Substrate Proton Exchange Catalyzed by γ-Cystathionase†

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ABSTRACT: Pulsed Fourier transform proton magnetic resonance was used to study the labilization of protons of various L-amino acids by the enzyme γ-cystathionase. In the course of the normal reaction, the enzyme labilizes the α and β protons of the substrate, L-homoserine, and promotes elimination of the γ substituent. It was found that γ-cystathionase also catalyzes the exchange of the α and β protons of L-amino acids which cannot undergo elimination reactions, but are competitive inhibitors of the enzyme. Both β protons of L-α-aminobutyrate, although not stereochemically equivalent, were exchanged at equal rates, whereas selectivity was shown for one of the β hydrogens when the carbon chain length was increased. The data also show that β-proton exchange cannot occur without α-proton exchange. The rate of α-proton exchange from amino acids containing a terminal hydroxyl group at the β, γ, or δ carbon is greater than from the corresponding unsubstituted amino acid. Exchange rates of the α proton for the inhibitors examined vary from one-seventh that of the normal enzymatic reaction to approximately the same rate as that for the elimination reaction with homoserine. An active site with two areas of substrate–enzyme interaction is proposed. One site contains pyridoxal 5′-phosphate and the base or bases involved in α- and β-proton exchange; the second site contains a base which normally facilitates removal of the γ substituent and can interact with the γ and δ carbons of the substrate molecule.

γ-Cystathionase is a pyridoxal 5′-phosphate containing enzyme which catalyzes several γ-elimination reactions:

$$\text{L-homoserine} \rightarrow \alpha\text{-ketobutyrate} + NH_4^+$$

$$\text{L-cystathionine} \rightarrow \alpha\text{-ketobutyrate} + NH_4^+ + \text{L-cysteine}$$

The mechanism of this reaction is thought to involve initial labilization of the α and β protons of the substrate, with subsequent elimination at the γ position (Davis and Metzler, 1972). During the course of our work with this enzyme, we decided to examine its ability to promote the labilization of the α and β protons of L-amino acids which can bind to the enzyme, but which cannot undergo the elimination reaction. The sensitivity of current ^1H NMR[1] techniques allowed us to easily follow the exchange of these protons with solvent-derived deuterium. From these studies, we were able to obtain rates of exchange for the α and β protons of several L-amino acids. In addition, the results provide some information concerning the relationship of substrate structure and catalytic activity.

Materials and Methods

L-Amino acids (norvaline, norleucine, alanine, α-aminobutyric acid) were purchased from Sigma Chemical Co. δ-Hydroxy-α-aminovaleric acid (nominally D,L) was purchased from Cyclo Chemical Co. Treatment of this compound with D- and L-amino acid oxidases (Sigma Chemical Co.), however, showed the preparation to be more than 99% L-isomer; in addition, γ-cystathionase catalyzed complete (>99%) deuterium incorporation into the α position of this compound. Deuterium oxide (99.8%) was purchased from Wilmad Glass Co. pH values were recorded directly and not corrected for the deuterium isotope effect.

γ-Cystathionase was prepared according to the method of Greenberg (1962). The enzyme was assayed by coupling the enzymatic reaction to lactate dehydrogenase. The reaction mixture contained pyridoxal 5′-phosphate (36 μM), β-mercaptoethanol (5.4 mM), EDTA (5 mM), potassium phosphate (75 mM, pH 7.5), D,L-homoserine (35 mM), NADH (0.2 mM), and LDH (Worthington Biochemicals, 26 units) in a volume of 1.4 ml. The assay was begun by addition of enzyme and the reaction was followed by the disappearance of absorbance at 340 nm. Purified enzyme (specific activity, 450 μmol h^{-1} mg^{-1}) was lyophilized and resuspended in D_2O prior to use. This procedure caused no loss of enzymatic activity. It was also determined that there was no solvent deuterium isotope effect on the enzymatic reaction with L-homoserine as substrate.

The ^1H NMR spectra were recorded with a Bruker WH 90 Fourier transform spectrometer modified as described (Redfield and Gupta, 1971).

Each experiment was performed as follows: 0.45 ml of a solution of the amino acid (100 mM) in potassium phosphate buffer (50 mM, pH 7.0) was lyophilized, taken up in an equal volume of D_2O, and placed in an NMR tube. Enzyme, previously lyophilized and resuspended in D_2O (50 μl), was added at zero time and the spectra were then recorded at regular intervals. Figure 1 shows a number of scans of the β protons of L-α-aminobutyrate at various times after the addition of enzyme. For each compound, peak integrals were also recorded and the log of these values was plotted as a function of time, as shown in Figure 2 for norvaline. The exchange rate of each proton species was determined from the slope of the line and is expressed in μmol h^{-1} μmol^{-1} of enzyme.

