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An enzymatic process to α-ketoglutarate from L-glutamate: the coupled system L-glutamate dehydrogenase/NADH oxidase

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Abstract—α-Ketoglutarate, employed to treat mild chronic renal insufficiency, was obtained through enzymatic oxidation of monosodium glutamate (MSG) catalyzed by L-glutamate dehydrogenase (L-gluDH) coupled with NADH oxidase for the regeneration of NADH back to NAD^+. The irreversible reduction of molecular oxygen to water by NADH oxidase is demonstrated to drive oxidation of MSG to α-ketoglutarate to completion. L-gluDH was found to be inhibited by all three oxidative deamination products, α-ketoglutarate, NADH, and ammonia. As the pH in the current system was balanced by sodium, not ammonia, and NADH was recycled to NAD^+, inhibition of L-gluDH by α-ketoglutarate is believed to present the biggest challenge to an efficient process. In a batch experiment, we achieved a volumetric productivity of 1 g/(L·d).

1. Introduction

Keto acids are the nitrogen-free analogs of amino acids, and are transaminated to form the respective amino acid in the body. This improves nitrogen balance at a lower nitrogen intake and corresponds with relief of the symptoms of uremia while maintaining good nutrition.[1] Thus, α-ketoglutarate is beneficial as a component of endoperitoneal solutions for the conservative treatment of mild chronic renal insufficiency in hemodialysis patients and, in combination with calcium carbonate, of hyperphosphatemia.[2–4] Long-term co-administration of keto acids, erythropoietin, and low-protein diet was also associated with a delay in progression of renal insufficiency and a reduction in proteinuria.

α-Ketoglutarate is most advantageously produced by oxidation of inexpensive L-glutamate (MSG), a flavor enhancer available at huge scale. The current process involves oxidation of MSG in air with a copper complex. The oxidation of MSG, however, can be afforded enzymatically, by catalysis with L-glutamate dehydrogenase (L-gluDH), with simultaneous reduction of NAD^+ to NADH. Herein, we demonstrate the feasibility to couple L-gluDH with NADH oxidase to regenerate the co-factor NADH back to NAD^+ and to drive the thermodynamically unfavorable equilibrium from L-glutamate to α-ketoglutarate. This is accompanied by the irreversible four-electron reduction of molecular oxygen to water. Recently, we published the characterization of a novel water-forming NADH oxidase from *Lactobacillus sanfranciscensis*, which accomplishes this task.[5,6] Herein, we use this protein in conjunction with L-gluDH from *Clostridium symbiosum*[7,8] expressed in *Escherichia coli*, to transform MSG to the sodium salt of α-ketoglutarate (Fig. 1).

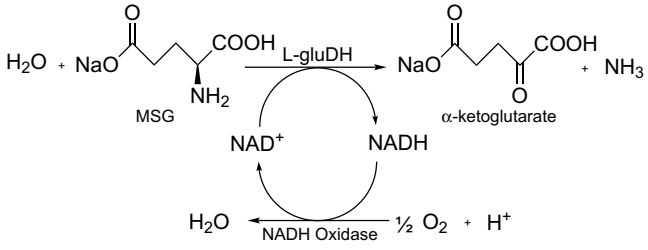

Figure 1. Process to α-ketoglutarate from monosodium glutamate (MSG) catalyzed by L-gluDH/NADH oxidase.

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Recently, in humans, L-glutamate dehydrogenase was implicated to also play a role in glucose homeostasis, linked to gain-of-function mutations in L-gluDH in connection with their function of oxidizing L-glutamate to α-ketoglutarate.[9]

While the thermodynamics of the oxidation of L-glutamate to α-ketoglutarate are very unfavorable, the irreversible reduction of molecular oxygen drives the oxidative biotransformation to completion, unlike regeneration schemes of NADH to NAD^+ based on acetone and an alcohol dehydrogenase functionality.[10,11] Previous work utilizing NADH oxidase for driving transformations with amino acid dehydrogenases include the recent separation of D,L-*tert*-leucine to trimethylpyruvate and D-*tert*-leucine catalyzed by L-leucine dehydrogenase (L-leuDH),[12] in which complete conversion is critical to achieve high enantiomeric excess of the remaining enantiomer.

A second advantage of cofactor regeneration is cost reduction of potentially expensive pyridine dinucleotide cofactor, if successful, to insignificant levels. Cycle numbers of up to 600,000 have been achieved for reductive direction (regeneration of NAD^+ back to NADH).[13] Assuming a typical price of NAD^+ (MW 663.4) of $10/g, the cost contribution per mole of product is $6.6 at a cycle number of 1000 and 1.1 cents at a number of 600,000.

2. Material and methods

2.1. Materials

L-Glutamate dehydrogenase (L-gluDH) from *C. symbiosum*, expressed in *E. coli*, was a kind gift of Roche Diagnostics (Mannheim/Germany). It was used as received and featured a specific activity of 12.5 U/mg protein at pH 7.5 and 30 °C (for assay, see below).

