Catalytic Activity of the N-Terminal Domain of *Escherichia coli* Asparagine Synthetase B Can Be Reengineered by Single-Point Mutation

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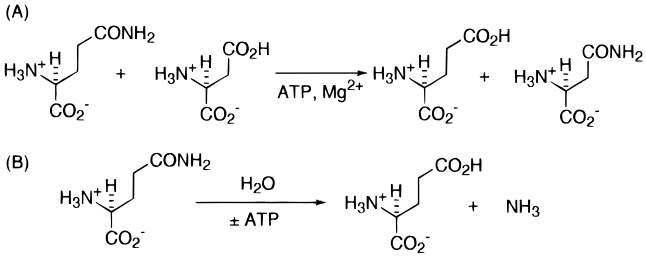
Abstract: The development of mechanistic strategies for the modification of enzyme function is of considerable biotechnological interest. We now report that replacement of the catalytically important residue Asn-74 by aspartic acid (N74D) in the N-terminal domain of *Escherichia coli* asparagine synthetase B (AS-B) confers nitrile hydratase activity upon the mutant enzyme. Furthermore, while wild type AS-B can efficiently catalyze the hydrolysis of glutamine to glutamate, the N74D AS-B mutant exhibits very low glutaminase activity. These results are consistent with similar experiments on papain, supporting the hypothesis that mutation of a critical active site residue to affect the partitioning of an intermediate common to multiple reaction mechanisms may represent an approach by which enzymes can be obtained with different catalytic function. Our experiments also provide the first direct chemical evidence for a close mechanistic relationship between papain, a thiol protease, and AS-B, a class II Ntn amidotransferase. These enzymes are not likely to have arisen by evolution from a common ancestral protein.

Introduction

General strategies for the modification of protein function are of considerable scientific and biotechnological interest.[1] One approach to creating proteins with novel function has been to combine domains from modular proteins.[2] For enzymes, the need to stabilize specific transition state structures or other intermediates along the reaction pathway places significant constraints on the chemical properties and spatial location of active site residues.[3] Most efforts to modify enzyme properties have therefore focused on altering protein stability by site-directed mutagenesis,[4] and directed evolution methods have yielded a subtilisin mutant possessing enhanced activity in aqueous dimethylformamide.[5] Mutational strategies to introduce new catalytic specificities by variation of residues in substrate-binding pockets have been investigated. While apparent changes in substrate specificity are observed, these usually arise by disrupting key interactions with the original substrate rather than by increasing the affinity of the mutant for alternate substrates.[6] Finally, the introduction of new catalyticc activity by mutagenesis has also been pursued, albeit with minor success. For example, subtilisin has been reengineered to yield an enzyme capable of synthesizing peptide bonds, although this required modification of multiple active site residues.[7] The observation that replacement of Gln-19 by glutamic acid in papain (Q19E) increased the turnover number for nitrile hydratase activity in the Q19E papain mutant by approximately 10^4 relative to that of the wild type enzyme is therefore significant for rational approaches to engineering new activities.[8] The identification

Scheme 1. Glutamine-Dependent Reactions Catalyzed by AS-B: (A) Glutamine-Dependent Asparagine Synthesis and (B) ATP-Stimulated Hydrolysis of Glutamine to Glutamate



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(4) It has been argued that redesigning enzymes for novel activities will be a difficult proposition given our limited understanding of the forces that govern protein stability and the interaction of enzymes and their substrates.[1d] On the other hand, there have been a number of successful attempts to modify substrate specificities using site-directed mutagenesis. For examples, see: (a) Nishiyama, M.; Birktoft, J. J.; Beppu, T. *J. Biol. Chem.* **1993**, *268*, 4656−4660. (b) Bocanegra, J. A.; Scrutton, N. S.; Perham, R. N. *Biochemistry* **1993**, *32*, 2738−2740. (c) Rheinnecker, M.; Baker, G.; Eder, J.; Fersht, A. R. *Biochemistry* **1993**, *32*, 1199−1203. (d) Hedstrom, L.; Szilagyi, L.; Rutter, W. J. *Science* **1992**, *255*, 1249−1253.

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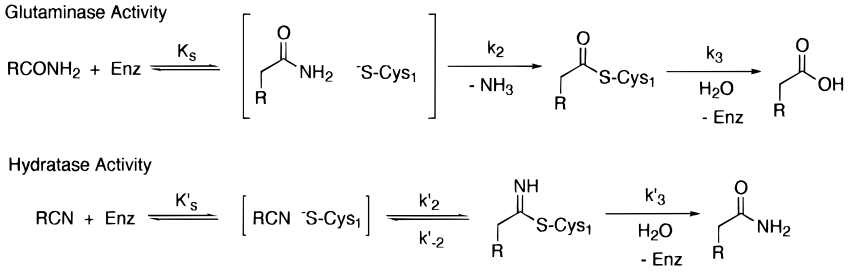
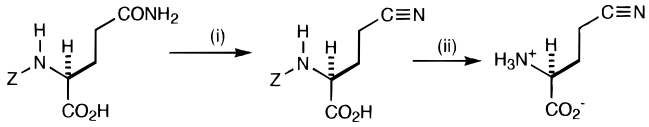
Scheme 2. Simple Kinetic Schemes for the Conversion of Amides to Acids (Glutaminase Activity) and Nitriles to Amides (Hydratase Activity)



of this key residue for mutagenesis, however, relied heavily on the delineation of critical residues involved in mediating the catalytic mechanism from extensive kinetic characterization of papain and a variety of site-specific mutants.[9] On the other hand, the Q19E papain mutant retains the ability to catalyze amide hydrolysis, albeit with a lower catalytic efficiency (k_{cat}/K_m) than the wild type (WT) enzyme.[8]

