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KYNURENINASE-TYPE ENZYMES AND THE EVOLUTION OF THE AEROBIC TRYPTOPHAN-TO-NICOTINAMIDE ADENINE DINUCLEOTIDE PATHWAY

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Summary

Kynureninase-type (L-kynurenine hydrolase, EC 3.7.1.3) activity has been found to be present in the livers of fish, amphibia, reptiles, and birds. In addition to past information concerning this enzyme activity in mammalian liver, it is now clear that all the major classes of vertebrates carry a highly specialized kynureninase-type enzyme, which we have termed a hydroxykynureninase. To compare the reactivities of these enzymes with L-kynurenine and L-3-hydroxykynurenine, ratios of τ values (K_m/V) were used. Based on this comparison, the bacterium *Pseudomonas fluorescens* carries the most efficient kynureninase, whereas the amphibian *Xenopus laevis* has the most efficient hydroxykynureniase. In these two cases, the ratio of τ values differs by a factor of 38 000. It is hypothesized that the tryptophan-to-nicotinamide adenine dinucleotide biosynthetic pathway evolved from a catabolic system of enzymes, and that the differences observed in the kynureninase-type enzymes between lower and higher organisms reflect the specialization of the function of these enzymes from a strictly catabolic role to an anabolic one during the course of evolution.

Introduction

The biosynthesis of the pyridine moiety of nicotinamide adenine dinucleotide (NAD) is known to occur by two distinct de novo pathways. One, originating from L-aspartate, is found in bacteria and plants [1,2]. The other,

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originating from L-tryptophan, is known to occur in fungi [3], higher animals [4], and in one bacterium, *Xanthomonas pruni* [5]. Although the enzymic reactions which transform L-aspartate to NAD are not known in their entirety [1,2], it is clear that the last three such reactions are identical to those which occur in the tryptophan-to-NAD pathway (cf. Fig. 1).

Recently we have shown that two forms of kynureninase-type enzymes(L-kynunenine hydrolase, EC 3.7.1.3) exist in various organisms [6]. One, an inducible kynureninase, is involved in the catabolism of L-tryptophan to anthranilate and other metabolites. The other, a constitutive hydroxykynureninase, is required for catalyzing the fourth reaction in the biosynthesis of NAD from L-tryptophan.

