94732-85-1; Cys, 52-90-4; Cys-Cys, 18048-87-8; cysteamine, 60-23-1; mercaptopropionic acid, 107-96-0.

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Studies of the Ammonia-Dependent Reaction of Beef Pancreatic Asparagine Synthetase†

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ABSTRACT: We have studied the asparagine synthetase reaction with regard to the ammonia-dependent production of asparagine. Hydroxylamine was shown to be an alternate substrate for the asparagine synthetase reaction, and some of its kinetic properties were examined. The ammonia-dependent reaction was examined with regard to inhibition by asparagine. It was found that asparagine inhibition was partial competitive with respect to ammonia, regardless of the concentration of aspartate. However, when MgATP was not saturating, the inhibition by asparagine became linear competitive. These results were interpreted to be consistent with a kinetic mechanism for asparagine synthetase where ammonia is bound to the enzyme followed by MgATP causing asparagine release.

A pathway for asparagine biosynthesis was first noted in bacteria (Al-Dawody & Varner, 1961; Ravel et al., 1962). In this system, asparagine synthetase catalyzes the amidation of aspartate from free ammonia with the concurrent hydrolysis of ATP to AMP and pyrophosphate. In mammalian tissues, however, it was found that, in addition to ammonia, glutamine could serve as the nitrogen source for asparagine synthetase (Patterson & Orr, 1968; Arfin, 1967). Patterson & Orr (1968) reported a K_m for NH_4Cl of 120 mM for asparagine synthetase from the Novikoff hepatoma. Since the activity of the ammonia-dependent reaction increased with pH, the substrate was considered to be the free ammonia species as opposed to the ammonium ion. In the glutamine-dependent reaction, the optimum pH range was found to be from 6.6 to 8.0.

The formation of β-aspartylhydroxamate by asparagine synthetase has been reported when ammonia or glutamine is

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replaced by hydroxylamine (Patterson & Orr, 1968; Cedar & Schwartz, 1969; Horowitz & Meister, 1972). Cedar & Schwartz (1969) reported that in *Escherichia coli*, 50 mM hydroxylamine produced the same rate of hydroxamate formation as asparagine formation by 20 mM ammonium chloride and that hydroxylamine concentrations of 0.1 M or greater produced substrate inhibition.

In the work presented here, the ammonia-dependent asparagine synthetase reaction is further characterized through the comparison of ammonia and hydroxylamine as substrates, and through the study of the kinetics of asparagine inhibition of the reaction.

Materials and Methods

Materials. Scintillation counting supplies were obtained from Research Products International (Elk Grove Village, IL). L-[4-^{14}C]Aspartic acid was purchased from Amersham (Arlington Heights, IL). The L-[4-^{14}C]aspartic acid was diluted with L-aspartic acid to a specific activity of 6.25×10^4 cpm/μmol. Aluminum sulfate and magnesium chloride were purchased from Mallinckrodt, Inc. (St. Louis, MO), ammonium sulfate was from Schwarz/Mann (Orangeberg, NY), and sodium acetate (buffer grade) was from Pierce Chemical Co. (Rockford, IL). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) and were of the highest quality available. Fresh bovine pancreata were obtained from American Stores Packing Co. (Lincoln, NE).

Asparagine Synthetase Assay. The method of Luehr & Schuster (1980) was used to measure the activity of asparagine synthetase. This assay is based on the Al^{3+}–pyridoxal-catalyzed β-decarboxylation of aspartic acid. The conditions used were shown (Luehr & Schuster, 1980) to cause β-decarboxylation of aspartic acid and not asparagine. All velocities were determined by measuring the production of [4-^{14}C]asparagine from [4-^{14}C]aspartate. The assay mixture was composed of the following: 83 mM tris(hydroxymethyl)aminomethane (Tris)–acetate, 10 mM L-Asp (6.25×10^4 cpm/μmol), 21 mM glutamine, 8.3 mM ATP, and 17 mM magnesium acetate, all at pH 8.0. The total incubation volume was 600 μL, 500 μL of which was pipetted into 1 mL of 1.2 N sodium acetate, pH 5.2, in a boiling water bath after a designated incubation period at 37 °C. Of this solution, 1 mL was added to a scintillation vial along with 1.0 mL of 500 mM pyridoxal hydrochloride and 5 mM $Al_2(SO_4)_3$. These vials were then incubated in a well-vented oven for 45 min at 80–90 °C; 500 μL of 5 N HCl and 10 mL of 3a70B scintillation cocktail were added before counting. All assays were performed in triplicate, and all rates were found to be linear within the time periods used.

To verify that the activity measured in the asparagine synthetase assay was indeed asparagine production, its sensitivity to asparaginase was tested. Two sets of duplicates were assayed as described above, but after the heat step, one set (0.9 mL) was incubated with 0.1 mL of asparaginase (1.0 mg/mL) for at least 2 h at 37 °C. The other set was incubated with 0.1 mL of H_2O. The difference in the two sets indicated the actual production of asparagine. It was found that after the ammonium sulfate fractionation, the observed activity was 100% asparaginase sensitive.