The K_m values for homoserine and serine were determined in the standard manner. K_I values of the other amino acids were determined from plots of the reciprocal of the velocity vs. the reciprocal of the inhibitor concentration at several concentrations of homoserine.

† This is publication No. 1127 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154. *Received July 19, 1976.* This work was supported by National Science Foundation Grant No. PCM 01807 (R.H.A.) and National Institutes of Health Grant No. GM 20168 (A.J.L.C.) and GM 00212 (W.W.). The ^1H NMR equipment was supported by National Science Foundation Grants GU 3825 and GP 37156.

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[1] Abbreviations used: NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; NADH, reduced nicotinamide adenine dinucleotide; LDH, lactic dehydrogenase.

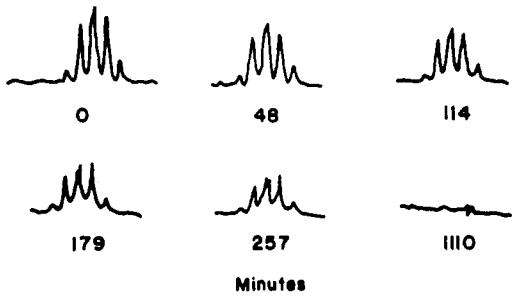


FIGURE 1: ^{1}H NMR spectra of the β protons of L-α-aminobutyrate. Spectra were recorded at various times, with zero time representing the spectrum prior to enzyme addition. The ^{1}H NMR tube contained L-α-aminobutyrate (100 mM), potassium phosphate buffer (50 mM, pH 7.0), and 19 units of γ-cystathionase in a total volume of 0.5 ml of D_2O. The time in minutes at which the spectra were recorded is noted below each spectrum. Resonance of β protons at 1.88 ppm.

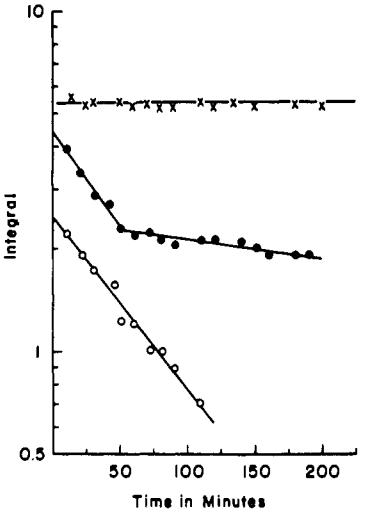
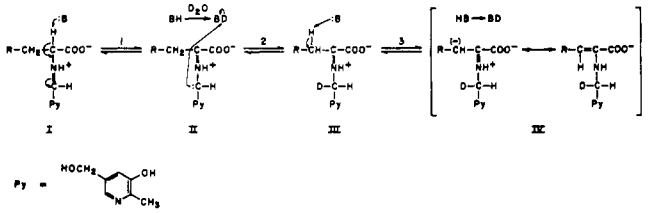


FIGURE 2: Disappearance of the α, β, and δ protons of L-norvaline. The signal areas under the peaks corresponding to the α, β, and γ protons of L-norvaline were recorded as the integral for each spectrum. The values are plotted as the log of the integral vs. time: (○) α protons, (●) β protons, and (X) δ protons. The ^{1}H NMR tube contained L-norvaline (100 mM), potassium phosphate buffer (50 mM, pH 7.0), and 45 units of γ-cystathionase in a total volume of 0.5 ml of D_2O. α protons, 3.71 ppm; β protons, 1.16–2.10 ppm; δ protons, 0.95 ppm.

Results and Discussion

Table I summarizes the rates of γ-cystathionase-catalyzed exchange of the α and β protons with solvent deuterons for several compounds possessing the structure $RCH(NH_3^+)COO^-$. In every case, α-proton abstraction was noted. For both L-alanine and L-α-aminobutyrate, each of the β protons is subject to exchange; moreover, the β protons (three for alanine; two for α-aminobutyrate) exchange at equal rates. For both amino acids, the rate of disappearance of the β-proton signal does not exceed the rate of disappearance of the α-proton signal, suggesting that (a) β proton exchange occurs only after α-proton removal and that (b) the α proton is not conserved after the initial abstraction. The data show that, for every α proton exchanged from L-alanine, an average of 2.8 β protons are replaced by deuterium. Therefore, once an α proton has been abstracted, several β-proton exchanges can occur before a deuteron is put back in the α position (in place of the original proton) and the amino acid dissociates from the enzyme. The following equation represents a reasonable mechanism for the γ-cystathionase-catalyzed exchange of α and β protons from amino acids.