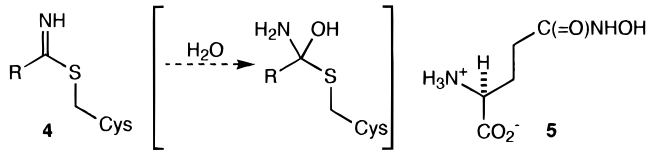
Asparagine synthetase B (AS-B) is a class II amidotransferase[10] encoded by the *asnB* gene of *Escherichia coli*,[11] which catalyzes the ATP-dependent synthesis of asparagine using glutamine as a primary nitrogen source (Scheme 1A). Sequence alignment studies in combination with the crystallographic structures of two related enzymes[12,13] suggest that AS-B is composed of N- and C-terminal domains that mediate glutamine utilization and activation of aspartic acid for nitrogen transfer, respectively.[10] In the absence of aspartate, AS-B catalyzes the hydrolysis of glutamine to glutamate, in a reaction that can be stimulated by ATP (Scheme 1B).[14] As for other class II amidotransferases,[10] the Cys-1 residue is essential for both of the glutamine-dependent activities exhibited by AS-B, presumably as the thiolate anion participates in the reaction leading to C−N bond cleavage.[15] In experiments employing glutamic acid γ-monohydroxamate as an alternate substrate, we have also identified an interaction between the side chain amides of glutamine and Asn-74 in the enzyme−substrate (ES) complex.[16] We now report that replacement of Asn-74 by aspartic acid (N74D) in the N-terminal domain of AS-B confers nitrile hydratase activity on the mutant enzyme (Scheme 2). Contrary to the observations on the Q19E papain mutant, however, the N74D AS-B mutant catalyzes glutamine hydrolysis with a specificity (k_{cat}/K_m) that is decreased 1000-fold relative to WT AS-B. The effects of replacing Asn-74 by aspartate on the reactions catalyzed by the mutant enzyme are therefore similar, but not identical, to those observed for the Q19E papain mutant and are the first direct evidence for a close mechanistic relationship between thiol proteases and class II Ntn amidotransferases.

Scheme 3. Preparation of Nitrile **1**[a]



[a] Z = $PhCH_2OC(=O)$. Conditions: (i) DCC, CH_2Cl_2; (ii) 10% Pd/C, 1,4-cyclohexadiene, EtOH, reflux.

Chart 1



Results and Discussion

Synthesis of Nitrile 1. The nitrile **1** was prepared in two steps from commercially available *N*-(carbobenzoxy)glutamine (**2**) in 76% overall yield (Scheme 3).[17] Initial treatment of glutamine derivative **2** with dicyclohexylcarbodiimide yielded 4-cyano-2-((carbobenzoxy)amino)butyric acid (**3**) after its purification from small amounts of **2** using reverse phase HPLC. Subsequent removal of the N-protecting group was then accomplished by catalytic hydrogen transfer using cyclohexadiene and 10% Pd/C in refluxing EtOH.

Inhibition of Wild-Type AS-B by Nitrile 1. Peptide nitriles are excellent inhibitors for thiol proteases such as papain.[18] NMR experiments with papain, employing labeled substrates, support the idea that the mechanism of inhibition involves attack of the thiolate anion of the active site cysteine on the nitrile to yield covalent adduct **4** (Chart 1) in a reversible reaction.[19]

Although the addition of water to **4** generates a tetrahedral intermediate identical in structure to that involved in amide

