Asparaginase

STEREOCHEMISTRY OF THE ACTIVE CENTER AS DETERMINED BY CIRCULAR DICHROISM OF SUBSTRATES*

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SUMMARY

Large Cotton effects associated with the $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions of asparaginase substrates have been shown in substrates containing a 2nd asymmetrical carbon atom at the $\beta$ position, dianominosuccinic acid monoamide and $\beta$-methylasparagine. Hydrolysis of the amide bond is accompanied by large changes in the rotatory strength of these transitions. These substrates have four possible isomers, L-L, D-D, L-D, and D-L. From the signs of the Cotton effects appearing on hydrolysis of mixtures of these isomers it can be determined which isomer is hydrolyzed. The enzyme has an almost absolute stereospecificity for the L-D isomer which defines the three-dimensional arrangement of the protein groups interacting with the substituents on the $\alpha$ carbon and the catalytic groups adjacent to the $\beta$-amide within rather narrow limits. While the L-L isomer of dianominosuccinic acid monoamide is not detectably hydrolyzed, the L-L isomer of $\beta$-methylasparagine is attacked at a rate 0.2 to 0.3% of that observed for L-asparagine, suggesting that a $\beta$-CH$_3$ group can be accommodated in this configuration while a $\beta$-NH$_2$ cannot.

The enzyme L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) has been the object of increasing interest since the purified enzyme has been found useful in the treatment of acute lymphoblastic leukemia (1-4). Its clinical utility is attributed to the asparagine-dependent nature of these neoplastic cells in contrast to normal cells which can synthesize asparagine (4). The physicochemical properties of the crystalline enzymes from Escherichia coli B and Erwinia carotovora have been reported on extensively (5-7). The protein is a tetramer of molecular weight ~133,000 (4, 5, 7).

A variety of alternate substrates for the enzyme have been described including $\beta$-cyanalanine and the $\beta$-hydroxamate of L-aspartic acid both of which are converted to L-aspartic acid (4). Catalytic decomposition of 5-diazo-L-oxo-L-norvaline as well as active site labeling by this reagent have been reported (8).

RESULTS

Substrate and Optical Properties.—The primary substrate employed in this study was the monoamide of dianominosuccinic acid (Scheme I). Since there are 2 asymmetrical carbon atoms the compound can exist as four different stereoisomers,

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has a combined molar ellipticity at the minimum of the CD
portions taken at longer pen periods with less random noise. Thus
pen response. The resulting large random noise does not, how-
erver, reduce the precision of the slope over that of determina-
tions taken at longer pen periods with less random noise. Thus
the change in ellipticity can be adapted as a satisfactory assay of
zyme activity. Unfortunately in the case of the mixture of
D-β-L- and D-β-L-diaminosuccinic acid monoamide there is
severe substrate inhibition at substrate concentrations > 5 x
10^{-3} M. This inhibition prevented a complete kinetic analysis.
The maximum turnover observed for diaminosuccinic acid mono-
amide was 4.75 x 10^{4} moles of substrate hydrolyzed per min per
mole of enzyme in 0.05 M Tris-HCl, pH 8.0, at a substrate con-
centration of 1 x 10^{-3} M. This number is the same order of
magnitude as the turnover numbers reported for L-asparagine as sub-
strate under similar conditions (4, 5). The highest maximum
velocity reported for the hydrolysis of L-asparagine is 8.95 x 10^{4}
moles per min per mole of enzyme, as assayed by the coupled
spectrophotometric assay (5). The substrate inhibition may rela-
to the presence of the unhydrolyzed isomer and it is possible
that with a pure isomer this CD assay could be developed into one
satisfactory for a complete kinetic analysis. It would have the
advantages of a direct spectrophotometric assay without the
addition of other enzymes as in the assay coupled to the reduction
of pyridine nucleotide (11) or the analytical problems of measur-
ing ammonia release.