Initial abstraction of the α proton from the aldimine (structure I) leads to the formation of the carbanion (structure II); exchange of the α proton with a solvent deuteron (BH → BD) and addition of this deuteron at the C-4′ position of pyridoxal phosphate give rise to the ketimine (structure III). The imino function at C-2 facilitates abstraction of a β proton, yielding the enamine–carbanion intermediate (structure IV). Reversal of steps 1 through 3 results in liberation of an amino acid deuterated in the α and β positions. It should be noted that the mechanism implies (a) the rapid exchange of the proton attached to base (B) with a solvent deuteron and (b) the rapid reversibility of step 3, which would allow more than one β proton to be exchanged for every α proton exchanged. The mechansim depicts one base as abstracting both α and β protons. However, the utilization of two basic groups, one for α proton abstraction and one for β proton abstraction, is equally feasible.

The finding that both of the β protons of L-α-aminobutyrate are exchanged at the same rate is surprising since these two protons are nonequivalent. It has been shown that in the conversion of L-homoserine to α-ketobutyrate the enzyme shows considerable stereoselectivity in the removal of one of the β protons from the substrate (Krongelb et al., 1968). Apparently, the ethyl group attached to the α carbon of L-α-aminobutyrate is sufficiently free to rotate within the active site, such that the β hydrogens appear equivalent and exhibit identical exchange rates.

When the carbon skeleton is extended by one more carbon (R = $CH_3CH_2CH_2$–), selectivity in the exchange of β hydrogens by γ-cystathionase is observed (Table I). One of the β protons now exchanges 24 times more slowly than the other. Presumably, extension of the carbon chain allows sufficient interaction between the enzyme and substrate to prevent rotation of the β carbon. Further extension of the molecule (R = $H_2COHCH_2CH_2$–) does not increase selectivity in the exchange of one β proton over the other.

Incorporation of a hydroxyl group into the substrate affects the exchange rate of the α proton. Thus, the α proton of L-serine exchanges four times faster than that of L-alanine. It was not possible to measure α-proton exchange with L-homoserine because this amino acid undergoes a rapid γ-elimination reaction. However, the elimination reaction proceeds three and a half times faster than exchange of the α proton of L-α-aminobutyrate, suggesting that substitution of a proton by a hydroxyl group on the γ carbon of L-α-aminobutyrate increases the rate of α-proton abstraction by at least threefold. An approximately sixfold increase in the α-proton exchange rate is seen between L-norvaline and L-δ-hydroxy-α-aminovaleric acid. The increase in exchange of the α hydrogen of serine as compared with alanine may reflect in part a polar effect. However, polar effects are probably not important with amino acids containing -OH groups on the γ or δ positions. The enhanced exchange is not simply due to bulk effects because the exchange rates of alanine and α-aminobutyrate are the same and that of norvaline only slightly less. The enhanced

TABLE I: Rates of Exchange of α and β Protons of L-Amino Acids Catalyzed by δ-Cystathionase.[a]

Compound $RCHNH_3^+COO^-$ R =	K_I or K_m (mM)	α-Proton Exchange ($\mu M\ min^{-1}\ (\mu M\ enzyme)^{-1}$)	β-Proton Exchange Rate ($\mu M\ min^{-1}\ (\mu M\ enzyme)^{-1}$)	No. of β Protons Exchanging
Alanine $-CH_3$	1.3	162	154[b]	3
Serine[c] $-CH_2OH$	15 (K_m)	613		
α-Aminobutyrate $-CH_2CH_3$	3.1	191	178	2
Homoserine $-CH_2CH_2OH$	20 (K_m)	673[d]		
Norvaline	25	92	96	1
$-CH_2CH_2CH_3$			3.75	1
δ-Hydroxy-α-aminovalerate	34	595	402	1
$-CH_2CH_2CH_2OH$			31	1

