RESOLUTION OF THE DIASTEREOISOMERS GENERATED BY THE CHEMICAL REDUCTION OF THE PYRUVATE-LYSINE SCHIFF'S BASE

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Summary - Borotritide reduction of a pyruvate L-lysine Schiff's base in solution produces an essentially random distribution of the diastereoisomeric pair N^6-{(1R)-1-[1-^{3}H] and N^6-{(1S)-1-[1-^{3}H]carboxyethyl}-L-lysine. Following formation of the di-n-butyl ester of the above diastereoisomers, turnover by leucine aminopeptidase gave results consistent with converting half of the radioactivity back to the free acid, and half to a monoester. Based upon the stereopreference and esterase activity of leucine aminopeptidase, the data suggest the conversion of the di-ester to N^6-{(1R)-1-[1-^{3}H]n-butylcarboxyethyl}-L-lysine monoester and N^6-{(1S)-1-[1-^{3}H]carboxyethyl}-L-lysine free acid which, followed by electrophoresis, resolves the diastereoisomers. This, then, provides a method for determining the stereochemistry of hydride anion attack at the ketimine carbon of the Schiff's base, revealing the absolute configuration of ES complexes as viewed from solvent among selected pyruvate enzymes.

INTRODUCTION

A number of Schiff's base-mediated pyruvate lyases have been studied as models of the aldolase reaction. Among these are 2-keto-3-deoxygluconate-6-P aldolase of Pseudomonas putida (1-4), 2-keto-3-deoxygalactonate-6-P aldolase of Ps. saccharophilia (5,6), 2-keto-4-hydroxyglutarate aldolase of both liver (7,8) and Escherichia coli (9), and N-acetylneuraminic acid aldolase of Clostridium perfringens (10). Among these enzymes, a protonated Schiff's base (ketimine) nitrogen is viewed as the sink directing electron flow resulting in catalysis (1). Presumptive evidence for Schiff's base formation at the catalytic site is the substrate (pyruvate)-dependent reductive inactivation of the enzyme. As a result of those cases which have been closely examined (7-11), it is now generalized that the Schiff's base results from reaction between the carbonyl carbon of pyruvate and the ε-amino group of an active site lysine. Chemical reduction of the ketimine carbon, then, generates a secondary amine, inacti-

vating the enzyme with concommitant incorporation of integral amounts of pyruvate (substrate), to form a unique amino acid in hydrolysates, viz. N^6-(1-carboxyethyl)-L-lysine.

A number of experiments have been carried out attempting to elucidate three-dimensional aspects of pyruvate aldolase catalysis (2,3,5,6,12). These studies have shown that the condensation between pyruvate and its acceptor aldehyde goes with retention of configuration at C-3 (3,5,12). As a consequence, among the four Schiff's base enzymes investigated, it is concluded that the aldehyde as well as the exchanging protons (3) must approach the same face of the bound trigonal pyruvate-lysyl eneamine. Further work has shown that two enzymes which must, respectively, orient opposite faces of the attacking aldehyde (3,5) have the same stereochemistry for turnover of a pyruvate analog prochiral at C-3 (6,13).

A key question leading to an overall view of the stereochemistry of reactions catalyzed by these enzymes is the absolute configuration of the pyruvate-lysine Schiff's base as viewed from solvent. Stereochemical details of Schiff's base reduction are shown in Fig. 1 where the ε-amino group of protein-bound lysine is depicted as Protein-NH_2. It is seen that if hydride anion attacks the si[1] face of the ketimine carbon with covalent bond formation, hydrolysates will yield N^6-[(1R)-1-carboxyethyl]-L-lysine while hydride attack at the re face yields N^6-[(1S)-1-carboxyethyl]-L-lysine. This pair could also be viewed as D-ala_α-NH_ε-L-lys and L-ala_α-NH_ε-L-lys, respectively, where the amino group of ala is a secondary amine nitrogen. Resolution of these diastereoisomers would reveal the stereochemistry of hydride ion attack, allowing one to deduce the absolute configuration of the Schiff's base as viewed from solvent.

Resolution of the diastereoisomers might be accomplished by taking advantage of the known stereopreference and esterase activity of leucine aminopeptidase (14-16) which can turnover esters of both L-lysine and the secondary

[1] The si face of the ketimine carbon is that described by the anticlockwise orientation of ligands substituted by imine nitrogen, carboxyl and methyl groups.