**Stereochemistry of Isomer of Diaminosuccinic Acid Monoamide
Hydrolyzed by Asparaginase**—The Cotton effect generated by
50% hydrolysis of the mixture of meso-isomers of diaminosuccinic
acid monoamide is negative (Fig. 1). Deduction of the stereo-
chemistry of the hydrolyzed and unhydrolyzed isomers from the
sign of this Cotton effect requires both an assignment of this
transition and a knowledge of its sign in environments of known
symmetry. This can readily be deduced from the CD of some
model compounds. Aliphatic amino acids of the L-configuration
including L-aspartic and L-glutamic acids have positive Cotton

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**Scheme I**

\[
\begin{align*}
\text{LL} & \quad \text{DL} \\
\text{DD} & \quad \text{DD} \\
\end{align*}
\]

For convenience in comparing this compound to asparagine the carbon adjacent to the carboxyl is designated as the α carbon and that carrying the amide as the β carbon. The parent compound, diaminosuccinic acid exists as three stereoisomers LL, DD, and the meso form (DL = LD). Since the first two isomers can be readily separated from the meso form, the meso form of diaminosuccinic acid has been used for synthesis of the amide substrate. The product should contain two meso-type isomers of diaminosuccinic acid monoamide, α-L-β-D and α-D-β-L (Scheme I). The product of this synthesis is not optically active (Fig. 1) confirming that the product must contain either two isomers showing equal and opposite rotations, or a single isomer with two asymmetrical centers (of opposite configuration) which make equal contributions to the rotation. Since the amide chromophore will not make the same contribution to rotation as the carboxyl chromophore (see below), the first alternative appears more likely and suggests the presence of the two isomers in equal concentrations as expected from the synthesis. Since hydrolysis of only one of the isomers is expected to be catalyzed by the enzyme, complete enzymatic hydrolysis should be accom-
panied by the appearance of optical activity at the wave
length of the absorption bands of the unhydrolyzed amide. A
solution of the diaminosuccinic acid amide treated with asparagi-
nase for 24 hours shows a large negative Cotton effect centered
near 222 nm (Fig. 1). This suggests that only 50% of the sub-
strate is hydrolyzed as has been confirmed by determining am-
nion release by Nesslerization (9). Enzymatic hydrolysis re-
results in the release of only 50% as much ammonia nitrogen as does complete acid hydrolysis of the substrate (9). Once maximum
rotation is achieved the CD spectrum remains the same indefi-
nitely suggesting that the other isomer is completely resistant to
hydrolysis.

**Rate of Change of Ellipticity as Function of Enzyme Concentra-
tion**—Enzymatic hydrolysis of 50% of the mixture of meso-is-
omers of diaminosuccinic acid monoamide yields a product which
has a combined molar ellipticity at the minimum of the CD spectrum of −6.3 x 10^{5} deg cm^{2} per decimole (Fig. 1). Com-
plete enzymatic hydrolysis of a 6.85 mM solution (1 mg per ml)
observed over a 1-cm path yields a change in observed ellipticity of −0.045° at 222 nm. Appearance of the 222 nm Cotton effect is
directly related to enzymatic hydrolysis of the substrate, since
the rate of appearance of this Cotton effect is directly propor-
tional to enzyme concentration (Fig. 2). Time courses for the
increase in negative ellipticity at 222 nm at three different en-
zyme concentrations are shown. The rate function over a 6-fold
range of enzyme concentration is shown in the inset to Fig. 2.
The pen period on the Cary 61 was set at 1 s to insure rapid
pen response. The resulting large random noise does not, how-
ever, reduce the precision of the slope over that of determina-
tions taken at longer pen periods with less random noise. Thus
the change in ellipticity can be adapted as a satisfactory assay of

