

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado
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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 09-112336- -00005-0000

Fecha: 2012-10-24 13:51:23 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 379 FASENACIONALII
Act. 519 TRASLADOINFORME Folios: 35

ASUNTO	Radicación	09-112336
	Trámite	011
	Evento	379
	Actuación	519
	Oficio	- - - 19195
	Folios	035

Patente de Invención	☒	Patente de Modelo de Utilidad	☐
Vía Nacional	☐		
Vía PCT (fase nacional)	☒	Cap. I	☐
		Cap. II	☒

No. Solicitud Internacional PCT/US2008/058286
Fecha de Presentación Internacional 26/Marzo/2008

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 8 a 58	09/Octubre/2012
Reivindicaciones:	Folios 59 a 66	09/Octubre/2012
	Folios 105 a 114	09/Octubre/2012
Dibujos:	Folios 67 a 69	09/Octubre/2012
Prioridad:	US 11/695.377	02/Abril/2007

2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486), y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Modificaciones no aceptadas (Art 34 D 486 y Art 19, 22 y 34 PCT)

No se aceptan las modificaciones al capítulo reivindicatorio en los folios 105 a 114 ya que el solicitante manifestó en el folio 118 que se pago la tasa para el primer capítulo reivindicatorio que consiste de 46 reivindicaciones en los folios 59 a 66, y sobre este se realiza el examen de patentabilidad.

4. Objeto de la invención

Título de la solicitud: "Catalizador de base biológica para retrasar los procesos de desarrollo de las plantas"

El problema planteado en esta solicitud consiste en encontrar métodos y aparatos para retrasar el desarrollo de plantas o partes de las mismas.

Para resolver este problema técnico la solicitud en estudio proporciona métodos para retrasar un proceso de desarrollo de las plantas que comprende exponer una planta o parte de la planta a una o más bacterias o enzimas. En formas de realización específicas, dichas una o mas bacterias se seleccionan del grupo que consiste de *Rhodococcus spp.*, *Pseudomonas chloroaphis*, *Brevibacterium ketoglutaricum*, y una mezcla que comprende cualquier combinación de estas bacterias. Comprende además aparatos para retrasar el proceso de desarrollo de las plantas que comprenden un catalizador que comprende una o mas de las bacterias anteriores.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A01N63/00; A01P21/00; A01N25/34; A01N37/44; A01N47/28; A01N59/16.

5. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

El método de la reivindicación 1 no es claro por que no especifica la secuencia de pasos necesarios para reproducir la invención. El método solo especifica una etapa, consistente en poner en contacto una planta con una o más bacterias, sin embargo no es claro:

- La forma adecuada en la cual dicho contacto se lleva a cabo.
- La cantidad suficiente de cada tipo de bacteria para alcanzar el objetivo del método.
- La cepa y número de deposito del microorganismo, y las modificaciones genéticas específicas realizadas sobre dichos microorganismos.

Por otra parte la expresión "*retrasar el proceso de desarrollo de la planta*" no es claro, el solicitante debe aclarar si se refiere a la germinación de las semillas, el crecimiento de las plántulas, crecimiento de partes de plantas particulares, la maduración de la fruta, producción de fruta, producción de flores u otras etapas del ciclo de vida de la planta. El solicitante debe especificar a cual etapa se refiere.

Las reivindicaciones 4 y 39 no son claras, ya que el termino "inducido" puede tener distintas interpretaciones, puede tratarse de inducir un cultivo de bacterias para que crezca hasta un cierto nivel, inducir las bacterias para producir un determinado producto o inducir las bacterias a realizar una determinada actividad. Además, el solicitante debe aclarar por que esta inducción es necesaria para el método que "retrasar el proceso de desarrollo"

Las reivindicaciones 7 y 8 no son claras ya que no especifican el método por el cual la planta es expuesta indirectamente o directamente al cultivo de bacterias, de manera que la actividad de las mismas retrase efectivamente el "proceso de desarrollo".

La reivindicación 18 y dependientes no son claras por que no definen la estructura del aparato, se limitan a identificar las bacterias contenidas en el aparato que están relacionadas al método de la reivindicación 1.

La reivindicación 26 no es clara por que no define las características estructurales del “dispositivo de control para ajustar la exposición del catalizador a una planta o parte de la planta”.

Las reivindicaciones 31 a 34 no son claras por que definen el aparato por resultados a alcanzar correspondientes a:

- Permitir que el aire fluya en el modulo catalizador.
- controlar el flujo del aire en el modulo catalizador
- controlar el nivel de humedad en la estructura física.
- estructura refrigerada.
- regular el nivel de dióxido de carbono en la estructura física.

Pero no indica las características estructurales del aparato que permiten alcanzar dichos resultados.

El aparato debe ser descrito por sus características estructurales necesarias para que cumpla la función indicada, dichas características deben estar completamente sustentadas en la descripción.

Las reivindicaciones 36, 41 a 43 no son claras por que no definen las características estructurales de la primera, tercera y cuarta capas adecuadas para el aparato reclamado.

El método de la reivindicación 46 no es claro por que no describe el método por la etapas sucesivas del mismo, la única etapa descrita no es suficiente para hacer el proceso reproducible, ya que no especifica como se obtiene el extracto enzimático, cual es la composición de dicho extracto y cual es la manera adecuada de poner este en contacto con la planta de forma que el método sea efectivo para “retrasar el proceso de desarrollo” de la planta. Además, no es claro por que se hace referencia a los agentes inductores de los cultivos de bacterias, si el método utiliza un extracto enzimático y no en el cultivo de bacterias.

Descripción (Art 28 D 486)

La descripción de las figuras en el folio 14 no es clara, ya que hace referencia a las capas designadas como A y B pero en la figura no se ven estas indicaciones. Por otra parte no se proporciona descripción para los números indicados en las figuras.

Según el Art. 28 D486 literal c una solicitud debe contener “*una descripción de la invención en términos que permitan la comprensión del problema técnico y de la solución aportada por la invención, exponiendo las diferencias y eventuales ventajas con respecto a la tecnología anterior*”, la presente solicitud describe el problema técnico objetivo como la necesidad de desarrollar métodos y aparatos para retrasar el proceso de desarrollo de una planta o una parte de la misma. Como antecedentes de la invención (folios 9 a 13) el solicitante expone el papel determinante del etileno en los procesos de desarrollo de las plantas y sus partes. Sin embargo a partir del folio 13, en donde inicia la descripción de la invención como tal, no se hace referencia a como los aparatos o los métodos descritos y reclamados controlan o inhiben la expresión de etileno, de manera que el problema técnico objetivo se vea resuelto.

Específicamente, la descripción no proporciona información sobre como los microorganismos (modificados o no) o los extractos enzimáticos, controlan o inhiben la producción de etileno en la planta o parte de planta. Las enzimas mencionadas como relevantes para la invención, hidratasa de nitrilo, amidasa y asparaginasa (folio 29 línea 11), que según el solicitante fueron introducidas en los microorganismos que hacen parte del aparato descrito, no se encuentran relacionadas ni con la producción o inhibición de la producción de etileno en plantas o con la degradación del mismo dentro del microorganismo. La hidratasa de nitrilo o nitrilohidratasa, tiene como actividad la hidrólisis de nitrilos orgánicos en microorganismos¹, la amidasa es una enzima que hidrolisa la urea y formamida en hojas de plantas², y asparaginasa cataliza la hidrólisis de asparagina a ácido aspártico³ y se encuentra presente tanto en microorganismos como en plantas.

En el estado de la técnica no se encuentra ninguna referencia a que dichas enzimas estén involucradas en vías metabólicas de producción o degradación de etileno. Ya que el etileno es la molécula que promueve el desarrollo de las plantas o de sus partes, y que el objetivo de la invención es retrasar dicho desarrollo, se esperaría que las enzimas y/o microorganismos de la invención estuvieran involucrados en vías metabólicas relacionadas con la producción o degradación del etileno.

De esta manera, se invita al solicitante aclarar como la invención soluciona el problema técnico objetivo que plantea, con el fin de que la solicitud cumpla con los requisitos del Art. 28 D486.

6. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 02/Abril/2007, y no se encontraron documentos que anticipen el objeto de esta solicitud.

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ

Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha

30 OCT. 2012

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1 <http://144.206.159.178/ft/243/51642/13866090.pdf>

2 Frankengerger Et al, Amidase and urease activities in plants, Plant and Soil 64, 153-166 (1982).

3 <http://www.worthington-biochem.com/aspr/default.html>

Structural and functional models of nitrile hydratase

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Received 28 March 2001; accepted 19 June 2001

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Abstract

Nitrile hydratase, the enzyme involved in microbial nitrile assimilation, comprises either one non-heme low-spin iron(III) or a non-corrinoid cobalt(III) center at the active site. Two carboxamido nitrogens, one cysteine-sulfur, and two sulfurs from a cysteine-sulfenic (Cys-SO) and a cysteine-sulfinic (Cys-SO₂) acid moiety respectively constitute the donor set around the metal center of this hydrolytic enzyme. Binding of one molecule of NO at the sixth site of the iron(III) center modulates the activity of the enzyme. It is suggested that a metal-bound hydroxide could be involved in the hydration of nitriles. Attack of water (or hydroxide) on a metal-bound nitrile is another possibility. During the past few years, modeling work by several groups have provided a great deal of insight into the structure and function of nitrile hydratase. Structural and spectroscopic studies on iron(III) and cobalt(III) complexes of various designed S,N-containing ligands have shown that the unusual coordination structure of the M(III) site (a) raises its potential and shuts off any redox activity, (b) allows binding of NO at the sixth site and oxidative modification of the bound S donors, and (c) brings the pK_a of metal-bound water close to 7. Attempts to synthesize catalytically active model complexes have met with limited success. This review includes the results and implications of the modeling studies reported so far. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Nitrile hydratase; Structural and functional models; Hydration studies

1. Introduction

The enzyme nitrile hydratase (NHase) is a key player in the process of assimilation of nitriles by microorganisms via hydration to amides [1–9]. Interest in this

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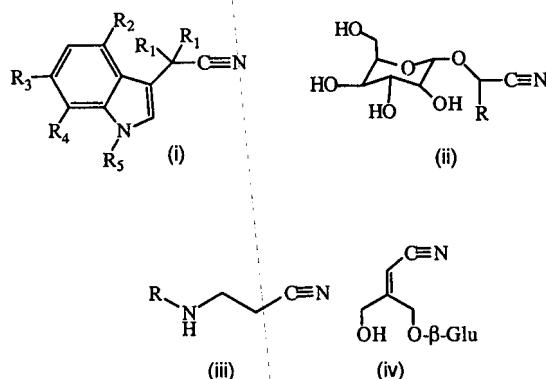
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enzyme has grown since its successful application in industrial production of amides. During the past ten years, a considerable amount of research effort has been directed toward studying smaller model complexes that mimic the active site(s) of NHase. The goal of such pursuit is to establish the role of the non-heme iron(III) (or non-corrinoid cobalt(III)) center of NHase in the process of hydration of organic nitriles (RCN) in the biosphere. It is also expected that selected model complexes could serve as synthetic catalysts in the production of amides. This review highlights the results of the investigations with model complexes reported so far. Brief discussions on nitriles in the environment and the enzyme have been included as the background of the review.

2. Background

2.1. Organic nitriles in the environment

Nitrile-containing compounds (RCN) are essential components in the metabolic cycle of higher plants and many soil microorganisms [1,2]. In plants, they have a widespread occurrence across several phyla where they serve as: (1) growth hormones during plant germination, (2) natural products of plant metabolites, and (3) constituents of a chemical defense system that guard plants from herbivores [10–12]. 3-Indoleacetonitrile and its derivatives (structure (i)) are abundant in plants. These compounds play a fundamental role in plant growth during the process of germination and in plant phototropism. Another family of organic nitriles in plants is the cyanoglycosides (structure (ii)) [12]. These nitriles release cyanide under mild conditions. The plants use them as chemical protective agents against casual herbivores.



To date, over 2000 species of plants have been identified to be cyanogenic by having cyanoglycosides in their roots, seeds or leaves. Plants also contain cyanoalkaloids, cyanolipids (structure (iii)), and other small molecular weight nitrile-containing natural products (an example is structure (iv)) that have other hitherto unknown functions [13,14]. Most plants release organic

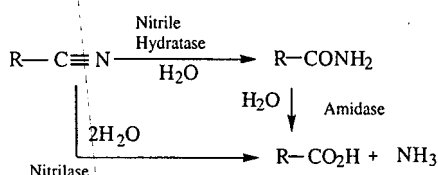
nitriles as a major portion of their root exudates [15]. This process has resulted in the evolution of microorganisms that assimilate nitriles from the rhizosphere and use them as their only source of carbon and nitrogen [2]. Most of these microorganisms utilize hydrolytic enzymes to hydrolyze the nitrile group of the plants exudates and obtain the corresponding carboxylic acid and ammonia products which they use as metabolites to make the essential elements of their biomass (DNA, RNA, and protein constituents). Microbes that assimilate organic nitriles from the environment belong to two Eubacterial phyla: the Purple bacteria and Gram-positive bacteria. It is interesting to note that these phyla share a common evolutionary origin as it was determined by a phylogeny study based on sequence comparisons of 16 S rRNA. This suggested that microorganisms that assimilate nitriles from the environment may have evolved from a common ancestor several million years ago and also advocates for a primeval presence of nitriles in the early environment of earth.

Organic nitriles are also produced in megaton scales for the manufacture of plastics, fibers, pesticides, reagents for water treatment, and other fine chemicals. Several nitriles of anthropogenic origin are, however, toxic and exhibit carcinogenic and teratogenic properties [16,17]. Acrylonitrile [18–20] and pesticides like bromoxynil [21,22] are now considered as threats to the environment. The industries also generate enormous quantities of nitrile-containing chemical wastes that are often difficult to dispose of. Most of the time, the nitriles are present in dilute aqueous solutions which cannot be incinerated. Presently, industrial wastes with toxic nitriles are either disposed at sea or pumped into deep pressure wells below the water table [23].

2.2. Enzymes involved in nitrile assimilation

Microbial enzymes that catalyze the hydrolysis of organic nitriles are divided into two main classes: the nitrilases and the nitrile hydratases. The nitrilases hydrolyze organic nitriles in one step to the corresponding carboxylic acid and ammonia products and they do not use metal cofactors for catalysis. On the other hand, the nitrile hydratases hydrolyze organic nitriles to the corresponding amide product [4–9]. Participation of other enzymes such as amidases completes the hydrolysis of the terminal amide to the corresponding acid and ammonia products (Scheme 1). Hereafter, the review will focus on NHase and more specifically on the models of NHases.

NHases contain either a non-heme iron(III) center or a non-corrinoid cobalt(III) center at their active site depending on the organisms and are subdivided into two classes: (1) the iron-containing NHases (Fe–NHases) [24–33]; and (2) the cobalt-containing NHases



Scheme 1.

(Co–NHases) [34–36]. The enzyme generally consists of α and β subunits and exists as $\alpha\beta$ 46 kDa heterodimers or $(\alpha\beta)_2$ 92 kDa tetramers. Several groups have reported that NHase functions as an $\alpha\beta$ heterodimer (with one metal center) and that its fragmentation into individual subunits inactivates the enzyme. Since NHases exhibit significant protein sequence homology, especially at their metal binding domain in the α subunit where a highly conserved -C-S-L-C-S-C- motif is noted, it is believed that all NHases possibly have the same structure and similar mechanism of catalysis. The relatively more robust Co–NHases have wider substrate specificity. In recent years, NHases from several organisms (and related mutants) have been employed as biocatalysts in industrial production of acrylamide in kiloton quantities [3–7,37,38] and in enantioselective syntheses of other fine chemicals with amide groups [39–45]. NHases have also been used in environmental remediation of nitriles [46–49].

2.3. Properties and structures of Fe–NHases

The Fe–NHases are readily characterized by their grayish green colors. They exhibit an electronic absorption spectrum with bands in the visible region at 700 and 420 nm, which arise from the iron center in these enzymes (Fig. 1). These enzymes also exhibit a rhombic electron paramagnetic resonance (EPR) spectrum with g -values at 2.28, 2.14, 1.97 which is characteristic of a low-spin ($S = 1/2$) iron(III) center (Fig. 1) [50]. Unlike other non-heme iron centers in biology [51,52] the iron(III) center of the NHase does not participate in redox reaction(s) and acts as a Lewis acid. The activity

of one subset of the Fe–NHases, namely *Rhodococcus* N-774 [30], N-771 [31], and *Brevibacterium* R-312 [27] is photoregulated in vivo by nitric oxide (NO) via a reaction that involves reversible binding of NO at the Fe(III) site. Binding of NO in dark results in deactivation of the enzyme, a process that is reversed by loss of NO upon illumination [3,53–56]. This phenomenon is not observed with Co–NHases. The Fe–NHases are also inactivated by N_3^- , CN^- , and by metal ions like Hg^{2+} and Ag^+ [29].

