Journal of Chromatography, 415 (1987) 104–109
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3491

Note

Gas chromatographic–mass spectrometric determination of tryptophan transaminase-catalyzed deuterium exchange*

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(First received July 1st, 1986; revised manuscript received November 10th, 1986)

Labilization of the α-hydrogen (C-2 hydrogen) of amino acids is characteristic of many pyridoxal 5′-phosphate-catalyzed reactions, including transamination [1]. This labilization has been demonstrated by incubating the enzymes with amino acid substrates in ^2H_2O and monitoring enzyme-catalyzed exchange of hydrogen for deuterium, or by monitoring release of tritium from α-tritiated substrates. Exchange of hydrogen in the β-position has been demonstrated for a number of transaminases, including L-alanine transaminase [2–4], aspartate transaminase [3], D-amino acid transaminase [5] and γ-aminobutyrate transaminase [5]. Most transaminases, however, labilize the hydrogen at the α-position only.

We have previously demonstrated the presence of an L-tryptophan:2-oxoglutarate aminotransferase in cell-free extracts of *Streptomyces flocculus* [6]. A tryptophan C-methyltransferase also competes for the tryptophan substrate. In studying the mechanism of C-methylation with partially purified enzyme, we found it necessary to determine whether the contaminating transaminase was capable of exchanging the α- and/or β-hydrogens of the substrate. To our knowledge, no tryptophan transaminase has been studied in this context. We chose to perform the study using gas chromatography–mass spectrometry (GC–MS) to monitor the incorporation of deuterium from a ^2H_2O environment into the substrate. MS determination of isotopic enrichment provides the ability to monitor both potential positions of incorporation simultaneously with high sensitivity, precision and accuracy [7].

*Presented in part at the 32nd Annual Conference on Mass Spectrometry and Allied Topics, San Antonio, TX, U.S.A., May 1984.

0378-4347/87/$03.50

EXPERIMENTAL

Deuterium oxide (99% ^2H), concentrated deuterium chloride in ^2H_2O (99% ^2H), aminooxyacetic acid (carboxymethoxylamine), N,N-dimethylformamide diethyl acetal and N,N-dimethylformamide dimethyl acetal were obtained from Aldrich (Milwaukee, WI, U.S.A.). L-Tryptophan and pyridoxal 5′-phosphate were obtained from Sigma (St. Louis, MO, U.S.A.). DL-[3,3-$^2H_2$]Tryptophan (>95% $^2H_2$) was a gift of Dr. Sanford Markey.

Deuterated buffers were prepared by evaporating solutions of Na_2HPO_4 and NaH_2PO_4 to dryness, reconstituting in ^2H_2O, drying again, and reconstituting in ^2H_2O for a final time. A 0.15 *M* buffer with a pD of 7.55, equivalent to pH 7.15 [8], was prepared by mixing the $Na_2{}^2HPO_4$ and the $Na^2H_2PO_4$ solutions prepared above in a 4:1 ratio.

Enzyme preparation

The tryptophan transaminase was prepared from 58-h cultures of *S. flocculus* and partially purified by ammonium sulfate precipitation as previously described [6]. The 35–60% ammonium sulfate precipitated protein was reconstituted in 10 m*M* sodium phosphate buffer, pH 8.0, dialyzed, and then lyophilized. Portions of the lyophilized enzyme preparation were reconstituted in pD 7.55, 10 m*M* sodium phosphate buffer.

Transaminase assay

The rate of transamination was determined in a parallel reaction in which the formation of the product, 3-indolepyruvate, was assayed by measuring the increase in absorbance at 305 nm using a Gilford System 2600 spectrophotometer [9]. The reaction mixture contained 0.5 ml of enzyme preparation, consisting of 2.1 mg protein reconstituted in 10 m*M* sodium phosphate buffer (pH 8.0), 5.0 m*M* α-ketoglutarate, 0.03 m*M* pyridoxal 5′-phosphate and 5.0 m*M* L-tryptophan in a total volume of 1.0 ml. All incubations were performed at 37°C.

Exchange reaction

The lyophilized enzyme preparation described above was reconstituted in 10 m*M* sodium phosphate buffer made up in ^2H_2O and allowed to incubate for 20 min to exchange dissociable protons on the enzyme for deuterium atoms. L-Tryptophan (5.0 μmol) and 0.06 μmol of pyridoxal 5′-phosphate, both dissolved in deuterated buffer, were added to 10.8 mg of protein in a total volume of 2.0 ml, and the reaction was allowed to proceed at 37°C for 90 min. Parallel control reactions were incubated with boiled enzyme in one and with 0.1 m*M* aminooxyacetic acid, which has been shown to completely inhibit the transaminase [10], in another. Aliquots (0.2 ml) were withdrawn after 0, 15, 30, 60 and 90 min of incubation.