Protein Determination. Layne's protein determination procedure was modified to incorporate an acid precipitation step (Layne, 1957). This was to remove interfering, nonproteinous compounds such as dithiothreitol and ammonia that interfere with the biuret protein assay. The protein samples were diluted to 0.75 mL, and 0.75 mL of 25% trichloroacetic acid was added. The protein was precipitated by centrifugation with a table-top centrifuge. The supernatant was drawn off with a Pasteur pipet, and the protein was resuspended in 2.0 mL of biuret reagent. The absorbance at 555 nm was taken for three different amounts of each protein sample, plus six different amounts of a standard bovine serum albumin (BSA) solution. The slopes of the unknown's line (from the three points) were compared to the standard solution's line by using a computer program to determine their protein concentrations.

Purification of Asparagine Synthetase. Eight fresh beef pancreata were cut into small pieces, discarding as much of the fat and connective tissues as possible, and then homogenized in a Waring blender for 1 min at high speed in buffer A [50 mM Tris-HCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM dithiothreitol, pH 7.85]. The total volume after homogenization was 3–4 times the volume of the original tissue. The homogenate was then centrifuged in a type GS-3 rotor with a Sorval RC-5 centrifuge at 7000 rpm for 45 min. The supernatant was passed through cheesecloth and frozen. After 3 days of being frozen, the precipitable material could be removed with a low-speed centrifugation.

The frozen fraction from the previous step was thawed and again centrifuged at 7000 rpm for 45 min. The resulting supernatant was brought to 32% saturation with solid ammonium sulfate (17.9 g of ammonium sulfate/100 mL of solution). The precipitated protein was removed by centrifugation at 7000 rpm for 20 min in a type GS-3 rotor and discarded. Solid ammonium sulfate was again added to the supernatant to bring the solution to 45% saturation (7.2 g/100 mL of solution). The precipitated protein was centrifuged as above, and the pellet was dissolved in a minimal amount of buffer A (usually 130–150 mL for eight pancreata). This solution could then be frozen prior to the next step.

Approximately 60 mL of the ammonium sulfate fractionated solution was processed at a time. The fraction was dialyzed against 4 L of 50 mM Tris–acetate, 0.5 mM EDTA, and 1 mM dithiothreitol, pH 7.85, for 5 h. The resulting precipitate was centrifuged at 15 000 rpm in a Sorval SS-34 rotor for 10 min. The precipitate was discarded, and the supernatant was applied to an A-50 diethylaminoethyl (DEAE) column (5 g of dry resin, 3.7 × 15 cm) equilibrated with the same buffer as the dialysis. The resin was washed with approximately 50 mL of the same buffer and then washed with approximately 80 mL of 50 mM Tris–acetate, 0.15 M sodium acetate, 0.5 mM EDTA, and 1 mM dithiothreitol, pH 7.85. Asparagine synthetase was eluted with 150–200 mL of 50 mM Tris–acetate, 0.3 M sodium acetate, 0.5 mM EDTA, and 1 mM dithiothreitol, pH 7.85, and concentrated by precipitation with ammonium sulfate. The solution was brought to 50% saturation with solid ammonium sulfate (29.1 g/100 mL of solution) and centrifuged, and the pellet was dissolved in a minimal amount of 50 mM Tris–acetate, 0.5 mM EDTA, and 1 mM dithiothreitol, pH 7.85. This fraction was then frozen for later use.

Enzyme from the DEAE purification step was desalted by using Sephadex G-25 chromatography prior to use. This preparation was free of ammonia, and all activity was 100% asparaginase sensitive.

For experiments where saturation of substrates was necessary, the previously published K_m values of Markin et al. (1981) were used. There values are the following: K_m(aspartate) = 0.6 mM; K_m(glutamine) = 1.4 mM; K_m(ATP) = 0.7 mM. The concentrations used for the substrates when saturation was needed were 10 mM aspartate, 22 mM glutamine, and 8.3 mM ATP.

Results

The Lineweaver–Burk plots of asparagine synthetase with

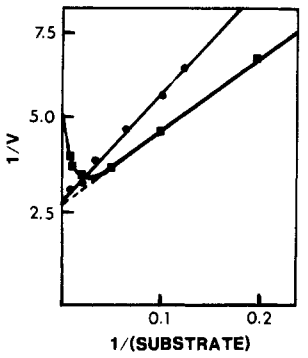

FIGURE 1: Double-reciprocal plot of $1/V$ for the asparagine synthetase reaction vs. $1/[NH_4^+]$ (●) and $1/V$ vs. $1/[NH_2OH]$ (■). Substrate concentration is in millimolar, and aspartate, ATP, and magnesium acetate were saturating at 10, 8.3, and 17 mM, respectively. Velocity is reported as nanomoles per minute per milligram.