The first structure of a NHase was determined by Nelson and coworkers in 1997 [57]. This group reported the structure of the Fe–NHase from *Brevibacterium* R312 (tetrameric $\alpha_2\beta_2$) at 2.66 Å resolution. The α subunit of this NHase consists of a long N-terminal arm and a rich α -helix region and it ends with an unprecedented fold at the C-terminal domain. The β subunit also contains a long N-terminal extension and it interacts strongly with the α subunit. A large subunit interface area of 3800 Å² and the tight contact suggest that a strong interaction between the two subunits is essential for the function of the enzyme. In a second crystallographic study, Endo and coworkers have determined the structure of the NO-inactivated Fe–NHase from *Rhodococcus* sp. N-771 at 1.7 Å resolution [58]. Since the two NHases are homologous by the sequence, the two structures are very similar. However, the latter structure is more precise and hence it offers additional structural information regarding the metal binding domain of NHase that was not revealed in the structure reported by Nelson and coworkers.

In both structures, the iron(III) center is located in a cleft situated at the interface of the two subunits of the $\alpha\beta$ heterodimer. The coordination structure of this active site is quite unusual. The low resolution structure shows that the iron(III) center is ligated to two *deprotonated* carboxamido nitrogens from the αCys113 - αSer114 peptide backbone and three sulfur thiolates from the αCys110 , αCys113 , and αCys115 amino acid residues [57]. The high resolution structure by Endo and coworkers [58] further reveals that two of the

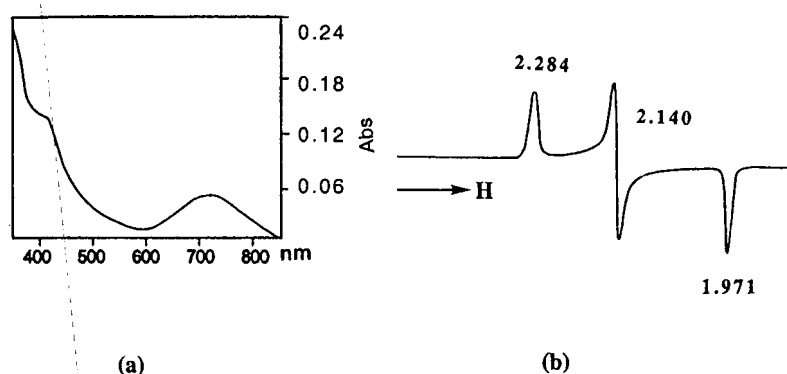


Fig. 1. (a) Electronic absorption spectrum and (b) EPR spectrum of Fe–NHase from *Brevibacterium* R312.

bound imminol intermediate that rearranges to the amide product and then the amide is released. This is known as the inner-sphere mechanism. The presence of a molecule of iodoacetonitrile in close proximity of the iron(III) center noted in crystallographic work by Nelson and coworkers provides some support to this mechanism. There is also precedence for platinum(II) and rhodium(II) complexes that bind nitriles and catalyze their hydrolysis via an inner-sphere mechanism [67,68].

The second postulated mechanism (Fig. 3b), known as the outer-sphere mechanism, involves a nucleophilic attack of the metal-bound hydroxide on the nitrile substrate nested at the active site pocket. The reaction generates a transient imminolate species that is O-bonded to the metal. This species then rearranges to amide and is released as product. In the third postulated mechanism (Fig. 3c), the metal-bound hydroxide causes deprotonation of a free water molecule near the active site. It is the newly generated hydroxide that carries out the hydrolysis of the nitrile substrate. The eventual rearrangement of the hydrolyzed intermediate leads to the amide product. This mechanism is known as the second outer-sphere mechanism of catalysis. Note that in this mechanism, no substitution occurs at the coordination sphere of the M(III) center.

Since the coordination structures of the M(III) sites of the Fe- and Co-NHase are assumed to be very similar or identical, one expects that the two M(III) sites function in very similar ways. Support for this argument comes from the kinetic data available for these enzymes. The Co-NHase from *Rhodococcus rhodochrous* sp. J1 and Fe-NHase from *Brevibacterium* R312 hydrolyze propionitrile at almost identical rates under the same reaction conditions (1600 and 1800 mmol min⁻¹, respectively). When one considers the distinctly different kinetic labilities of low-spin iron(III) (d⁵) and cobalt(III) (d⁶) centers, a similar reactivity of the two NHases suggests that an outer-sphere mechanism of catalysis (i.e. one involving no ligand exchange) is most possibly in operation. However, there is no evidence that supports this hypothesis so far.

3. Model complexes and their reactivities

3.1. Required features and synthetic difficulties

The desire to understand the unusual hydrolytic chemistry of the iron(III) and cobalt(III) sites of the NHases has prompted efforts to synthesize smaller model complexes that reproduce both the structure and function of the active sites of Fe- and Co-NHases. Such efforts, often dubbed as 'synthetic analogue approach' have provided great insights into the

intrinsic properties of the active sites of metallo-biomolecules [69] and to date, research on analogues of the Fe- and Co-NHases has similarly afforded valuable information on the function of the metalloenzyme. The following sections highlight the results of the model studies reported so far.

In principle, good structural and functional models of NHase should include the following structural and functional features.

1. The mononuclear iron(III) or cobalt(III) center is ligated to carboxamido N and thiolato S donor centers with one or more of the thiolato S in the sulfenato and/or sulfinato form.
2. The reduction potential of the iron(III) site is high and hence it does not exhibit redox behavior and just functions as a Lewis acid.
3. The metal center has an open or solvent-bound coordination site. When this site is occupied by water, one expects that the pK_a of the bound water is close to 7. Whether nitriles bind to this site or not remains to be established.
4. The iron(III) center exhibits reactivity toward NO. Binding of NO converts the low-spin iron(III) site to a diamagnetic iron(III)-NO adduct. Upon illumination, the iron center loses NO readily.

If indeed one could synthesize such iron(III) and/or cobalt(III) model complex(es), then it is reasonable to expect that studies on the hydrolysis of nitriles with such species will provide important clues relating to the mechanism of the hydrolysis by the NHases.

In general, monomeric iron(III) complexes that comprise thiolato S and carboxamido N donor centers are quite scarce [70]. Most reactions with ferric ions and thiolates either afford S-bridged polymers or undergo auto-redox reactions that generate thiyl radicals (RS[•]) and iron(II) species. These reactions impede the isolation of the iron(III) species [71–73]. Moreover, coordination of carboxamido N to iron(III) centers was believed to be 'improbable' for a long time [74]. Since the pH necessary for deprotonation of the carboxamido N is too high for iron(II) and iron(III) to exist in aqueous solution (both ions precipitate as hydroxides at pH ≥ 3) [75], it had generally been concluded that ligation of the deprotonated peptide N to iron was improbable. This paradigm discouraged many research groups from synthesizing such complexes. The situation has, however, been changed in recent years following isolation of several iron complexes with coordinated carboxamido nitrogens [70]. Overall, the scarcity of good models of NHase stems from the inherent difficulties one encounters in the synthesis of iron(III) (and cobalt(III)) complexes with carboxamido N and thiolato S donors. As described below, the majority of the NHase models have so far been synthesized with S-containing Schiff-base ligands.

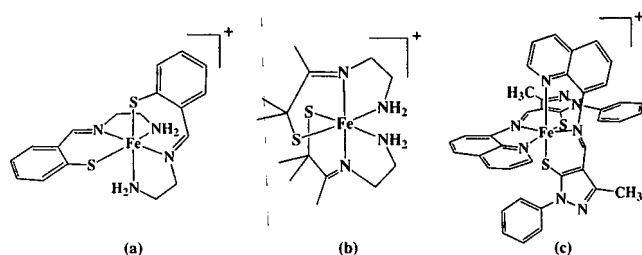


Fig. 4. Structures of: (a) $[\text{Fe}^{\text{III}}(\text{S-sal:N}^*(\text{CH}_2)_2\text{NH}_2)_2]^+$ (cation of 1); (b) $[\text{Fe}^{\text{III}}(\text{ADIT})_2]^+$ (cation of 2); and (c) $[\text{Fe}^{\text{III}}(\text{L}^{\text{N}_2\text{S}})_2]^+$ (cation of 3).

3.2. Models of Fe–NHases: complexes with thiolato S and amine/imine N donors

In 1975, Gatehouse and coworkers reported the first iron(III) complex with a mixed N, S coordination sphere although it was not reported as a NHase model [76]. The complex, bis- $[\text{N}-(2\text{-aminoethyl})\text{thiosalicylideneiminato}]\text{iron(III) chloride}$, $[\text{Fe}^{\text{III}}(\text{S-sal:N}(\text{CH}_2)_2\text{NH}_2)_2]\text{Cl}$ (**1**), was synthesized via a metal-initiated template reaction in which ethylenediamine and 2-mercaptobenzaldehyde were added to iron(III) chloride in methanol. Complex **1** consists of a six-coordinate iron(III) center in a distorted octahedral geometry with two ligated 2-aminoethylsalicylidene-iminato tridentate units (Fig. 4a) [76]. The average length of the Fe–S bonds in this complex, 2.21 Å, is significantly shorter than the sum of the appropriate covalent radii (Fe, 1.34 and S, 1.04 Å). This suggests that a significant interaction exists between the p and d orbitals of sulfur and the d orbitals of iron. Both the magnetic moment (2.00 μ_{B}) and the rhombic EPR signal with *g*-values of 2.16, 2.12, 2.00 are consistent with a low-spin ($S = 1/2$) electronic configuration of the iron(III) center in **1** [77].

Kovacs and coworkers synthesized a similar iron(III) complex in 1995 and introduced it as a model for the low-spin non-heme iron(III) site in Fe–NHase [78]. This group followed the same in situ condensation on a metal template developed by Gatehouse. However, they used α -mercaptoacetone with ethylenediamine and iron(III) chloride in basic methanol and isolated bis(6-amino-2,3-dimethyl-4-azahex-3-ene-2-thiolato)iron(III) chloride, $[\text{Fe}^{\text{III}}(\text{ADIT})_2]\text{Cl}$, (**2**). The iron(III) center in $[\text{Fe}^{\text{III}}(\text{ADIT})_2]^+$ contains two thiolato sulfurs, two imino nitrogens, and two amine (NH_2) nitrogens in the first coordination sphere (Fig. 4b). The average Fe–S bond distance in **2** is 2.20 Å and is significantly shorter than that reported for Fe–NHase (2.32 Å) [57,58]. This difference could be due to the small bite of the ligand. Complex **2** comprises an iron(III) center which is low-spin ($S = 1/2$) at all temperatures as indicated by its magnetic moment (1.85 μ_{B}) and its EPR spectrum in methanol glass ($g = 2.19, 2.13, 2.01$). The electronic absorption spectrum of this complex in acetonitrile (two bands with maxima, λ_{max} , at 718 and 438 nm)

resembles the absorption spectrum of the Fe–NHase in aqueous buffer.

Nivorozhkin and coworkers have reported a mononuclear iron(III) complex with the tridentate ligand $[\text{N},\text{N}'\text{-bis}(5\text{-mercapto-3-methyl-1-phenylpyrazol-4-ylmethylene})\text{ethylenediamine}]$ as a spectroscopic model for the iron(III) site in Fe–NHase [79]. The structure of this complex, namely $[\text{Fe}^{\text{III}}(\text{L}^{\text{N}_2\text{S}})_2](\text{ClO}_4)$ (**3**) reveals that the iron(III) center has a distorted octahedral geometry with two thiolato sulfurs, two imine nitrogens, and two quinoline ring nitrogens as donors (Fig. 4c). The average Fe–S bond distance in **3** (2.28 Å) compares well with those reported for the other iron(III) complexes described above. Complex **3** is also low-spin ($S = 1/2$) and exhibits a magnetic moment of 1.95 μ_{B} . In chloroform glass (77 K), it displays a rhombic EPR signal with *g*-values 2.19, 2.13, and 1.98. The electronic absorption spectrum of this blue–green complex in chloroform consists of two bands with λ_{max} at 995 and 750 nm.

Mascharak and coworkers have studied the chemistry of the iron(III) complexes of two Schiff-base ligands namely, *N*-2-mercaptophenyl-2'-pyridylmethylenimine (PyASH) and *N*-2-mercapto-2-methylpropyl-2'-pyridylmethylenimine (PyMSH) [80]. The iron(III) complexes $[\text{Fe}^{\text{III}}(\text{PyAS})_2](\text{BPh}_4)$ (**4**) and $[\text{Fe}^{\text{III}}(\text{PyMS})_2](\text{BPh}_4)$ (**5**) have been synthesized by oxidizing the corresponding iron(II) complexes by ferrocenium salts [81]. In **4** and **5**, the deprotonated ligands are coordinated in *mer* fashion with two thiolato sulfurs *cis* to each other (Fig. 5). Both complexes are low-spin and exhibit rhombic EPR spectra (Fig. 6).

It is important to note at this point that six-coordinate iron(III) Schiff-base complexes with N_xO_y donor sets like $[\text{Fe}(\text{N}_2\text{O})_2]^+$, $[\text{Fe}(\text{N}_3\text{O}_2)\text{L}]^+$ and $[\text{Fe}(\text{N}_4\text{O}_2)]^+$ are either high-spin ($S = 5/2$) or exhibit spin equilibria [82,83]. Even the hexadentate Schiff-base ligands with N_4O_2 donor set do not afford low-spin ($S = 1/2$) iron(III) complexes [84]. Thus the O-analogue of tridentate Schiff-base ligands like AMITH, ADITH and PyASH (Fig. 7) give rise to iron(III) complexes of the type $[\text{Fe}(\text{N}_2\text{O})_2]^+$ which are either high-spin or exhibit spin equilibria. In contrast, Schiff-base ligands with N_2S donor set like ADITH, AMITH, PyASH and PyMSH all afford iron(III) bis complexes that are

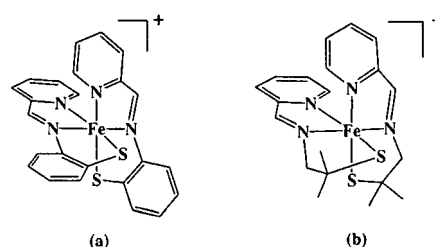


Fig. 5. Structures of: (a) $[\text{Fe}^{\text{III}}(\text{PyAS})_2]^+$ (cation of 4) and (b) $[\text{Fe}^{\text{III}}(\text{PyMS})_2]^+$ (cation of 5).

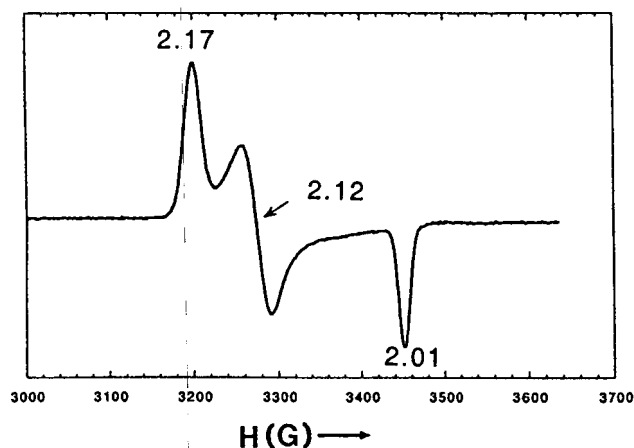


Fig. 6. X-band EPR spectrum of $[\text{Fe}^{\text{III}}(\text{PyAS})_2]^+$ in DMF glass at 8 K.

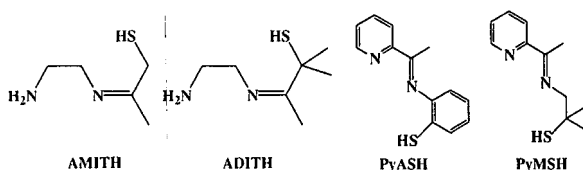


Fig. 7. Schiff-base ligands used for nitrile hydratase models.

low-spin at all temperatures. The change in spin configuration due to replacement of O with S in these ligands clearly indicates stronger interaction between thiolato sulfur and iron(III) center. Most of these species also exhibit thiolate-to-iron(III) charge transfer bands in the 550–850 nm range.