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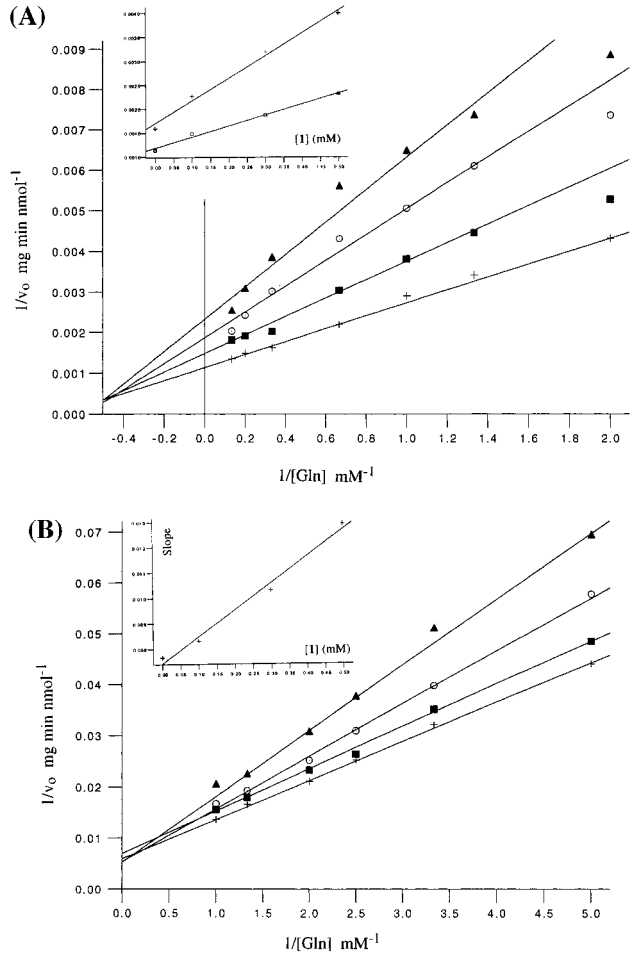

Figure 1. (A) Inhibition of WT AS-B glutaminase activity by nitrile **1**. Double reciprocal plot of initial velocities of AS-B with glutamine (0.5, 0.75, 1.0, 1.5, 3.0, 5.0, and 7.5 mM) at various fixed concentrations of **1**: (+) 0 mM, (■) 0.1 mM, (○) 0.3 mM, (▲) 0.5 mM. The inset shows the replot of the slopes and intercepts versus nitrile concentration (mM). (B) Inhibition of the N74A AS-B mutant glutaminase activity by nitrile **1**. Double reciprocal plot of initial velocities of N74A with glutamine (0.2, 0.3, 0.4, 0.5, 0.75, and 1.0 mM) at various fixed concentrations of **1**: (+) 0 mM, (■) 0.1 mM, (○) 0.3 mM, (▲) 0.5 mM. The inset shows the replot of the slopes versus nitrile concentration (mM). Each initial velocity is the average of three parallel experiments.

hydrolysis, conversion of nitriles into the corresponding acids by papain is not observed unless extreme conditions are employed.[20] In view of the possible mechanistic similarity between the AS-B glutaminase activity and papain-catalyzed peptide hydrolysis, nitrile **1** was assayed against wild type (WT) AS-B and shown to be a weak, noncompetitive inhibitor of the enzyme at pH 6.5 (Figure 1A). At pH 8.0, however, **1** became a competitive inhibitor of glutamine (data not shown). The molecular basis for this pH-dependent change in inhibition kinetics remains to be determined. Importantly, for our subsequent studies, no glutamate formation was observed by amino acid analysis when **1** was incubated with WT AS-B for up to 24 h at pH 6.5 and 37 °C, even at high concentrations of enzyme. The assay employed in these experiments involved derivatizing the amino acids in solution with phenylisothiocyanate (PITC), after quenching the reaction and removal of the protein, and determining the amounts of each fluorescent derivative using reverse phase HPLC (RP-HPLC). The inability

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Table 1. Kinetic Parameters for the Nitrile Hydratase and Glutaminase Activities of WT AS-B and the N74A and N74D AS-B Mutants at pH 6.5 and 37 °C

enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
	Nitrile Hydratase Activity		
WT AS-B	0.36[a]	$<5.5 \times 10^{-6}$	0.015
N74D	0.3 ± 0.04	0.013 ± 0.001	39.3
	Glutaminase Activity		
WT AS-B[b]	1.7	1.0	588
N74A[b]	0.45	0.14	311
N74D	1.7[c]	3.3×10^{-4}	0.19

[a] Value estimated from the inhibition constant for **1** with WT AS-B. [b] Data taken from ref 14 and included here for purposes of comparison. [c] Estimated assuming that K_m is unaltered in the mutant enzyme. Given that glutamine binding is likely to be less favorable, this value represents a lower limit on this kinetic parameter.

of WT AS-B to employ **1** as a substrate is entirely consistent with previous studies on the inhibition of thiol proteases by nitriles. Given the sensitivity of the RP-HPLC assay, our measurements set an upper limit on the turnover number, k_{cat}, of 5.5×10^{-6} s^{-1} for the hydratase activity of WT AS-B. Assuming that the K_m for the nitrile can be approximated by the observed K_I, the catalytic efficiency, k_{cat}/K_m, for the intrinsic hydratase activity of the wild type enzyme is therefore less than 0.015 M^{-1} s^{-1} at pH 6.5. Efforts to incubate WT AS-B with nitrile **1** for longer times could not be carried out given technical complications arising from protein instability under the reaction conditions. Hence, the inhibition, albeit weak, of the AS-B glutaminase activity by **1** is strikingly similar to that observed for peptide nitriles with papain, especially as WT AS-B appears to lack a catalytically important histidine residue in its glutamine-utilizing, N-terminal domain.[21]

