Hydrolysis of N-Acetyl-L-Glutamine by Acylase I

J. BAXTER, Y. KIM, AND M. SNOWDEN

ABSTRACT: Parenteral administration of N-acetyl-L-glutamine (NAQ) produces substantial urinary losses. To evaluate enteral utility, we examined NAQ hydrolysis by acylase I, a critical first step in biological utilization. NAQ was quantitatively hydrolyzed to glutamine in vitro. Enzyme kinetic parameters were compared for NAQ (Km = 11.4 mM, Vmax = 5.54 nmole glutamine/min/μg enzyme) and an approved food additive, N-acetyl-L-methionine (NAM) (Km = 1.36 mM, Vmax = 7.48 nmole methionine/min/μg enzyme). These data indicated preference for NAM in substrate recognition (Km), but similar relative catalytic ability (Vmax). While NAQ is possibly a suitable enteral glutamine source, utility will depend on intestinal acylase I levels and intestinal residence times not yet determined.

Keywords: nutrition, N-acetyl-L-glutamine, glutamine, acylase, bioavailability

Introduction

GLUTAMINE IS THE MOST ABUNDANT AMINO ACID IN BLOOD and intracellular fluid. It is approximately half of the whole-body pool of free amino acids (Souba and others 1985). Also, glutamine is a conditionally essential nutrient (Swails and others 1992) in high stress (Ziegler 1996) or rapid growth, and serves as a primary source of energy for the gut (Klein and Fleming 1993; Kuhn and others 1996). It is thus a desirable component in nutritional products intended for use in trauma patients. However, glutamine is relatively unstable in aqueous solutions. Like all free amino acids, it is susceptible to the Maillard browning reactions, which can generate undesirable flavors and aromas (William and others 1996), reduce bioavailability, inhibit some digestion enzymes, and produce carcinogens (Nagao and others 1983; Skog and others 1994). Free glutamine also undergoes a rapid internal nucleophilic reaction forming pyroglutamic acid (PGA), a possible neurotoxin (Shih 1985). This reaction is pH-dependent (Acree and Lee 1975), and occurs at substantial rates (0.7 to 0.9% glutamine loss per day at room temperature) in parenteral solutions containing approximately 70 mmole/L (Kahn and others 1991). There is obviously a need, then, for an alternative to free glutamine that avoids these undesirable reactions.

One compound, N-acetyl-L-glutamine (NAQ) seems a likely candidate for such an alternative. NAQ is easily synthesized via simple, high-yield chemistries, and is thus potentially available on a large-scale commercial basis (Magnusson and others 1989). Also, NAQ is stable in solution even after heat sterilization (Schäfer and others 1967; Ollenschlager and others 1989). NAQ is not "GRAS" (that is, generally recognized as safe), although several N-acetylated amino acids such as N-acetyl-L-tyrosine, N-acetyl-L-cysteine, and N-acetyl-L-methionine (NAM) are used in foods as approved food additives (Federal Register 1978). This fact consequently suggests that a related compound such as NAQ could also be metabolically available. The addition of NAM as a methionine substitute in protein-containing but methionine-deficient products does improve nutritional quality, while avoiding the off-flavors and odors produced during the processing of foods containing free methionine (Damaco 1975). The literature concerning use of NAM as a food ingredient is plentiful, and can serve as a guide to demonstrate NAQ's acceptability as an additive which can provide glutamine as a form stable in aqueous solution.