The first NHase model that comprises three thiolato sulfurs in a six-coordinate environment has been reported by Wiegardt and coworkers [85]. This iron(III) complex is derived from the hexadentate ligand 1,4,7-tris(4-*tert*-butyl-2-mercaptobenzyl)-1,4,7-triazacyclononane which provides three thiolato sulfurs and three amine-type nitrogens as the donor set. To avoid the auto-redox process known to take place between iron(III) salts and organic thiolates, this group either reacted the ligand with $[\text{Fe}^{\text{III}}(\text{acac})_3]$ or with $[\text{Fe}^{\text{II}}(\text{OAc})_2]$ in methanol and exposed the reaction mixture to air 'carefully' to produce the desired complex (1,4,7-tris(4-*tert*-butyl-2-mercaptobenzyl)-1,4,7-triazacyclononane)iron(III), $[\text{Fe}^{\text{III}}(\text{L}^{\text{N}_3\text{S}_3})]$ (**6**). The structure of this complex revealed a six-coordinate pseudo-octahedral iron(III) center with three amine nitrogens from the triazacyclononane ring and three thiolato S atoms coordinated in a *fac*-fashion around the metal center (Fig. 8). In $[\text{Fe}^{\text{III}}(\text{L}^{\text{N}_3\text{S}_3})]$, the average Fe–S distance is 2.28 Å and compares well with the corresponding distance reported for the other complexes that have only two thiolato S donors. Interestingly, the average Fe–N_{amine} distance in **6** is 2.08 Å and is very close to the Fe–N_{amido} distance noted for NHase (2.07 Å). How-

ever, its spectroscopic parameters are very different from those reported for NHase. For example, **6** exhibits a solution magnetic moment of $2.4 \mu_{\text{B}}$ at 77 K which rises to $3.7 \mu_{\text{B}}$ at 300 K. Also, the EPR spectrum of solid **6** at 10 K displays an isotropic signal with $g = 4.3$ corresponding to a typical high-spin Fe(III) ($S = 5/2$) species. The electronic absorption spectrum of **6** in acetonitrile consists of two bands in the visible region with λ_{max} at 775 and 651 nm. It is interesting to note that although **6** contains three thiolato sulfur donors in the coordination sphere, the iron(III) center remains high-spin. Wiegardt and coworkers have also reported the synthesis and properties of the corresponding iron(III) complex with alkylthiolato groups connected to the triazacyclononane frame. This Fe(III) complex is also high-spin at all temperatures and displays one electronic absorption band at 551 nm.

Recently, in a series of papers, Kovacs and coworkers have reported the synthesis and reactivity of a coordinatively unsaturated iron(III) complex that comprises two thiolato sulfurs, two imine-type nitrogens, and one amine-type nitrogen in the coordination sphere. This group synthesized the iron(III) complex 2,3,13,14-tetramethyl-4,8,12-triaza-3,12-pentadecadiene-2,14-dithiolato iron(III) via ferrocenium oxidation of the corresponding iron(II) species prepared by in situ condensation of 3-methyl-3-mercapto-2-butanone and 3,3'-iminobis(propylamine) in presence of Fe(II) chloride [86]. The structure of the pentacoordinate iron(III) complex, namely $[\text{Fe}^{\text{III}}\text{S}_2^{\text{Me}_2}\text{N}_3(\text{Pr},\text{Pr})]\text{PF}_6$ (**7**), consists of an iron(III) center in a distorted trigonal bipyramidal geometry in which the thiolates are positioned in the basal plane and the two imine nitrogens are at the axial positions (Fig. 9a). The average Fe–S distance noted for

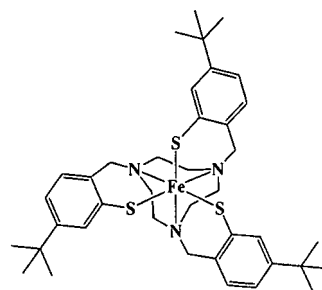


Fig. 8. Structure of $[\text{Fe}^{\text{III}}(\text{L}^{\text{N}_3\text{S}_3})]$ (**6**).

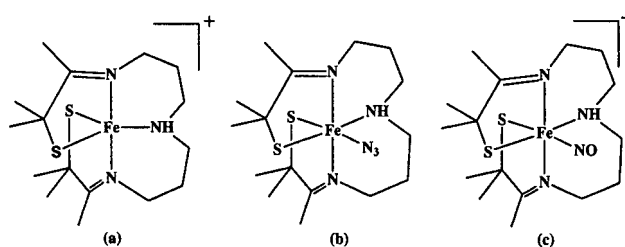


Fig. 9. Structures of: (a) $[\text{Fe}^{\text{III}}\text{S}_2^{\text{Me}_2}\text{N}_3(\text{Pr},\text{Pr})]^+$ (cation of **7**); (b) $[\text{Fe}^{\text{III}}\text{S}_2^{\text{Me}_2}\text{N}_3(\text{Pr},\text{Pr})(\text{N}_3)]^+$; and (c) $[\text{Fe}^{\text{III}}\text{S}_2^{\text{Me}_2}\text{N}_3(\text{Pr},\text{Pr})(\text{NO})]^+$.

7 (2.14 Å) is significantly shorter than the corresponding distance in the other complexes and in NHase. It is quite possible that the absence of the carboxamido nitrogens (negatively charged) in the coordination sphere allows the ligated thiolates to interact more strongly with the iron(III) center of the complex and gives rise to short Fe(III)–S_{thio} distances. This complex is high-spin at room temperature as evident from its magnetic moment of 5.0 μ_B at 300 K. However, the magnetic moment drops to 2.0 μ_B below 50 K and the low temperature EPR spectrum in methanol glass (< 150 K) displays a rhombic signal (*g* = 2.20, 2.15, 2.00). These results indicate that the low-spin state is significantly populated at low temperatures. The electronic absorption spectrum of [Fe^{III}S₂^{Me2}N₃(Pr,Pr)]⁺ in methanol consists of one band with λ_{max} at 416 nm which is quite different from the electronic spectrum of Fe–NHase (λ_{max} at 700 and 420 nm). However, when [Fe^{III}S₂^{Me2}N₃(Pr,Pr)]⁺ binds azide (N₃[−]) in acetonitrile to form the corresponding azide adduct namely [Fe^{III}S₂^{Me2}N₃(Pr,Pr)(N₃)] (Fig. 9b), the electronic absorption spectrum becomes very similar to that of the Fe–NHase. Thus, [Fe^{III}S₂^{Me2}N₃(Pr,Pr)(N₃)] has absorbances with λ_{max} at 708 and 460 nm. Also, the azide adduct is low-spin and exhibits a rhombic EPR signal in methanol glass (*g* = 2.23, 2.16, 1.99). These spectral features make the azide adduct a good model of the azide-inhibited form of the Fe–NHase. The relatively open S–Fe–N angle (132.3(2)°) in the basal plane of 7 (Fig. 9a) allows binding of azide to the iron(III) center of 7 and in [Fe^{III}S₂^{Me2}N₃(Pr,Pr)(N₃)], azide is *trans* to a thiolato sulfur (Fig. 9b). Binding of azide is reversible and the extent of binding depends both on the temperature and the nature of the solvent. Interestingly, 7 does not bind nitriles under any conditions.

The five-coordinate complex [Fe^{III}S₂^{Me2}N₃(Pr,Pr)]⁺ deserves further attention due to its ability to bind one molecule of NO to generate the corresponding diamagnetic (*S* = 0) nitrosyl adduct [Fe^{III}S₂^{Me2}N₃(Pr,Pr)(NO)]⁺ [87]. Structural characterization of this species reveals that the iron(III) center has a distorted octahedral geometry in which NO is coordinated *trans* to a thiolato sulfur (Fig. 9c). The X-ray absorption spectrum of this NO-adduct resembles that of the dark-adapted (NO-bound) Fe–NHase from *Rhodococcus* sp. R312 [88]. Also, the NO-adduct [Fe^{III}S₂^{Me2}N₃(Pr,Pr)(NO)]⁺ displays the IR stretch corresponding to the nitrosyl group (ν_{NO}) at 1822 cm^{−1} close to the ν_{NO} of NO-inactivated enzyme (1853 cm^{−1}) [54,55]. The Fe–N–O moiety in [Fe^{III}S₂^{Me2}N₃(Pr,Pr)(NO)]⁺ is, however, quite linear (172.3°). It thus appears that there is greater backbonding in the Fe–NO unit of this model complex compared to that in the Fe–NO moiety in the NO-inactivated Fe–NHase where the Fe–N–O angle is 158.6°. This difference could arise from the absence of sulfenic

(RSO) and/or sulfinic groups (RSO₂) at the coordination sphere of the model complex. Interestingly, exposure of [Fe^{III}S₂^{Me2}N₃(Pr,Pr)(NO)]⁺ to a strong UV-light source (Hg lamp) for 6 h results in the loss of NO from the complex [87]. However, the researchers also observed that the metal complex undergoes irreversible decomposition.

3.3. Models of Fe–NHases: complexes with thiolato *S* and carboxamido *N* donors

The model complexes described above do not include carboxamido nitrogens and/or sulfe(i)nato (RSO/RSO₂) moieties in the coordination sphere of the iron(III) centers. Since these are some essential features of the metal site in NHases, several groups have undertaken the task of isolation and characterization of iron(III) complexes of designed ligands with varying number of thiolato sulfur and carboxamido nitrogen donor centers. Studies on such complexes have provided valuable insight into the role(s) of such coordination in the overall reactivity of the iron(III) centers in NHases. A major portion of work in this area has been completed by Mascharak and coworkers during the period 1997–2001.

The first model complex with thiolato sulfurs and carboxamido nitrogens was reported by Mascharak and coworkers in 1998. Reaction of (Et₄N)[FeCl₄] with deprotonated designed ligand PyPepS^{2−} (where PyPepSH₂ = *N*-2-mercaptophenyl-2'-pyridinecarboxamide, Hs denote the dissociable peptide and thiol H) in DMF affords (Et₄N)[Fe^{III}(PyPepS)₂] (8) in good yield. The geometry around the iron(III) center in 8 is octahedral with the two essentially planar tridentate ligand frames coordinated in *mer* fashion (Fig. 10a) [89]. Complex 8 exhibits an Fe(III)–N_{amido} distance of 1.954(2) Å which is very similar to that observed in other iron(III) complexes with coordinated carboxamido nitrogens [70,90–93]. Coordination of carboxamido nitrogens to iron(III) in 8 is readily indicated by the shift of ν_{CO} to 1612 from 1666 cm^{−1} in free PyPepSH₂ [70]. The iron(III) center of this model complex is low-spin in the temperature range 8–300 K and its EPR spectrum (*g* = 2.22, 2.14, 1.98) resembles that of the enzyme very closely. In

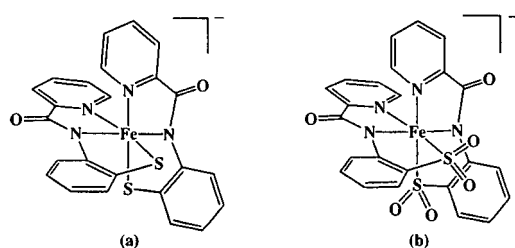


Fig. 10. Structures of: (a) [Fe^{III}(PyPepS)₂]^{2−} (anion of 8) and (b) [Fe^{III}(PyPepSO₂)₂]^{2−} (anion of 9).

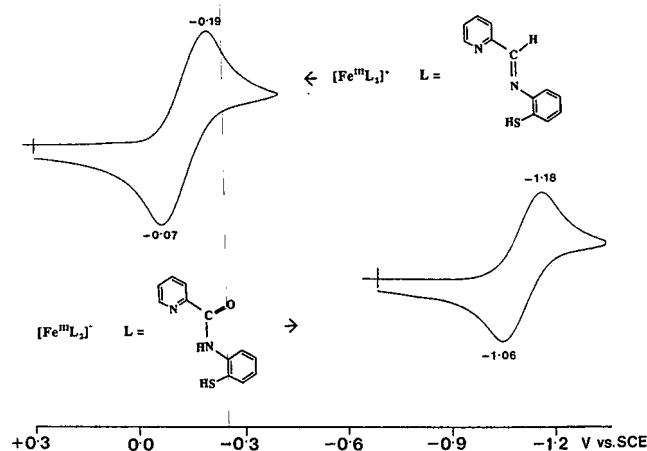


Fig. 11. Cyclic voltammograms of $(\text{Et}_4\text{N})[\text{Fe}^{\text{III}}(\text{PyPepS})_2]$ (**8**) and $[\text{Fe}^{\text{III}}(\text{PyAS})_2](\text{BPh}_4)$ (**4**) in DMF (0.1 M $(\text{Et}_4\text{N})(\text{ClO}_4)$, Pt electrode, 50 mV s^{-1} scan rate). Potentials are shown vs. aqueous saturated calomel electrode.

addition, **8** exhibits a thiolate-to-iron(III) charge transfer band at 850 nm in water.

The synthesis of the model complex **8** affords some interesting clues regarding ligation of carboxamido nitrogens to iron. First, no reduction of iron(III) takes place upon mixing of PyPepS^{2-} with iron(III) salts. Under similar conditions, addition of PyAS^- (Fig. 7) to iron(III) salts causes immediate reduction of iron and only the iron(II) complex is obtained from the reaction mixture. It thus appears that ligands like PyPepSH_2 that employ carboxamido nitrogens in addition to thiolato sulfurs to bind iron prefer iron(III) centers. Indeed, PyPepSH_2 does not react with iron(II) salts at all. Second important point to note is the stability of **8** in aqueous medium. Although the complex is synthesized in DMF, it is quite stable in protic solvents and does not afford oxo- or hydroxo-bridged dimeric or polymeric iron(III) species which are often recognized as thermodynamically very stable entities in the hydrolytic chemistry of iron [75]. Finally, formation of **8** as the only product in the reaction mixture indicates that thiolate-bridged multinuclear iron complexes are not very common when the ligands contain additional carboxamide groups [70].

The electrochemical properties of **8** provides the most important clue regarding the role of carboxamido nitrogens in the redox behavior of the iron(III) center in Fe–NHase. In DMF, $(\text{Et}_4\text{N})[\text{Fe}^{\text{III}}(\text{PyPepS})_2]$ (**8**) and $[\text{Fe}^{\text{III}}(\text{PyAS})_2](\text{BPh}_4)$ (**4**) exhibit their half-wave potentials ($E_{1/2}$) at -1.12 and -0.13 V (vs. SCE, saturated calomel electrode), respectively (Fig. 11). This dramatic shift of 1 V in $E_{1/2}$ clearly indicates that the coordination of negatively charged strong-field carboxamido nitrogens provide significant stability to the +3 oxidation state of iron in non-heme iron species [70,89,94]. The presence of carboxamido nitrogens and thiolato

sulfurs around the iron(III) site in NHase thus raises the redox potential of the iron site. The high reduction potential in turn does not allow the iron(III) site of NHase to participate in any redox process; rather it acts as a Lewis acid in the process of hydration of nitriles.

When H_2O_2 is added to a cold solution of **8** in DMF, a deep green solution (λ_{max} 690 nm) results from which $\text{Na}[\text{Fe}^{\text{III}}(\text{PyPepSO}_2)_2]$ (**9**) has been isolated [95]. Crystallographic studies on $[\text{Fe}^{\text{III}}(\text{PyPepSO}_2)_2]^-$ reveal that the process of oxidation affords the corresponding sulfinato complex (Fig. 10b). This model complex from Mascharak's group is the first example of a iron(III) species with both carboxamido nitrogen and S-bonded sulfinato groups around the metal center. The S–O distance in **9** (average $1.472(4) \text{ \AA}$) compares well with the same observed with other known sulfinato species and the coordinated sulfinato groups display ν_{SO} at 1184 cm^{-1} . Complex **9** is low-spin and it dissolves in water, acetonitrile, and DMF to produce green solutions. The aqueous solution is indefinitely stable and exhibits an electronic absorption spectrum (λ_{max} at 700 and 420 nm) almost identical to that of the Fe–NHase. This work demonstrates for the first time, that oxidation of iron(III) complexes with coordination sphere resembling that of the active site of Fe–NHase can be oxidized to give sulfinato species. Interestingly, attempts to oxidize $[\text{Fe}^{\text{III}}(\text{PyAS})_2](\text{BPh}_4)$ (**4**) under similar conditions only result in decomposition of the iron complex.

Very recently, Mascharak and coworkers have synthesized the iron(III) complex of the designed pentadentate ligand PyPSH_4 (Fig. 12) [96]. Despite coordinative unsaturation, the model complex $(\text{Et}_4\text{N})[\text{Fe}^{\text{III}}(\text{PyPS})]$ (**10**) remains mononuclear. In **10**, the iron(III) center is coordinated to two carboxamido nitrogens, two thiolato sulfurs and one pyridine ring nitrogen (Fig. 12). The average Fe– N_{amido} and Fe– S_{thio} distances are $2.038(2)$ and $2.312(2) \text{ \AA}$, respectively. This blue–green model complex is particularly notable since it allows one to examine for the first time, the reactivity of the iron(III) center toward various molecules including nitriles. The iron(III) center in **10** binds to a variety of ligands (methanol, PhO^- , PhS^- , py, *N*-Me-imidazole) at low temperature and gives rise to green solutions with a band around 700 nm. Such bindings are re-

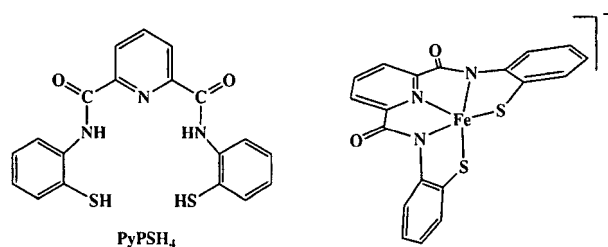


Fig. 12. Structures of PyPSH_4 and $[\text{Fe}^{\text{III}}(\text{PyPS})]^-$ (anion of **10**).