Probing the Role of Asn-74 in Mediating AS-B Inhibition by Nitrile 1. Site-directed mutagenesis studies on papain have implicated Gln-19 in stabilization of various reaction intermediates in peptide hydrolysis.[22] Using a number of site-directed AS-B mutants and their interaction with the alternate substrate, glutamic acid γ-monohydroxamate (**5**, Chart 1), we have also shown that Asn-74 plays a catalytically important role in the glutamine-dependent activities of AS-B.[14,16] In light of these results, it seemed likely that Asn-74 in AS-B and Gln-19 in papain were functioning in a similar manner. We therefore investigated the ability of nitrile **1** to inhibit the glutaminase activity of the N74A As-B mutant. At pH 6.5, **1** was a weak, competitive inhibitor with respect to glutamine, of the glutaminase activity of the N74A AS-B mutant (Figure 1B), displaying a decreased affinity for the mutant, relative to WT enzyme (Table 1). In the glutaminase reaction at pH 6.5, the N74A AS-B mutant has a lower catalytic efficiency, as defined by k_{cat}/K_m, than WT AS-B,[16] corresponding to an increased free energy barrier of 0.39 kcal/mol for the reaction of 37 °C.[23] The reduced ability of **1** to inhibit the N74A AS-B mutant arises

(21) The question of whether there is a catalytically important histidine in the GAT-domain of AS-B and other Class II amidotransferases remains the subject of discussion. In the crystal structure of glutamine PRPP amidotransferase, another member of the *purF* family, the histidine originally implicated in catalysis by site-directed mutagenesis (Mei, B.; Zalkin, H. *J. Biol. Chem.* **1989**, *264*, 16613−16617) appears to be placed incorrectly to interact with Cys-1.[12] Neither His-29 nor His-80 in AS-B appear important for either glutamine-dependent activity.[11b] On the other hand, there is evidence for a Cys-His pair in Class I amidotransferases. See: (a) Tesmer, J. J. G.; Klem, T. J.; Deras, M. L.; Davisson, V. J.; Smith, J. L. *Nat. Struct. Biol.* **1996**, *3*, 74−86. (b) Chaparian, M.; Evans, D. R. *J. Biol. Chem.* **1991**, *266*, 3387−3395.

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Table 2. Inhibition Constants for Nitrile **1** in the Glutaminase Activity of AS-B

enzyme	pH	inhibition pattern	K_{is} (mM)	K_{ii} (mM)
WT AS-B	6.5	noncompetitive	0.36	0.51
WT AS-B	8.0	competitive	0.10	
N74A	6.5	competitive	0.70	

from a decrease in the stability of the complex by 0.41 kcal/mol. The similarity in the free energy changes observed for catalysis and inhibition supports the hypothesis that Asn-74 plays essentially the same functional role in mediating glutamine hydrolysis and nitrile inhibition. Given the steric and electronic properties of the thioimidate intermediate **4** (Chart 1), the side chain of Asn-74 is probably involved in stabilizing the thioacylenzyme intermediate in the mechanism of AS-B-catalyzed glutamine hydrolysis.

Characterization of the N74D AS-B Mutant. Having determined that the Asn-74 residue in AS-B was playing a similar role in catalyzing amide hydrolysis to that reported for Gln-19 in papain, we characterized the catalytic properties of the AS-B mutant in which Asn-74 was replaced by aspartic acid (N74D). The structural integrity of the N74D AS-B mutant was confirmed by the similarity in the kinetic parameters for the ammonia-dependent synthetase activity of the mutant and the WT enzyme. On the other hand, both glutamine-dependent activities of the N74D AS-B mutant were severely diminished relative to WT AS-B (Table 1). In a series of experiments, the residual levels of glutaminase activity were determined by incubating glutamine with the N74D AS-B mutant for varying periods of time up to 24 h. Glutamate production was assayed by RP-HPLC, the detection limits in this procedure corresponding to specific activities of less than 1 nmol min^{-1} mg^{-1} of protein. Although very low levels of glutaminase activity for the N74D AS-B mutant could be detected under these stringent assay conditions, the upper limit on k_{cat} was found to be approximately 3.3×10^{-4} s^{-1}. Efforts to increase the sensitivity of these assays were again hindered by protein instability. In order to rule out the possibility that conversion of glutamine to glutamate was being catalyzed by a contaminating enzyme, asparagine was added to the reaction mixtures at concentrations (500 μM) known to inhibit the glutaminase activity of WT AS-B. At these levels, asparagine caused a decrease in the rate of production of glutamic acid. Assuming that K_m for glutamine is unaffected by the mutation, the effect of replacing the Asn-74 side chain by the carboxylate of aspartate is to raise the energetic barrier for the glutaminase reaction by *at least* 4.9 kcal/mol, relative to WT enzyme. In contrast, nitrile **1** proved to be a substrate for the N74D AS-B mutant yielding glutamine by the addition of water across the carbon−nitrile triple bond. Due to the inability of the mutant enzyme to function as a potent glutaminase, it was straightforward to monitor glutamine production by PITC derivatization and RP-HPLC of the reaction mixture. The pH-dependence of the kinetic parameters associated with the nitrile hydratase activity of the N74D AS-B mutant could then be determined using standard techniques (Table 3). The N74D-catalyzed nitrile hydratase reaction exhibited Michaelis−Menten kinetics in all experiments, the highest catalytic efficiency ($k_{cat}/K_m = 39.3$ M^{-1} s^{-1}) being observed at pH 6.5 and 37 °C (Table 3). These data indicate that the site-specific mutation decreases the energy barrier for the pathway leading to hydration of **1** to form glutamine by approximately 4.8 kcal/mol under our reaction conditions.