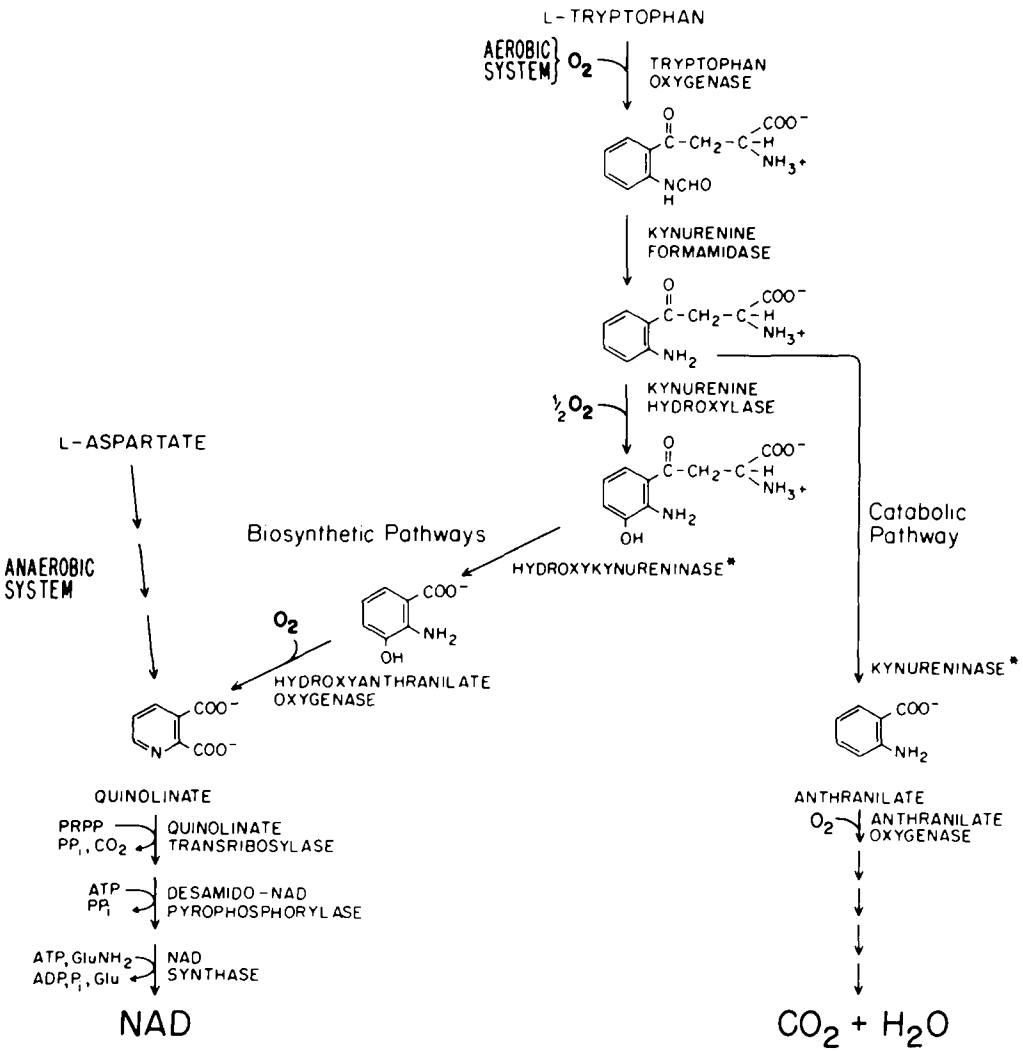

Fig. 1. Pathways for the biosynthesis of NAD and the catabolism of L-tryptophan. The anaerobic pathway for the synthesis of NAD begins with L-aspartate. However, depending on the organism, either L-aspartate and dihydroxyacetone phosphate or *N*-formyl-L-aspartate and acetyl-coenzyme A form the carbon skeleton of quinolinate [1]. The respective roles of kynureninase and hydroxykynureninase are emphasized by asterisks.

In the present report additional evidence is provided for the occurrence of distinct hydroxykynureninases in higher organisms. In conjunction with previous findings, these results have led us to an hypothesis which connnects the evolution of the kynureninase-type enzymes with the origins of the biosynthetic pathways for NAD.

Materials and methods

Enzyme preparation

Pseudomonas fluorescens was grown in modified Vogel's and Bonner's minimal salts medium as was previously described [6]. The colorless mutant of *Xanthomonas pruni* (a gift from Dr. Conrad Wagner, Veterans Administration Hospital, Nashville, Tennesse) was grown and maintained in the minimal medium defined by Brown and Wagner [5]. Both of the bacterial cells were homogenized by passing them through a French pressure cell. Enzyme extraction and purification were done by protamine sulfate precipitation, ammonium sulfate precipitation, Sephadex G-25 gel filtration, and DEAE-cellulose chromatography as described in an earlier report [6].

Growth of yeast cells, and extraction and purification of enzyme up to DEAE-cellulose chromatography were described previously by Shetty and Gaertner [7].

The fungi *Neurospora crassa*, *Penicillium roqueforti*, *Aspergillus niger*, and *Rhizopus stolonifer* were grown in Vogel's minimal medium containing 1 g of yeast extract and 20 g of sucrose per 1. The inoculation and growth of mycelia, as well as extraction and purification of enzymes, were as described earlier [6].

Livers of mouse, chicken, frog, turtle, and trout were each minced and homogenized with 10 vols of 0.05 M potassium phosphate (pH 7.0) in prechilled ground-glass homogenizers. In each case the homogenate was centrifuged at 10 000 × *g* for 30 min to remove the particulate material. The supernatant was subjected to protamine sulfate precipitation, ammonium sulfate precipitation, and Sephadex G-25 gel filtration as was done with fungi and bacteria.