Product isolation

The reaction was terminated by precipitation of protein with 5 μl of 1.0 *M* ^{2}HCl. Following centrifugation at 5000 *g* for 10 min, the supernatant was transferred to

another test tube and dried under a nitrogen atmosphere. The residue was reconstituted with 200 μl of water and extracted with 200 μl of chloroform. The chloroform layer was discarded and the aqueous fraction was dried under nitrogen.

The residue was derivatized by dissolving in 100 μl of warm N,N-dimethylformamide diethyl acetal–acetonitrile–ethanol (3:2:1). The corresponding methyl ester derivative was formed by this method by substituting N,N-dimethylformamide dimethyl acetal for N,N-dimethylformamide diethyl acetal in the reagent mixture.

Gas chromatography–mass spectrometry

Aliquots of derivatized L-tryptophan, ethyl 2-(N,N-dimethyl-N′-formamidino)-3-(3-indolyl)propionate, were analyzed on a Carlo Erba 4130 gas chromatograph with a 15 m×0.25 mm I.D. methyl silicone capillary column with helium as carrier gas. After a 1-min solvent delay, the column was heated from 150 to 250°C at 12°C/min and maintained at 250°C for 5 min. The capillary column was directly interfaced with an Extrel Simulscan mass spectrometer operating either in the electron ionization mode at an ionizing voltage of 70 eV or in the chemical ionization mode in which 1 Torr of isobutane or methane was used as reagent gas. The system was under the control of an Extrel 1000 data system scanning at 500 a.m.u./s over a mass range of 45–502, or in the selected-ion monitoring (SIM) mode. In the electron ionization SIM mode, *m/z* 130, 131, 132, 157, 158 and 287 were monitored for dwell times of 100 ms and the peak areas integrated by the data system. A low signal-to-noise ratio was observed in the molecular ion region which precluded determination of accurate and precise isotope enrichment measurements at *m/z* 288. The relative areas of *m/z* 157 and 158 were used to indicate that α-exchange had occurred, while the areas of ions 130, 131 and 132 were used as a measure of β-exchange.

RESULTS AND DISCUSSION

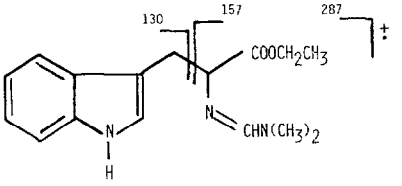
In order to determine the extent and location of deuterium incorporation in tryptophan arising from solvent exchange catalyzed by a tryptophan transaminase, the polar functionalities of tryptophan were derivatized prior to analysis by GC–MS. Thenot and Horning [11] have reported that dialkyl acetals of N,N-dimethylformamide can be used to derivatize both the amine and carboxyl moieties of various amino acids in one step. We have previously used this method to derivatize 4-aminobutanoic acid (GABA) and related compounds [12]. Here we report the use of N,N-dimethylformamide diethyl acetal for the conversion of tryptophan to the derivative, ethyl 2-(N,N-dimethyl-N′-formamidino)-3-(3-indolyl)propionate. Since dialkyl acetals of dimethylformamide react with secondary amines to form N-formyl derivatives [13], there was the possibility that the indole nitrogen may be a reactive site. GC–chemical ionization MS of the derivative yielded a protonated molecular species (MH^+, *m/z* 288) which indicated that the indole nitrogen had not been formylated.

Under electron ionization conditions, ethyl 2-(N,N-dimethyl-N′-formamidino)-3-(3-indolyl)propionate fragments at the α-carbon yielding the equivalent of an indolylmethylene cation or a quinolinium ion (*m/z* 130) (Table I).

TABLE I

DEUTERIUM ENRICHMENT IN THE α-POSITION OF TRYPTOPHAN

Values derived from relative intensities of fragment ions m/z 130, 131 and 132, corrected for natural abundance isotopes, were consistent with $<2\%$ incorporation in the β-position.



Incubation time (min)	Atom percent excess* (%)
0	0
15	2
30	8
60	15
90	24

*Values for incorporation of deuterium in the α-position were derived from duplicate determination of relative intensities of fragment ions m/z 157 and 158 corrected for natural abundance isotopes.