Acylase I (aminoacylase; N-acetylamino-acid amidohydrolase, E.C. 3.5.1.14.) hydrolyzes NAM to L-methionine and acetate. This enzyme participates in the intracellular catabolism of terminal N-acyl-peptides, and in the salvage of N-acetylated amino acids (Endo 1980; Gade and Brown 1981). It also participates in xenobiotic bioactivation (Anders and Dekant 1994). The enzyme is highly specific for the L-isomer of N-acetylmethionine (Birnbaum and others 1952), and has the requirement for divalent metal ions such as Co^{2+}, which is most often used in assays (Bommarius and others 1992). Acylase I has been found at high levels in both kidney and intestinal tissues of several animals, including humans (Endo 1978; Ziomek and Szwczuk 1978). Ferjancic-Biagini and others (1998) proposed that acylase I plays an important role in the biological utilization of N-acylated food proteins. Supporting this hypothesis is the finding that N-acetyl-D-methionine, when fed to rats, is excreted in the urine intact, and is not a bioavailable source of dietary methionine (Rotruck and Boggs 1975; Boggs and others 1975), even though free D-methionine is available as a methionine source (Baker and Boebelo 1980; Friedman and Gumbmann 1984), through the action of D-amino acid oxidase (Konno and Yasumura 1984). This suggests that the dietary utility of acetylated amino acids is heavily dependent on the ability of acylase I to release the free amino acid. Therefore, the bioavailability of acetylated amino acids may be predicted, based on the ability of acylase I to catalyze their hydrolyses.

Consequently, the purpose of this study is to compare the ability of acylase I to hydrolyze NAQ and NAM. A secondary goal was to estimate the D-isomer content in NAQ preparations.

Materials and Methods

Chemicals used

L-methionine, L-glutamine, L-glutamic acid, L-pyroglutamic acid, NAM, NAQ, acylase I from porcine kidney, fluorescamine, D-glutamine, boric acid, $CoCl_2 \cdot 6H_2O$, pyridine, and acetic anhydride were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). HCl (37% w/v) and NaOH were obtained from Mallinckrodt Specialty Chemicals Co. (Paris,

Ky., U.S.A.). NaH_2PO_4 was obtained from Aldrich Chemical Co. (Milwaukee, Wis., U.S.A.). Water was deionized using a MilliQ Plus™water purification system (Millipore Laboratory Water Division, Cranberry Twp., Penn., U.S.A.).

N-acetyl-D-glutamine was prepared using a previously published and patented procedure (Orgamol SA 1966) with an overall yield of 86%, and its identity as N-acetylglutamine was confirmed on reverse-phase high-performance liquid chromatography (RP-HPLC) analysis against standard NAQ. (RP-HPLC cannot separate the isomers).

Assay of L-methionine and L-glutamine

Free amino acid was estimated, using the fluorescamine reaction (Udenfriend and others 1972; Roche Diagnostics 1973). Solutions of L-methionine or L-glutamine (0.1 to 100 nmole/mL) in 0.3 M sodium borate buffer (pH 9.0) were prepared, and 1.0 mL aliquots were placed in 12 × 75-mm test tubes. One-half mL of a solution containing 2.5% (w/v) glycerol, 0.25 mM $CoCl_2$, and 0.1 M sodium phosphate buffer (to match enzyme incubation buffer contribution) were added to the test tubes. A fluorescamine solution (0.5 mL, 0.25 mg/mL in acetone) was then added rapidly with vigorous mixing in a vortex mixer (Thermolyne Maxi Mix II, Thermolyne Corp., Dubuque, Iowa, U.S.A.). Fluorescence was measured by spectrofluorometer (Perkin-Elmer LS50B Luminescence Spectrofluorometer, Perkin-Elmer Co. Norwalk, Conn., U.S.A.) with excitation at 390 nm and emission monitored at 480 nm. Plots of fluorescence against concentration of amino acid were used as standard curves for the experiments outlined below.

General enzyme assay details and stock solutions

The enzyme was prepared as a 2 μg/mL stock solution in 10% (w/v) glycerol, 1 mM $CoCl_2$, 0.1 M sodium phosphate buffer (pH 7.0), and stored on ice until needed. Various enzyme concentrations were used during these experiments, and preliminary dilutions as necessary were performed into the same buffer, immediately prior to use. The enzyme was stable for several h under these conditions.