Enzyme assay

The kynureninase and hydroxykynureninase activities were assayed as described by Gaertner et al. [8], except that the reaction mixture did not contain $MgSO_4$ and the L-3-hydroxykynurenine concentration was reduced to 0.1 mM in routine assays. For V and K_m studies, reaction mixtures similar to those described above were used, except that the L-kynurenine and L-3-hydroxykynurenine concentrations were varied.

Results

It was found earlier that kynureninase-type enzymes of mouse liver [9] and rat liver [10] had the catalytic properties of hydroxykynureninase. In both mammals the enzyme in question had a K_m for L-3-hydroxykynurenine (2—5 μM) 50—100 times less than the K_m for L-kynurenine. In order to determine whether the presence of such hydroxykynureninases is of general importance, and therefore whether they are widely distributed in the animal kingdom, we chose to study examples of each of the other major classes of vertebrates.

Extracts were made from the livers of a fish (*Salmo gairdneri*), amphibian (*Xenopus laevis*), reptile (*Terrapene carolina*), and bird (*Gallus gallus*). As can be seen in Table I, in each case a kynureninase-type enzyme was found which efficiently catalyzes L-3-hydroxykynurenine; K_m values (4—9 μM) were 50- to 415-fold less than for L-kynurenine; V values varied from 1.6- to 5-fold greater for L-3-hydroxykynurenine than for L-kynurenine. Also in Table I, these results can be compared directly with those obtained for examples of kynureninases and hydroxykynureninases isolated from other organisms.

In Table II, the kinetic data have been condensed to a single value — the ratio of transient times (τ). The derivation of τ and its value in estimating the amount of enzyme required in coupled enzyme assays has been described by Easterby [11]. Here we show another use for this parameter in making comparisons between enzymes catalyzing two different substrates. In our comparison, the longer the transient ($\tau = K_m/V$), the less efficient the enzyme in catalyzing a particular substrate. Thus, an enzyme that catalyzed L-3-hydroxkynurenine more efficiently than L-kynurenine will have a τ(kynurenine)/τ(hydroxykynurenine) or (τ_k/τ_{hk}) significantly greater than 1.0. All of the constitutive kynureninase-type enzymes (hydroxykynureninases) isolated to date have τ_k/τ_{hk} values ranging from 19 to 1150, whereas all of the isolated inducible kynureninase-type enzymes (kynureninases) have τ_k/τ_{hk} values of 1.05 or less.

TABLE I

KINETIC PROPERTIES OF KYNURENINASE-TYPE ENZYMES FROM MICROORGANISMS AND ANIMALS

Enzyme assays were performed as described in Methods, and the data presented in this table are from Lineweaver-Burk plots.

Source of enzymes	Inducible enzyme (kynurenine/hydroxykynurenine)		Constitutive enzyme (kynurenine/hydroxykynurenine)	
	K_m (μM)	V (relative units)	K_m (μM)	V (relative units)
Bacteria				
Pseudomonas fluorescens *	71/500	10/21	Not present	
Xanthomonas pruni *	18/220	10/10	Not present	
Fungi				
Neurospora crassa *	67/250	10/10	250/5.0	10/4
Penicillium roqueforti *	71/59	10/9	100/4.6	10/9
Aspergillus niger *	80/140	10/7	80/3.9	10/13
Rhizopus stolonifer **	Not present		250/6.7	10/25
Saccharomyces cerevisiae *	Not present		540/6.7	10/14
Vertebrates				
Frog liver (*Xenopus laevis*) **	Not present		1660/4.0	10/23
Chicken liver (*Gallus gallus*) **	Not present		2550/11.0	10/16
Mouse liver (*Mus musculus*) *	Not present		300/6.0	10/25
Trout liver (*Salmo gairdneri*) **	Not present		2000/14	10/50
Turtle liver (*Terrapene carolina*) **	Not present		3330/19	10/23

* DEAE-cellulose peak fraction.

