Protein Carboxyl Methyltransferase Facilitates Conversion of Atypical L-Isosaartyl Peptides to Normal L-Aspartryl Peptides*

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Recent studies with adrenocorticotropic hormone (ACTH) and related synthetic peptides indicate that the protein carboxyl methyltransferases from bovine brain have this unusual specificity appear to be present in a wide range of cell types including rat PC12 cells, murine 70Z/3 lymphoma cells, Xenopus oocytes, and the bacterium Salmonella typhimurium (O'Connor and Clarke, 1985). This class of protein methyltransferase is distinct from the methyltransferase which modifies the y-carboxy group of normal glutamyl residues of methyl-accepting proteins of chemotactic bacteria (Clarke, 1985).

At physiological pH and temperature, the ACTH iso-Asp methyl ester formed by the enzyme undergoes a rapid (t_m = 4–8 min), spontaneous demethylation resulting in formation of an aspartyl cyclic imide (Johnson and Aswad, 1985; Murray and Clarke, 1986). The imide subsequently becomes hydrolyzed (t_m = 3–4.2 h) to generate a mixture of isoaspartyl (70–80%) and aspartyl (20–30%) peptides. The overall reaction pathway is shown in Fig. 1. This scheme predicts that prolonged incubation of an isoaspartyl peptide with the methyltransferase and AdoMet should lead to extensive conversion to the normal aspartate-containing form, since the isopeptide bond will be continually recycled through the pathway by the methyltransferase. The ability of protein carboxyl methyltransferase to facilitate conversion of atypical isoaspartyl peptides to normal aspartyl peptides has important implications for the function of this unusual enzyme. In the present paper, we have investigated the effects of incubating three unrelated synthetic isoaspartyl peptides with bovine brain protein carboxyl methyltransferase and AdoMet for periods up to 48 h under conditions of physiological pH and temperature. A preliminary report of this work has been published previously in abstract form (Aswad et al., 1986).

**Experimental Procedures**

RESULTS

General Strategy for Demonstrating Conversion of L-Isosartryl Peptides to Normal L-Aspartryl Peptides—Attempts to demonstrate methylation-dependent conversion of L-Isosartryl peptide residues to normal L-Asp residues were made with three unrelated synthetic L-Isosartryl-containing peptides. We compared these peptides in order to determine possible effects of varying amino acid sequence on the specificity of protein carboxyl methyltransferase for L-Isosartryl and on the overall rate of...
conversion of L-iso-Asp to L-Asp. The structures of the L-iso-Asp peptides and their methylation kinetic constants are shown in Table I.

The ACTH(22-27) peptide was chosen for study because the synthetic L-iso-Asp, L-Asp, and L-cyclic imide peptides were available and because a considerable amount of information on the kinetics of the methylation and demethylation of the L-iso-Asp form had already been obtained in previous studies (Murray and Clarke, 1984; Aswad, 1984a; Murray and Clarke, 1986). Speract was chosen because the L-iso-Asp and L-Asp forms can be prepared easily from the commercially available native peptide (see "Appendix") and because it has a sequence distinct from that of the ACTH peptide except for the iso-Asp-Gly linkage. The LDH peptide was chosen because it was available in both the L-iso-Asp and L-Asp forms from a previous study (Aswad et al., 1987), because it has a low Kₘ for the methyltransferase, and because it provides additional information regarding sequence dependence, containing Asp or iso-Asp in a carboxyl linkage with Ser rather than Gly.

The general approach used for test for conversion of L-iso-Asp to L-Asp was to incubate pure L-iso-Asp peptides (6–15 μM) with purified methyltransferase (2.5 μM) and excess AdoMet (200 μM) at 37 °C, pH 7.4, for varying periods of time, up to 48 h. Samples of the reaction mixtures were subjected to reversed-phase HPLC to separate the various intermediates and end products. After determining the identity of the HPLC peaks produced, we plotted the time course of each intermediate or product as a percentage of total peptide. In the following section, we describe in detail our results with the LDH L-iso-Asp peptide because it displayed the most rapid and extensive conversion of the three peptides. We then present a summary of our results with the speract and ACTH(22-27) L-iso-Asp peptides.