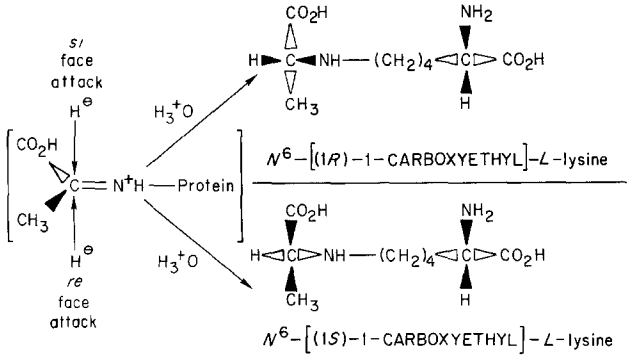

Fig. 1. Stereochemical events in the reduction of a pyruvate-lysine azomethine.

amino acid L-proline (16). By analogy, then, the peptidase might turnover the substituted alanine moiety in the S, i.e., L-configuration but not that in the R, i.e., D-configuration. Consequently, the enzyme might convert the di-n-butyl ester of the carboxylethyl-L-lysine[2] diastereoisomers into N^6-[(1R)-1-n-butylcarboxyethyl]-L-lysine monoester and N^6-[(1S)-1-carboxyethyl]-L-lysine free acid. The separation of the monoester from the free acid could be readily accomplished. With this goal in mind, it was proposed to test the use of leucine aminopeptidase as the resolving agent for the diastereoisomers. The results of such experiments are reported in this paper.

MATERIALS AND METHODS

Authentic tritiated carboxyethyl-lysine was synthesized in the following manner. One equivalent (based on lysine) of L-polylysine was reacted with five equivalents of ethylpyruvate at pH 8 to allow Schiff's base formation. This was then reduced with ten equivalents of $NaBH_4(^3H)$ to form the ethyl ester of tritiated carboxyethyl(poly)-L-lysine which on hydrolysis would yield tritiated carboxyethyl-L-lysine. Since, during reduction in solution, hydride anion would randomly attack either diastereotopic face of the Schiff's base carbon (see Fig.

[2] Abbreviation: Carboxyethyl-lysine, N^6-(1-carboxyethyl)lysine.

1), one expects synthesis of both diastereoisomers in about random distribution, viz. N^6-{(1S)-1-[1-^{3}H] and N^6-{(1R)-1-[1-^{3}H]carboxyethyl}-L-lysine. After synthesis, the derivatized polylysine was adjusted to pH 4 with acetic acid to destroy residual borotritide, dialyzed against water and then hydrolyzed 48 hr in 6 N HCl at 110° in a vial hermetically sealed in vacuo. The HCl was then removed in vacuo. The hydrolysate, in water, was transferred to a 2.5 x 20 cm column of Dowex-50 (H^+) equilibrated against 0.1 N HCl. The column was washed with 0.1 N HCl to elute the residual tritiated-lactate resulting from reduction of excess ethylpyruvate during synthesis. Then, the column was washed with 0.1 M pyridine. Two radioactive peaks were observed, one at and the other following the pyridine front. Tritiated carboxyethyl-lysine was the latter peak. The product was obtained in 60% yield (based on lysine), and the material used in this study had a specific activity of 3.0 x 10^6 cpm/μmole, assuming carboxyethyl-lysine has 1.07 the extinction of leucine in the ninhydrin assay (17). The product of this synthesis was found to exhibit both ninhydrin reactivity and radioactivity coincident with that of carboxyethyl-lysine isolated after the reductive inactivation of the Schiff's base mediated (11) 2-keto-3-deoxy-gluconate-6-P-aldolase by ^{14}C-pyruvate and borohydride, followed by hydrolysis.

The tritiated product, in 25 mM NH_4HCO_3 (pH 8), was further purified by chromatography on a 3 x 15 cm column of DEAE-Sephadex A-25 using a linear 25 mM - 250 mM NH_4HCO_3 gradient. The pooled peak tubes containing the bulk of the tritium were repeatedly dried in vacuo, after dissolving the residue in water, to remove the bulk of NH_4HCO_3.

Radioactive carboxyethyl-lysine was converted to its di-ester by suspending the free acid in 2 ml of anhydrous n-butyl alcohol and bubbling HCl gas through the reaction mixture (18) for 2 hr. Solvents were then removed in vacuo.