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**Fig. 1.** ORD (Curve 1) and CD (Curve 2) spectra of a solution of
α-D-β-L- and α-L-β-D-diaminosuccinic acid monoamide (1.84 x
10^{-4} M) after 24 hours incubation with 10 μl of asparaginase (0.161
mg per ml). The error bars on the axis indicate the range of a
base-line CD spectrum taken on the same solution untreated with
enzyme. Conditions: 0.05 M Tris, pH 8.0, 25°, 0.2-cm path length.
effects near 200 nm due largely to the overlapping $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions of the carboxyl chromophore at the $\alpha$ position (Fig. 3). The $n \rightarrow \pi^*$ transition of the carboxyl group is usually near 204 nm (10). If the $\beta$- or $\gamma$-carboxyl is changed to an amide chromophore as in L-asparagine there is a small but significant change in the rotation (Fig. 3). The change in ellipticity from the amide to the acid is relatively small, since it is due to the vicinal effect of the asymmetrical center located 2 carbon atoms away. The relatively small change in optical rotatory strength and the low wave length of the change near 210 nm, make it unsatisfactory to follow asparagine hydrolysis with optical rotation (Fig. 3). However, if an amide is substituted for the $\alpha$-carboxyl, the $n \rightarrow \pi^*$ transition shifts to the region of 220 nm with a resultant change in the CD pattern in the region of 220 nm. This is illustrated in Fig. 4 by the CD spectra of L-leucine and L-leucine amide. The free acid shows the typical positive Cotton effect at 198 nm, but a prominent negative band appears at 227 nm in the amide. The latter can be assigned to the $n \rightarrow \pi^*$ transition which undergoes a pronounced red shift in amides (10). The $n \rightarrow \pi^*$ Cotton effect is negative when the amide is adjacent to a carbon in the $L$ configuration. Thus in the case of the diaminosuccinic acid monoamide hydrolyzed by asparaginase, the Cotton effect at 222 nm is due to the unhydrolyzed amide and that amide is of the $L$ configuration (Fig. 1), hence the $\alpha$-$D$ isomer must be the one hydrolyzed. The relationship of this finding to the stereochemistry of the active site of the enzyme will be discussed below (see discussion).

If a mixture of $\alpha$-$L$-$\beta$-$L$- and $\alpha$-$D$-$\beta$-$D$-diaminosuccinic acid monoamide 2HBr (2.7 mg per ml) is treated with up to 0.10 mg per ml of asparaginase no optical rotatory changes are observed over a period of 72 hours. Furthermore, the enzyme did not release a significant amount of ammonia from the mixture of $\alpha$-$L$-$\beta$ and $\alpha$-$D$-$\beta$ isomers over this time period.

Hydrolysis of $\beta$-Methylasparagine Although no hydrolysis of the $\alpha$-$L$-$\beta$-$L$- and $\alpha$-$D$-$\beta$-$D$ mixture of diaminosuccinic acid amide was detected, a very slow hydrolysis of the $\alpha$-$L$-$\beta$-$L$- and $\alpha$-$D$-$\beta$-$D$ isomers of $\beta$-methylasparagine was observed. This mixture of isomers can be prepared by amidation of the commercially available three-$L$-$N$-$\beta$-methylaspartic acid. The Cotton effects appearing upon treating a mixture of $\alpha$-$L$-$\beta$-$L$- and $\alpha$-$D$-$\beta$-$D$-methylasparagine with asparaginase are shown in Fig. 5A. A large negative ellipticity band appears at 204 nm and an apparent smaller negative ellipticity band at $\sim$220 nm (Fig. 5A).

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**Fig. 2.** Changes in observed ellipticity, $\theta_{obs}$, at 222 nm as a function of time after additions of 0.88, 1.76, and 2.64 units of asparaginase (0.164 mg per ml) to a solution of $\alpha$-$L$-$\beta$-$L$ -diaminosuccinic acid monoamide 2HBr $\times 10^{-2}$ M contained in 0.05 M Tris, pH 8.0, 25°C. Inset, $\theta_{obs}$ per min as a function of asparaginase concentration, 0.44 unit/10 $\mu$L. To insure adequate pen response the time constant on the Cary 61 was set at 1 s.