Conversion of the L-iso-Asp LDH Peptide to a Normal, L-Asp Peptide—Fig. 2 shows sample HPLC profiles obtained after the L-iso-Asp LDH peptide (6 μM) was incubated for various times at pH 7.4, 37 °C with 2.5 μM methyltransferase and 200 μM AdoMet. Peak II, which became detectable after an hour of incubation, was by far the predominant form of the peptide present after 24 h. Because this peak comigrated with the authentic L-Asp-containing LDH peptide, it seemed that extensive conversion of L-iso-Asp to L-Asp had occurred.

In order to verify the identity of peak II, it was collected from a 24-h reaction mixture and subjected to proteolysis using two proteases of differing specificity, pepsin and the Staphylococcus aureus V8 protease, endoproteinase Glu-C. For comparison, authentic L-iso-Asp- and L-Asp-containing forms of the LDH peptide were incubated with the proteases under identical conditions. Each of the proteolysis mixtures was then subjected to reversed-phase HPLC in order to obtain a profile of the fragments produced. The results obtained using pepsin are shown in Fig. 3. The profiles obtained for peak II and for the authentic L-Asp peptide were virtually the same. In contrast, the L-iso-Asp peptide yielded a proteolytic profile clearly different from that of the L-Asp peptide. Similar results were obtained using endoproteinase Glu-C (not shown). These results indicate that peak II is indeed the L-Asp-containing form of the LDH peptide and hence that carboxyl methylation can convert L-iso-Asp residues to normal L-Asp.

Peak I in Fig. 2 consists of a mixture of two forms of LDH(231-242), the methylated and the cyclic imide forms. As shown elsewhere (Aswad et al., 1987), peak I at early times (~10 min) consists mainly of the methylated isopeptide, since it alone carries the ³H label and the labeling is nearly stoichiometric. As the time of reaction increases, the amount of radiolabel relative to UV-absorbing material in the peak steadily decreases, indicating the presence of another form of the peptide in peak I. Because the hydrolysis of aspartyl carboxyl esters in a variety of Asp-X linkages, including Asp-Ser, are known to generate cyclic imides (Battersby and Robinson, 1955; Bernhard et al., 1962; Bodanszky and Kwei, 1978; Ondetti et al., 1968; Johnson and Aswad, 1985; Murray and Clarke, 1986), it seemed likely that the peptide remaining in peak I after the disappearance of methyl groups was a cyclic imide-containing LDH peptide. The peak I peptide was collected from a 1-h methylation reaction and incubated at

| Peptide                  | Kₘ  | Vₘax |
|-------------------------|-----|------|
| [iso-Asp]³H-LDH(231-242)| 0.54| 22.0 |
| Lys-Gln-Val-val-iso-Asp-Ser-Ala-Tyr-Glu-Val-Ile-Lys | 3.2 | 10.5 |
| Lys-Val-iso-Asp-Ser-Ala-Tyr-Glu-Val-Ile-Lys | 6.3 | 10.7 |

**Note:** Values for the LDH(231-242) isoepitope are from Aswad et al. (1987); values for the speract isoepitope are from this study (see "Appendix"); and values for the ACTH(22-27) isoepitope are from Murray and Clarke (1984). All amino acids are the L-isomer.
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peptide was omitted which water was substituted for peptide. The peaks at the "Experimental Procedures." The and were then subjected to reversed-phase HPLC as described under "Experimental Procedures." Peak I coeluted with to that of the unincubated L-iso-Asp peptide. If the L-iso-Asp peptide was omitted from the reaction mixture, no peaks were observed in the retention range shown here.