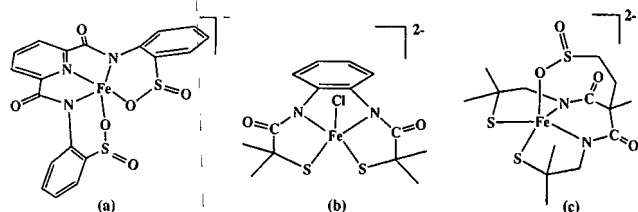


Fig. 13. Structures of: (a) $[\text{Fe}^{\text{III}}(\text{PyP}\{\text{SO}_2\}_2)]^-$ (anion of **11**); (b) $[\text{Fe}^{\text{III}}(\text{N}_2\text{S}_2)\text{Cl}]^{2-}$ (anion of **12**); and (c) $[\text{Fe}^{\text{III}}(\text{L}^{\text{N}_2\text{S}_3\text{O}_2})]^{2-}$ (anion of **13**).

versible and the six-coordinate $[\text{Fe}^{\text{III}}(\text{PyPS})\text{L}]^n-$ species are all low-spin with g -values very close to those of the Fe–NHase. These results indicate that the iron(III) site of the functional enzyme is six-coordinate. The pK_a of the water bound at the iron(III) site of **10** has been determined (at -30°C) to be 6.3 ± 0.4 . It is important to note that **10** (like **7**) does *not* show any affinity toward nitriles. It thus appears that at physiological pH, a metal-bound hydroxide promotes hydration of nitriles nested at the active site pocket of Fe–NHase (mechanism 3b or 3c).

Limited exposure of a solution of **10** (in acetone) to dioxygen results in the formation of a bis-sulfenic species. This reaction mimics the post-translational modification of the Cys-S center in Fe–NHase. The structure of the more stable O-bonded sulfinato complex $(\text{Et}_4\text{N})[\text{Fe}^{\text{III}}(\text{PyP}\{\text{SO}_2\}_2)]$ (**11**, Fig. 13a) has been determined [96]. It appears that oxidation of coordinatively unsaturated $[\text{Fe}^{\text{III}}(\text{PyPS})]^-$ (**10**) gives rise to the O-bonded sulfinato complex **11** while coordinatively saturated species $[\text{Fe}^{\text{III}}(\text{PyPepS})_2]^-$ (**8**) affords the S-bonded sulfinato complex **9**. This is further supported by the fact that six-coordinated low-spin cyanide adducts of the S-bonded and O-bonded sulfinato complexes namely, $\text{Na}_2[\text{Fe}^{\text{III}}(\text{PyP}\{\text{SO}_2\}_2)(\text{CN})]$ and $(\text{Et}_4\text{N})_2[\text{Fe}^{\text{III}}(\text{PyP}\{\text{SO}_2\}_2)(\text{CN})]$ have been isolated by starting from six- and five-coordinate species, respectively. These low-spin cyanide adducts afford green solutions in water and other solvents. The $E_{1/2}$ values of $(\text{Et}_4\text{N})[\text{Fe}^{\text{III}}(\text{PyPS})]$ and $(\text{Et}_4\text{N})[\text{Fe}^{\text{III}}(\text{PyP}\{\text{SO}_2\}_2)]$ in DMF (-0.65 and -0.36 V vs. SCE) indicate that the iron(III) centers in these two model complexes are quite stabilized much like the iron(III) site in Fe–NHase ($E_{1/2}$ in aqueous buffer = -0.48 V vs. SCE [97]). Comparison of the structure, properties, and reactivity of $(\text{Et}_4\text{N})[\text{Fe}^{\text{III}}(\text{PyPS})]$ (**10**) with those of the iron(III) site of Fe–NHase suggests that **10** is indeed a very good model of the active site of the Fe–NHase.

Two additional iron(III) complexes with ligands containing carboxamido nitrogen and thiolato sulfur donors have recently been reported. The first one has been reported by Artaud et al. in a short review on nitrile hydratase [97]. These researchers have employed the previously known ligand *N,N'*-1,2-phenylenebis-(2-

mercapto-2-methylpropionamide), developed by Hans and Krüger who synthesized its copper complex [98]. The structure of the iron(III) species, namely, $(\text{Et}_4\text{N})[\text{Fe}^{\text{III}}(\text{N}_2\text{S}_2)\text{Cl}]$ (**12**) reveals that the iron(III) center exists in a square pyramidal geometry in which two carboxamido nitrogens and two thiolato sulfurs are bonded in the basal plane while a chloride ion is coordinated at the axial position (Fig. 13b). In $[\text{Fe}^{\text{III}}(\text{N}_2\text{S}_2)\text{Cl}]^-$, the average Fe– N_{amido} distance is 1.92 Å which is somewhat shorter than the corresponding distance in NHase (2.07 Å). Also, the average Fe– S_{thio} distance in **12** (2.19 Å) is shorter than the corresponding distance in NHase. Despite the fact that the iron(III) center in $[\text{Fe}^{\text{III}}(\text{N}_2\text{S}_2)\text{Cl}]^-$ has structural features similar to the active site of Fe–NHase, its physical parameters are very different from those reported for the enzyme. For example, **12** displays a broad EPR spectrum in DMF at 10 K with g -values of 5 and 3.5 which is indicative of an intermediate spin state ($S = 3/2$) for the iron(III) center. Also, the electronic absorption spectrum of **12** in CH_2Cl_2 consists of only one band in the visible region with λ_{max} at 500 nm. The red color of this model complex is distinctly different from that of the green enzyme (λ_{max} at 700 and 420 nm).

In a recent communication, Chottard and coworkers have reported the synthesis and structure of the second iron(III) complex that comprises two carboxamido nitrogens, two thiolato sulfurs and one O-bonded sulfinato SO_2 donor in its coordination sphere [99]. The iron(III) center in this model complex is ligated to 2-methyl-2-thiomethylmalonylenebis-(2-mercapto-2-methylpropionamide) forming $(\text{Et}_4\text{N})_2[\text{Fe}^{\text{III}}(\text{L}^{\text{N}_2\text{S}_3\text{O}_2})]$ (**13**), a square pyramidal complex with two carboxamido nitrogens and two thiolato sulfurs in the basal plane (Fig. 13c). The axial position is occupied by one oxygen atom of the sulfinato group. In $[\text{Fe}^{\text{III}}(\text{L}^{\text{N}_2\text{S}_3\text{O}_2})]^{2-}$, the average Fe– N_{amido} and Fe– S_{thio} distances are 1.95 and 2.22 Å, respectively. The Fe– SO_2 bond distance in **13** (2.00 Å) compares well with the corresponding distance in **11** (1.91 Å). Accidental exposure to air most possibly gives rise to the sulfinato group in **13** although the researchers have so far been unable to isolate the tris-thiolato species. It is important to note that here again, one obtains the O-bonded sulfinato isomer of the coordinatively unsaturated iron(III) precursor much like the oxidation of **10** leading to **11**.

Although $(\text{Et}_4\text{N})_2[\text{Fe}^{\text{III}}(\text{L}^{\text{N}_2\text{S}_3\text{O}_2})]$ (**13**) is a good structural model for the active site of Fe–NHase, its spectroscopic properties are surprisingly very different from those of the enzyme. Indeed, the spectroscopic parameters of **13** are more similar to those of **12**. For example, **13** exhibits an intermediate spin state ($S = 3/2$) as is evident from its solid state magnetic moment of $3.8 \mu_B$ at all temperatures (3–300 K). This was also supported by its broad EPR spectrum with g -values centered at

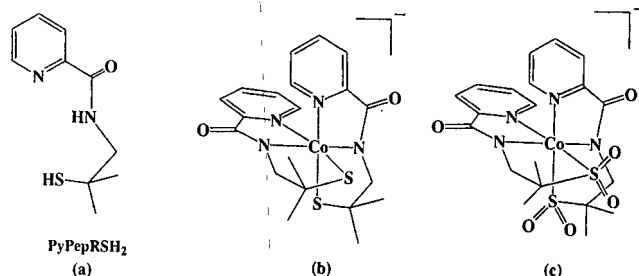


Fig. 14. Structures of: (a) PyPepRSH₂; (b) [Co^{III}(PyPepRS)₂]⁻ (anion of 15); and (c) [Co^{III}(PyPepRSO₂)₂]⁻ (anion of 17).

3.75 and 2.01 (DMF glass at 10 K). Furthermore, the electronic absorption spectrum of 13 in MeCN consists of only one band with λ_{max} at 475 nm. Collectively, these results suggest that proper disposition of the carboxamido nitrogens and the thiolato sulfurs (including the modified ones) around the iron(III) center is crucial in determining the overall spectroscopic (and electronic) parameters of the metal center in the model complexes. As revealed in the X-ray studies (Fig. 2), the iron(III) center of a good model complex should be coordinated to a thiolato sulfur at the axial position while the carboxamido nitrogens and the modified sulfe(i)nato groups should be ligated in the basal plane.

3.4. Models of Co–NHases: complexes with thiolato S and carboxamido N donors

Modeling work in this area began with the assumption that the coordination structures of the active sites of the Fe–NHase and the Co–NHase are very similar or identical. Mascharak and coworkers reported the first set of cobalt(III) complexes of ligands containing both carboxamido nitrogen and thiolato sulfur donors. The cobalt(III) complex of the designed ligand PyPepSH₂ namely, (Me₄N)[Co^{III}(PyPepS)₂] (14) is structurally very similar to the corresponding iron(III) complex 8 (see Fig. 10a) [95]. This octahedral diamagnetic cobalt(III) complex has two thiolato sulfurs in *cis* positions while the two carboxamido nitrogens are *trans* to each other. In 14, the average Co(III)–N_{amido} and Co(III)–S_{thio} bond distances are 1.917(3) and 2.222(3) Å, respectively. A similar cobalt(III) complex with a ligand with alkyl thiolates PyPepRSH₂ (Fig. 14a) has also been reported by this group [100]. The presence of *gem*-dimethyl group next to the thiolate donor allows isolation of the monomeric bis complex Na[Co^{III}(PyPepRS)₂] (15, Fig. 14b) in which the coordination structure around the cobalt(III) center is similar to that in 14. Both these complexes have been synthesized via reaction of [Co(NH₃)₅Cl]Cl₂ with the deprotonated ligands in DMF at 60 °C. The unusual use of a kinetically inert cobalt(III) starting material in these syntheses is noteworthy.

Reaction of H₂O₂ with solutions of 14 or 15 in methanol at 0 °C readily affords the corresponding sulfinato complexes Na[Co^{III}(PyPepSO₂)₂] (16) and Na[Co^{III}(PyPepRSO₂)₂] (17, Fig. 14c) in high yield [100]. Similar oxidation is also observed when methanolic solutions of 14 and 15 are exposed to oxygen. These reactions are comparatively slow. However, the process of oxidation can be accelerated by the addition of activated charcoal to the reaction mixtures. The clean conversion of the thiolato sulfurs to the S-bonded sulfinato groups can be followed by absorption spectroscopy and mass spectrometry. The reddish brown color of the solutions of 14 and 15 arises from a strong thiolate-to-cobalt(III) charge transfer transition around 500 nm. This band disappears upon oxidation of the thiolato sulfurs to sulfinic groups.

A functional model of Co–NHase has recently been reported by Mascharak and coworkers who utilized the designed ligand PyPSH₄ (Fig. 12) in synthesizing a mononuclear cobalt(III) species with hydroxide as one of the ligand [101]. Reaction of PyPS⁴⁻ with [Co(NH₃)₅Cl]Cl₂ in DMF results in the formation of the dimeric complex (Et₄N)₂[Co₂^{III}(PyPS)₂] (16) in which each cobalt(III) center attains six coordination by forming one thiolato bridge (Fig. 15a). Further reaction of 16 with (Et₄N)(CN) in acetonitrile affords the monomeric cyanide-adduct (Et₄N)₂[Co^{III}(PyPS)(CN)] (17) in which the cobalt(III) center is bonded to two carboxamido nitrogens (*trans* to each other), two thiolato sulfurs (*cis* to each other), one pyridine nitrogen, and a cyanide (Fig. 15b). This model complex resembles the proposed cyanide-inhibited cobalt(III) site of Co–NHase in terms of the coordination structure. The Co–C and C–N distances of the Co–CN unit (1.896(12) and 1.149(13) Å, respectively) of 17 compare well with those in other cobalt(III) complexes with cyanide ligands. Both 16 and 17 are diamagnetic and the latter complex displays its ν_{CN} at 2111 cm⁻¹.

Complex 17 is stable when dissolved in acetonitrile or DMF. However, when it is dissolved in water (pH 7), CN⁻ is immediately lost and [Co^{III}(PyPS)(H₂O)]⁻ is formed (reaction b, Scheme 2). This facile replacement of CN⁻ by water indicates that the enzyme-like coordination structure of the cobalt(III) center of 17 is *not* substitutionally inert. Clearly, the conventional wisdom of kinetic inertness of low-spin cobalt(III) centers is not

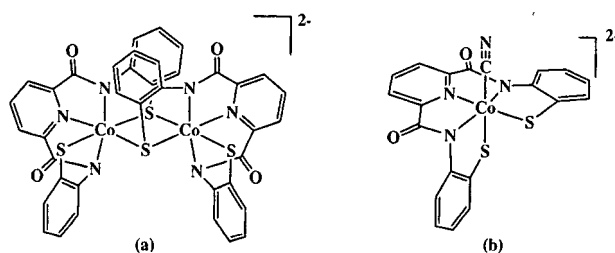
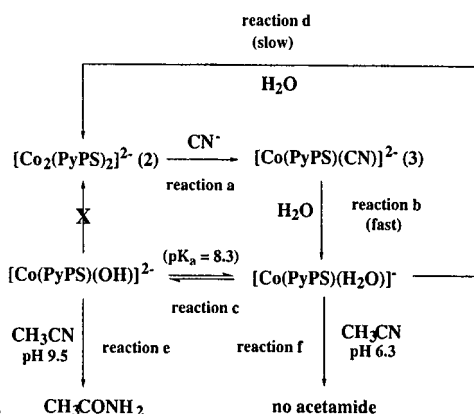


Fig. 15. Structures of: (a) [Co₂^{III}(PyPS)₂]²⁻ (anion of 16) and (b) [Co^{III}(PyPS)(CN)]²⁻ (anion of 17).



Scheme 2.

applicable to all cobalt(III) species and exceptions are found in complexes with specific donor centers (vide infra). The pK_a of the bound water in $[\text{Co}^{\text{III}}(\text{PyPS})(\text{H}_2\text{O})]^-$ has also been measured by Mascharak and coworkers [101]. Comparison of this pK_a value (8.3) with that of cobalt(III) complex $[\text{Co}^{\text{III}}(\text{L})(\text{H}_2\text{O})]^+$ (L = very similar pentacoordinate peptide ligands but with all nitrogen donors, $pK_a = 7$) [102] indicates that the thiolato sulfurs in $[\text{Co}^{\text{III}}(\text{PyPS})(\text{H}_2\text{O})]^-$ raise the pK_a of the bound water. The reactivity of $[\text{Co}^{\text{III}}(\text{PyPS})(\text{OH})]^{2-}$, the species present in pH 9 buffer is very noteworthy. As shown in Scheme 2, this species promotes rapid hydration of acetonitrile to acetamide at room temperature with multiple turnovers. Since the $[\text{Co}^{\text{III}}(\text{L})(\text{H}_2\text{O})]^+$ complex does not show such activity at pH 9, it is evident that the presence of thiolato sulfurs around the cobalt(III) center is crucial in promoting hydration of nitriles.

To date, $[\text{Co}^{\text{III}}(\text{PyPS})(\text{H}_2\text{O})]^-$ (obtained from 17) is the only functional model of NHase that catalyzes hydration of nitriles at $\text{pH} \geq 8$. Reaction e in Scheme 2 suggests that the enzyme-mediated hydration of nitriles could proceed via intermolecular attack of cobalt-bound hydroxide on nitriles nested in the active site pocket (mechanism b or c, Fig. 3). Since $[\text{Co}^{\text{III}}(\text{PyPS})(\text{H}_2\text{O})]^-$ does not initiate hydration at pH 6, the alternative mechanism in which nitriles first coordinate to cobalt(III) by replacing water and then get hydrolyzed (mechanism a, Fig. 3) is not supported.