Substrate solutions were prepared in 0.1 M sodium phosphate buffer (pH 7.0). Reactions were initiated by mixing appropriate volumes of enzyme and substrate stock solutions. Incubations occurred at 37 °C, and were stopped by rapid addition of 2 volumes of 0.3 M sodium borate buffer (pH 9.0). Hydrolysis was assessed by reaction of 1.5 mL of the resulting mixture with 0.5 mL fluorescamine solution, comparing to standards (glutamine or methionine), as detailed above.

Hydrolysis of NAQ and NAM by acylase I (varying enzyme concentration and varying incubation time)

With respect to varying enzyme concentration, reaction mixtures containing various concentrations of acylase I (0 to 0.5 μg/mL) in 2.5% (w/v) glycerol, 0.25 mM $CoCl_2$, and 25 mM substrate (NAM or NAQ) in sodium phosphate buffer (0.1 M, pH 7.0), in a final volume of 0.5 mL, were incubated at 37 °C for 30 min. The reaction was stopped and overall primary amine release was assessed (as detailed above). Blanks were prepared for each of the samples prepared in the description above, except that substrate solution was added after stop buffer, and a final result was then calculated, after subtracting the blank result.

With respect to varying incubation time, reaction mixtures were prepared containing acylase I (0.075 μg/mL) in 2.5% glycerol, 0.25 mM $CoCl_2$, 25 mM substrate (NAM or NAQ) in sodium phosphate buffer (0.1 M, pH 7.0), in a final volume of 0.5 mL, and incubated at 37 °C for various times (0 to 120 min). The incubation was stopped, and extent of the reaction was measured, using the fluorescamine reaction (as detailed above).

Km and Vmax values for NAM and NAQ

Reaction mixtures containing acylase I (0.5 μg/mL) in 2.5% glycerol, 0.25 mM $CoCl_2$, 0.1 M sodium phosphate (pH 7.0), with varying concentrations of either substrate (NAM or NAQ) in a final volume of 0.5 mL were prepared and incubated at 37 °C for 30 min. The reaction was stopped, and free amine generation was measured, as detailed above. The data were then plotted as 1/V against 1/S, and fitted using a weighted linear regression analysis (Wilkinson 1961). Data are expressed as the fit value and 95% confidence interval.

Complete reaction and estimation of maximum content of D-isomer

A reaction mixture containing 0.08 mg/mL acylase I, 50 mM NAQ in 10% glycerol, 1 mM $CoCl_2$, 0.1 M sodium phosphate buffer (pH 7.0) was prepared, and 8-mL aliquots were immediately transferred to 2 10-mL incubation vials in a 37 °C bath. (Note: Reaction was started by addition of solid enzyme; no stock was prepared). After 95 min of incubation, extra acylase I (0.64 mg) was added to one of the vials. At various times (0 to 155 min), a 20-μL aliquot was removed and diluted to 25 mL with sodium borate buffer (0.3 M, pH 9.0). Blanks were prepared for either vial by incubating identical solutions without substrate in parallel, removing 20-μL aliquots at the designated times, diluting with sodium borate buffer (0.3 M, pH 9.0) to 20 mL volume, then adding 20 μL of 50-mM NAQ solution in sodium borate buffer (0.3 M, pH 9.0), then diluting to a final volume of 25 mL with sodium borate buffer (0.3 M, pH 9.0). Hydrolysis was assessed, using the fluorescamine reaction described above, with a 1.5-mL aliquot of the stopped reaction mixture.

A second reaction mixture containing 0.08 mg/mL acylase I, 50 mM N-acetyl-D-glutamine in 10% glycerol, 1 mM $CoCl_2$, 0.1 M sodium phosphate buffer (pH 7.0) was prepared, and an 8-mL aliquot was immediately transferred to a 10-mL incubation vial in a 37 °C bath. At various times (0 to 120 min), a 200-μL aliquot (to enhance sensitivity of the assay) was removed, and diluted to 25 mL with sodium borate buffer (0.3 M, pH 9.0). Blanks were prepared by incubating an identical mixture without added substrate in parallel, removing 200-μL aliquots at the designated times, diluting with borate buffer to 20 mL volume, then adding 200 μL of 50-mM NAQ D-isomer solution in sodium borate buffer (0.3 M, pH 9.0), and diluting to a final volume of 25 mL with sodium borate buffer (0.3 M, pH 9.0). The time course of hydrolysis was assessed using the fluorescamine reaction with a 1.5-mL aliquot of each stopped reaction mixture sample.