FIG. 2. Reversed-phase HPLC of LDH L-iso-Asp peptide methylation reactions. Incubations and HPLC were performed as described under "Experimental Procedures." Peak II coeluted with the authentic L-Asp-containing LDH peptide. HPLC profiles of 24-h reaction mixtures lacking methyltransferase or AdoMet were identical to that of the unincubated L-iso-Asp peptide. If the L-iso-Asp peptide was omitted from the reaction mixture, no peaks were observed in the retention range shown here.

FIG. 3. Proteolytic mapping of LDH peptides using pepsin. LDH peptides (25 mM) were incubated with 5 mg/ml pepsin at pH 2 and were then subjected to reversed-phase HPLC as described under "Experimental Procedures." The bottom panel is a protease blank, in which water was substituted for peptide. The peaks at the far right of the top three panels elute at the same retention time as the undigested peptides. The correspondence between the profiles obtained with peak II and the L-Asp peptide strongly suggests that these two peptides are identical.

37 °C in 50 mM K-HEPES (pH 7.4) for varying periods of time, followed by reversed-phase HPLC. Cyclic imides become hydrolyzed with a half-life of several hours under these conditions into a mixture of L-iso-Asp and L-Asp peptides, the iso-Asp form predominating (Sondheimer and Holley, 1954; Batterby and Robinson, 1955; Bodanszky and Kwei, 1978; Murray and Clarke, 1984, 1986; Blodgett et al., 1985; Johnson and Aswad, 1985). The peak I peptide became hydrolyzed with a t1/2 of 3.2 h and resulted in a 5.3:1 ratio of isoaspartyl peptide to aspartyl peptide. Thus, it appeared that the conversion of L-iso-Asp to L-Asp in this peptide occurred via the mechanism outlined in Fig. 1.

After a 24-h methylation of the L-iso-Asp LDH peptide (Fig. 2), it was clear that, in addition to the L-Asp peptide, another form of the peptide (peak III) had accumulated. Although peak III appeared to be a minor product, it seemed important to determine the nature of this peptide, which may represent the product of a physiologically significant side reaction. It has been proposed recently that the formation of a cyclic imide could greatly accelerate racemization at the aspartyl α-carbon (McFadden and Clarke, 1982; Clarke, 1985). This racemization reaction would ultimately lead to the formation of a D-iso-Asp peptide as the major product of D-cyclic imide hydrolysis (Fig. 1). Peak III was therefore collected from a 24-h methylation reaction and analyzed for the presence of D-Asp after acid hydrolysis as described under “Experimental Procedures.” D-Asp was found to comprise 75.3% ± 3.8% of the aspartate released. The peptide was also subjected to thin layer electrophoresis at pH 3.5, a method which allows one to distinguish between the presence of iso-Asp and Asp on the basis of the pK_a of the free carboxyl group (see “Experimental Procedures”). It coeluted with the authentic L-iso-Asp LDH peptide, indicating that the peptide contained D-iso-Asp rather than D-Asp.

The collected D-iso-Asp LDH peptide was tested for its ability to accept methyl groups from the methyltransferase under conditions which result in stoichiometric methylation of the L-iso-Asp peptide. These conditions included 10 μM peptide, 4 μM methyltransferase, 200 μM [methyl-3H]AdoMet and were carried out at pH 6 and 30 °C for 1 h. The methylation barely exceeded 0.10 mol of methyl group/mol of peptide, indicating that the D-iso-Asp LDH peptide is, at best, a very poor methyl acceptor. These results provide further support for the stereospecificity of protein carboxyl methyltransferase previously indicated in studies on the methylation of ACTH(22–27) analogues (Murray and Clarke, 1984).

Because significant amounts of D-iso-Asp peptide were formed in the conversion reactions, it was important to determine whether peak II, previously identified as an L-Asp-containing peptide by proteolytic mapping, contained significant amounts of D-Asp. The peak therefore was collected from a 24-h methylation and analyzed in the same manner used for peak III. This hydrolyzed peptide was found to contain 93.8% ± 1.0% L-Asp, indicating that very little D-Asp peptide had been formed.