Leucine aminopeptidase was purchased from Worthington Biochemical Co. and had a specific activity of 128.5 units per mg protein. Poly-L-lysine was purchased from Sigma Chemical Co., ethylpyruvate from K and K Laboratories, Inc., and $NaBH_4$(^{3}H) from New England Nuclear Corp. Radioactivity was determined by

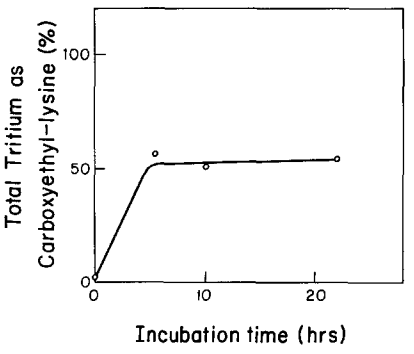

Fig. 2. Leucine aminopeptidase turnover of the di-n-butyl ester of (1RS)-carboxyethyl-L-lysine. The incubation mixture consisted of di-ester (400,000 cpm), 10 mM $MnCl_2$, 50 mM phosphate pH 7.4, and 0.35 mg of leucine aminopeptidase in a total volume of 0.5 ml.

liquid scintillation counting in an ethanol-toluene cocktail. Counting was corrected for quench. Electrophoresis was carried out using thin layer (250 or 500 μ) cellulose plates. Radioactivity was located by cutting out and transferring 1 cm sections of the cellulose to scintillation vials and counting.

RESULTS AND DISCUSSION

Turnover of n-butylcarboxyethyl-L-lysine di-ester by leucine aminopeptidase - The results of incubation of the mixed diastereoisomers of the di-n-butyl ester of tritiated carboxyethyl-L-lysine with leucine aminopeptidase are shown in Fig. 2. In this experiment carboxyethyl-lysine is monitored as radioactivity washing through a 0.75 x 4 cm column of Dowex-50 (Na) at pH 5. A sample was applied to and then washed through the column with 5 ml of 10 mM sodium acetate pH 5.0. A portion of the wash was counted. The data show that the di-n-butyl ester which was hydrolyzed to free carboxyethyl-lysine during peptidase incubation equilibrated at 50% turnover. In a control without enzyme, free carboxyethyl-lysine accumulated to < 2% over the course of the experiment. Since the synthesis of tritiated carboxyethyl-L-lysine is expected to form the

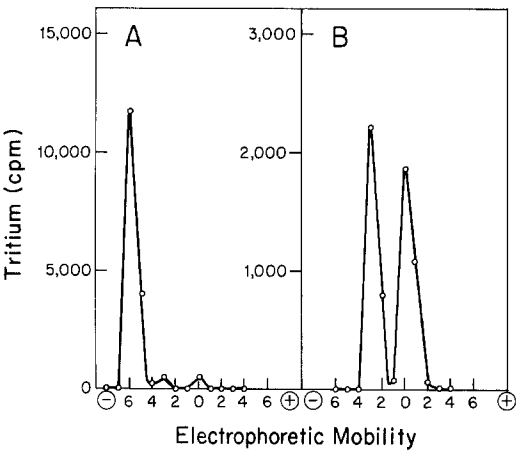

Fig. 3. Paper electrophoresis of the products of leucine aminopeptidase turnover of the n-butyl-di-ester of tritiated carboxyethyl-lysine. Electrophoresis was at 600 V for 30 min in a buffer composed of (in volumes) butanol 2, pyridine 1, acetic acid 1 and water 36 at pH 4.75.

(1R) and (1S) diastereoisomers in random distribution (Methods), and from the known stereospecificity of leucine aminopeptidase for hydrolyzing esters of L-amino acids (1-3), we can conclude the free acid produced is N^6-{(1S)-1-[1-^{3}H] carboxyethyl}-L-lysine, while its (partially) esterified (1R),L-diastereoisomer is retained on the Dowex-50 column. This conclusion is supported by paper electrophoretic examination of the reaction mixture. These results are in Fig. 3. The electrophoretic behavior of authentic di-n-butyl ester is seen in Fig. 3A where the predominant peak migrates 6 cm towards the cathode in 30 min at 600 V and at pH 4.75. Associated with this is a minor component at the origin consistent with the electrophoretic behavior of carboxyethyl-lysine which behaves as isoelectric at the pH used, and a second minor component migrating 3 cm towards the cathode. This latter component could be a mixed monobutyl ester produced by spontaneous deesterification, which could also produce the trace of ^{3}H-free acid observed. It is seen in Fig. 3B, after 22 hr incubation with leucine aminopeptidase, that the radioactivity of the di-ester had been converted to two com-