** Sephadex G-25 eluate of ammonium sulfate fraction (0—60% saturation).

TABLE II

COMPARISON OF TRANSIENT TIME-RATIO FOR INDUCIBLE AND CONSTITUTIVE KYNURENINASES FROM VARIOUS ORGANISMS

Source of enzyme	Inducible enzyme τ_k/τ_{hk} *	Constitutive enzyme τ_k/τ_{hk} *
Bacteria		
Pseudomonas fluorescens	0.03	Not present
Xanthomonas pruni	0.08	Not present
Fungi		
Neurospora crassa	0.27	19
Penicillium roqueforti	1.05	20
Aspergillus niger	0.36	27
Rhizopus stolonifer	Not present	93
Saccharomyces cerevisiae	Not present	113
Vertebrates		
Mouse liver (*Mus musculus*)	Not present	125
Turtle liver (*Terrapene carolina*)	Not present	378
Chicken liver (*Gallus gallus*)	Not present	398
Trout liver (*Salmo gairdneri*)	Not present	716
Frog liver (*Xenopus leavis*)	Not present	1150

* τ_k is K_m/V for L-kynurenine. τ_{hk} is K_m/V for L-3-hydroxykynurenine.

Discussion

As shown in Table I, a variety of organisms have been found to contain a constitutive kynureninase-type enzyme having a high specificity for L-3-hydroxykynurenine. The specificity of these constitutive enzymes, termed hydroxykynureninases, is emphasized by the fact that a second group of inducible enzymes, termed kynureninases, have been demonstrated in a variety of microorganisms (Table I). The kynureninases have a greater reactivity with L-kynurenine than do the hydroxykynureninases, whereas the opposite is true for L-3-hydroxykynurenine. Quantitatively, the specificity of the two types of enzymes is seen most readily by the fact that the relative reactivities, expressed as a ratio of τ values ($\tau = K_m/V$, see ref. 11) of the enzymes (Table II), range from 19 to 1150 for the constitutive enzymes and from 0.03 to 1.05 for the inducible ones. Thus, based on this comparison, the bacterium *Pseudomonas fluorescens* has the most efficient kynureninase whereas the amphibian *Xenopus laevis* has the most efficient hydroxykynureninase. The enzymes from these two organisms differ in their relative catalytic efficiencies (expressed as a ratio of τ values) by a factor of 38 000. We contend that this difference reflects that these enzymes have evolved to serve different physiological roles.

Since those organisms containing an inducible kynureninase excrete anthranilate [7,8], and since induction is characteristic of catabolic enzymes [12], we conclude that the kynureninases are mainly, if not exclusively, involved in the degradation of L-tryptophan to anthranilate or other catabolites. In addition, because those organisms containing a constitutive hydroxykynureninase synthesize NAD from L-tryptophan via 3-hydroxyanthranilate, and because constitutivity is a characteristic of biosynthetic enzymes, we believe that the main physiological function of hydroxykynureninase is as an essential

catalytic component in the transformation of L-tryptophan to NAD.

Since the cofactor NAD is a key component in both anaerobic and aerobic metabolism, a pathway for synthesizing it must have existed as one of the first features in biochemical evolution. However, because the earth was initially anaerobic, reactions leading to NAD synthesis could not have involved molecular oxygen. It can therefore be concluded that the aspartate-to-NAD pathway, which does not require molecular oxygen, is most closely related to the primordial NAD biosynthetic system. The fact that plants (photosynthetic auxotrophs) and most bacteria (primitive heterotrophs) utilize the anaerobic pathway and lack the ability to transform L-tryptophan to NAD is consistent with this view.