Since results of previous experiments using the fluorescamine assay suggested incomplete hydrolysis of NAQ, we wished to confirm these results with an independent assay. At several time points (0, 30, 60, and 120 min) during the incubation of the 2 reaction mixtures described above, the content of both glutamine and NAQ was estimated by HPLC analysis, using a modification of a previously developed method (Palmerini and others 1981). The system used consisted of a HPLC pump (Model 126), a diode array detector (Model 168), an autosampler (Model 508), and the System Gold Neuveau controller, all from Beckman Coulter of Fullerton, Calif., U.S.A.). An InertSil® C_8, 5 μm column, 250 × 4.6 mm (Keystone Scientific, Inc., Bellefonte, Pa., U.S.A.) was

eluted isocratically at 1 mL/min, with water adjusted to pH 2.2 with HCl. Absorption was monitored at 220 nm, with a run-time of 15 min. The sample was removed directly from the incubation mixture (no borate buffer addition), diluted with an equal volume of mobile phase, and immediately injected (10 μL) onto the column. Standards (NAQ at 0.1, 0.2, 2.5 mM, and L-glutamine at 25 mM) were used to calibrate the instrument response using peak- height measurements. Other elution times were determined by duplicate injections of single 25-mM standards to eliminate the possibility of coelution of other compounds potentially present in the reaction mixture (that is, pyroglutamic acid, glutamic acid, N-acetylglutamic acid, acetate).

Results and Discussion

Assay of L-methionine and L-glutamine

As expected, the fluorescence yield was not linear with concentration for either L-methionine or L-glutamine. Nonlinear regression fits (Second-Order quadratic) were adequate to allow calculation of concentrations from unknown samples (Prism© Version 2.0, GraphPad Software, Inc., San Diego, Calif., U.S.A.).

Hydrolysis of NAQ and NAM by acylase I

Using acylase I, hydrolysis of either NAQ or NAM was linearly dependent on enzyme concentration (Figure 1) and time (Figure 2), indicating that incubation conditions did not denature the enzyme, and that the enzyme recognizes either compound as a substrate. Under these conditions, NAM is more readily hydrolyzed by acylase I than NAQ.

Km and Vmax values for NAM and NAQ

To assess the relative ability of acylase I to hydrolyze NAM and NAQ, the Michaelis-Menten constants Km and Vmax (Michaelis and Menten 1913) were determined for each substrate. Enzyme activity was determined at several substrate concentrations, plotted (1/V against 1/S), and Km and Vmax values calculated, using a weighted linear regression analysis (Wilkinson 1961). Substrate depletion was never greater than 7.5% for any of the incubation mixtures. Enzyme constant data are presented in Table 1, and expressed as the fit values and 95% confidence intervals.

Table 1—Comparison of Km, Vmax of acylase I for NAM and NAQ

	Km (mM)	Vmax (nmole Met or Gln/min/μg acylase I)
N-acetyl-L-methionine	1.36 ± 0.15	7.48 ± 0.28
N-acetyl-L-glutamine	11.41 ± 1.05	5.54 ± 0.28