Detection of isotope enrichment in this fragment would indicate incorporation of deuterium in the β-position (C-3) of tryptophan. In support of this structure assignment of the m/z 130 ion, [3,3-$^2H_2$]tryptophan was derivatized, a mass spectrum recorded and a prominent m/z 132 ion observed. Another major fragment in the electron ionization spectrum at m/z 157, which arises from cleavage at the indolylic methylene, separates the α- (C-2) and β- (C-3) carbon atoms. This fragmentation is analogous to a benzylic cleavage (between C-2 and C-3) with charge retention on both fragments (m/z 130 and 157). Deuterium enrichment in the α-position shifts the signal from m/z 157 to 158. Support for the identity of the side-chain fragment was derived from analysis of the methyl ester derivative. Derivatization of tryptophan with the dimethyl acetal of dimethylformamide yielded on MS analysis an analogous fragment at m/z 130 for the quinolinium ion and a major ion at m/z 143, 14 a.m.u. ($-CH_2$) lower mass than the ion at m/z 157, the side-chain fragment ion observed for the ethyl ester derivative.

The isotope enrichment data shown in Table I, derived from monitoring m/z 157 and 158, show a linear increase with time up to 90 min, at which time the calculated atom percent excess of deuterium was 24%. The rate of exchange was calculated to be 0.13 μmol min^{-1} mg^{-1} protein.

In addition to providing a measure of the progress of the reaction, deuterium incorporation in re-isolated tryptophan yields mechanistic information regarding the transaminase reaction. These data are consistent with a mechanism in which

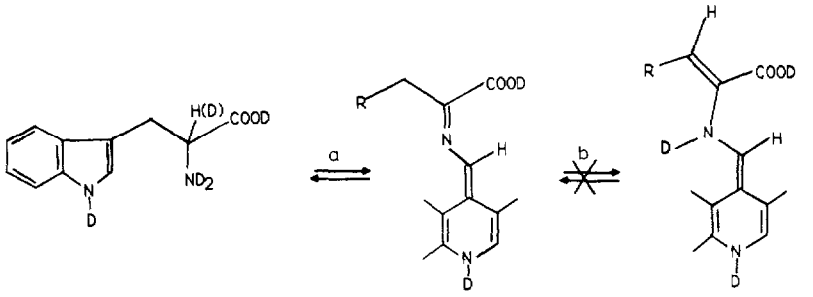


Fig. 1. Proposed mechanism for deuterium exchange in the α-position of tryptophan. (a) In the presence of enzyme, ^2H_2O and pyridoxal 5′-phosphate a ketimine–aldimine tautomeric pair is formed leading to α-exchange. (b) Pathways leading to the formation of enamine intermediates, and subsequent β-exchange, were not observed. R = indole.

pyridoxal 5′-phosphate reacts with the aliphatic nitrogen of tryptophan to form an aldimine (Fig. 1). When the reaction was carried out in deuterated buffer, the tryptophan isolated from the incubation mixture contained deuterium in the α-position only. This result is consistent with the proposed mechanism in which deuterium is incorporated during the prototropic shift.

Aminooxyacetic acid inhibits pyridoxal 5′-phosphate-mediated reactions by condensing with the carbonyl carbon of pyridoxal [14]. Thus, in the presence of this agent, tryptophan transaminase activity was decreased by removal of a required cofactor [10]. The experimental results showed a lack of α-exchange when tryptophan was incubated in the presence of aminooxyacetic acid. These results suggest that the enzyme responsible for the exchange requires pyridoxal as a cofactor, and help confirm that the transaminase is responsible for the exchange.

Transamination mechanisms that would require tautomerization of a ketimine intermediate to an enamine conjugated with the indole nucleus have been ruled out since no β-exchange was observed by MS analysis of re-isolated tryptophan. Similarly, mechanisms involving deuterium incorporation through keto–enol tautomerization of the product, 3-indolepyruvic acid, which could be re-aminated to give deuterated tryptophan, were also ruled out by the absence of β-exchange.

The results of the exchange experiments are supportive of the classical transaminase mechanism which involves aldimine formation with pyridoxal phosphate followed by a prototropic shift of the α-proton and subsequent hydrolysis of the imine to form the product, in this case 3-indolepyruvate. Furthermore, the steps of the reaction up to and including the prototropic shift are reversible and the abstracted proton can be exchanged with deuterium atoms in the aqueous medium. Since the tryptophan transaminase is not capable of labilizing hydrogen from the β-position of tryptophan, small amounts of transaminase will not interfere with future attempts to determine C-methyltransferase-catalyzed β-exchange.

In summary, the combined instrumentation techniques of quantitative GC–MS provided a useful means of analysis of a regio-specific deuterium exchange catalyzed by a pyridoxal dependent transaminase.

ACKNOWLEDGEMENT

We thank Dr. Sanford P. Markey of the National Institutes of Health for providing a sample of [3,3-$^2H_2$]tryptophan.

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