Artaud and coworkers have reported the cobalt(III) complex of the ligand *N,N'*-1,2-phenylenebis-(2-mer-

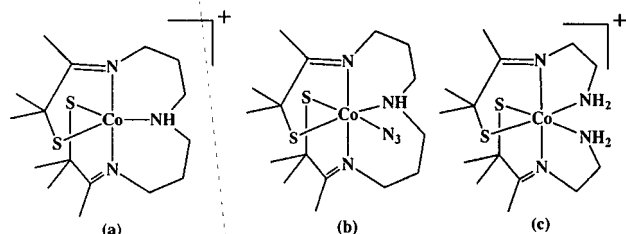


Fig. 16. Structures of: (a) $[\text{Co}^{\text{III}}\text{S}_2^{\text{Me}_2}\text{N}_3(\text{Pr},\text{Pr})]^+$ (cation of 19); (b) $[\text{Co}^{\text{III}}\text{S}_2^{\text{Me}_2}\text{N}_3(\text{Pr},\text{Pr})(\text{N}_3)]^+$; and (c) $[\text{Co}^{\text{III}}(\text{ADIT})_2]^+$ (cation of 20).

capto-2-methylpropionamide) namely, $(\text{Et}_4\text{N})[\text{Co}^{\text{III}}(\text{N}_2\text{S}_2)\text{Cl}]$ (18) [103]. This distorted square planar complex with short Co–N and Co–S bonds (1.882 and 2.134 Å, respectively) and triplet ($S = 1$) ground state does not interact with nitriles, H_2O , imidazole or OH^- . However, it binds two CN^- or two NO molecules. The IR spectrum of the dinitrosyl product exhibits two ν_{NO} at 1765 and 1820 cm^{-1} . The reactivity of 18 with NO is interesting although Co–NHases are not known to bind NO.

3.5. Models of Co–NHases: complexes with thiolato S and amine/imine N donors

Kovacs and coworkers have employed their in situ condensation technique to synthesize the cobalt(III) analogue of 7 namely $[\text{Co}^{\text{III}}\text{S}_2^{\text{Me}_2}\text{N}_3(\text{Pr},\text{Pr})]\text{PF}_6$ (19) [104]. This five-coordinate trigonal bipyramidal cobalt(III) complex (Fig. 16a) is structurally very similar to 7 with Co– S_{thio} distance of 2.16(2) Å, which is shorter than that noted for most cobalt(III) thiolates. Complex 19 is intermediate spin ($S = 1$) and is reversibly reduced at $E_{1/2} = -0.46$ V versus SCE. The cobalt(III) center in 19 binds azide and SCN^- quantitatively at room temperature *trans* to one of the thiolates but exhibits no affinity toward NO, nitriles, and butyrate. The structure of the dark pink azide-adduct $[\text{Co}^{\text{III}}\text{S}_2^{\text{Me}_2}\text{N}_3(\text{Pr},\text{Pr})(\text{N}_3)]$ has been determined (Fig. 16b) [105]. As expected, the spectroscopic and structural parameters of $[\text{Co}^{\text{III}}\text{S}_2^{\text{Me}_2}\text{N}_3(\text{Pr},\text{Pr})(\text{N}_3)]$ resemble those of the six-coordinate cobalt(III) model complex $[\text{Co}^{\text{III}}(\text{ADIT})_2](\text{PF}_6)_2$ (20, Fig. 16c) which has also been synthesized by this group via oxidation of the cobalt(II) species with ferrocenium salts [105]. Kinetic studies on azide- and thiocyanate binding by 19 reveals that the rate of temperature-dependent reversible binding of these ligands to the cobalt(III) center of 19 is of the order of 10^{-1} to 10^{-2} s^{-1} . Since these rates are comparable to those observed for the corresponding iron(III) complexes, it appears that the unique coordination environment at the active site of NHases gives rise to lability to both iron(III) and cobalt(III) centers despite low-spin configuration. This in turn provides an answer to why a kinetically inert low-spin cobalt(III) ion could be present at the active site of NHase and promote catalytic hydration of nitriles at significant speed [105].

Prolonged exposure to air converts 19 into the square pyramidal sulfonato/thiolato complex $[\text{Co}^{\text{III}}(\text{S}^{\text{Me}_2}(\text{S}^{\text{O}_2})\text{N}_3(\text{Pr},\text{Pr}))]\text{PF}_6$ (21) in which only one of the thiolato sulfur is oxidized (Fig. 17a). Complex 21 is low-spin and exhibits S–O distance (1.453(2) Å) comparable to that noted for 17. Interestingly, 21 does not bind azide or SCN^- to its open site, presumably due to strong *trans*-effect of the sulfonato group [104]. Addition of H_2O_2 to 21 results in the oxidation of the remaining thiolate to a sulfenato and the oxygen atom of the sulfenato group occupies the sixth site on cobalt in

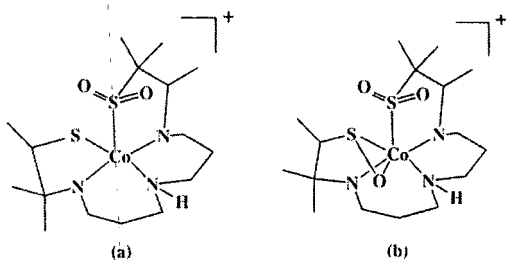


Fig. 17. Structures of: (a) $[\text{Co}^{\text{III}}(\text{S}^{\text{Me}_2}(\text{S}^{\text{O}_2})\text{N}_3(\text{Pr},\text{Pr}))]^+$ (cation of **21**) and (b) $[\text{Co}^{\text{III}}(\eta^2\text{-SO})(\text{S}^{\text{O}_2})\text{N}_3(\text{Pr},\text{Pr}))]^+$ (cation of **22**).

$[\text{Co}^{\text{III}}(\eta^2\text{-SO})(\text{S}^{\text{O}_2})\text{N}_3(\text{Pr},\text{Pr})]\text{PF}_6$ (**22**, Fig. 17b). No further oxidation of this sulfur center is observed, even upon prolonged treatment with H_2O_2 . Complex **22** is the first example of a NHase model with sulfenato coordination. The S–O distance in **22** (1.548(3) Å) falls in the usual range. Unfortunately, lack of reactivity of both **21** and **22** prevents one from exploring the changes in reactivity of the cobalt(III) center upon incremental oxidation of the coordinated sulfur atoms.

4. Conclusions

To date, results of the modeling work have provided valuable insight into the unusual properties of the non-heme iron(III) site in NHase. For example, it is quite evident that coordination of carboxamido nitrogens to the iron(III) center of NHase raises its reduction potential and hence this iron site does not participate in any redox reaction in conjunction with dioxygen. It has also become clear that iron(III) and cobalt(III) centers with coordination structures similar to that in NHase are kinetically active and exhibit similar binding properties. In addition, the pK_a of water bound to such M(III) center is around 7. The model studies have also suggested that iron(III) centers with N, S coordination could bind NO although it is yet to be shown that such iron(III) nitrosyls are photolabile. Hydration studies with models like $[\text{Co}^{\text{III}}(\text{PyPS})(\text{H}_2\text{O})]^-$ (obtained from **17**) indicate that a metal-bound hydroxide could be responsible for the hydration of nitriles at the active site. It is expected that future studies with more robust and active model complexes will afford additional clues related to the unusual non-heme iron(III) (and non-corrinoid cobalt(III)) site in NHase.

Acknowledgements

Research in Mascharak's lab was supported by grants from the National Institute of General Medical Sciences and the National Science Foundation. P.M. thanks Dr Juan Noveron and Dana Marlin for help in preparing this manuscript.

References

- [1] B. Vennesland, E.E. Conn, C.J. Knowles, J. Westley, F. Wissing, *Cyanide in Biology*, Academic Press, London, 1981.
- [2] E.E. Conn, *Cyanide Compounds in Biology*, Wiley, Chichester, UK, 1988.
- [3] I. Endo, M. Odaka, M. Yohda, *Trends Biotechnol.* 17 (1999) 244.
- [4] M. Kobayashi, S. Shimizu, *Nature Biotechnol.* 16 (1998) 733.
- [5] H. Yamada, M. Kobayashi, *Biosci. Biotechnol. Biochem.* 60 (1996) 1391.
- [6] M. Kobayashi, T. Nagasawa, H. Yamada, *Trends Biotechnol.* 10 (1992) 402.
- [7] T. Nagasawa, H. Yamada, *Trends Biotechnol.* 7 (1989) 153.
- [8] C.M. Hjort, S.E. Godtfredsen, C. Emborg, *J. Chem. Technol. Biotechnol.* 48 (1990) 217.
- [9] T. Nagasawa, H. Yamada, *Trends Biotechnol.* 7 (1989) 153.
- [10] N. Takahashi, *Chemistry of Plant Hormones*, CRC Press, Boca Raton, FL, 1986.
- [11] E.E. Conn, in: E.A. Ebell, B.V. Charlwood (Eds.), *Secondary Plant Products*. In: *Encyclopedia of Plant Physiology*, vol. 8, Springer, Berlin, 1980, pp. 461–492.
- [12] R.D. Gibbs, *Chemotaxonomy of Flowering Plants*, McGill-Queens University Press, Montreal, 1974.
- [13] F.F. Fleming, *Nat. Prod. Rep.* 16 (1999) 597.
- [14] A.D. Robira, *Bot. Rev.* 35 (1969) 35.
- [15] E.A. Paul, F.E. Clark, *Soil Microbiology and Biochemistry*, Academic Press, San Diego, CA, 1989.
- [16] A. Leonard, G.B. Gerber, C. Stecca, J. Rueff, H. Borba, P.B. Farmer, R.J. Sram, A.E. Czeizel, I. Kalina, *Mut. Res.* 436 (1999) 263.
- [17] J.M. Wyatt, C.J. Knowles, *Biodegradation* 6 (1995) 93.
- [18] R.A. Woutersen, *Scand. J. Work. Environ. Health* 24 (1998) 5.
- [19] E. Delzell, R.R. Monson, *J. Occup. Med.* 24 (1982) 767.
- [20] A.E. Czeizel, S.L. Timar-Hegedus, *Mut. Res.* 427 (1999) 105.
- [21] H. Kidd, D.R. James, *The Agrochemicals Handbook*, 3rd ed., Royal Society of Chemical Information Services, Cambridge, UK, 1998.
- [22] US National Library of Medicine, *Hazardous Substances Database*, Bethesda, MD, 1995.
- [23] J.M. Wyatt, E.A. Linton, *Cyba Found. Symp.* 140 (1988) 32.
- [24] Y. Asano, K. Fujishiro, Y. Tani, H. Yamada, *Agric. Biol. Chem.* 46 (1982) 1165.
- [25] T. Amarant, Y. Vered, Z. Bohak, *Biotechnol. Appl. Biochem.* 11 (1989) 49.
- [26] Y. Tani, M. Kurihara, H. Nishise, *Agric. Biol. Chem.* 53 (1989) 3151.
- [27] T. Nagasawa, K. Ryuno, H. Yamada, *Biochem. Biophys. Res. Commun.* 139 (1986) 1305.
- [28] J.L. Moreau, S. Azza, A. Arnaud, P. Galzy, *J. Basic Microbiol.* 33 (1993) 323.
- [29] T. Nagasawa, H. Nanba, K. Ryuno, K. Takeuchi, H. Yamada, *Eur. J. Biochem.* 162 (1987) 691.
- [30] M. Kobayashi, M. Nishiyama, T. Nagasawa, S. Horinouchi, T. Beppu, H. Yamada, *Biochim. Biophys. Acta* 1129 (1991) 23.
- [31] T. Nagamune, J. Honda, W.D. Cho, N. Kamiya, Y. Teratani, A. Hirata, H. Sasabe, I. Endo, *J. Mol. Biol.* 220 (1991) 221.
- [32] M.R. Kaakeh, J.L. Legras, R. Duran, A. Chion-Arnaud, P. Galzy, *Zentbl. Mikrobiol.* 146 (1991) 89.
- [33] W.Z. Li, Y.Q. Zhang, H.F. Yang, *Appl. Biochem. Biotechnol.* 36 (1992) 171.
- [34] T. Nagasawa, K. Takeuchi, H. Yamada, *Eur. J. Biochem.* 196 (1991) 581.
- [35] M.S. Payne, S. Wu, R.D. Fallon, G. Tudor, B. Stieglitz, I.M. Turner, M.J. Nelson, *Biochemistry* 36 (1997) 5447.
- [36] S. Wu, R.D. Fallon, M.S. Payne, *Appl. Microbiol. Biotechnol.* 48 (1997) 704.
- [37] T. Nagasawa, K. Ryuno, H. Yamada, *Experientia* 45 (1989) 1066.