Thus, the major products of the extended methylation of the LDH L-iso-Asp peptide are the corresponding L-Asp- and D-iso-Asp-containing peptides, which are formed in a reaction involving the L-iso-Asp α-carboxyl methyl ester and a cyclic imide as intermediates (Fig. 1). The kinetics of the conversion are shown in Fig. 4. The reaction progressed steadily, approaching completion by 24 h, at which point the L-Asp peptide represented 80% of the total peptide and the D-iso-Asp peptide comprised 13%, the remainder being present as cyclic imide or methyl ester.

Conversion of L-Iso-Asp Speract to L-Asp Speract—[L-Iso-Asp]Speract (15 μM) was incubated with methyltransferase and AdoMet in the same manner described for the LDH peptide, and reaction mixtures of varying duration were subjected to reversed-phase HPLC. Fig. 5A shows HPLC profiles obtained upon injection of purified L-iso-Asp speract (top panel) and upon injection of a 48-h methylation of the L-iso-Asp peptide (bottom panel). The major product of the reaction coeluted with the authentic L-Asp peptide. Further support for the identity of this peak was obtained by thin layer electrophoresis at pH 3.5 and by determining the stereocon-
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At early time points in the conversion of L-iso-Asp speract, two additional peaks representing significant proportion of the total peptide were observed (retention times given in Table II). Using the same strategy outlined for the LDH peptide, it was determined that one of the peaks contained the L-iso-Asp α-carboxyl methyl ester and the other contained the corresponding cyclic imide. The minor peak in Fig. 5A is the cyclic imide-containing peptide which is still present after 48 h of reaction. Upon incubation at pH 7.4, 37°C, the isolated speract cyclic imide became hydrolyzed with a t½ of 4.4 h into a 5.7:1 ratio of iso-Asp speract and Asp speract.

Thus, the conversion of L-iso-Asp speract generated the same intermediates and products that were formed in the conversion of the L-iso-Asp LDH peptide. After 48 h, the speract conversion reaction resulted in 65% L-Asp peptide, 31% D-iso-Asp peptide, and 4% imide.

Conversion of the L-iso-Asp ACTH Peptide to an L-Asp Peptide—The [L-iso-Asp53]ACTH(22-27) peptide (15 μM) was incubated with methyltransferase and AdoMet as described for the LDH and speract peptides. Fig. 5B shows that a 48-h reaction produced an HPLC profile almost identical to that obtained with the iso-Asp speract peptide. The 22-min peak (70% of the total) was identified as the L-Asp53 peptide: it coeluted with authentic L-Asp peptide and the Asp was found to be 90.6% ± 1.1% in the L-enantiomer. The 20.5-min peak (26% of the total) was identified as a mixture of about 30% L-iso-Asp and 70% D-iso-Asp peptides. The small peak at 24.5 min (4% of the total) was identified as the imide based on its coelution with authentic cyclic imide and its rate of appearance and disappearance during early time points (not shown). Formation of a cyclic imide intermediate has been shown previously to occur subsequent to methylation of L-iso-Asp-containing forms of ACTH(22-27) (Murray and Clarke, 1986) and ACTH(1-39) (Johnson and Aswad, 1985).

Comparison of the Rates of Conversion of the Three L-iso-Asp Peptides to Normal, L-Asp Peptides—The rates of conversion of L-iso-Asp to L-Asp in each of the three peptides are compared in Fig. 6. The LDH peptide displayed the most rapid, as well as the most extensive, conversion. The ACTH L-iso-Asp peptide and L-iso-Asp speract had similar kinetics of conversion, the ACTH peptide conversion proceeding somewhat more rapidly than that of L-iso-Asp speract after 12 h of reaction.