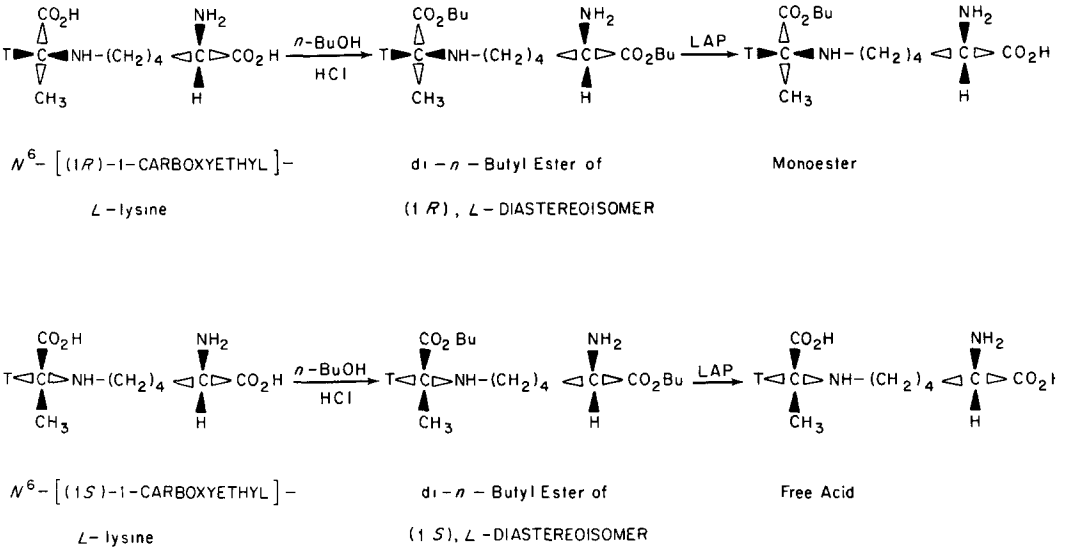

Fig. 4. Scheme for conversion of the N^6-{(1RS)-1-[1-^{3}H] carboxyethyl}-L-lysine diastereoisomers to their corresponding di-n-butyl esters which are then resolved by leucine aminopeptidase (LAP). In the Figure tritium is shown as T.

pounds of equal radioactivity. At the origin is found 2900 cpm exhibiting the non-mobility of free carboxyethyl-lysine. The other peak (3000 cpm) exhibits migration towards the cathode slower than that of the di-n-butyl ester, consistent with this new radioactive component being a monobutyl ester of carboxyethyl-lysine. It is assumed that this monobutyl ester was retained on the Dowex-50 column in the experiment shown in Fig. 1. Similar results were observed by electrophoretic examination of the incubation at 5.5 and 10 hrs (Fig. 2). The distribution of radioactivity between these two components is affected neither by extending the incubation time nor by increasing the leucine aminopeptidase concentration. Subsequent to the elution of the radioactivity, presumed to be the monoester, from cellulose and hydrolysis by acid, all of the radioactivity exhibited the electrophoretic behavior of carboxyethyl-lysine. Further, elution of the isoelectric component (Fig. 3B) followed by its reester-

ification and incubation with leucine aminopeptidase showed that more than 90% of the radioactivity was hydrolyzed back to the free-acid by the enzyme. Consequently, the derivatization step does not perturb the configuration of the two chiral centers of (1S)-carboxyethyl-L-lysine. These data are consistent with leucine aminopeptidase converting the di-n-butyl ester of tritiated (1RS)-carboxyethyl-L-lysine into (1S)-carboxyethyl-L-lysine free acid and (1R)-carboxyethyl-L-lysine monoester as shown in Fig. 4, which followed by electrophoresis resolves the two diastereoisomers produced by the chemical reduction of a pyruvate-L-lysine-Schiff's base in solution.

The existence of a technique for resolving the diastereoisomers of chemically reduced pyruvate-lysine Schiff's bases will make it possible to resolve the stereochemistry of borohydride (or its analogs) reduction of selected enzyme-substrate complexes, thereby revealing their absolute configuration as viewed from solvent. In addition, by taking advantage of the ability of enzymes such as D- or L-amino acid oxidase to both turnover a variety of amino acids, and to form N^6-lysyl secondary amines *via* attack of the corresponding product imino acids by an ε-amino of protein-bound lysine and borohydride reduction (17,19), one might "custom" synthesize N^6-analogs of carboxyethyl-lysine. Using these derivatives as model compounds, one could then extend stereochemical studies to the reduction of complexes other than the pyruvate-lysine azomethine. Consequently, methodology is now available promising a view of the absolute configuration of a number of Schiff's base-mediated ES complexes.

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