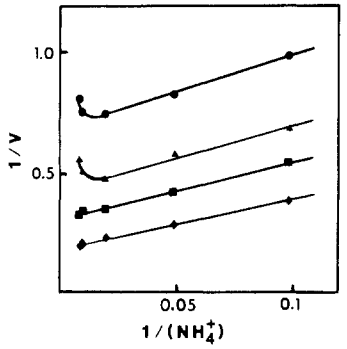

FIGURE 2: Double-reciprocal plot of initial velocity of asparagine synthetase with ammonium acetate (10, 20, 50, 100, and 120 mM) at various fixed levels of aspartate: (●) 0.9 mM; (▲) 1.67 mM; (■) 3.33 mM; (♦) 10.0 mM. MgATP was saturating at 10 mM. Velocity is reported as nanomoles per minute per milligram.

Table I: Kinetic Constants for Asparagine Inhibition of Asparagine Synthetase with Respect to Hydroxylamine and Ammonia

substrate	K_m (mM)	V_{max} (nmol min^{-1} mg^{-1})	α^b	K_I(asparagine) (mM)
ammonium acetate	6.61 ± 0.15[a]	4.23 ± 0.03	5.72 ± 1.3	0.369 ± 0.14
hydroxylamine	4.42 ± 0.54	3.56 ± 0.12	2.34 ± 1.3	0.115 ± 0.07

[a] The K_m value given is for ammonia plus ammonium. At pH 8.0, the K_m for ammonia was calculated to be 0.31 mM. [b] The value of α is as described under Results.

each of the substrates, ammonia and hydroxylamine, are shown in Figure 1. As previously reported (Cedar & Schwartz, 1969), hydroxylamine produces substrate inhibition which is not seen with ammonia when MgATP and aspartate are saturating (Figure 1). This is interesting since it is assumed that both substrates act in an analogous manner. However, it should be noted that the true substrate is probably NH_3 and not the ammonium ion, resulting in a corrected K_m for free ammonia of approximately 0.31 mM (see Table I). This makes ammonia a much better substrate than hydroxylamine even though their apparent V_{max}'s are virtually identical.

Figure 2 shows that ammonia can produce substrate inhibition when aspartate is nonsaturating and MgATP is saturating. As the aspartate concentration is raised to saturating concentrations, inhibition by ammonia disappears in the concentration range investigated. Ammonia did not produce substrate inhibition over the same range of concentrations when MgATP was nonsaturating but aspartate was saturating (data not shown).

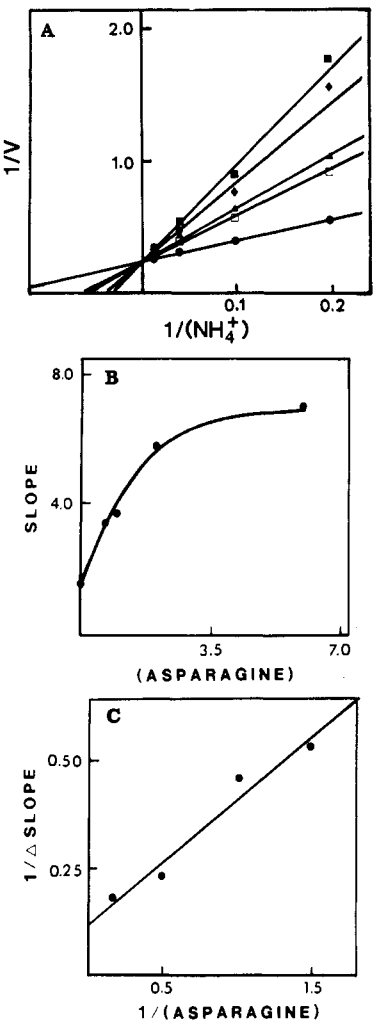

FIGURE 3: (A) Double-reciprocal plot of initial velocity of asparagine synthetase with ammonium acetate (5, 10, 25, and 75 mM) at various fixed levels of asparagine: (●) 0; (□) 0.667 mM; (▲) 1.0 mM; (♦) 2.0 mM; (■) 6.0 mM. ATP, aspartate, and magnesium acetate were saturating at 8.3, 10, and 17 mM, respectively. Velocity is reported as nanomoles per minute per milligram. (B) Replot of the slopes of the double-reciprocal plot in (A). (C) Replot of the slope vs. asparagine concentration plot shown in (B).