DISCUSSION

Enzymatic carboxyl methylation of three unrelated L-isoaspartyl peptides has been shown in each case to promote
conversion of the atypical L-isoaspartyl residue to a normal L-aspartyl residue with a high degree of efficiency. Conversion occurs through the formation of isoaspartyl methyl ester and cyclic imide intermediates, supporting the mechanism proposed in Fig. 1 (Aswad, 1984a; Murray and Clarke, 1984, 1986; Johnson and Aswad, 1985). As a side reaction, slow racemization of the imide and subsequent hydrolysis of the d-imide causes the formation of significant amounts of D-isoaspartate-containing peptide.

Support for the completeness of the reaction scheme shown in Fig. 1 was obtained by modeling the conversion of the ACTH isopeptide (see "Appendix" for details). This peptide was chosen for modeling because the kinetic constants for each of the individual reactions in the pathway have been determined independently. Fig. 7 shows that the modeled time course of the conversion agrees well with the experimentally determined points. The conversion of L-iso-Asp to L-Asp fails to reach completion primarily because racemization of the imide intermediate occurs. Another factor is inactivation of the methyltransferase caused by accumulation of the product inhibitor AdoHcy and by depletion of AdoMet. Enzyme inactivation was a limitation of our in vitro analysis which presumably does not apply in vivo where AdoMet and AdoHcy levels should each remain near some regulated, steady state level. When enzyme inactivation was eliminated from the model, there was an additional 2% conversion to the imide form by 48 h. Racemization may also occur at a much greater rate than explained by this model.

The overall imide hydrolysis must be sufficiently rapid to approximate the loss of methyltransferase activity which occurred in Fig. 6. Two-fold changes in $K_n$ or $k_i$ (ester breakdown) have relatively little effect on conversion efficiency. A 2-fold change in the $V_m$ (not shown) also has negligible effect. Decreasing $k_1$ (l-imide to d-imide) by a factor of 2 increases conversion slightly from 53% to about 57%. Variations in the rate of l-imide hydrolysis have, by far, the most profound effect. Increasing the rate of hydrolysis by a factor of 2 increases L-Asp formation from 53 to 74%. This effect is expected, since L-imide hydrolysis is the rate-limiting step in the overall conversion cycle. The ratio of L-Asp/L-iso-Asp formed from L-imide hydrolysis (not shown in Fig. 8) also has a very large influence on conversion efficiency. When this ratio is changed from 1/2 (the value for the ACTH imide) to 1/5,3 (the value for the LDH imide) conversion drops from 53 to 39%. It appears from this analysis that the enhanced conversion of iso-Asp to Asp observed with the LDH peptide is probably due to a greater instability of the imide form of this peptide. The overall imide hydrolysis must be sufficiently rapid to overcome the unfavorable Asp/iso-Asp ratio produced by imide breakdown. Because the LDH cyclic imide and iso-Asp methyl ester forms could not be separated by HPLC, we have

**Fig. 7. Modeled kinetics of the methylation-dependent conversion of the L-iso-Asp ACTH peptide.** The kinetics of the conversion of this isopeptide were modeled according to the scheme in Fig. 1 using the independently determined rate constants given in Table III (see "Appendix"). The model also included an equation approximating the loss of methyltransferase activity which occurred during the incubations (see "Appendix"). The curves represent the results of the reaction modeling. The points are the means of duplicate experimental determinations showing the percentage of peptide present in each form as judged by reversed-phase HPLC analysis performed as in Fig. 5B. Curves and points both indicate mixtures of D- and L-stereoisomers. At the 48-h time point of the modeled reaction, 93% of the Asp peptide was in the L-form, as was 84% of the cyclic imide. At the same time point, 86% of the iso-Asp peptide was present as the D-stereoisomer.