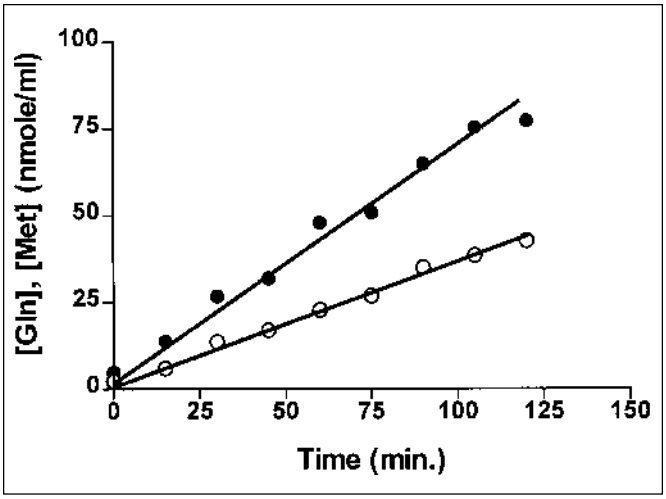

Figure 2—Hydrolysis of NAM and NAQ by acylase I as a function of incubation time. Reaction mixtures containing acylase I (0.075 μg/mL) at pH 7.0, and either 25 mM NAM (●) or 25 mM NAQ (○), were incubated at 37 °C for various times (0 to 120 min). Zero-time was measured immediately after adding enzyme. Hydrolysis was assessed using fluorescamine.

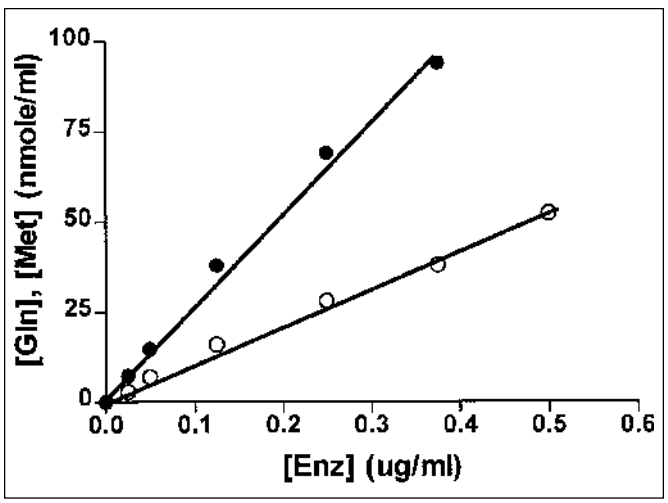

Figure 1—Hydrolysis of NAQ and NAM by acylase I as a function of enzyme concentration. Reaction mixtures containing various concentrations of acylase I (0 to 0.5 μg/mL) at pH 7.0, and either 25 mM NAM (●) or 25 mM NAQ (○), were incubated at 37 °C for 30 min and hydrolysis assessed using fluorescamine as detailed in "Materials and Methods."

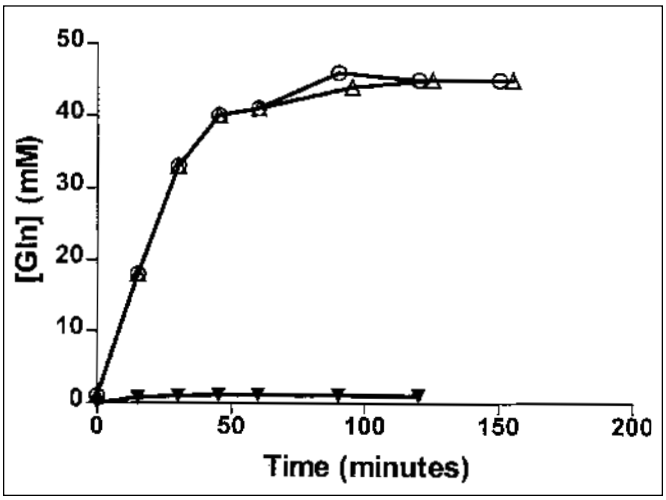

Figure 3—Hydrolysis of NAQ and N-acetyl-D-glutamine by acylase I. Reaction mixtures containing 0.08 mg/mL acylase I at pH 7.0, and either 50 mM NAQ (○) or N-acetyl-D-glutamine (▼), were incubated at 37 °C. Extra acylase I was added after 90 min incubation (△). Zero-time was measured immediately after adding enzyme. Hydrolysis was assessed using fluorescamine.