In efforts to develop a mechanistic model for the change in enzyme activity, the kinetic data for the hydratase reaction catalyzed by the N74D AS-B mutant were fit to the following expression, which assumes that the catalytic activity is modulated by a single residue:[8]

$$k_{cat}/K_m = (k_{cat}/K_m)_{lim}/(1 + K_a/[H^+])$$

A value of 7.0 ± 0.4 was obtained for the pK_a of the functional group mediating nitrile hydratase activity, with $(k_{cat}/K_m)_{lim}$ being equal to 52.7 ± 0.8 M^{-1} s^{-1}. Although the structural nature of the glutamine-utilizing active site in the N-terminal (GAT) domain of the N74D AS-B mutant could, in principle, decrease the acidity of the Asp-74 side chain by a significant factor, we tentatively propose that this pK_a reflects the thiol group of Cys-1.

Table 3. Kinetic Parameters for the Hydratase Activity of the N74D AS-B Mutant as a Function of pH

pH	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
6.5[a]	0.3 ± 0.04	0.013 ± 0.001	39.3
7.5	1.2 ± 0.2	0.015 ± 0.001	12.0
8.5	5.1 ± 0.9	0.012 ± 0.0004	2.25

[a] Kinetic parameters included for ease of comparison.

Proposed Mechanism for the N74D-Catalyzed Nitrile Hydratase Activity. Although the molecular basis of the hydratase activity of the N74D AS-B mutant remains to be definitively established, a simple mechanistic scheme can be proposed (Scheme 4). We note that this proposal is consistent with an unperturbed pK_a for the side chain of Asp-74, as suggested by the pH-dependence of k_{cat}/K_m for the hydratase reaction. On the other hand, this contrasts with the observation that the acid side chain of Glu-19 in the Q19E papain mutant is protonated during hydration of peptide nitriles.[22] Initially, the nitrile **1** binds to the active site of the N74D AS-B mutant to form an ES complex. Given that nitriles generally have a pK_a of approximately −10,[24] and that there does not appear to be a catalytically important histidine in the AS-B GAT domain,[11b,21] protonation from solvent to give **5** becomes energetically favorable due to the proximity of the ionized side chain of Asp-74. The electrophilic species **5** can then undergo reaction with the Cys-1 thiolate to yield the thioimidate intermediate **6** that has an increased pK_a due to the proximity of the N74D carboxylate. Subsequent reaction of the protonated thioimidate with water forms the tetrahedral intermediate **7**, which is common to both the hydratase and glutaminase reaction mechanisms. Importantly, **7** can then partition to products via one of two pathways. In the first, which is most favorable in the WT enzyme, protonation of the amine followed by C−N bond cleavage yields thioacylenzyme **8** that can react with water to yield a carboxylic acid. We note that recent heavy-atom kinetic isotope effect (KIE) determinations are consistent with solvent being the proton donor in the WT AS-B glutaminase mechanism.[25] The N74D AS-B mutant, however, lacks the asparagine residue capable of stabilizing thioester **8** through hydrogen bonding, thereby making ejection of ammonia kinetically unfavorable and preventing conversion of glutamine or **1** into glutamate. In the second pathway, reaction of **7** therefore proceeds via C−S bond cleavage to give the resonance-stabilized amide moiety of glutamine, avoiding formation of the thioester intermediate **8**.

Although the details of the underlying mechanism of nitrile hydration may differ, the observed modification of catalytic activity in the N74D AS-B mutant is consistent with that reported for the Q19E papain mutant.[8] Moreover, the relative