Scheme I

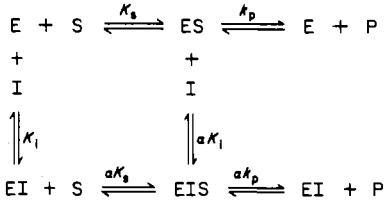

Figure 3A reveals that when MgATP and aspartate are saturating, asparagine is a competitive inhibitor with respect to ammonia but, as is shown in Figure 3B, the replot of the slope vs. asparagine concentration is not linear as would have been expected for a simple competitive inhibitor. The plot of $1/\Delta$ slope vs. $1/$[asparagine] for the ammonia-dependent reaction is shown in Figure 3C.

When hydroxylamine is the substrate, the double-reciprocal plot again indicates that asparagine is a competitive inhibitor, but, as in the ammonia-dependent reaction, the slope vs. asparagine concentration replot is nonlinear, and the $1/\Delta$ slope plot is linear (data not shown).

The significance of the Δ slope plot is that, when it can be shown to be linear, consistency with a partial competitive

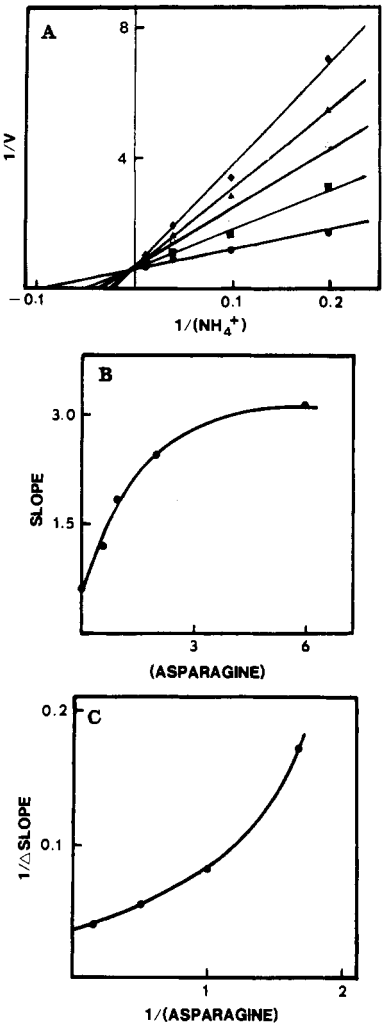

FIGURE 4: (A) Double-reciprocal plot of initial velocity studies of asparagine synthetase with ammonium acetate (5.0, 10.0, 25.0, and 75.0 mM) at various fixed levels of asparagine: (●) 0; (■) 0.6 mM; (△) 1.0 mM; (▲) 2.0 mM; (♦) 6.0 mM. Aspartate was nonsaturating at 2.0 mM; ATP and magnesium acetate were saturating at 10 and 17 mM, respectively. Velocity is reported as nanomoles per minute per milligram. (B) Replot of the slopes of the double-reciprocal plot shown in (A). (C) Replot of the slope vs. asparagine concentration plot shown in (B).

inhibition mechanism is presumed. A mechanism exhibiting a partially competitive inhibitor model is shown in Scheme I (Segel, 1975) where α is greater than 1. As is shown in eq 1, the reciprocal velocity equation, as [I] increases to infinity the slope increases to a constant ($\alpha K_m/V_m$), but the y intercept is not affected by [I].

$$\frac{1}{V} = \frac{K_m}{V_m}\left(\frac{1 + [I]/K_I}{1 + [I]/\alpha K_I}\right)\frac{1}{[S]} + \frac{1}{V_m} \qquad (1)$$

The replot of slope vs. [I] is nonlinear, but the plot of $1/\Delta$ slope vs. $1/[I]$ is linear as shown in eq 2 and 3. The K_m and V_m

$$\Delta \text{ slope} = \frac{K_m}{V_m}\left(\frac{1 + [I]/K_i}{1 + [I]/\alpha K_I}\right) - 1 \qquad (2)$$

$$\frac{1}{\Delta \text{ slope}} = \left[\frac{\alpha k_I V_{max}}{K_m(\alpha - 1)}\right]\frac{1}{[I]} + \frac{V_{max}}{K_m(\alpha - 1)} \qquad (3)$$

are calculated from the Lineweaver–Burk plot in the absence of inhibitor. Using the plot of eq 3, we calculated α from the y intercept, $V_m/K_m(\alpha - 1)$, and K_I from the x intercept

