β-carboxymuconic semialdehyde, and 3-hydroxyanthranilate eluted after 3.7 min, 4.5 min, and 11.2 min, respectively.

Identification of the HAD Reaction Product by NMR Analysis

A typical reaction mixture consisted of 0.1 mg of protein (100 μl of gel-filtered cell-free extract from the HAD overexpression strain, 5% HAD, in 10 mM potassium phosphate buffer containing 20 mM DTT, pH 7.2) and 7.5 μmol 3-hydroxyanthranilate dissolved in 25 μl d_6-DMSO in 1 ml D_2O. The reaction mixture was analyzed using a 400 MHz Varian NMR instrument. The intermediate α-amino-β-carboxymuconic semialdehyde shows a doublet of doublets at 6.4 ppm, a doublet at 7.25 ppm, and a doublet at 8.8 ppm. These signals disappear over time and are replaced by signals that are characteristic of quinolinate: a doublet of doublets at 7.33 ppm, a doublet at 7.88 ppm, and a doublet at 8.27 ppm.

Kinetics Studies on 3-Hydroxyanthranilate-3,4-Dioxygenase

For kinetic studies, the activity of HAD was monitored over time by measuring the absorbance increase at 360 nm resulting from the production of α-amino-β-carboxymuconic semialdehyde (ϵ_{360} = 47,500 $M^{-1}cm^{-1}$; [44]). A typical reaction mixture consisted of 4 μg of gel-filtered cell-free extract (5% HAD) in 495 μl of oxygen-saturated buffer (10 mM phosphate, 20 mM DTT, pH 7.2) and 5 μl of a solution of 3-hydroxyanthranilate in DMSO (0.05 mM to 6.0 mM).

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Accession Numbers

The following DNA sequences have been deposited in GenBank. *Gemmata str. Wa1-1* tryptophan-2,3-dioxygenase (AY308027), N-formylkynurenine formamidase (AY308028), kynurenine-3-monooxygenase (AY305284), 3-hydroxy-kynureninase (AY308030), 3-hydroxyanthranilate-3,4-dioxygenase (AY308032), quinolinate phosphoriobsyl transferase (AY308034), and NaMN adenylyl transferase (AY308036). *Polaribacter filamentus* tryptophan-2,3-dioxygenase (AY304519), N-formylkynurenine formamidase (AY308029), kynurenine-3-monooxy genase (AY305285), 3-hydroxy-kynureninase (AY308031), 3-hydroxyanthranilate-3,4-dioxygenase (AY308033), quinolinate phosphoriobsyl transferase (AY308035), NaMN adenylyl transferase (AY308037), NAD synthase (AY308038), and NAD kinase (AY308039).