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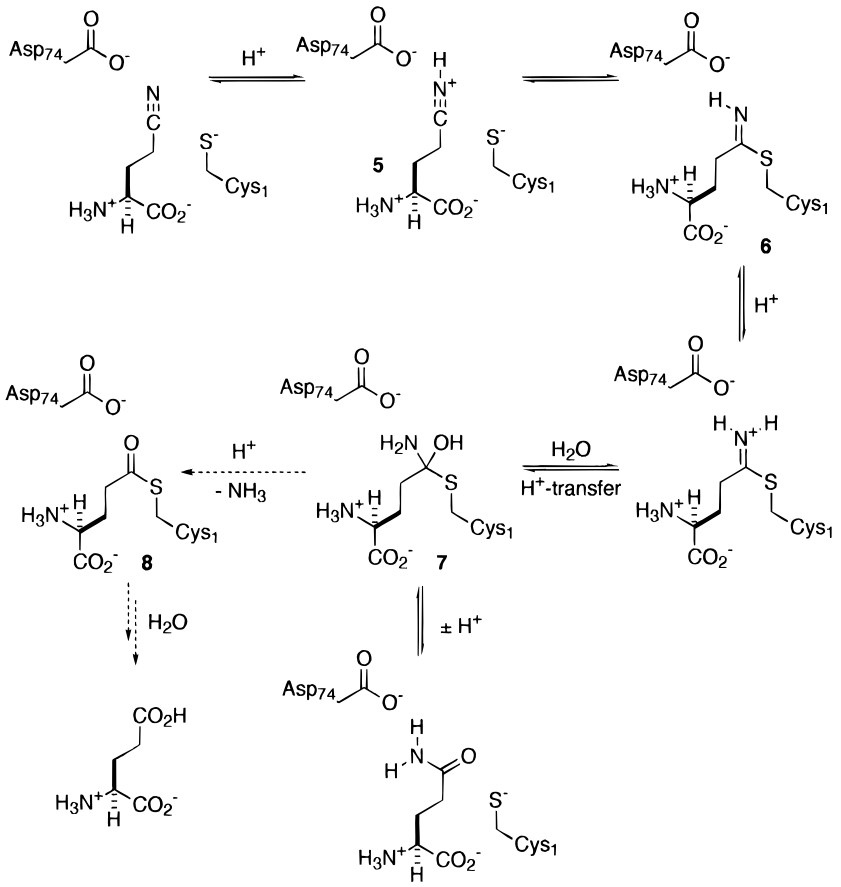
Scheme 4. Putative Mechanism for the Nitrile Hydratase Reaction Catalyzed by the N74D AS-B Mutant



changes in $[(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{WT}]$ for the hydratase activity of the mutant and WT enzymes are very similar, being 10^4 and 2×10^3 for papain and AS-B, respectively. Much of the difference between these values, however, is probably associated with the K_m values of the substrates for papain and AS-B. Hence, the K_m for the peptide nitriles employed in the experiments on papain and its Q19E mutant are in the micromolar range. Therefore, our observations appear to provide the first direct chemical evidence for a close mechanistic relationship between the amidohydrolase mechanisms of papain and class II Ntn amidotransferases. On the other hand, despite the probable similarity in catalytic role of the amide side chains of Gln-19 and Asn-74 in papain and AS-B, respectively, differences in the chemistry arising from the active site structures of these two enzymes are evident. First, the pH-dependence of k_{cat}/K_m for the nitrile hydratase activity of the Q19E papain mutant can be associated with a single residue with a pK_a of 6.03. This has been assigned to the involvement of the protonated form of Glu-19 in the catalytic mechanism. For the N74D AS-B mutant, it seems likely that Asp-74 is unprotonated in the active form of the catalyst. Second, the Q19E papain mutant retains the ability to convert the product amides into the corresponding acids, albeit with a 6-fold reduction in amidohydrolase activity (k_{cat}/K_m) relative to WT enzyme when CBZ-Phe-Ala-NH_2 is the substrate.[8] The glutaminase activity of the N74D AS-B mutant is diminished over 1000-fold compared to that of the WT AS-B (Table 1), which may reflect that the catalytic histidine in papain is absent in AS-B.

Comparison of the properties of the N74D AS-B mutant with enzymes from natural sources reveals a surprising difference in activity. While detailed mechanistic studies remain to be carried out, nitrile hydratases that convert nitriles into amides employ metal centers and, possibly, the pyrroloquinoline quinone cofactor in catalysis.[26] For example, recent studies on the nitrile hydratase from *Rhodococcus* sp. 312 using resonance Raman, ENDOR, and EXAFS techniques have shown that this enzyme contains a non-heme iron that is complexed in a low-spin state by three histidine and two cysteine ligands, the sixth coordination site being occupied by hydroxide derived from solvent water.[27] The role of the iron may be to enhance the nucleophilicity of water in attacking the nitrile, although NO-binding to the metal in place of the hydroxide in regulating enzyme activity has been discussed.[26d] In contrast, the nitrilases that possess an essential cysteine residue, not only catalyze the hydration of nitriles but also hydrolyze the resulting amides to form the corresponding carboxylic acids.[28] The N74D AS-B mutant therefore appears unique in its ability to employ cysteine in catalyzing hydration of nitrile **1** without subsequently hydrolyzing the product amide to glutamate at any significant rate.