Complete reaction and estimation of maximum content of D-isomer

Acylase I hydrolyzes NAQ, producing a fluorescamine reactive product (Figure 3). The total glutamine released reaches a plateau after approximately 90 min, at an estimated 90% of theoretical glutamine release when fluorescence is calibrated against standard glutamine. Adding additional enzyme after hydrolysis stopped (95 min) produced no additional release, eliminating the possibility of enzyme instability generating the plateau. Incubation of N-acetyl-D-glutamine with acylase I resulted in a very slight hydrolysis plateau at approximately 2% of theoretical completion. This is consistent with a small amount of racemization during the production of this derivative, which was previously observed for the acetylation chemistries by du Vigneaud and Meyer (1932).

During this attempt to generate complete hydrolysis, HPLC analysis was applied to aliquots at various time points during hydrolysis to: (1) further evaluate the completeness of the hydrolysis; (2) confirm that glutamine is the product; (3) determine residual NAQ (Figure 4). Contrary to the fluorescence measurement data, HPLC analysis estimated essentially quantitative conversion of NAQ to glutamine, detecting less than 2% residual NAQ (Table 2). Again, additional enzyme added late in the incubation had no effect.

Table 2—HPLC analysis – Production of glutamine from NAQ, and estimation of maximum D-isomer content

Reaction #	Initial NAQ[a] (mM)	Final NAQ (mM)	Final Gln (mM)	Final Glu (mM)
1 (no extra enz)	50	0.8	50.7	ND[b]
2 (extra enz added)	50	0.4	50.0	ND

a is N-acetyl-L-glutamine
b is Not Detected (ND)

Conclusions

OUR EXPERIMENTS CONFIRM PREVIOUS RESULTS (GALAEV AND Svedas 1982), which showed that NAM is hydrolyzed to L-methionine by acylase I. We also demonstrated that the same enzyme hydrolyzes NAQ, releasing L-glutamine. Because acylase I participates in the intracellular catabolism of N-acyl-peptides, and in the salvage of other N-acetylated