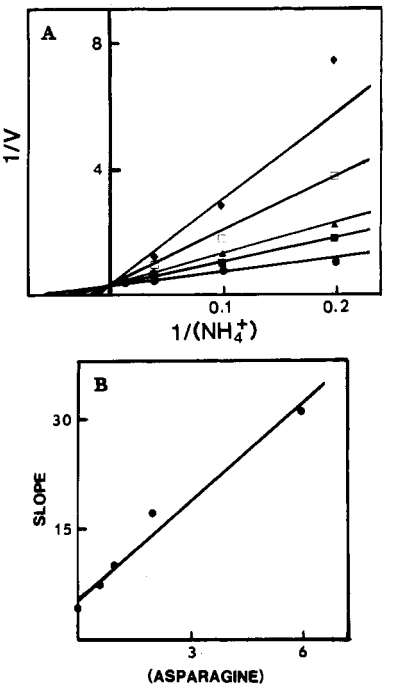

FIGURE 5: (A) Double-reciprocal plot of initial velocity of asparagine synthetase with ammonium acetate (5.0, 10.0, 25.0, and 75.0 mM) at various fixed levels of asparagine: (●) 0; (■) 0.6 mM; (▲) 1.0 mM; (□) 2.0 mM; (♦) 6.0 mM. ATP was nonsaturating at 1.67 mM; aspartate and magnesium acetate were saturating at 10 and 17 mM, respectively. Velocity is reported as nanomoles per minute per milligram. (B) Replot of the double-reciprocal plot shown in (A).

($-1/\alpha K_I$). The appropriate kinetic constants obtained for both the ammonia-dependent and the hydroxylamine-dependent reactions are summarized in Table I. The very low K_I for asparagine with respect to ammonia and its affect on the apparent K_m for ammonia indicate that asparagine is a potentially important physiological regulator of the ammonia-dependent reaction.

To explore the mechanism of the inhibition of asparagine with respect to ammonia, the above experiments were repeated at nonsaturating concentrations of either MgATP or aspartate. The data shown in Figure 4A were obtained when asparagine was the inhibitor of the ammonia-dependent synthesis of asparagine when MgATP was saturating and aspartate less than saturating. As can be seen, asparagine is still a competitive inhibitor, but the replot in Figure 4B reveals hyperbolic inhibition. When these data were replotted as $1/\Delta$ slope vs. $1/[Asn]$, the results were as shown in Figure 4C. When the experiment was repeated with aspartate saturating and ATP nonsaturating, the results were as shown in Figure 5A. Again, asparagine is a competitive inhibitor, but in this case, the replot (Figure 5B) reveals more linear inhibition. When neither aspartate nor MgATP is present in saturating concentrations, the inhibition by asparagine is as shown in Figure 6A. While asparagine is again competitive with respect to ammonia, the replot in Figure 6B reveals marked hyperbolic inhibition. Under these circumstances, it appears that even a plot of $1/\Delta$ slope vs. [asparagine] does not become linear (see Figure 6C). Therefore, it appears that the only case under which asparagine is a *linear* competitive inhibitor of ammonia-dependent asparagine synthesis is when the MgATP concentration is less than saturating but aspartic acid is saturating.

Discussion

For the ammonia-dependent reaction of asparagine synthetase, the order of substrate binding and product release

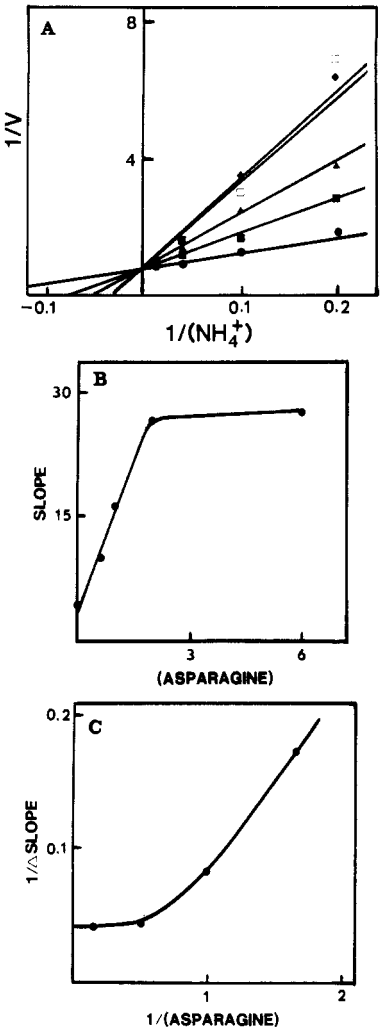

FIGURE 6: (A) Double-reciprocal plot of initial velocity of asparagine synthetase with ammonium acetate (5.0, 10.0, 25.0, and 75.0 mM) at various fixed levels of asparagine: (●) 0; (■) 0.6 mM; (▲) 1.0 mM; (□) 2.0 mM; (♦) 6.0 mM. Aspartate and ATP were nonsaturating at 2.0 and 2.5 mM, respectively, and magnesium acetate was saturating at 17 mM. Velocity is reported as nanomoles per minute per milligram. (B) Replot of slopes of the double-reciprocal plot shown in (A). (C) Replot of the slope vs. asparagine concentration plot shown in (B).

can be described independent of any consideration of the number of sites for amino acid binding. While previous work has described the kinetic mechanism of asparagine synthetase (Markin et al., 1981), the data presented here attempt to define the effects of asparagine on the ammonia-dependent reaction. Therefore, a consideration of ammonia as a substrate as well as asparagine as an inhibitor will be emphasized.