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The results outlined here raise the question of whether the class II amidotransferases and thiol proteases are related by common ancestry. Sequence analysis does not, however, appear to support such a hypothesis for these two protein families, and the three-dimensional folds of papain and the N-terminal glutamine-utilizing domain of glutamine PRPP (5′-phosphoribosylpyrophosphate) amidotransferase are clearly different. Therefore, it seems likely that the active sites of these two families of enzymes has arisen through convergent evolution and that the functional equivalence of Gln-19 in papain and Asn-74 in AS-B is dictated by the chemical nature of the transition states and intermediates that are formed during thiol-catalyzed amide hydrolysis. Our studies provide further evidence for the hypothesis that chemistry places strict limits on the nature of catalytic residues in enzyme active sites and are consistent with recent observations concerning members of the enolase superfamily, in which anions are formed adjacent to carboxylic moieties using chemically equivalent residues.[29]

Finally, our ability to reengineer the catalytic activity of the N-terminal AS-B domain by mutation of a critical active site residue, in a similar manner to that reported for papain,[8] suggests a strategy by which the modification of a single residue might be employed to obtain enzymes with altered activity. Hence, if an intermediate in the mechanism underlying WT activity can be identified that is common to an alternate pathway, then replacement of the protein side chain can be used to bias partitioning of the intermediate to introduce a new activity. In the example reported here, mutagenesis of Asn-74 affects the tetrahedral intermediate that is common to the mechanisms of nitrile hydration and amide hydrolysis. It is possible that such an evolutionary mechanism has been operative in generating the mechanistic diversity observed in the enzymes of the enolase superfamily.

Experimental Section

Melting points were recorded using a Fisher−Johns melting point apparatus and are uncorrected. Chemical shifts are reported in ppm (δ) downfield of tetramethylsilane as an internal reference (δ 0.0). Splitting patterns are abbreviated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Combustion analyses for C, H, and N were determined in the Microanalysis Facility in the Department of Chemistry, University of Florida. Analytical thin-layer chromatography (TLC) was performed on silica gel 60F-245 plates, unless otherwise stated. Flash chromatography was performed by standard methods[30] on Davisil grade 633 type 60 Å silica gel (200−425 mesh). All solvents were purified by distillation before use. Chemical compounds were purchased from Aldrich and used without further purification.

2-((Carbobenzoxy)amino)-4-cyanobutyric Acid (3). This material was prepared, from *N*-(carbobenzoxy)glutamine **2**, using the literature procedure,[17a] as a colorless oil in quantitative yield: IR ($CHCl_3$) ν 3421, 3026, 2954, 2250, 1720, 1509, 1453, 1344, 1228, 1051 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 1.99−2.08 (1 H, m), 2.20−2.32 (1 H, m), 2.39−2.42 (2 H, m), 4.41−4.44 (1 H, m), 5.10 (2 H, s), 5.79 (1 H, d, J = 7.8 Hz), 7.31−7.37 (5 H, m), 10.39 (1 H, br s); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 13.53 (t), 27.93 (t), 52.55 (d), 67.39 (t), 118.71 (s), 128.06 (d), 128.29 (d), 128.50 (d), 135.65 (s), 156.24 (s), 173.94 (s); HRMS (FAB H^+) exact mass calcd for $C_{13}H_{15}N_2O_4$ requires 263.1032, found 263.1031; *m*/*e* (rel intensity) 263 (36), 91 (100).

2-Amino-4-cyanobutyric Acid (1). The protected nitrile **3** (1.33 g, 5.07 mmol) was dissolved in dry EtOH (10 mL) together with 1,4-cyclohexadiene (2.4 mL). After 10% Pd/C (680 mg) as catalyst was added, the mixture was gently heated until solvent reflux was observed. Heating was then stopped, and the reaction was allowed to proceed at ambient temperature for 1 h. The suspension was filtered through Celite, which was subsequently washed with water. The resulting solution was then lyophilized to yield **1** as a white solid, which was recrystallized from dioxane/water: 1.2 g, 76%; mp 227−229 °C [lit.[17a] 227.5−229 °C]; $[\alpha]^{22}_D$ +38.8° (c = 1.28, 1 M AcOH) [lit.[17b] $[\alpha]^{21}_D$ +32.2° (c = 0.54, 1 M AcOH)]; IR (KBr) ν 2959, 2122, 1618, 1588, 1414, 1342, 1314, 1210, 1150, 1085, 1011 cm^{-1}; ^1H NMR (D_2O, 500 MHz) δ 2.17−2.29 (2 H, dq, J = 7.2, 10.6 Hz), 2.69 (2 H, dt, J = 4.3, 7.5 Hz), 3.79 (1 H, t, J = 6.7 Hz); ^{13}C NMR (D_2O, 75.4 MHz) δ 13.24 (t), 26.00 (t), 53.04 (d), 119.79 (s), 172.74 (s); HRMS (CI H^+) exact mass calcd for $C_5H_9N_2O_2$ requires 129.066, found 129.063; *m*/*e* (rel intensity) 129 (100), 83 (42). Anal. Calcd for ($C_5H_8N_2O_2$): C, 46.87; H, 6.29; N, 21.86. Found: C, 46.85; H, 6.45; N, 21.70.