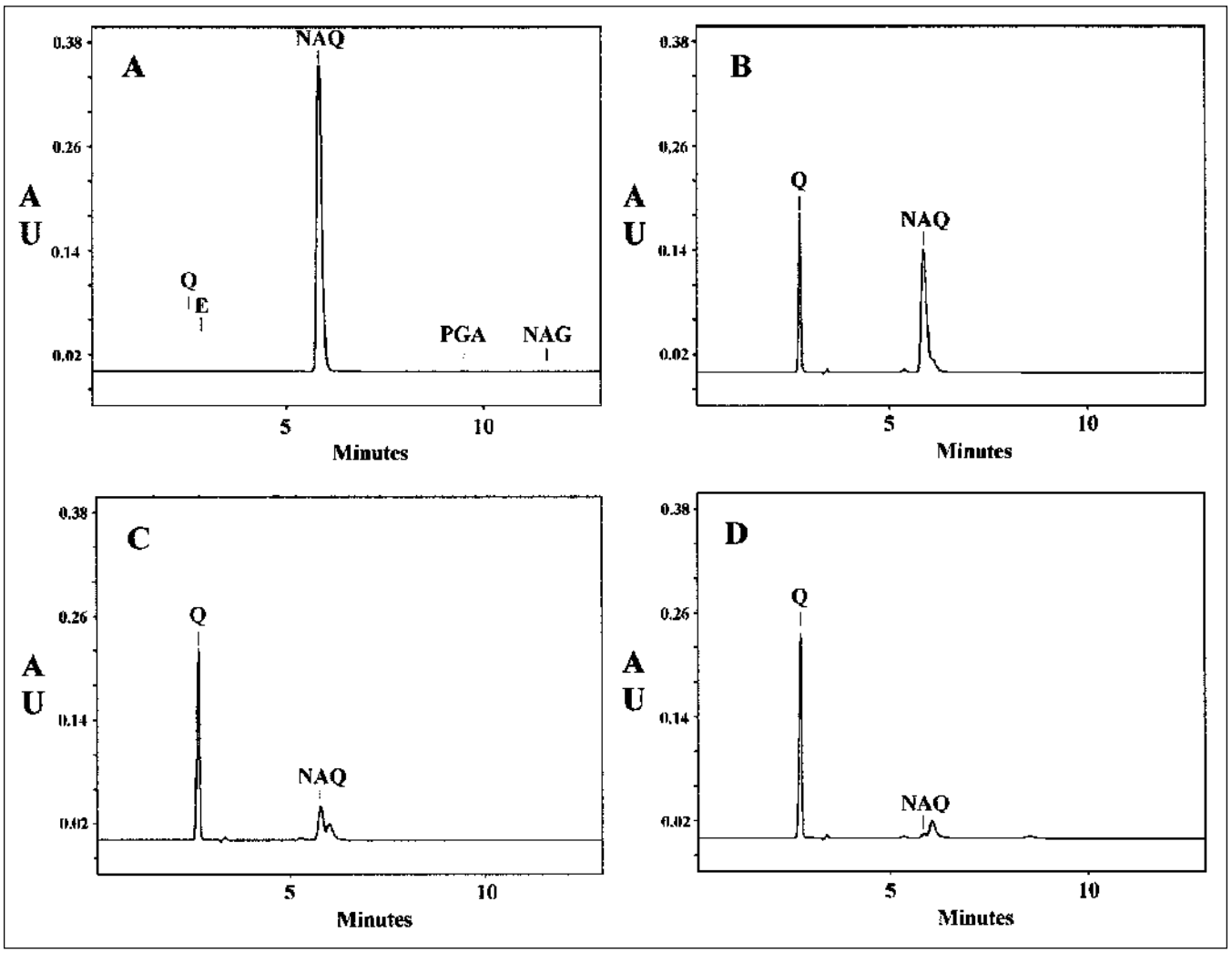

Figure 4—Hydrolysis of NAQ by Acylase I - Identification of glutamine product. NAQ (50 mM) was hydrolyzed using 0.08 mg/mL acylase I and aliquots taken at 0-time (A), 30 min. (B), 60 min. (C), and 120 min. (D), and were analyzed by isocratic RP-HPLC, as detailed in "Materials and Methods." Elution times for various potential hydrolysis and/or degradation products are indicated on Panel A. Q = glutamine, E = glutamic acid, NAQ = N-acetyl-glutamine, PGA = pyroglutamic acid, NAG = N-acetyl-glutamic acid. In Panels B, C, and D, the small peak immediately following NAQ is acetate.

amino acids (Endo 1980; Gade and Brown 1981), as well as the biological utilization of N-acylated food proteins (Ferjancic-Biagini and others 1998), the demonstrated ability of this enzyme to hydrolyze NAQ suggests that this compound may be an acceptable alternative nutritional glutamine source.

We also determined the classic enzyme kinetic constants (Km and Vmax) for both NAM and NAQ in order to quantitatively assess the relative ability of acylase I to hydrolyze these substrates (Table 1). The Km value obtained for NAM in this study (1.36 ± 0.15 mM) is within the range of previously published values (0.67 mM to 7.9 mM) (Bruns and Schulze 1962; Endo 1978; Szajni and others 1980; Cho and others 1987), and is substantially lower than the value for NAQ (11.41 ± 1.05 mM). The Vmax values for the two substrates are only slightly different (NAQ Vmax is 75% of NAM Vmax). So from a very simple point of view, Km is a measure of how easily and quickly the enzyme binds a particular substrate, while Vmax assesses how fast the enzyme can catalyze the reaction to yield products (Dixon and Webb 1958). With acylase I, therefore, recognition and binding of NAM compared to NAQ are different. But once the enzyme active site is saturated with substrate, hydrolyses occur at similar rates for the 2 substrates.