[a] The rates of exchange for each compound were determined as described in the Materials and Methods section. Each value represents the average of two independent determinations. Each ^1H NMR experiment was performed using L-amino acid (100 mM), potassium phosphate buffer (50 mM, pH 7.0), and varying amounts of enzyme in a total volume of 0.5 ml of D_2O. [b] This represents exchange of a single β proton. Total loss of protons from the β position is 2.8× that from the α position. [c] α-Keto acid production via β elimination of -OH from serine occurs at a rate 10^3 slower than the γ-elimination reaction with homoserine. Under the conditions employed in this experiment, the rate of pyruvate production was negligible. No β-hydrogen exchange was detected. The K_m value for serine was determined in a separate experiment using a larger amount of enzyme. [d] This value represents the rate of conversion of homoserine to α-ketobutyrate. For each turnover, a single α and β proton will be removed.

exchange rates of the α hydrogen of L-amino acids possessing a hydroxyl group over those that do not is probably due to specific interaction of the hydroxyl group with a group on the enzyme. This interaction may lead to a more favorable positioning of the α-hydrogen relative to a base at the enzyme active site or may help to strain or distort the substrate and thereby promote catalysis. The latter behavior could represent an example of induced destabilization (Jencks, 1975).

It is interesting to note that, while the rate of α-proton exchange increases with the addition of a hydroxyl group (as compared with the unsubstituted amino acid), the dissociation constant (K_I) of the substrates increases. The increased binding energy which may be available from the presence of the hydroxyl function appears to result in rate enhancement, rather than in tighter binding (Jencks, 1969). The binding energy obtainable from the presence of a hydroxyl group could readily account for the rate increases seen with these compounds.

The results reported here can best be interpreted if one assumes an active site for γ-cystathionase with two areas of substrate–enzyme interaction. The major site of interaction contains pyridoxal 5′-phosphate and the base or bases involved in α- and β-proton exchange. Another site contains the base which must normally facilitate elimination of the γ substituent. Interaction of the compounds at this site is responsible for (a) the appearance of selectivity in the exchange of β protons in going from L-α-aminobutyrate to L-norvaline and (b) the increase in the α-proton exchange rate when a terminal proton at the β, γ, or δ carbon is replaced by a hydroxyl group.

These data can be compared with the results obtained with two other pyridoxal phosphate containing enzymes where the rates of α- and β-proton exchange have been studied. An enzyme of similar catalytic capabilities, cystathionine γ-synthase, normally catalyzes a γ-substitution reaction, but will also catalyze γ-elimination reactions when the second substrate is absent (Guggenheim and Flavin, 1969). It has been found that the enzyme catalyzes stereospecific β-proton exchange from L-α-aminobutyrate. Examination of tritium incorporation into this position from the solvent indicated that exchange of the second β proton occurs over 100 times more slowly than the first (Posner and Flavin, 1972). The active site of this enzyme must hold α-aminobutyrate in a more constrained fashion than does γ-cystathionase, where no selectivity in the exchange of these two protons is observed. Presumably, the γ carbon of the β–γ unsaturated enzyme intermediate formed with cystathionine γ-synthase must be held in such a way as to facilitate attack by the incoming nucleophile, whereas no such positioning is required in the reaction with γ-cystathionase.

Another pyridoxal enzyme, glutamate-alanine transaminase, also catalyzes the incorporation of solvent-derived deuterium into the α and β positions of L-alanine (Oshima and Tamiya, 1959), although β-proton removal is not required in the normal catalytic reaction. Recent ^1H NMR studies (Cooper, 1976) indicate that, during the course of this reaction, alanine species deuterated solely in the α position or solely in the β position were detectable, suggesting considerable conservation of the α proton during the exchange reactions. In the γ-cystathionase-catalyzed exchange reaction with L-alanine, however, alanine species containing deuterium solely in the β position could not be detected. These findings suggest that for the γ-cystathionase reaction there is little if any conservation of the α proton during each turnover, a fact in keeping with a fairly open active site, with little protection from solvent.

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Quantitative Analysis of the Digestion of Yeast Chromatin by Staphylococcal Nuclease†

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ABSTRACT: The DNA in intranuclear yeast chromatin is protected from rapid staphylococcal nuclease degradation so as to yield an oligomeric series of DNA sizes. The course of production and disappearance of the various oligomers agrees quantitatively with a theory of random cleavage by the enzyme at uniformly susceptible sites. The sizes of the oligomers are integral repeats of a basic size, about 160 base pairs, and 80–90% of the yeast genome is involved in this repeating structure. Within this repeat there exists a 140 base pair core of more nuclease-resistant DNA. During the course of digestion, the sizes of the oligomers decrease continuously. The widths of the distribution of DNA sizes increase in the order: monomer (1 × repeat size, half width = 5–7 base pairs) < dimer (2 × repeat size, half width = 30 base pairs) < trimer (3 × repeat size, half width = 40–45 base pairs). The yeast genome thus seems to have variable spacing of the nuclease-resistant cores, to produce the average repeat size of about 160 base pairs. Also, the presence of more than one species of monomer and dimer at certain times of digestion suggests a possible heterogeneity in the subunit structure.