Expression and Purification of WT AS-B and the N74A and N74D AS-B Mutants. Full experimental details of the construction, expression, and purification of the proteins used in these studies have been published previously.[11b,14]

Kinetic Characterization of the Glutaminase Activities of WT AS-B and the N74A and N74D AS-B Mutants. (a) The glutaminase activities of WT AS-B and the N74A AS-B mutant were assayed using a spectroscopic procedure[31] as previously described[14] except that all reactions were carried out using 100 mM Bis-Tris/100 mM Tris-HCl as buffer. (b) Glutamine was incubated at 37 °C with 8 mM $MgCl_2$, and the N74D AS-B mutant (80 μg) was incubated at a concentration of 2.5 mM in 100 mM Bis-Tris/100 mM Tris-HCl adjusted to pH 6.5. The total reaction volume was 150 μL. At various times (0, 45, 90, 135, and 180 min), an aliquot (25 μL) of the mixture was taken and reaction terminated by boiling. Samples were filtered through a 2 μm filter and derivatized with phenylisothiocyanate (PITC). The glutamine and glutamate derivatives were separated by HPLC and quantitated spectroscopically. This assay procedure can detect less than 100 pmol of product under standard conditions.

Enzyme Inhibition Assays. (a) The ability of wild type AS-B to employ **1** as a substrate in asparagine synthesis was assayed by incubating 10 mM nitrile **1** with 5.6 μg of enzyme, 10 mM aspartate, 5 mM ATP, and 8 mM $MgCl_2$ in 100 mM HEPES buffer at pH 7.0 (100 μL total reaction volume) for 15 min at 37 °C. After the reaction was terminated by boiling, no pyrophosphate production could be detected in a coupled assay system (Sigma Technical bulletin number B1-100), indicating that **1** was not a substrate for the synthetase activity of enzyme. (b) The inhibition of the glutaminase activity of WT AS-B by **1** was determined by measuring glutamate production using the reaction of glutamate dehydrogenease in the presence of NAD^+.[31,14] Standard curves were obtained in the presence and absence of nitrile **1** and showed that the nitrile did not affect the activity of glutamate dehydrogenase. In these studies, WT AS-B (1.5 μg) was incubated for 15 min at 37 °C with 8 mM $MgCl_2$ in HEPES buffer, pH 7 (total volume 100 μL), at various glutamine concentrations (0.5, 0.75, 1.0, 1.5, 3.0, 5.0, and 7.5 mM). Nitrile concentrations of 0, 0.1, 0.3, and 0.5 mM were employed in determining the inhibition constants for **1**. Reaction mixtures were added to 380 μL of the coupling reagent (300 mM glycine, 250 mM hydrazine pH 9, 1 mM ADP, 1.6 mM NAD^+, and 2.2 U glutamate dehydrogenase) and incubated for 10 min at room temperature. The solution absorbance was measured at 340 nm, and the amount of glutamate present was determined from a standard curve. All assays were performed in triplicate. In the inhibition studies at pH 8, identical conditions were employed except that the buffer was 100 mM Tris-HCl and 2.5 μg of WT AS-B was present. (c) The inhibition of the glutaminase activity of the N74D AS-B mutant by **1** was determined as described for the WT enzyme. In these studies, the N74A AS-B mutant (14.7 μg) was incubated for 15 min at 37 °C with 8 mM $MgCl_2$ in HEPES buffer, pH 7 (total volume 100 μL), at various glutamine concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.75, and 1.0 mM). Nitrile concentrations of 0, 0.1, 0.3, and 0.5 mM were employed in determining the inhibition constants for **1**. All assays were performed in duplicate.

Kinetic Characterization of the Nitrile Hydratase Activity of WT AS-B and the N74D AS-B Mutant. (a) Nitrile **1** was incubated with

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8 mM $MgCl_2$ and the N74D AS-B mutant (7.5 μg) at varying concentrations (0.1−3.0 mM) in 100 mM Bis-Tris/100 mM Tris-HCl adjusted to pH 6.5, 7.5, or 8.25. The total reaction volume was 50 μL. After 20 min, the reaction was terminated by boiling for 3 min, and the samples were filtered through a 2 μm filter and derivatized by reaction with phenylisothiocyanate (PITC). The PITC derivative of glutamine was separated by HPLC, and the amount was quantitated spectroscopically. Values of K_M and k_{cat} for **1** were obtained by nonlinear regression fitting of initial velocity data to the Michaelis−Menten equation using the Prism software package (Graphpad Inc., San Diego, CA). In control experiments, the production of glutamine was found to be linear at reaction times of up to 30 min. (b) Nitrile **1** was incubated with 8 mM $MgCl_2$ and WT AS-B (25 μg) at a concentration of 2.5 mM in 100 mM Bis-Tris/100 mM Tris-HCl, adjusted to pH 6.5, for 3, 6, and 24 h. The total reaction volume was 50 μL. Reactions were terminated by the addition of 4% trichloroacetic acid (TCA), and the resulting samples were filtered through a 2 μm filter and derivatized by reaction with phenylisothiocyanate (PITC). The PITC derivative of glutamine was separated by HPLC, and the amount was quantitated spectroscopically. This assay procedure can detect less than 100 pmol of product under our standard conditions.

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