**Fig. 3.** CD spectra of L-asparagine (- - -), L-aspartic acid (--), and L-asparagine after complete hydrolysis by asparaginase (-----). Conditions: 0.05 M Tris, 3.75 $\times 10^{-4}$ M amino acid, 25°C, 0.2-cm path.

**Fig. 4.** CD spectra of L-leucine (- - -) and L-leucine amide (-----). Conditions: 0.05 M Tris, 3.8 $\times 10^{-2}$ M amino acid, 25°C, 0.2-cm path.
At the enzyme concentration required to observe significant hydrolysis, however, the enzyme itself makes a significant contribution to the CD spectrum and the ellipticity at 220 nm has a large contribution from the enzyme. *Curve 1*, Fig. 5A, is largely due to the enzyme. In the case of β-methylasparagine no clear \( n \rightarrow \pi^* \) amide transition is separated out between 220 and 230 nm as in the case of the diaminosuccinic acid amide (Fig. 1). Thus in order to be certain of the stereochecmistry of the isomer hydrolyzed, it was necessary to separate the product acid from the unhydrolyzed amide. This was accomplished with a Dowex I-X2 column and the CD spectra of the resolved compounds are shown in Fig. 5B. The unhydrolyzed amide is of the \( \alpha-L-\beta-D \) configuration, giving rise to large negative Cotton effects, while the hydrolyzed isomer is of the \( \alpha-L-\beta-L \) configuration, giving rise to large positive Cotton effects. The net increase in negative ellipticity observed on hydrolysis (Fig. 5A) is due to a decrease in magnitude and slight blue shift of the positive Cotton effects for the \( \alpha-L-\beta-L \)-acid compared to the \( \alpha-L-\beta-L \)-amide. These shifts in CD between the acid and the amide are more like those occurring on hydrolysis of asparagine (Fig. 2) where no clear separation of the \( \pi \rightarrow \pi^* \) and \( n \rightarrow \pi^* \) transitions is present.

The \( \alpha-L-\beta-L \) isomer of β-methylasparagine is hydrolyzed only 0.3% as fast as the \( \alpha-L-\beta-L \)-diaminocarboxylic acid monoamide as shown by the time course for development of the Cotton effects in Fig. 5A. The turnover number calculated from this data is only 150 moles of substrate hydrolyzed per min per mole of enzyme. This is significantly faster, however, than the hydrolysis of the \( \alpha-L-\beta-L \) isomer of diaminocarboxylic acid amide.

**Discussion**

The bond hydrolyzed in the case of the natural substrate for asparaginase is part of a chromophile located 2 carbon atoms away from the asymmetrical center, and hence the rotatory changes accompanying this hydrolysis are qualitatively less significant than if the amide bond hydrolyzed involved a carbon adjacent to an asymmetrical center (Figs. 3 and 4). The diaminocarboxylic acid derivative maintains the substrate requirements for asparaginase, but introduces an asymmetrical carbon adjacent to the carbon of the hydrolyzed amide bond. This greatly enhances the rotatory change near 225 nm on hydrolysis as well as moving the change to the more specific \( \pi \rightarrow \pi^* \) transition of the amide (Fig. 1). Such a procedure may be applicable to the substrates of other enzymes where the development of assays based on change in optical activity are desirable.

Since the natural substrate of the enzyme is \( L \)-asparagine and \( D \)-asparagine is poorly hydrolyzed (4,11), the proper relationship between the enzyme-binding groups of the substrate and the catalytic centers on the enzyme resulting in the hydrolysis of the \( \beta \)-amide presumably requires the \( L \)-configuration about the \( \beta \) carbon.

Since the \( \beta \) carbon is not asymmetrical the amide can assume all positions of the \( D \) or \( L \) configuration of the \( \beta \) carbon. Whether there is a strict stereospecificity for the \( \beta \)-amide once the substrate is in place on the enzyme surface cannot be determined.