**Fig. 8. Effect of varying individual rate constants on the amount of L-Asp produced after 24 h of a modeled reaction.** Values for $K_n$ (C), the methyl ester hydrolysis rate constant ($k_1$, ○), the L-imide → D-imide rate constant ($k_2$, □), and the L-imide hydrolysis rate constant ($k_3$, △) were individually varied in the reaction model over a 4-fold range centered around the value of the corresponding rate constant which had been determined for the ACTH(22-27) isopeptide. Throughout these modeled reactions, the methyltransferase activity was held constant. The abscissa label (k/k') indicates the ratio of the rate constant chosen for the calculation (k) to the corresponding rate constant observed for the ACTH peptide (k'). The latter values are taken from Table III (see "Appendix"). Symbols indicate the values of log(k/k') used in the model. For each modeled rate constant, the predicted amount of L-Asp peptide present at 24 h is shown on the ordinate as the percentage of L-iso-Asp starting material. The greater slope observed upon varying the L-imide hydrolysis rate constant indicates that it is a major factor in determining the rate of conversion of L-iso-Asp to L-Asp.

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The fact that only the L-iso-Asp form of this peptide is methylated adds further evidence that the eucaryotic protein carboxyl methyltransferase modifies L-isoaspartyl residues and not normal aspartyl residues. More recently, McMullen and Clarke (1987) have shown that protein carboxyl methyltransferase from bovine erythrocytes promotes conversion of L-isoaspartyl tetragastrin (Thr-Met-iso-Asp-Phe-NH₂) to the L-aspartyl form.

The LDH isopeptide exhibited a significantly faster conversion to the L-Asp form than did the other two isopeptides. We have used our kinetic model to assess the possible sources of this enhancement. Fig. 8 shows how independently varying several of the kinetic constants for the ACTH isopeptide conversion affects the amount of L-Asp peptide formed by 24 h. Two-fold changes in $K_n$ or $k_i$ (ester breakdown) have relatively little effect on conversion efficiency. A 2-fold change in the $V_m$ (not shown) also has negligible effect. Decreasing $k_1$ (l-imide to d-imide) by a factor of 2 increases conversion slightly from 53% to about 57%. Variations in the rate of l-imide hydrolysis have, by far, the most profound effect. Increasing the rate of hydrolysis by a factor of 2 increases L-Asp formation from 53 to 74%. This effect is expected, since L-imide hydrolysis is the rate-limiting step in the overall conversion cycle. The ratio of L-Asp/L-iso-Asp formed from L-imide hydrolysis (not shown in Fig. 8) also has a very large influence on conversion efficiency. When this ratio is changed from 1/2 (the value for the ACTH imide) to 1/5,3 (the value for the LDH imide) conversion drops from 53 to 39%. It appears from this analysis that the enhanced conversion of iso-Asp to Asp observed with the LDH peptide is probably due to a greater instability of the imide form of this peptide. The overall imide hydrolysis must be sufficiently rapid to overcome the unfavorable Asp/iso-Asp ratio produced by imide breakdown. Because the LDH cyclic imide and iso-Asp methyl ester forms could not be separated by HPLC, we have
not accurately determined the imide hydrolysis rate for this peptide; however, our estimates indicate that this imide has a half-life of <3.2 h (see Footnote 3), definitely less than the half-life of 4.2 h determined for the ACTH imide. In the LDH peptide, the iso-Asp residue is linked to a serine rather than a glycine. This may account, at least in part, for the more labile nature of the cyclic imide in the LDH peptide.

In proteins, secondary and tertiary structure might favor the formation of L-Asp upon imide hydrolysis. As discussed above, the L-Asp/L-iso-Asp ratio has a profound effect on the efficiency of the conversion. Therefore, if the reaction occurs in vivo, the L-Asp/L-iso-Asp ratio has a profound effect on the labile nature of the cyclic imide in the LDH peptide.

A glycine. This may account, at least in part, for the more labile nature of the cyclic imide in the LDH peptide.

l-isoaspartate is linked to a serine rather than a glycine. This may account, at least in part, for the more labile nature of the cyclic imide in the LDH peptide.

To protein-free diets, suggesting a metabolic, rather than a glycine. This may account, at least in part, for the more labile nature of the cyclic imide in the LDH peptide.