Prior to discussing the mechanism in terms of the data presented in this paper, it is necessary to summarize briefly the mechanism of the asparagine synthetase reaction as it is known to occur when glutamine functions as the nitrogen source. This work has been presented in detail previously (Markin et al., 1981). The reaction proceeds by the mandatory binding of glutamine followed by the binding of ATP. After this occurs, glutamate is released (presumably leaving NH_3 bound) and asparate is bound. After the central complex is formed (presumably the aspartyl-AMP is attacked by the NH_3), the products are released. The order of product release requires pyrophosphate to be first, followed by AMP, and finally followed by asparagine. In contrast to the mandatory order found for the glutamine-dependent reaction, the ammonia-dependent reaction exhibits much more tolerance for random binding of substrates. In the ammonia-dependent

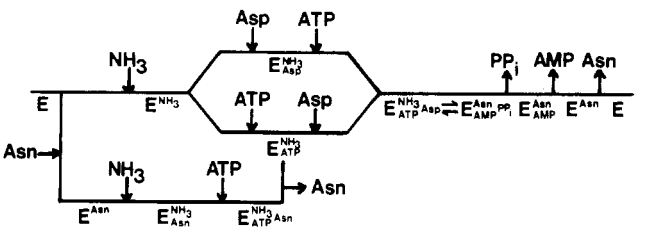

FIGURE 7: Mechanism for asparagine inhibition of the ammonia-dependent asparagine synthetase reaction.

reaction, ammonia binding is a necessary first step. After this occurs, the binding of aspartate and ATP can occur randomly. The order of product release is the same as that described for the glutamine-dependent reaction.

As is shown in Figure 1, the rate of catalytic turnover for the ammonia-dependent asparagine synthetase reaction and that for the hydroxylamine-dependent reaction are virtually identical. Nonetheless, when one takes into account the small ratio of NH_3/NH_4^+ at pH 8.0, it can be seen that the binding of the two substrates is quite different. Also, at saturating aspartate and MgATP concentrations, hydroxylamine exhibited substrate inhibition but ammonia did not. Hydroxylamine's substrate inhibition becomes apparent at 50 mM, but for NH_3 to be 50 mM at pH 8, ammonium acetate would have to be at a concentration of 1.4 M. Perhaps at this concentration ammonia would show substrate inhibition as does hydroxylamine.

At nonsaturating concentrations of aspartate (MgATP saturating), ammonia is shown to produce substrate inhibition which disappears when the aspartate concentration is raised to saturation. When aspartate is saturating, but MgATP is nonsaturating, ammonia produces no substrate inhibition over the same ammonia concentration range. This seems to indicate that ammonia can bind competitively with respect to aspartate, causing a dead-end complex since saturating aspartate can overcome this inhibition.

The data of Markin et al. (1981) show that in the glutamine-dependent asparagine synthetase reaction, asparagine acts as a simple competitive inhibitor with respect to glutamine. The data presented here (Figure 3) show that in the ammonia-dependent reaction, asparagine exhibits partial competitive inhibition with respect to ammonia. One explanation that would be consistent with both of these findings is that asparagine could bind to the active site of the free enzyme form at the site where glutamine would usually bind. This would block the glutamine-dependent reaction but still allow ammonia to bind to some extent, perhaps by virtue of its size alone. Once ammonia does bind, the reaction can proceed to completion so that even with infinite amounts of asparagine present the reaction velocity will not be reduced to zero. Thus, a partial competitive inhibition pattern is observed (see Figure 3).

If asparagine does indeed bind to the active site as described, in order for the reaction to proceed to completion, asparagine would have to be released at some point prior to product formation. Figure 7 shows one possible mechanism that could account for the data presented. In the top portion of the mechanism without asparagine, ammonia binds first followed by a random addition of aspartate and MgATP. In the presence of asparagine (i.e., the lower pathway shown in Figure 7), the mechanism becomes ordered—the binding of ammonia must be followed by MgATP. When MgATP binds, asparagine is released and aspartate can bind, with product formation and release occurring as it would in the absence of the inhibitor.

To test the possible existence of the mechanism shown in Figure 7, a detailed kinetic analysis must be done. Therefore, the replots of the Lineweaver–Burk plots were examined for kinetic experiments performed under a variety of conditions. The mechanism in Figure 7 predicts that when both MgATP and aspartate are saturating, partial competitive inhibition by asparagine should result. This is because when the MgATP concentration is high enough, the $E^{NH_3}_{Asn}$ complex can be pushed toward a productive enzyme form. Therefore, in this case, even infinite asparagine concentrations will not cause a dead-end complex. This prediction is shown to be verified by the data in Figure 3. When the MgATP concentration is saturating and the aspartate concentration is below saturation, the mechanism predicts hyperbolic inhibition by asparagine because asparagine release is still occurring. This prevents a dead-end complex from forming. This is shown to be the case in Figure 4. The mechanism predicts, however, that when the MgATP concentration is below saturation and the aspartate concentration is saturating, inhibition by asparagine will become more linear. This is because in the presence of asparagine the enzyme is forced to form a dead-end complex, i.e., the $E^{NH_3}_{Asn}$ complex. It may require very high MgATP concentration to force the release of asparagine from this complex. These predictions are borne out by the data of Figure 5A,B. The data presented showing that the inhibition by asparagine remains hyperbolic when both MgATP and aspartate are nonsaturating (see Figure 6) also support this notion.