In the case of diaminocarboxylic acid amide, stereospecificity is conferred on the \( \beta \) carbon, and thus the enzyme active site might accept only one of the \( \beta \) carbon configurations. When the mixture of isomers is of the \( \alpha-D-\beta-L-NH_2 \) and \( \alpha-L-\beta-D-NH_2 \) configurations, the \( \alpha-L-\beta-D-NH_2 \) configuration is hydrolyzed (Fig. 1). This is in keeping with the analogy to asparagine where the configuration around the carbon carrying the carboxyl must be \( L \).

The significance of the \( D \) configuration at the amide in the rapidly hydrolyzed substrate was not apparent until enzymatic hydrolysis of the alternate pair of isomers, \( \alpha-L-\beta-L-NH_2 \) and \( \alpha-D-\beta-D-NH_2 \) was attempted. No significant hydrolysis was detected and thus the \( \alpha-L-\beta-L-NH_2 \) isomer does not meet the requirements. Hence the catalytic region of the enzyme interacting with the...
The hypothetical catalytic groups of the enzyme are represented as a base B and an acidic group AH. The isomers are both shown in an extended form with the NH2 groups on opposite sides of the C-2—C-3 bond and occupying the same positions in the α-L-β-D and α-β-L configurations. This results in the susceptible amide bond pointing away from the catalytic groups in the α-L-β-L isomer.

It appears that the L configuration of substituents at the β carbon interferes with the proper positioning of the substrate if the binding groups at the α carbon are confined to the L configuration. If the amide group of the α-L-β-L isomer is to be placed in the same position as that of the effectively hydrolyzed α-L-β-D isomer, then the β-amino group occupies the position occupied by the β-hydrogen in the α-L-β-D isomer. This may accommodate a hydrogen in this position but not an amine group. Alternatively, if binding requires that the substituents on the α carbon be in the L configuration and that the amino group of the β carbon be in the position it occupies in the α-L-β-D isomer, then the placement of the β-amino group in this same position for the α-L-β-L isomer results in rotation of the amide away from the site that must contain the catalytic groups on the protein. These relationships for the α-L-β-D, α-L-β-L pair are illustrated in the stereodrawing in Fig. 6.

The α-L-β-D isomer is shown in the extended form, since this is the likely form in solution with the amino groups on opposite sides of the C-2—C-3 bond. The relationship of the binding points on the protein receiving the groups around the α carbon (carboxyl and amino) and the catalytic groups (A and B) attacking the amide bond is then shown by the α-L-β-D isomer in Fig. 6.

This stereochemistry is not absolutely fixed in the sense that the configuration of the α-L-β-D isomer can change from that shown in Fig. 6 by rotation about the α-β C—C bond. Although extensive deviation from the extended form does not appear likely, the enzyme-substrate dissociation constant appears to be relatively small and binding could force some changes from the extended configuration. Such rotation does not change the general features of substrate-enzyme stereochemistry pictured in Fig. 6.

While the β-amino group in the α-L-β-L isomer of diaminosuccinic acid amide appears to interfere with the proper positioning of the substrate, a methyl group can be accommodated in this position, although the rate of hydrolysis is much decreased over the rate observed if a hydrogen occupies this position. Stereo factors would appear to be primarily responsible for the decreasing rate of hydrolysis in the order H > CH3 > NH2 when those groups in the β position are placed in the L-configuration. These steric factors may influence the binding step or alter the relationship between the catalytic groups and the substrate once the latter is bound. The catalytic groups are represented in purely hypothetical fashion as a base, B, attacking the carbonyl carbon, perhaps assisted by donation of a proton from a protein group, AH, to the amide. A specific nucleophilic attack is pictured, since data on 13C exchange (12) and on the formation of β-aspartohydroxamic acid catalyzed by the enzyme (12, 13) have suggested but not proved the formation of an acyl enzyme intermediate during hydrolysis by asparaginase.

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