One can see examples of at least five possible stages in the evolution of the NAD biosynthetic pathways and of the kynureninase-type enzymes. As summarized in Table III, these examples progress from the most primitive bacteria

TABLE III

POSSIBLE EXTANT EXAMPLES IN THE STAGES OF EVOLUTION OF THE NAD BIOSYNTHETIC PATHWAYS AND THE KYNURENINASE-TYPE ENZYMES

Evolutionary stage		Examples	Anaerobic NAD synthesis	Catabolic (inducible) kynureninase	Aerobic NAD synthesis	Biosynthetic (constitutive) hydroxykynureninase	Reference
I	a	*Euglena gracilis*	+	0	0	0	S/G *
		Spinach	+	0	0	0	S/G
	b	*Bacillus subtilis*	+	0	0	0	(13)
		Escherichia coli	+	0	0	0	(13)
II		*Pseudomonas fluorescens*	+	+	0	0	(6)
III		*Xanthomonas pruni*	0	+	+	0	S/G
		Blue-green bacterium (*Agmenellum quadruplicatum*)	?	+	?	0	S/G
IV	a	*Neurospora crassa*	0	+	+	+	(3)
		Aspergillus niger	0	+	+	+	(6)
		Penicillium notatum	0	+	+	+	(6)
	b	*Saccharomyces cerevisiae*	+	0	+	+	(7)
		Mucor hiemalis	?	0	+	+	(6)
		Rhizopus stolonifer	?	0	+	+	S/G
	c	Mouse liver	0	0	+	+	T **
		Rat liver	0	0	+	+	(10)
		Chicken liver	0	0	+	+	T **
		Fish liver	0	0	+	+	T
		Frog liver	0	0	+	+	T
		Turtle liver	0	0	+	+	T
V		*Drosophila melanogaster*	0	0	0	0	S/G
		Crab heptopancrease (*Gecarcinus lateralis*)	?	0	?	0	S/G

* S/G, Shetty and Gaertner, unpublished results.

** T, data from Table I.

in Stage I to the most advanced animals in Stage IV, and show the following evolutionary steps: development of the anaerobic NAD biosynthetic pathways (Stage I); appearance of the inducible kynureninases (Stage (II); formation of the aerobic NAD pathway from inducible catabolic enzymes (Stage III); further refinement of the synthetic capacity of the aerobic NAD-pathway enzymes, accompanied by the elimination of the inducible kynureninase and the anaerobic NAD pathway (Stage IV); and complete elimination of the capacity to synthesize NAD by either de novo pathway (Stage V).

With both the anerobic biosynthetic pathway and the aerobic pathway of tryptophan catabolism present in an organism, only two additional enzymic reactions would be required to complete the tryptophan-to-NAD synthetic sequence. As can be seen in Fig. 1, tryptophan oxygenase, kynurenine formamidase, kynureninase, and anthranilate oxygenase are present as catalytic components of the catabolic system. Quinolinate transribosylase, desamido-NAD pyrophosphorylase, and NAD synthase are essential catalysts in both the aerobic and anaerobic NAD biosynthetic pathways. Hence, only kynurenine hydroxylase and hydroxyanthranilate oxygenase would be needed to complete the synthetic chain from tryptophan to NAD.

In connection with the evolution of the tryptophan catabolic system and the aerobic NAD biosynthetic pathway, it is apparent, in a teleonomic sence, that the kynureninase-type enzymes developed in the course of evolution to hydrolyze specifically either L-kynurenine or L-3-hydroxykynurenine. The fungi, with their two forms of kynureninase-type enzymes, suggest how this specialization may have occurred. At some point in Stage III of the evolutionary process there may have been a gene duplication (see, for example, ref. 14) providing an organism with two copies of a gene encoding kynureninase. With simultaneous selective advantage to synthesize NAD from L-tryptophan and to catabolize L-tryptophan as an energy source, one can conceive how mutational events in the duplicate enzymes could have led to their eventual specialization. The loss of the catabolic kynureninase and anerobic NAD biosynthetic pathway at Stage IV could have occurred with the advent of alternative routes for degrading L-tryptophan via 3-hydroxyanthranilate and for synthesizing NAD aerobically from L-tryptophan.

We believe our data shed light on the question of when and how the anaerobic and aerobic pathways for synthesizing NAD developed during the course of evolution and that our model explains the diversity of extant examples of NAD synthetic systems and kynureninase-type enzymes. What remains unclear are the possible selective pressures that brought about the elimination of the kynureninases and the anaerobic NAD synthetic system in higher organisms and that permitted the retention of the aerobic NAD synthetic pathway from L-tryptophan.

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