- [38] T. Nagasawa, H. Shimizu, H. Yamada, *Appl. Microbiol. Biotechnol.* 40 (1993) 189.
- [39] R. Padmakumar, P. Oriel, *Appl. Biochem. Biotechnol.* 77 (1999) 671.
- [40] K. Matoishi, A. Sana, N. Imai, T. Yamazaki, M. Yokoyama, T. Sugai, H. Ohta, *Tetrahedron: Asymmetry* 9 (1998) 1097.
- [41] T. Sugai, T. Yamazaki, M. Yokoyama, H. Ohta, *Biosci. Biotechnol. Biochem.* 61 (1997) 1419.
- [42] A. Stolz, S. Trott, M. Binder, R. Bauer, B. Hirrlinger, N. Layh, H.-J. Knackmuss, *J. Mol. Catal. B—Enzymatic* 5 (1998) 137.
- [43] R. Bauer, H.-J. Knackmuss, A. Stolz, *Appl. Microbiol. Biotechnol.* 49 (1998) 89.
- [44] S.J. Maddrell, N.J. Turner, A. Kerridge, A.J. Willetts, J. Crosby, *Tetrahedron Lett.* 37 (1996) 6001.
- [45] H. Kakeya, N. Sakai, T. Sugai, H. Ohta, *Tetrahedron Lett.* 32 (1991) 1343.
- [46] M. Kobayashi, S. Shimizu, *Nat. Biotechnol.* 16 (1998) 733.
- [47] L.Y. Young, C.E. Cerniglia, *Microbial Transformation and Degradation of Toxic Organic Chemicals*, Wiley-Liss Inc, New York, 1995.
- [48] J.M. Wyatt, K.C. Knowles, *Int. Biodeterior. Biodegrad.* 35 (1995) 227.
- [49] G.R.V. Babu, C.S. Chetty, J.H. Wolfram, K.D. Chapatwala, *J. Environ. Sci. Health Part A—Environ. Sci. Eng.* 29 (1994) 1957.
- [50] Y. Sugiura, J. Kuwahara, T. Nagasawa, H. Yamada, *J. Am. Chem. Soc.* 109 (1987) 5848.
- [51] L. Que Jr., R.Y.N. Ho, *Chem. Rev.* 96 (1996) 2607.
- [52] A.L. Feig, S.J. Lippard, *Chem. Rev.* 94 (1994) 759.
- [53] T. Noguchi, J. Honda, T. Nagamune, H. Sasabe, Y. Inoue, *I. Endo, FEBS Lett.* 358 (1995) 9.
- [54] T. Noguchi, M. Hoshino, M. Tsujimura, M. Odaka, Y. Inoue, *I. Endo, Biochemistry* 35 (1996) 16777.
- [55] M. Tsujimura, M. Odaka, S. Nagashima, M. Yohda, I. Endo, *J. Biochem.* 119 (1996) 407.
- [56] M. Odaka, K. Fuji, M. Hoshino, T. Noguchi, M. Tsujimura, S. Nagashima, M. Yohda, T. Nagamune, Y. Inoue, I. Endo, *J. Am. Chem. Soc.* 119 (1997) 3785.
- [57] W. Huang, J. Jia, J. Cummings, M. Nelson, G. Schneider, Y. Lindqvist, *Structure* 5 (1997) 691.
- [58] S. Nagashima, M. Nakasako, N. Dohmae, M. Tsujimura, K. Takio, M. Odaka, M. Yohda, N. Kamiya, I. Endo, *Nat. Struct. Biol.* 5 (1998) 347.
- [59] Endo and coworkers [58] pointed out that the electron densities of the oxygen atoms corresponding to the post-translationally modified cysteine residues did not appear until the resolution in the structure refinement was higher than 2.3 Å and this explains why Nelson and coworkers [57] could not identify such groups in their study at 2.65 Å resolution.
- [60] M. Tsujimura, N. Dohmae, M. Odaka, M. Chijimatsu, K. Takio, M. Yohda, M. Hoshino, S. Nagashima, I. Endo, *J. Biol. Chem.* 272 (1997) 29454.
- [61] J.W. Peters, M.H.B. Stowell, S.M. Soltis, M.G. Finnegan, M.K. Johnson, D.C. Rees, *Biochemistry* 36 (1997) 1181.
- [62] T. Murakami, M. Nojiri, H. Nakayama, M. Odaka, M. Yohda, N. Dohmae, K. Takio, T. Nagamune, I. Endo, *Protein Sci.* 9 (2000) 1024.
- [63] M.J. Nelson, H. Jin, I.M. Turner Jr., G. Grove, R.C. Scarrow, B.A. Brennan, L. Que Jr., *J. Am. Chem. Soc.* 113 (1991) 7072.
- [64] P.E. Doan, M.J. Nelson, H. Jin, B.M. Hoffman, *J. Am. Chem. Soc.* 118 (1996) 7014.
- [65] M. Kobayashi, S. Shimizu, *Eur. J. Biochem.* 261 (1999) 1.
- [66] B.A. Brennan, G. Alms, M. Nelson, L.T. Durney, R.C. Scarrow, *J. Am. Chem. Soc.* 118 (1996) 9194.
- [67] C.M. Jensen, W.C. Trogler, *J. Am. Chem. Soc.* 108 (1986) 723.
- [68] R.A. Michelin, M. Mozzon, R. Bertani, *Coord. Chem. Rev.* 147 (1996) 299.
- [69] J.A. Ibers, R.H. Holm, *Science* 5 (1980) 157.
- [70] D.S. Marlin, P.K. Mascharak, *Chem. Soc. Rev.* 29 (2000) 69.
- [71] R.H. Holm, J.A. Ibers, *Iron–Sulfur Proteins*, vol. III, Academic Press, New York, 1977 (chap. 7).
- [72] E. Deutsch, M.J. Root, D.L. Nosco, *Adv. Inorg. Bioinorg. Mech.* 1 (1982) 269.
- [73] K.J. Ellis, A.G. Lappin, A.J. McAuley, *J. Chem. Soc. Dalton Trans.* (1975) 1931.
- [74] M. Sigel, R.B. Martin, *Chem. Rev.* 82 (1982) 385.
- [75] S.J. Lippard, *Angew. Chem. Int. Ed. Engl.* 27 (1988) 344.
- [76] G.D. Fallon, B.M. Gatehouse, *J. Chem. Soc. Dalton Trans.* (1975) 1344.
- [77] P.J. Marini, K.S. Murray, B.O. West, *J. Chem. Soc. Dalton Trans.* (1983) 143.
- [78] S.C. Shoner, D. Barnhart, J.A. Kovacs, *Inorg. Chem.* 34 (1995) 4517.
- [79] A.L. Nivorozhkin, A.I. Uraev, G.I. Bondarenko, A.S. Antsyshkina, V.P. Kurbatov, A.D. Garnovskii, C.I. Turta, N.D. Brashoveanu, *Chem. Commun.* (1997) 1711.
- [80] L.F. Lindoy, S.E. Livingstone, *Inorg. Chim. Acta* 1 (1967) 365.
- [81] J.C. Noveron, R. Herradora, M.M. Olmstead, P.K. Mascharak, *Inorg. Chim. Acta* 285 (1999) 269.
- [82] J.-P. Costes, F. Dahan, J.-P. Laurent, *Inorg. Chem.* 29 (1990) 2448.
- [83] K. Ramesh, R. Mukherjee, *J. Chem. Soc. Dalton Trans.* (1992) 83.
- [84] M. Tweedle, L.J. Wilson, *J. Am. Chem. Soc.* 98 (1976) 4824.
- [85] C. Butzlaff, A.X. Trautwein, T. Beissel, K.S. Burger, V. Gottfried, K. Wiegardt, *Inorg. Chem.* 32 (1993) 124.
- [86] J.J. Ellison, A. Nienstedt, S.C. Shoner, D. Barnhart, J.A. Cowen, J.A. Kovacs, *J. Am. Chem. Soc.* 120 (1998) 5691.
- [87] D. Schweitzer, J.J. Ellison, S.C. Shoner, S. Lovell, J.A. Kovacs, *J. Am. Chem. Soc.* 120 (1998) 10996.
- [88] R.C. Scarrow, B.S. Strickler, J.J. Ellison, S.C. Shoner, J.A. Kovacs, J.G. Cummings, M.J. Nelson, *J. Am. Chem. Soc.* 120 (1998) 9237.
- [89] J.C. Noveron, M.M. Olmstead, P.K. Mascharak, *Inorg. Chem.* 37 (1998) 1138.
- [90] S.J. Brown, M.M. Olmstead, P.K. Mascharak, *Inorg. Chem.* 29 (1990) 3229.
- [91] X. Tao, D.W. Stephan, P.K. Mascharak, *Inorg. Chem.* 26 (1987) 754.
- [92] M. Ray, D. Ghosh, Z. Shirin, R. Mukherjee, *Inorg. Chem.* 36 (1997) 3568.
- [93] D.S. Marlin, M.M. Olmstead, P.K. Mascharak, *Inorg. Chim. Acta* 297 (2000) 106.
- [94] D.S. Marlin, M.M. Olmstead, P.K. Mascharak, *Inorg. Chem.* 38 (1999) 3258.
- [95] L.A. Tyler, J.C. Noveron, M.M. Olmstead, P.K. Mascharak, *Inorg. Chem.* 38 (1999) 616.
- [96] J.C. Noveron, M.M. Olmstead, P.K. Mascharak, *J. Am. Chem. Soc.* 123 (2001) 3247.
- [97] I. Artaud, S. Chatel, A.S. Chauvin, D. Bonnet, M.A. Kopf, P. Leduc, *Coord. Chem. Rev.* 190–192 (1999) 577.
- [98] J. Hans, H.J. Krüger, *Angew. Chem.* 35 (1996) 2827.
- [99] L. Heinrich, Y. Li, J. Vaissermann, G. Chottard, J.C. Chottard, *Angew. Chem. Int. Ed. Engl.* 38 (1999) 3526.
- [100] L.A. Tyler, J.C. Noveron, M.M. Olmstead, P.K. Mascharak, *Inorg. Chem.* 39 (2000) 357.
- [101] J.C. Noveron, M.M. Olmstead, P.K. Mascharak, *J. Am. Chem. Soc.* 121 (1999) 3553.
- [102] F.A. Chavez, C.V. Nguyen, M.M. Olmstead, P.K. Mascharak, *Inorg. Chem.* 35 (1996) 6282.
- [103] S. Chatel, M. Rat, S. Dijols, P. Leduc, J.P. Tuchagues, D. Mansuy, I. Artaud, *J. Inorg. Biochem.* 80 (2000) 239.
- [104] I. Kung, D. Schweitzer, J. Shearer, W.D. Taylor, H.L. Jackson, S. Lovell, J.A. Kovacs, *J. Am. Chem. Soc.* 122 (2000) 8299.
- [105] J. Shearer, I.Y. Kung, S. Lovell, W. Kaminsky, J.A. Kovacs, *J. Am. Chem. Soc.* 123 (2001) 463.

Amidase and urease activities in plants

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Key words Amides Foliar fertilization Nitrogen fertilizers Plant enzymes Urea

Summary Foliar fertilization has received considerable attention in recent years. Because of the importance of amides and urea as N sources, this work was carried out to study the enzymes that catalyze the hydrolysis of these compounds in plant leaves. The methods developed for assay of these enzymes in plants involve determination by steam distillation of the $\text{NH}_4^+ - \text{N}$ produced by amidase or urease activity when plant materials are incubated at 37°C with buffered (0.1 M THAM pH 8.0) amide solution or buffered (0.1 M THAM pH 7.5) urea solution, respectively. Amidase and urease were detected in 21 diverse plants in the families of Gramineae and Leguminosae. Results showed that amidase and urease have optimum activities at buffer pH values of 8.0 and 7.5, respectively. Both amidase and urease activities were decreased significantly upon freezing or air-drying of plant samples before enzyme assay. These differences were proportional to the original activities of fresh plant materials. Studies on the effect of temperature on amidase and urease activities showed that these enzymes are inactivated at temperatures above 60 and 70°C, respectively. The energy of activation of the reaction catalyzed by amidase and urease in plants, expressed in $\text{kJ} \cdot \text{mole}^{-1}$, ranged from 44.0 to 51.2 (avg. = 47.1) and from 43.1 to 56.5 (avg. = 51.2) when formamide and urea were used as substrates, respectively. The apparent K_m constants of these enzymes varied among the plant samples studied. By using the Lineweaver-Burk plot, the K_m values for amidase when formamide was used as a substrate ranged from 2.0 to 9.4 (avg. = 5.8 mM) and for urease ranged from 0.4 to 1.6 (avg. = 0.8 mM). The $V_{\max}$ values of 7 plant samples, expressed in μg of $\text{NH}_4^+ - \text{N}$ produced/0.1 g of plant materials/2 h, ranged from 137 to 514 for amidase and from 29 to 123 for urease. The importance of these enzymes in application of amides and urea to plant leaves is discussed.

Introduction

Foliar application of fertilizer nutrients on field grain crops has received attention in recent years. Among the various nutrients required for plant growth and development, application of N, P, K, and S solutions, designed to avoid excessive depletion of these nutrients from leaves during the seed-filling period of soybeans, has been tested with encouraging results^{9,21}. Recent attempts in applying N for this purpose have included urea and formamide solutions¹⁰. Application of such N compounds, however, has resulted in contradictory results; some have reported increases in yield of grains, and others have reported damage to leaves (burning of leaves)²¹. Urea and formamide are among the N compounds most likely used for foliar fertilization with N. The possible hydrolysis of urea and formamide and concomitant production of high levels of

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NH_4^+ , resulting in the observed damage, have not been investigated. Consequently, little information is available about the extent of urea and amides hydrolysis by plant leaves. This lack of information stems mainly from the limited information available about the activity and catalytic properties of the enzymes, urease and amidase, responsible for hydrolysis of urea and formamide or other amides, applied to plant leaves. Understanding the properties of the reactions catalyzed by these enzymes in leaves of plants should aid in better use of urea and formamide or other amides as foliar fertilizers.

The popular assays of urease in plant tissues include electrochemical⁴, spectrophotometric¹², fluorometric¹⁴, radiochemical¹⁵, potentiometric¹⁶, and diffusion methods³. Each of these methods has some disadvantages including low sensitivity, cost of material, lengthy procedures, and handling of hazardous reagents. To our knowledge, little information is available about amidase activity in plant tissues. For this purpose, simple and sensitive methods are needed to assay urease and amidase activities of plant materials.

Therefore, the objectives of this work were: (i) to develop simple and sensitive methods for assay of the activities of amidase and urease in leaves of a variety of plants, (ii) to study the factors affecting the activities of these enzymes in plant leaves, and (iii) to assess the properties of the reactions catalyzed by these enzymes in plant materials.

Materials and methods

Plant materials

The plant materials used were leaf samples, and only green leaves were assayed. Leaves with yellow and brown regions were discarded. With the exception of the results in Table 3 and Fig. 5, all assays for amidase and urease activities were performed on air-dried plant samples. The air-dried samples were obtained by spreading the plants on a clean paper in the laboratory and allowing them to dry at room temperature (*ca* 23°C) for 5 days. The leaves were then crushed and ground to pass <40-mesh sieve. Frozen plant materials were prepared for assay by packing 2- to 5-cm leaf cuttings (obtained immediately from the field) in a 50-ml Pyrex test tube and freezing the samples in liquid nitrogen for 30 seconds. The samples were then macerated by a stirring rod and assayed immediately for amidase and urease activities. Enzyme activities in both the fresh and air-dried samples were expressed on a moisture-free basis; moisture being determined from loss in weight after drying at 65°C for 48 h.

Amidase assay

The reagents required for assay of plant amidase included the THAM*-sulfuric acid buffer (0.1 M, pH 8.0), 0.5 M formamide (Aldrich certified), 0.5 M acetamide (Sigma certified), 0.5 M propionamide (Aldrich certified), and potassium chloride (2.5 M)-uranyl acetate (0.005 M) solution used by Frankenberger and Tabatabai⁶. The reagents used for determination of the $\text{NH}_4^+ - \text{N}$ produced were those described by Tabatabai and Bremner²⁰. To assay amidase activity, 0.1 g of plant material in a 50-ml volumetric flask was treated with 9-ml of THAM buffer (pH 8.0). The flask was swirled for a few seconds to mix the contents and treated with 1 ml of 0.5 M amide solution. The flask was swirled

* THAM = tris(hydroxymethyl)aminomethane.

again to mix the contents, stoppered, and incubated at 37°C. The incubation time varied with the substrate used. Hydrolysis of formamide was relatively rapid; therefore, the incubation time was 2 h. The rates of hydrolysis of acetamide and propionamide were somewhat slower than that of formamide, and the incubation time was 24 h. After incubation, the flask was unstoppered, and approximately 35 ml of the $\text{KCl-UO}_2(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 2\text{H}_2\text{O}$ were added and allowed to cool to room temperature (ca 5 min). Then the volume was adjusted to 50 ml by adding $\text{KCl-UO}_2(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 2\text{H}_2\text{O}$ solution. The stoppered flask was inverted several times to mix the contents, and an aliquot (20 ml) of the mixed suspension was taken for $\text{NH}_4^+ - \text{N}$ analysis by steam distillation with MgO^{20} . Controls were performed in each series of analysis to allow for $\text{NH}_4^+ - \text{N}$ not derived from the amide through amidase activity. To perform controls, the procedure just described was followed, but the 1 ml of 0.5 M amide solution was added after the addition of 35 ml $\text{KCl-UO}_2(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 2\text{H}_2\text{O}$ (i.e., before making up the volume).

Urease assay

The reagents required for assay of plant urease included THAM-sulfuric acid buffer (0.1 M, pH 7.5), 0.5 M urea (Fisher Scientific certified) solution, potassium chloride (2.5 M)-silver sulfate (100 ppm) solution, and the reagents used for determination of $\text{NH}_4^+ - \text{N}^{20}$. The procedure used for assay of urease was the same as that used for the assay of amidase except that the pH of the buffer used was 7.5, urea was used as a substrate, and $\text{KCl-Ag}_2\text{SO}_4$ was used to inactivate urease²⁰.

Enzyme stability

In studies of the effect of temperature on the stability of amidase and urease activities in plant samples, fresh tissue and air-dried samples were incubated for 2 h at temperatures ranging from 30 to 100°C. The samples were then brought to room temperature (23°C) before assay of the enzyme activities.

Kinetic properties

For determination of the K_m and V_{max} values, amidase and urease activities were assayed at substrate concentrations ranging from 1 to 5 mM formamide and 0.2 to 5 mM urea. The K_m and V_{max} values were calculated by plotting the enzyme activity according to the Lineweaver-Burk plot. The activation energies were calculated from amidase and urease activities obtained at a substrate concentration of 50 mM, but the temperature of incubation was varied from 10 to 50°C. The activation energies were calculated by using the Arrhenius equation plot⁵. All results reported are averages of duplicate determinations expressed on a moisture-free basis, moisture being determined after drying at 65°C for 48 h.

Results and discussion

Optimum pH and choice of buffer

Studies of the effect of buffer on enzyme-catalyzed reactions are essential because hydrogen ion concentration in the reaction system affects the ionization groups of enzyme proteins and influences the ionization state of the substrate. The ionizable groups of both the enzyme and substrate must be in their proper states to maintain the conformation of the enzyme active sites to bind with the substrate and thus catalyze the reaction. Fig. 1 shows the effect of varying the pH of 0.1 M THAM- H_2SO_4 buffer used in the assay of amidase and urease as described. The urease-pH profile displays a bell-shaped curve showing optimal

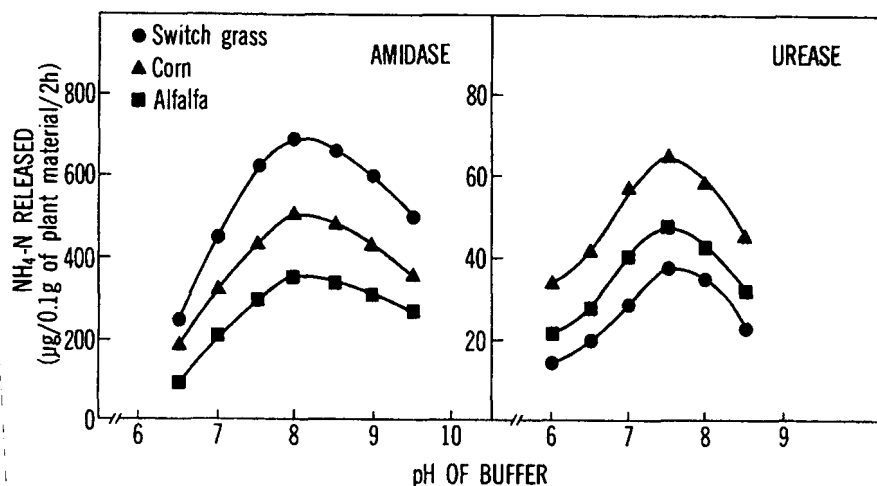


Fig. 1. Effect of pH of buffer on amidase and urease activities of plant materials.

activity at 7.5, which is somewhat lower than that reported ($\text{pH} = 8.0$) by Wall and Laidler²² for the activity of jack-bean urease in THAM buffer. Activity of plant amidase also displayed a bell-shaped curve showing optimal activity at $\text{pH} 8.0$ (Fig. 1). The optimal pH is dependent on the buffer used. Jespersen¹¹ reports that the maximal activity usually occurs at a pH value close to the pK_a of the buffer used. In this case, the pK_a of THAM is 8.0, which corresponds to the optimal activity of both plant amidase ($\text{pH} = 8.0$) and urease ($\text{pH} = 7.5$).