When chromatins from higher eukaryotes are digested by staphylococcal nuclease, the DNA is cleaved into a series of size classes which represent integral multiples of a basic size (cf. Hewish and Burgoyne, 1973), about 200 base pairs (Noll, 1974; Shaw et al., 1976). Approximately half of the DNA in chromatin is ultimately accessible to digestion with staphylococcal nuclease (Clark and Felsenfeld, 1971) and at this limit consists of a distinctive distribution of DNA sizes smaller than the basic repeat size (Axel et al., 1974).

We have previously shown that nuclease digestion of baker's yeast chromatin produces integral DNA size classes as in more complicated eukaryotes, but that the repeat size is smaller (Lohr and Van Holde, 1975). In this study we have followed the time course of nuclease digestion of yeast chromatin using several confluent analyses: accurate sizing of the nuclease-resistant size classes of yeast DNA using PM2 DNA restriction fragments as internal standards; study of the acid solubility to assess susceptibility to nuclease; quantitative analysis of the size classes to assess the amount of the genome involved in the repeating structure; comparison with a random-hit theory of nuclease action. We have also obtained evidence for possible nonuniformity of the repeating structure.

Materials and Methods

Growth of Yeast and Isolation of Nuclei. Baker's yeast, strain Y55, was grown in 1% yeast extract–2% Bactopeptone–2% glucose to a density of about 5×10^7 cells/ml. Cells were harvested, spheroplasts made according to Cabib (1971), and nuclei isolated essentially according to Wintersberger et al. (1973), procedure b, with minor modifications in the HM step. These are: use of 7% Ficoll instead of 5% polyvinylpyrrolidone according to Sajdel-Sulkowska et al. (1974); three "slow" spins at 7000, 6500, and then 6000 rpm in a Sorvall SS34 rotor; a "fast" spin at 20 000*g*, 25 min.

Nuclease Digestion and DNA Isolation. Nuclei were resuspended in 1 M sorbitol, 0.5 mM Ca^{2+}, or 0.05 mM Ca^{2+}, pH 6.3, at a concentration of about 150 μg/ml DNA (about 3×10^9 nuclei/ml). Digestion was done at concentrations of 130 to 180 U/ml staphylococcal nuclease (Worthington Biochemical Corp.) at 37 °C. Samples were removed at designated times and reaction was stopped by addition of cold 8 mM Tris[1]–35 mM Na_2EDTA (pH 6.2) to a final concentration of 11 mM Na_2EDTA. Samples were put immediately on ice and divided into two aliquots for subsequent analysis. (a) An aliquot of each sample was made to 0.3 N in $HClO_4$–0.3 M in NaCl on ice, shaken for 10 min, and centrifuged at 15 000 *g* for 15 min. The pellet was washed once with 0.3 N $HClO_4$–0.3 M NaCl and recentrifuged. The supernatants constituted the acid-soluble and the pellet constituted the acid-precipitable samples, respectively, for DNA analysis. (b) The rest of the digestion sample was made to 100 μg/ml in pancreatic RNase (Schwarz/Mann), 100 U/ml in T_1 RNase (Sigma), and 0.1 U/ml in T_2 RNase (Sigma) and incubated at 37 °C for 15 min. One-tenth volume of 21% Sarkosyl, 1.6 M NaCl, and 50 μl of 10 mg/ml Pronase per ml of sample were added and the samples incubated overnight at 37 °C. DNA was extracted by a modified Marmur procedure (Britten et al., 1974) and pre-

† From the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331. *Received May 25, 1976.* Supported in part by grants from the National Science Foundation (PCM75-23461, PCM75-19420, and BMS75-07746). A preliminary report of some of these results was presented at the Biophysical Society Meeting, Seattle, Washington, February, 1976.

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[1] Abbreviations used: bp, base pairs of DNA; DABA, diaminobenzoic acid dihydrochloride; Hae III, endonuclease R *Hae* III obtained from *Haemophilus aegyptius;* PM2, *Pseudomonas* bacteriophage 2; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.