It is possible that asparagine could be released after ammonia binds but before either MgATP or aspartate bind. In that case, no difference would be expected between the replots when only aspartate was nonsaturating and when only MgATP was nonsaturating. This is clearly not the case (see Figures 4 and 5).

If asparagine were released after both ammonia and aspartate were bound but before MgATP, a reversal of the results shown in Figures 4 and 5 would be expected. If asparagine were not released prior to product formation, nonsaturating concentrations of either MgATP or aspartate, or both, should have no effect. The data of Figures 4–6 show this clearly not to be the case.

In order to explain the data presented here in molecular terms, it is necessary to postulate two possible models for the active site of asparagine synthetase. The simplest model would involve only one amino acid binding site that in turn binds glutamine and then aspartate during glutamine-dependent asparagine synthesis. It is possible that this site first makes the reactive ammonia and then (after glutamate release) allows the formation of the aspartyl-AMP intermediate. With the two necessary reactive intermediates formed in close proximity, product formation would be facilitated. A more complex model would have two separate sites for amino acid binding. That is to say, there is a site where glutamine is bound and hydrolyzed and the ammonia "held". There would be a separate (but close) site where aspartate would be bound so that the aspartyl-AMP intermediate could be formed. When the two intermediates were formed in their respective sites, the attack of the nucleophilic ammonia could occur by some sort of conformational change bringing the two sites closer together. For the purpose of discussion, the models will be referred to as the one-site and two-site models, respectively. Since the structure of the asparagine synthesis is not known, it is impossible to eliminate either of these models. Therefore, the data will be interpreted in terms of both models.

One-Site Model. It is necessary to envision the reaction as occurring with only one amino acid binding site. It is essential to reiterate the point that (in the glutamine-dependent reaction) ATP binding is necessary for the release of the glutamate formed from hydrolysis of glutamine. For the ammonia-dependent reaction, there is nothing that fills the amino acid binding site that must be displaced by ATP binding. When the product asparagine is present (as an added inhibitor), the amino acid binding site is filled even during the ammonia-dependent reaction. Therefore, when asparagine is present, the ammonia-dependent reaction will become ordered with respect to the binding of aspartate and ATP. This is because asparagine is binding to the amino acid binding site and must be displaced by the binding of ATP, just as glutamine had to be displaced during the glutamine-dependent reaction. The data of Figure 3 indicate that asparagine is a partial competitive inhibitor with respect to ammonia. Recalling that ammonia must be bound prior to ATP binding (Markin et al., 1981), it is clear that ammonia can bind to some extent even in the presence of asparagine. It is also expected that asparagine would be a pure competitive inhibitor with respect to glutamine. This has been shown to be the case previously (Markin et al., 1981).

Two-Site Model. This model is envisioned as a pair of amino acid binding sites with an ammonia site as the junction between them. One of the amino acid sites is that which binds glutamine and the other binds aspartate and the aspartyl-AMP intermediate. To have this model fit the kinetic data presented, it is necessary to propose that when the glutamine site is filled nothing can bind to the aspartate site. If this were not the case, random addition of substrates (ATP and aspartate) would occur during the glutamine-dependent reaction. This does not occur [see Markin et al. (1981)]. This model can accommodate a random substrate addition of ATP and aspartate during the ammonia-dependent reaction. This is because the glutamine site would not normally be filled. If asparagine is present, ammonia can still bind because asparagine does not completely cover the glutamine site. This is consistent with the fact that asparagine is competitive with respect to glutamine (Markin et al., 1981) and is a partial competitive inhibitor with respect to ammonia (Figure 3). From the kinetic evidence presented here, a consistent interpretation would be that asparagine inhibition occurs only by binding to the glutamine binding site. For the alternative to occur (i.e., asparagine binding to the aspartate site), asparagine inhibition would have to be a linear competitive inhibitor with respect to both glutamine and ammonia. The data of Figure 3 show that this is not the case. This model also accommodates an interpretation for the substrate inhibition by ammonia and hydroxylamine (Figure 1). That is, ammonia could bind both to the ammonia site and to the aspartate site. The fact that substrate inhibition by ammonia does not occur until aspartate becomes nonsaturating (see Figure 2) is consistent with this interpretation.