The choice of buffer in the methods described was based on the findings that the $\text{NH}_4\text{-N}$ released by both amidase and urease activities was greater in the presence of THAM than in the presence of citrate and phosphate buffers. Similar findings have been reported for the assay of amidase and urease in soils^{6,20}. The use of THAM buffer was investigated because Wall and Laidler²² found that, unlike other buffers used in studies of urease activity (*e.g.*, sodium or potassium phosphate), THAM buffer has no activating or inhibitory effect on the hydrolysis of urea by jack-bean urease. The expression 'THAM- H_2SO_4 ' is used throughout this paper to describe a buffer prepared by treatment of THAM solution with sulfuric acid. Wall and Laidler²² found that buffers prepared by treatment of THAM solutions with hydrochloric acid had an activating effect on the hydrolysis of urea by jack-bean urease.

Temperature of incubation

Enzyme-catalyzed reactions proceed at a faster velocity with increasing temperature of incubation within a certain temperature range as long as the enzyme is stable and retains its full activity. At some temperatures above this range, the activity begins to decrease because of enzyme inactivation. Studies of

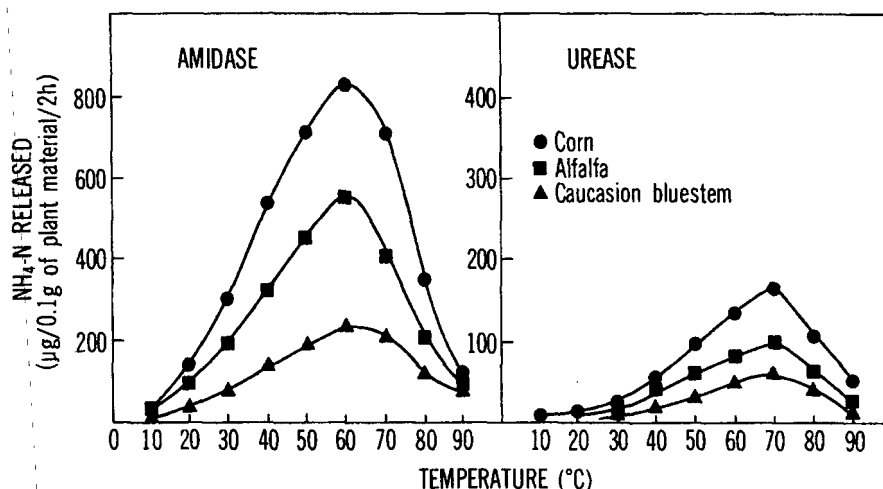


Fig. 2. Effect of temperature of incubation on amidase and urease activities of plant materials.

the effect of temperature on amidase and urease activities in plant tissues showed that inactivation occurred after 60 and 70°C, respectively (Fig. 2). Baker and Mustakas² obtained similar results for urease and reported that the first decrease in urease activity in soybeans was detected at 74°C. The inactivation temperatures of amidase and urease found in plants are similar to those found in soils⁷.

Temperature has a significant effect on chemical hydrolysis of urea; chemical hydrolysis of urea occurred at temperatures above 50°C. Thus, the experiments for effect of temperature on urease activity in plant materials were carried out with appropriate controls. For controls, urea was incubated with the THAM-H₂SO₄ as described in the assay procedure, but without plant material, and the amount of NH₄-N released at each temperature (60-90°C) was used for correction of urease activity of plant materials. The rate of chemical hydrolysis of urea below 50°C is not significant, but it increases as the incubation temperature increases. The chemical hydrolysis of urea with temperature, however, is reproducible. No chemical hydrolysis of formamide was observed at the incubation temperatures used.

The assay of amidase and urease was performed at 37°C because this temperature has been used extensively for the assay of enzymes in biological materials and because preliminary tests with the plant tissues listed in Table 1 showed that it was not necessary to use a higher temperature to obtain precise results under the conditions of the method described.

Activation energies

The rate of enzyme reactions increases with increasing temperature and decrease upon reaching a temperature of thermal inactivation. As shown

previously, temperature inactivation of plant amidase and urease occurred above temperatures of 60° and 70°C, respectively. The temperature dependence of the rate constant at a temperature below inactivation can be described by the Arrhenius equation:

$$k = A \cdot \exp(-E_a/RT)$$

where A is the pre-exponential factor, E_a is the energy of activation, R is the gas constant, and T is the temperature in °K. The Arrhenius equation also can be expressed in the log form:

$$\log k = (-E_a/2.303 RT) + \log A;$$

log A and E_a can be determined from the intercept and slope, respectively, of a linear plot of the dependence of log k on 1/T. The Arrhenius plot for both plant amidase and urease activities were linear from 10 to 50°C. A plot of the results for 3 plants (corn, soybean, and caucasian bluestem) is shown in Fig. 3. The slope of the lines for the plants used and, thus, the energies of activation of amidase and urease were similar. The energy of activation of the reaction catalyzed by amidase and urease, expressed in kJ · mole⁻¹, ranged from 44.0 to 51.2 (avg. = 47.1) and

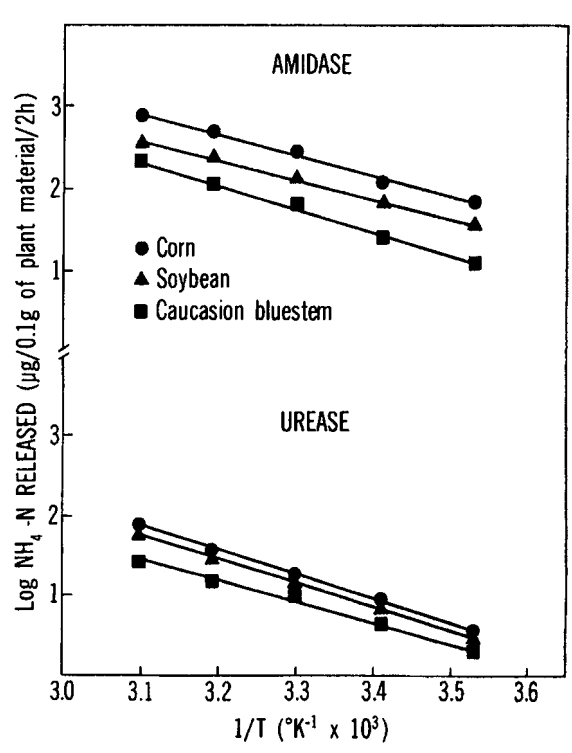


Fig. 3. Arrhenius equation plot of amidase and urease activities of plant materials.

Table 1. Activation energy values (kJ · mole⁻¹) of amidase and urease in various plant materials

Plant material	Amidase*	Urease
Alfalfa	46.6	54.4
Bermuda grass	51.2	51.7
Caucasian bluestem	50.4	48.3
Corn	47.7	54.1
Oat	45.7	56.5
Soybean	44.0	50.3
Tall fescue	44.2	43.1
Average	47.1	51.2

* Formamide as a substrate.

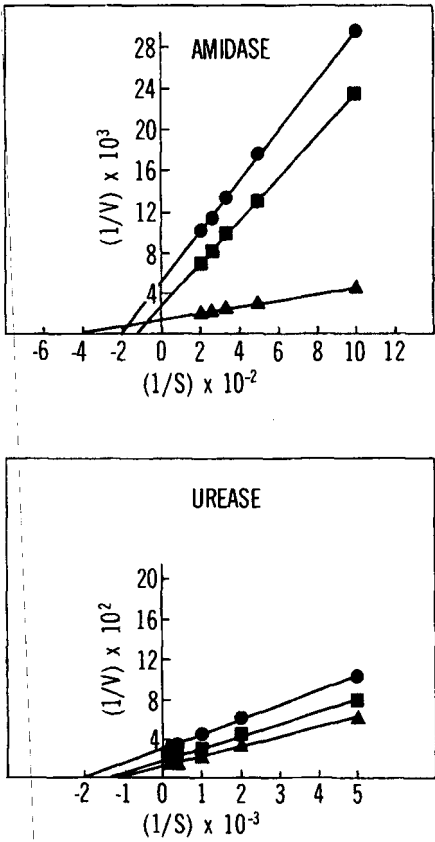


Fig. 4. Lineweaver-Burk plot of amidase and urease activities of plant materials. Velocity (V) is expressed as $\mu\text{g NH}_4\text{-N released} \cdot 0.1 \text{ g}^{-1}$ of plant material $\cdot 2 \text{ h}^{-1}$ and substrate concentration (S) is in M. ●, soybean; ▲, corn; ■, alfalfa.

43.1 to 56.5 (avg. = 51.2) when formamide and urea were used as substrates, respectively (Table 1).

The average energy of activation ($51.2 \text{ kJ} \cdot \text{mole}^{-1}$) obtained for urease is within the range reported for this enzyme (41 to $51 \text{ kJ} \cdot \text{mole}^{-1}$) by Lynn and Yankwich¹³. The average energy of activation ($47.1 \text{ kJ} \cdot \text{mole}^{-1}$) obtained for plant amidase is almost the same as that obtained for this enzyme (avg. = $46.9 \text{ kJ} \cdot \text{mole}^{-1}$) in soils⁷ when determined under similar conditions. Jespersen¹¹ reported that determination of urease activation energies by using THAM and phosphate buffers differs significantly and that, with THAM buffer, the energy of activation increased with increasing urea concentration while the phosphate buffer tends to decrease the energy of activation of urease. Therefore, activation energies should be compared only under similar conditions such as optimal pH, choice of buffer, substrate concentration, and temperature of incubation. All these conditions were observed in this work.

K_m and V_{max} values

Figure 4 shows the plots of a linear transformation of the Michaelis-Menten equation applied to amidase and urease activities obtained as a function of the concentration of formamide and urea. The lines shown in Fig. 4 are those calculated by regression analysis. The Michaelis constant,

$$K_m = \frac{(V_{\max} - v)S}{v} = \frac{k_2 + k_3}{k_1}$$

was expressed in the linear form representing the Lineweaver-Burk equation:

$$1/v = K_m/V_{\max} \cdot 1/S + 1/V_{\max}$$

Where *v* is the initial velocity of the reaction, *K_m* is the Michaelis constant, *V_{max}* is the maximum initial velocity, *S* is the substrate concentration, and *k₁*, *k₂*, and *k₃*

Table 2. *K_m* and *V_{max}* values of amidase and urease in various plant materials

Plant material	Amidase		Urease	
	<i>K_m</i> , mM	<i>V_{max}</i> *	<i>K_m</i> , mM	<i>V_{max}</i> *
Alfalfa	8.21	341	0.69	52
Corn	2.27	514	0.77	70
Oat	7.68	264	0.74	68
Reed canary grass	2.02	205	0.72	123
Ryegrass	9.42	137	1.63	33
Soybean	4.75	164	0.50	29
Sudan grass	6.01	294	0.38	39
Average	5.76		0.78	

* $\mu\text{g NH}_4\text{-N released} \cdot 0.1 \text{ g}^{-1} \text{ plant material} \cdot 2 \text{ h}^{-1}$ by using formamide or urea as a substrate.

are the rate constants. Both K_m and V_{max} are constants for the enzyme, but they may vary independently of each other under different conditions. The K_m value is independent of enzyme concentration and is very valuable in studies of enzyme kinetics. For example, it permits calculation of the minimum substrate concentration needed for maximum enzyme reaction velocity (V_{max}) and allows calculation of enzyme activity (v) as a percentage of V_{max} at any substrate concentration. Table 2 shows the K_m and V_{max} values of amidase and urease in 7 plant samples. The K_m values for amidase, with formamide as a substrate, ranged from 2.0 (reed canary grass) to 9.4 mM (ryegrass) (avg. = 5.8 mM). The K_m values for urease, with urea as a substrate, ranged from 0.4 (sudan grass) to 1.6 mM (ryegrass) (avg. = 0.8 mM). The average affinity ($1/K_m$) of urease for urea is 7 times greater than that of amidase for formamide. Expressed in $\mu\text{g NH}_4\text{-N}$ released/0.1 g plant tissue/2 h for formamide and urea, the V_{max} values ranged from 137 to 514 and 29 to 123, respectively.

The average K_m values for plant amidase (5.8 mM) and urease (0.8 mM) are somewhat lower than those found in soils (amidase, with formamide as a substrate averaged 12.3 mM⁷ and urease with urea as a substrate, averaged 3.3 mM¹⁹). It often is difficult to compare kinetic data with those reported in the literature because preparation, storage, handling of samples, variation in age of the samples, type of buffer used, pH, and incubation temperature may influence the kinetic constants. Therefore, the K_m values of plant urease determined in this investigation are one order of magnitude lower than those reported for jack-bean urease¹⁹. A search of the literature did not reveal any kinetic properties of amidase in plant materials.

Effects of sample pretreatments

Table 3 shows the effects of sample pretreatments on amidase and urease activities in various plant materials. These enzymes were assayed in freshly sampled homogenized materials and after freezing the material in a test tube placed in liquid nitrogen or air-dried at room temperature (23°C) for 48 h. Results showed that amidase activity was greater than urease activity in the fresh plant samples, especially when formamide or propionamide was used as a substrate (Table 3). Urease activity of the fresh homogenized plant tissues shown in Table 3 may not represent the actual enzyme content in the plants under field conditions because the samples were leaf macerates, and the information available indicates that maceration decreases urease activity. For example, Smith *et al.*¹⁸ reported that maceration can decrease urease activity by as much as 50%. They attributed this decrease to inactivation of urease by an air-oxidation effect accelerated by the cavitation action of maceration. Urease has greater activity in the reduced state, and to preserve its structure, agents such as cysteine⁸ and glutathione¹⁸ often are added in preparation of the sample before assay. No information is available about the effect of tissue maceration on amidase activity.

Freezing resulted in significant decrease in amidase and urease activities.

Table 3. Effect of pretreatments on activity* of amidase and urease in plant materials

Plant material	Pre-treatment**	Amidase activity by using the substrate specified			Urease activity
		Formamide	Acetamide	Propionamide	
Soybean	H	1,267	93	203	123
	F	336(27)	22(24)	51(25)	94(76)
	AD	166(13)	18(19)	41(20)	32(26)
Oat	H	2,131	214	520	225
	F	570(27)	57(27)	135(26)	171(76)
	AD	246(12)	38(18)	99(19)	57(25)
Alfalfa	H	3,019	291	607	174
	F	702(23)	75(26)	150(25)	133(76)
	AD	365(12)	55(19)	113(19)	44(25)
Corn	H	3,740	369	1,041	235
	F	880(24)	97(26)	245(24)	184(78)
	AD	505(14)	73(20)	187(18)	59(25)

* $\mu\text{g NH}_4\text{-N released} \cdot 0.1 \text{ g}^{-1} \text{ plant material} \cdot 2 \text{ or } 24 \text{ h}^{-1}$ by using the substrates, formamide or acetamide and propionamide, respectively. Urease activity is expressed in $\mu\text{g NH}_4\text{-N released} \cdot 0.1 \text{ g}^{-1} \text{ plant material} \cdot 2 \text{ h}^{-1}$. Figure in parentheses is activity expressed as percentage of activity of a fresh, homogenized sample (treatment H).

** H, homogenized (fresh); F, frozen; AD, air-dried plant material.

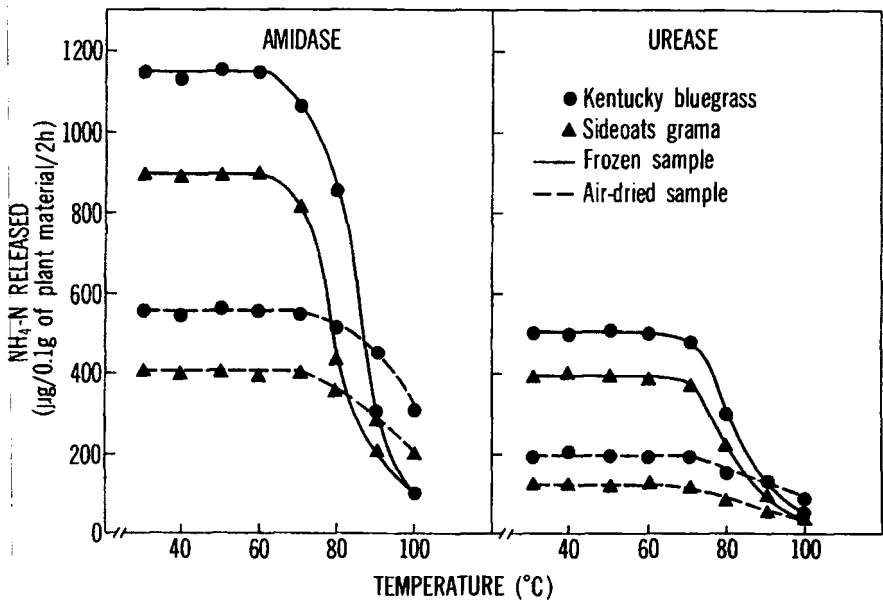


Fig. 5. Effect of preheating temperature on amidase and urease activities of frozen and air-dried plant materials.