Acylase I is able to catalyze the hydrolysis of NAQ, and the reaction proceeds to completion in less than 90 min under the conditions reported (Figure 3). Using the fluorescamine test, we calculated a 90% release (final content of 45 mM) of glutamine expected at the reaction plateau. Addition of a further aliquot of enzyme after 95 min of hydrolysis does not increase L-glutamine release, indicating that enzyme inactivation is not the cause of the plateau in glutamine production. This apparent lack of quantitative hydrolysis could be due to any number of factors, including impurities in either the NAQ preparation or the standards used, other analytical errors or bias, or the attainment of reaction equilibrium (though NAQ hydrolysis should not be easily reversible). Acylase I does not appear to catalyze the hydrolysis of N-acetyl-D-glutamine (hydrolysis plateaus at about 2% of theoretical completion), so potential contamination of the NAQ preparation with the D-isomer could also explain the lack of quantitative hydrolysis.

In order to resolve this issue, the amount of NAQ and glutamine resulting after complete hydrolysis was determined, using an independent RP-HPLC technique. Using this approach, analysis determined less than 2% residual NAQ after reaction completion. This analysis also confirmed that glutamine was the final hydrolysis product of the reaction, and (unlike the fluorescamine test) found quantitative glutamine recovery. It is concluded that the fluorescamine test, under the conditions applied here, is only semi-quantitative.

Acylase I is capable of hydrolyzing NAQ *in vitro*, completely releasing the glutamine content.

This suggests (based solely on the ability of acylase I to quantitatively release glutamine) that NAQ is a suitable material to provide glutamine-enriched nutrition. However, in previous studies, when NAQ was administered intravenously to either dogs or humans, a relatively high urinary secretion of the intact compound was observed, sometimes accounting for 30 to 40% of the administered dose (Magnusson and others 1989; Abumrad and others 1989). This data shows that NAQ may not be a desirable glutamine precursor for use in parenteral nutrition, due to low actual bioavailability (Coudray-Lucas and others 1999). However, because acylase I has been found in the small intestinal mucosa (in total enzyme amounts roughly equivalent to that found in the kidney), it is possible that NAQ bioavailability would be adequate for use in an orally- administered product. We are unaware of any intravenous studies on NAM bioavailability, but it has been approved as a dietary additive to improve protein nutriture, and is thus adequately available via an oral route of administration. Further work *in vivo* needs to be done to adequately assess the potential use of NAQ as an orally-administered food product.

In conclusion, these data indicate that a major step in the metabolism of acetylated amino acids, the hydrolytic removal of acetate by acylase I, is functional for NAQ *in vitro*. This study demonstrated that acylase I can hydrolyze not only NAM (as previously demonstrated by others), but also NAQ, quantitatively yielding the free amino acid. However, affinity of acylase I for the two substrates is different, though maximal velocity is similar. Hydrolysis of NAQ by acylase I goes to completion in approximately 90 min under the test conditions, which is certainly within any reasonable human intestinal residence time. However, the rate of NAQ hydrolysis *in vitro* is less than that observed for NAM, and an *in vivo* assessment of the metabolic fate of orally-administered NAQ is needed to complete the evaluation of this material as a potential food ingredient. This work is in progress. Finally, as a secondary result of analysis of the final products by RP-HPLC, the NAQ preparation used in this study at most contained less than 2% of the D-isomer, an important consideration for quality assurance in nutritional product manufacture.

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Author Kim is with Tufts University's Antioxidants Research Laboratory, Boston, Mass., U.S.A. Authors Baxter and Snowden are with Ross Products Division of Abbott Laboratories, Dept. 105670-RP4-3, 625 Cleveland Ave., Columbus, OH 43215-1724, U.S.A. Address inquiries to author Baxter (E-mail: jeffrey.baxter@rossnutrition.com).