There is not sufficient evidence to eliminate either the one-site or the two-site model for asparagine synthetase. However, there is ample precedent for either mechanism when examining the literature regarding transferase reactions. As an example, Knowles (1980) describes both types of models as being operative for phosphoryl transfer reactions. The work on carbamoylphosphate synthetase (which is highly analogous to asparagine synthetase) clearly indicates that the glutaminase reaction and the transferase reaction actually occur on separate subunits (Trotta et al., 1974). Further studies will be required to resolve these possibilities.

Registry No. Ammonium, 14798-03-9; ammonia, 7664-41-7; hydroxylamine, 7803-49-8; asparagine, 70-47-3; asparagine synthetase, 9023-69-2.

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γ-Butyrobetaine Hydroxylase: Stereochemical Course of the Hydroxylation Reaction†

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ABSTRACT: The stereochemical course of the aliphatic hydroxylation of γ-butyrobetaine by calf liver and by *Pseudomonas* sp AK1 γ-butyrobetaine hydroxylases has been determined. With [3(*RS*)-3-^{3}H]-γ-butyrobetaine or [3(*R*)-3-^{3}H]-γ-butyrobetaine as substrate, a rapid and significant loss of tritium to the medium occurred. On the other hand, with [3(*S*)-3-^{3}H]-γ-butyrobetaine, only a negligible release of tritium to the aqueous medium was observed. Indeed, on hydroxylation of [3(*S*)-3-^{2}H]-γ-butyrobetaine by either the calf liver or bacterial hydroxylase, the isolated product L-carnitine was found to have retained all of the deuterium initially present in the 3(*S*) position. Since the absolute configuration of the product L-carnitine has been determined to be *R*, such results are only compatible with a hydroxylation reaction that proceeded with retention of configuration. With [*methyl*-^{14}C,3(*R*)-3-^{3}H]-γ-butyrobetaine as substrate for the calf liver hydroxylase, the percentage of tritium retained in the [*methyl*-^{14}C]-L-carnitine product was determined as a function of percent reaction. The results of these studies indicated that *pro-R* hydrogen atom abstraction exceeded 99.9%. Experiments using racemic [*methyl*-^{14}C,3(*RS*)-3-^{3}H]-γ-butyrobetaine as substrate yielded similar results and additionally allowed us to estimate α-secondary tritium kinetic isotope effects of 1.10 and 1.31 for the bacterial and calf liver enzymes, respectively. These results are discussed within the context of the radical mechanism for γ-butyrobetaine hydroxylase previously proposed [Blanchard, J. S., & Englard, S. (1983) *Biochemistry 22*, 5922], and the required topographical arrangement of enzymic oxidant and substrate is illustrated.

The enzyme γ-butyrobetaine hydroxylase [4-trimethylaminobutyrate,2-oxoglutarate:oxygen oxidoreductase (3-hydroxylating), EC 1.14.11.1] catalyzes the hydroxylation of γ-butyrobetaine to form L-carnitine in accordance with the reaction

γ-butyrobetaine + O_2 + α-ketoglutarate → L-carnitine + CO_2 + succinate

The enzyme has been obtained in highly purified form from bacteria (Lindstedt et al., 1977), calf liver (Kondo et al., 1981), and human kidney (Lindstedt et al., 1982) and is representative of that group of non-heme iron dioxygenases in which hydroxylation of the substrate is coupled to the oxidative decarboxylation of α-ketoglutarate [for reviews, see Abbott & Udenfriend (1974) and Hayaishi et al. (1975)]. α-Ketoglutarate is the only α-keto acid known to support hydroxylation, and its oxidative decarboxylation supplies two of the four electrons for reduction of molecular oxygen.

We recently reported on the primary and secondary tritium kinetic isotope effects exhibited by the bacterial and calf liver γ-butyrobetaine hydroxylases and proposed that the hydroxylation reaction proceeds via a homolytic carbon–hydrogen bond cleavage to yield a carbon radical (Blanchard & Englard, 1983). We also previously reported preliminary results suggesting that hydroxylation proceeds with retention of configuration (Englard & Midelfort, 1978), but those data were unable to distinguish unequivocally between a stereoselective process and a stereospecific hydrogen atom abstraction. Such a stereoselective process operates during the P-450$_{CAM}$-catalyzed aliphatic hydroxylation of camphor (Gelb et al., 1982).

We report here an analysis of the stereochemical course of aliphatic hydroxylation catalyzed by the calf liver and bacterial γ-butyrobetaine hydroxylases. We show that the hydroxylation reaction proceeds with retention of configuration and occurs with greater than 99.9% stereospecificity. The pertinence of these results to the mechanism of action of the two enzymes and the relative topography of the substrate and enzymic oxidant are discussed.

MATERIALS AND METHODS

γ-Butyrobetaine hydroxylase, isolated and purified from *Pseudomonas* sp AK1 (Linstedt et al., 1970, 1977), was generously provided by Dr. Göran Lindstedt (University of

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