Amidase was more susceptible to freezing than was urease. Whereas amidase activity decreased by about 75%, urease activity decreased by about 25% upon freezing of the plant materials. Air-drying the fresh plant materials resulted in further reduction in amidase and urease activities. Amidase activity remaining after air-drying ranged from 12 to 14% of the initial activity by using formamide, from 18 to 20% by using acetamide, and from 18 to 20% by using propionamide as substrates. Urease activity remaining after air-drying was about 25%. The decreases observed in amidase and urease activities upon freezing or air-drying plant materials were proportional to the initial activities of the fresh homogenized samples. This was independent of the type of plant material studied (Table 3). The results reported in Table 3, however, reflect the relative activities of amidase and urease in plant tissues as affected by various pretreatments.

The effect of temperature on the stability of plant amidase and urease in frozen tissues and air-dried samples is shown in Fig. 5. In this work, we exposed the plant tissue to different temperatures for 2 h, then brought the samples to room temperature (23°C) and assayed amidase and urease activities at 37°C. In both frozen tissues and air-dried samples, amidase and urease were stable up to 60 and 70°C, respectively. Although the air-dried samples contained much less amidase and urease activities, at temperatures above 60°C, the rate of inactivation of these enzymes was much faster in the frozen tissues than in the air-dried samples. Amidase and urease activities in frozen tissues decreased by 90 and 94%, respectively, when the samples were exposed to 100°C for 2 h before assay. Air-dried samples contained more residual activity than the frozen tissues and averaged 49 and 71% loss in amidase and urease activities, respectively, when exposed to 100°C. In studies of the activation energies of plant enzymes, it is assumed that the incubation temperature has no effect on the stability of the enzymes. The data in Figure 5 support the validity of this assumption.

Distribution of amidase and urease among plants

Table 4 shows the activities of amidase and urease detected in 21 diverse plant samples. These samples represent the Gramineae and Leguminosae families. Among the Gramineae, the following tribes were represented: Festuceae, Aveneae, Chlorideae, Phalarideae, Agrostideae, Paniceae, Andropogoneae, and Triticeae. The Leguminosae included the Trifolieae (alfalfa) and Phaseoleae (soybean) tribes.

For convenience and because the results reported in Table 3 showed that proportional amounts of amidase and urease activities are remaining after air-drying plant samples, the results reported in Table 4 were obtained with air-dried samples. Because the concentration of the substrates (urea, formamide, acetamide, and propionamide) were the same and both amidase and urease were saturated with their substrates (zero-order reaction), the activities of these enzymes could be used to reflect their relative concentrations in the leaf tissue of the plants studied. Activities for urease, expressed in μg of $\text{NH}_4\text{-N}$ released/0.1 g

Table 4. Activity* of amidase and urease in air-dried leaves of various plants

Plant material	Amidase activity by using the substrate specified			Urease activity
	Formamide	Acetamide	Propionamide	
Alfalfa (<i>Medicago sativa</i> L.)	331 (246)	44 (35)	114 (91)	47 (49)
Barnyard grass (<i>Echinochloa muricata</i> (Beauv.) Fern.)	165 (130)	24 (16)	63 (47)	81 (84)
Bermuda grass (<i>Cynodon dactylon</i> (L.) Pers.)	114 (70)	18 (11)	45 (31)	62 (63)
Big bluestem (<i>Andropogon gerardii</i> Vitman)	487	41	162	87
Canada bluegrass (<i>Poa compressa</i> L.)	413	53	137	144
Caucasian bluestem (<i>Bothriochloa cavcasica</i> Hubb)	123	14	38	11
Corn (<i>Zea mays</i> L.)	495 (327)	59 (43)	167 (133)	64 (65)
Kentucky bluegrass (<i>Poa pratensis</i> L.)	559 (483)	78 (53)	198 (145)	163 (159)
Oat (<i>Avena sativa</i> L.)	257 (166)	39 (26)	96 (74)	63 (62)
Orchard grass (<i>Dactylis glomerata</i> L.)	102 (63)	14 (9)	37 (29)	40 (38)
Quackgrass (<i>Agropyron repens</i> (L.) Beauv.)	254	33	84	28
Reed canary grass (<i>Phalaris arundinacea</i> L.)	189	22	56	117
Ryegrass (<i>Lolium perenne</i> L.)	127	18	41	32
Sideoats grama (<i>Bouteloua curtipendula</i> (Michx.) Torr.)	395 (323)	56 (38)	155 (124)	138 (138)
Smooth brome (<i>Bromus inermis</i> Leyss.)	263	31	81	11
Sorghum (<i>Sorghum bicolor</i> L. Moench)	218	28	73	74
Soybean (<i>Glycine max</i> (L.) Merr.)	162 (115)	18 (11)	52 (37)	28 (28)
Sudan grass (<i>Sorghum sudanense</i> (Piper) Stapf)	263 (208)	31 (24)	81 (65)	38 (37)
Switch grass (<i>Panicum virgatum</i> L.)	680	91	241	38
Tall fescue (<i>Festuca arundinaceae</i> L.)	195	28	76	66
Timothy (<i>Phleum pratense</i> L.)	303	28	108	93

* $\mu\text{g NH}_4\text{-N}$ released $\cdot 0.1 \text{ g}^{-1}$ plant material $\cdot 2$ or 24 h^{-1} by using the substrates, formamide or acetamide and propionamide, respectively. Urease activity is expressed in $\mu\text{g NH}_4\text{-N}$ released $\cdot 0.1 \text{ g}^{-1}$ plant material $\cdot 2 \text{ h}^{-1}$. Activities obtained in the presence of toluene are given in parentheses.

plant material/2 h, ranged from 11 (*Bromus inermis* Leyss. and *Bothriochloa cavasica* Hubb.) to 163 (*Poa pratensis* L.). Amidase activity ranged from 102, 14, and 37 (*Dactylis glomerata* L.) to 680, 91, and 241 (*Panicum virgatum* L.) μg of $\text{NH}_4\text{-N}$ released/0.1 g plant material/2 h when formamide, acetamide, and propionamide were used as substrates, respectively. Although amidase and urease activities were detected in all 21 diverse plant samples, there have been reports that some families of the plant kingdom contain no urease¹. For example, urease is absent in the Cruciferae and Tiliaceae families. Because the activities of these enzymes in air-dried samples were proportional to the activities of fresh samples (Table 3), from the activity of amidase and urease found in air-dried plant materials one can estimate the original activity in fresh samples of the plants reported in Table 4.

The presence of toluene had no effect on urease, but it reduced the activity of amidase (Table 4). Toluene is used to inhibit microbial activity during determination of extracellular enzyme activities in soils¹⁷.

Use of amide and urea as foliar fertilizers may lead to NH_4^+ toxicity, especially when an amide such as formamide is used as a source of N. The parameters, especially temperature, that affect the activities of these enzymes in plant leaves should be considered as a means of reducing the substrate hydrolysis and subsequent NH_4^+ toxicity. The possible use of specific inhibitors to reduce the rate of hydrolysis of the substrates of these enzymes by plant leaves deserves investigation.

Acknowledgements Journal Paper No. J-10262 of the Iowa Agriculture and Home Economics Experiment Station, Ames. Projects 2082 and 2112.

Received 12 May 1981. Revised October 1981

References

- 1 Bailey C J and Boulter D 1971 Urease, a typical seed protein of the Leguminosae. pp 485-502. In Chemotaxonomy of the Leguminosae. Eds. J B Harborne, D Boulter and B L Turner. Academic Press, New York.
- 2 Baker E C and Mustakas G C 1973 Heat inactivation of trypsin inhibitor, lipoxgenase, and urease in soybeans: Effect of acid and base additives. J. Am. Oil Chem. Soc. 50, 137-141.
- 3 Blain J A and Caskie M 1965 Urease by diffusion assay. Chem. Ind. London 3, 17-18.
- 4 Croston C B, Smith A K and Cowan J C 1955 Measurement of urease activity in soybean oil meal. J. Am. Oil Chem. Soc. 32, 279-282.
- 5 Dick W A and Tabatabai M A 1978 Inorganic pyrophosphatase activity of soils. Soil Biol. Biochem. 10, 59-65.
- 6 Frankenberger W T Jr and Tabatabai M A 1980 Amidase activity in soils: I. Method of assay. Soil Sci. Soc. Am. J. 44, 282-287.
- 7 Frankenberger W T Jr and Tabatabai M A 1980 Amidase activity in soils: II. Kinetic parameters. Soil Sci. Soc. Am. J. 44, 532-536.
- 8 Freiberg S R and Payne P 1957 Foliar absorption of urea and urease activity in banana plants. J. Am. Soc. Hortic. Sci. 69, 226-234.

- 9 Garcia R and Hanway J J 1976 Foliar fertilization of soybeans during the seed-filling period. *Agron. J.* 68, 653-657.
- 10 Hunter A S 1974 Field comparisons of formamide, urea-ammonium nitrate solution and ammonium nitrate as nitrogen sources for grasses and wheat. *Agron. J.* 66, 540-543.
- 11 Jespersen N D 1975 A thermochemical study of the hydrolysis of urea by urease. *J. Am. Chem. Soc.* 97, 1662-1667.
- 12 Kyllingsbaek A 1975 Extraction and colorimetric determination of urea in plants. *Acta Agric. Scand.* 25, 109-112.
- 13 Lynn K R and Yankwich P E 1962 ¹³C kinetic isotope effects in the urease-catalyzed hydrolysis of urea. I. Temperature dependence. *Biochim. Biophys. Acta* 56, 512-530.
- 14 Roch-Ramel F 1967 An enzymic and fluorophotometric method for estimating urea concentrations in nanoliter specimens. *Anal. Biochem.* 21, 372-381.
- 15 Schatalova A and Meerov G 1963 A radiometric method for urease assay. *Biokhimiya* 28, 384-387.
- 16 Shapiro I A 1976 Determination of urease activity in lichens by a direct potentiometric method. *Sov. Plant Physiol.* 23, 712-714.
- 17 Skujins J 1976 Extracellular enzymes in soil. *Crit. Rev. Microbiol.* 4, 383-421.
- 18 Smith A K, Belter P A and Anderson R L 1956 Urease activity in soybean meal products. *J. Am. Oil Chem. Soc.* 33, 360-363.
- 19 Tabatabai M A 1973 Michaelis constants of urease in soils and soil fractions. *Soil Sci. Soc. Am. Proc.* 37, 707-710.
- 20 Tabatabai M A and Bremner J M 1972 Assay of urease activity in soils. *Soil Biol. Biochem.* 4, 479-487.
- 21 Vasilas B L, Legg J O and Wolf D C 1980 Foliar fertilization of soybeans: Absorption and translocation of ¹⁵N-labeled urea. *Agron. J.* 72, 271-275.
- 22 Wall M C and Laidler K J 1953 The molecular kinetics of the urea-urease system: IV. The reaction in an inert buffer. *Arch. Biochem. Biophys.* 43, 299-306.

Asparaginase
I.U.B.: 3.5.1.1
C.A.S.: 9015-98-3

Enzymatic Reaction (*image will open in a new window*)

Asparaginase catalyzes the hydrolysis of L-asparagine to produce L-aspartic acid and ammonia. Bacterial type L-asparaginases are classified into subtypes I and II, which is defined by their intra- or extracellular localization (Michalska and Jaskolski 2006). Type I (cytosolic) has a lower affinity for L-asparagine, while type II (periplasmic) has a high substrate affinity. Plant type L-asparaginases differ structurally and have a different evolutionary origin than bacterial L-asparaginases (Michalska *et al.* 2006)

History:

In 1904, Lang became the first to detect asparaginase activity in beef tissues. Fürth and Friedmann confirmed these results in 1910. Fürth and Friedmann believed asparaginase activity was found at the same level in all animal tissues, which was later refuted by Clementi (Clementi 1922) whose experiments concluded the enzyme was active in practically all tissues of herbivores, only in the liver of omnivorous animals, and absent in the organs of carnivorous animals, amphibians, and reptiles (Michalska and Jaskolski 2006).

In 1953, Kidd reported anti-tumor properties of guinea pig serum, which was later attributed to asparaginase activity (Broome 1961). In 1963, Mashburn and Wriston found that the *E. coli* enzyme had anti-tumor activity. Work proceeded in several laboratories leading to clinical trials, making *E. coli* asparaginase II the first antileukemic L-asparaginase to be used clinically (Roberts *et al.* 1966, Old *et al.* 1967, Boyse *et al.* 1967, Hill *et al.* 1967, Clarkson *et al.* 1970, and Oettgen *et al.* 1970). It was soon discovered that in addition to the secreted EcAII, *E. coli* (and other bacteria) also produce cytosolic asparaginase, asparaginase I (EcAI), which has a lower affinity for L-asparagine and is not effective against cancer (Willis and Woolfolk 1974).

Preliminary efforts to determine the primary structure were carried out in 1974, and the sequence was confirmed in 1990 (Maita *et al.* 1974, Maita and Matsuda 1980, and Bonthron 1990). After crystallographic studies by Epp *et al.* in 1971, a partial model of the EcAII crystal structure was published in 1988 by Ammon *et al.*. Although not completely accurate, this structure was used as a stepping stone by Swain *et al.* who elucidated the true structure in 1993. Many crystal structures of L-asparaginase from a wide variety of organisms have been determined (Lubkowski *et al.* 2003).

Current research with L-asparaginase continues to focus on its oncological importance, and the specific transcription factors EcAII targets (Suto *et al.* 2010). Structure-function relationships of L-asparaginase from a variety of sources are also currently under investigation (Labrou and Papageorgiou 2010, and Bansal *et al.* 2010).

Specificity:

Asparaginase

Manual Text

- Assay
- References
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EcAII exhibits a broad pH activity profile and higher substrate affinity than EcAI. The reaction consists of two steps. In the first step, the nucleophilic threonine attacks the carbonyl of the amide substrate to generate an acyl-enzyme intermediate. A molecule of ammonia is released. In the second step, water attacks the acyl-enzyme intermediate to produce L-aspartate (Michalska and Jaskolski 2006).

Composition:

EcAII is a tetrameric protein composed of four identical subunits, each subunit contains 326 amino acid residues. The two threonine residues present at the active site are required for activity (see PDB 3ECA). Debate still continues over which threonine residue supplies the nucleophile. Most believe that it is the N-terminal threonine. The C-terminal threonine is part of a triad that resembles the typical SHD catalytic triad of serine proteases (Carter and Wells 1988).

Molecular Characteristics:

The promoter of the gene encoding L-asparaginase II, *ansB*, is coactivated by cAMP receptor protein (CRP) and the FNR protein (Scott *et al.* 1995).

CRP is activated in response to carbon sources, and the FNR protein, the *fnr* gene product, in response to anaerobiosis. The FNR site is located 41.5 bp upstream of the major start site, and 28 bp upstream of the CRP site is the CRP regulation site (Jennings and Beacham 1993).

Protein Accession Number: P00805

CATH Classification (v. 3.2.0):

- Class: Alpha Beta
- Architecture: 3-Layer(aba) Sandwich
- Topology: Rossmann Fold

Molecular Weight:

- Monomer: 36.8 kDa (Theoretical)
- Tetramer: 141,000 (Maita *et al.* 1974 and Shifrin *et al.* 1974)

Optimal pH:

- 8.6 (Zhang *et al.* 2004)
- 8.0 (Wriston 1971)

Extinction Coefficient:

- 21,290 cm⁻¹ M⁻¹
- E_{1%, 278} = 7.1 (Ho *et al.* 1971)

Active Site Residues:

- Threonine (T34, T111)
- Aspartate (D112)
- Lysine (K184)

Activators:

- Anaerobic conditions (Cedar and Schwartz 1967)

- Amino acids (not specifically asparagine) (Cedar and Schwartz 1968)

Inhibitors:

- 5-diazo-4-oco-L-norvaline (DONV) (Wriston and Yellin 1973)
- Dimethylsulfoxide (Wriston 1971)
- Glutaraldehyde (Zhang *et al.* 2004)
- L-Asparagine and L-aspartate (Wriston and Yellin 1973)
- NH₄⁺ (Wriston 1971)
- Trypsin (Zhang *et al.* 2004)

Applications:

- Treatment of acute lymphoplastic leukemia (Zhang *et al.* 2004)
- L-asparagine concentration determination (Gentili *et al.* 1994)

Please email any suggestions/corrections for this manual entry to Krystal Worthington: krystal@worthington-biochem.com

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