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Purification and Peptides: The final set of de novo synthesized methyltransferase was purified from bovine tissue as described previously (Andersen and Beavis, 1985). The preparation was fractionated by gel filtration and affinity chromatography with ligand-bound porcine liver 

Experiments: Determination of Methyltransferase Activity During the Conversion of L-Asp to L-Asp by Methylation.

The reaction mixture contained 50 mM Tris-HCl (pH 7.4), 1 mM DTT, 0.5 mM MgCl2, 0.25% bovine albumin, 100 uM unlabeled AdoMet, and 200 uM L-Asp. The reaction was quenched with 100 mM NaOH, and the inorganic phosphate was removed by dialysis against water. The reaction products were analyzed by reverse phase HPLC, using a Waters C18 column of 25 mm × 0.46 cm and a gradient of 60% acetonitrile (pH 3.0) and 40% water, containing 0.1% trifluoroacetic acid, over 60 minutes. The HPLC chromatogram of the reaction products is shown in Figure 5.
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Table III

| Kinetic Constant | Value |
|------------------|-------|
| $k_{1}$          | 10.7 mol min$^{-1}$ mg$^{-1}$ |
| $k_{2}$          | 2.3 h$^{-1}$ |
| $k_{3}$          | 0.76 h$^{-1}$ |
| $k_{4}$          | 0.124 h$^{-1}$ |
| $k_{5}$          | 0.041 h$^{-1}$ |
| $k_{6}$          | 0.014 h$^{-1}$ |
| $k_{7}$          | 0.095 h$^{-1}$ |
| $k_{8}$          | 0.072 h$^{-1}$ |

The initial value problem was solved by Euler integration using time steps of 0.001 h for the first 2 h of reaction and steps of 0.05 h thereafter (Spain, 1966). The solution of the differential equations described the time course of the enzymatic changes in the kinetic profiles of all reaction intermediates and products. As described below, the values of $v_{2}$ drop dramatically during the reaction due to depletion of Aspartate and accumulation of Asparginase, a potent competitive inhibitor at the Aspartate binding site (L92726). Therefore, the value of $v_{2}$ was counted after each time step using equation 8, where $v_{0}$ is the velocity of the methyrase reaction under conditions of saturating Aspartate and L-Isoaspartate and $t$ is the elapsed time in hours.

$$v_{0} = \frac{v_{2} \cdot [\text{Asp}]}{K_{M_{\text{Asp}}} + [\text{Asp}]}$$

Equation 8 was derived empirically from measurements of methyrase activity at various rates obtained during a conversion reaction with initial L-Isoaspartate as the conversion substrate in 'Experimental Procedures.' The results of the experiment were fitted to expressions that reflect methyrase saturation kinetics. The enzyme inactivation was detected when methyrase was incubated at $37^\circ$ C for 8 h in a control reaction in which Aspartate and Asparagine were omitted. Thus, it appears that the loss of enzyme activity is due entirely to deactivation of Aspartate as an enzymatic and non-enzymatic methylation of Aspartate. The rapid phase of enzyme inactivation is expected from the faster change of kinetic parameters, which refers to the hydroxylation of the L-Aspartate peptide which refers to the hydrolysates of the L-Isoaspartate peptide.

![Figure 10: Loss of methyrase activity during the conversion of L-Isoaspartate.](image)

The activity of the methyrase to metabolize dipeptide Aspartate was determined under initial rate conditions as described in 'Experimental Procedures.' The measured activities, expressed as a percentage of the original activity, are shown by the following equation 9, where $v_{0}$ is the activity at time zero, $t$ is the time of measurement, $k_{n}$ is the first-order rate constant and $t$ is the elapsed time in hours.

$$v_{t} = v_{0} e^{-kt}$$

In the biphasic inactivation curve reported in 8 h of the incubation has a half time of 0.18 h, and 50% has a half time of 13.2 h (equation 8 as the reaction model).