L-GluDH from bovine liver was either obtained from Sigma or was a kind gift of Biocatalytics (Pasadena, CA). Each was used as received and featured a specific activity of 1.1–1.2 U/mg protein at pH 9.0 and 30 °C.

NADH oxidase from *L. sanfranciscensis* was obtained from our own stock[5,6] (for preparation and assay, see below).

Monosodium glutamate (MSG), α-ketoglutarate, Tris, and other components were obtained from Sigma–Aldrich–Fluka (Milwaukee, WI) and used as received.

2.1.1. GluDH activity assay. GluDH activity was measured by monitoring the production of NADH in a UV/vis spectrophotometer at 340 nm, with temperature control set at 30 °C. The assay solution was 150 mM Tris buffer pH 7.5 with 250 mM MSG and 4 mM NAD^+. After allowing 990 μL of the solution to reach temperature, 3 μg L-gluDH (10 μL of 300 μg/mL stock) was added to yield a final volume of 1 mL, the solution was mixed and data collection was started.

2.1.2. Determination of protein concentration. Protein concentration was determined by the Bradford method utilizing Coomassie Plus Protein assay reagent, prediluted protein assay standards-BSA (Pierce Chemical, Rockford, IL).

2.2. Purification of NADH oxidase

Enzyme preparation (growth, expression, purification, and activity assay) was performed with a modified version of the literature methods.[5,6]

2.2.1. Cell growth and protein expression. Cells of *E. coli* JM101 containing the sfnox2 gene in the pKK223-3 vector were grown in 5 mL cultures at 37 °C and 250 rpm in 15 mL disposable culture tubes to 1.0 OD 600 nm in LB media + 100 μg/mL ampicillin. One-liter cultures of LB medium were seeded with a 5 mL starter culture and grown at 30 °C and 200 rpm in baffled 2.8 L Fernbach shake flasks. At $OD_{600} = 0.7$, protein production was induced by addition of 1.0 mM IPTG final concentration and cells allowed to grow for an additional 3 h. Additional ampicillin, 200 μg/mL, was added at induction and 1.5 h later to maintain selection pressure on the culture. Cultures were harvested by centrifugation at 5000 rpm in 1 L centrifuge containers (Beckman J2-M) for 15 min at 4 °C and the resulting cell pellet was frozen at −80 °C.

2.2.2. Enzyme purification. Frozen cell pellets, 31.5 g WCP, were thawed and resuspended in 30 mL of 100 mM 1-methylpiperazine buffer pH 5.0 + 1 mM EDTA + 5 mM DTT + 20 mM Spermine. The resulting cell slurry was sonicated with a Fisher Scientific 60 Sonic dismembrator for 6 × 2 min while floating the tube in ice/water for cooling. The resulting lysate was centrifuged at 16,000 rpm in a Beckman J2-21 M for 45 min at 4 °C. For acid precipitation, the clarified lysate was then loaded into Specto/Por® regenerated cellulose dialysis membrane tubing (60 K MWCO) and dialyzed with 1.5 L of 20 mM 1-methylpiperazine pH 5.0 at 30 °C + 1 mM EDTA + 5 mM DTT. The sample was dialyzed versus 1.5 L of buffer for 2 h at 30 C and 200 rpm stirring before exchanging the dialysis buffer and dialyzing for two more hours under the same conditions. Temperature and stirring conditions were maintained by digital stir plate with an external temperature probe. The sample was then transferred and centrifuged at 16,000 rpm for 45 min at 4 °C. The resulting clarified solution was then loaded onto an Amersham Pharmacia Hiprep 16/10 Source™ 30Q column on an AKTA explorer system at 4 °C. After sample loading, the column was washed with 10 column volumes of 20 mM 1-methylpiperazine pH 5.0 at 4 °C + 5 mM DTT. The protein was then eluted with a linear gradient from 0–100% of 1 M NaCl in the same buffer. Fractions (5 mL) were collected at a flow rate of 5 mL/min. The most concentrated fraction had a specific activity of 221 U/mg with 10 kU total activity.

2.2.3. NADH oxidase activity assay. NADH oxidase activity was assayed at 30 °C in 0.1 M triethanolamine (TEA), pH 7.5 in a total volume of 1 mL at 340 nm using

a final concentration of 0.2 mM NADH and adding 10 μL enzyme solution. Enzyme reaction was followed for 1 min and activity was calculated using an extinction coefficient ε of NADH of 6.22 L/(mmol·cm).

2.3. Cofactor regenerating assay

Runs with the coupled L-gluDH/NADH oxidase reaction system were conducted in 2 mL Eppendorf tubes at 30 °C and pH 7.5 in air-saturated 150 mM Tris buffer. The initial concentrations of MSG and NAD^+ were 5 and 0.5 mM, respectively. Enzyme levels in the reaction solution were 0.25 mg/mL L-gluDH (3.2 U/mL) and 0.1 mg/mL (22 U/mL) NADH oxidase; 5 mM DTT were added to stabilize NADH oxidase.

Samples were taken periodically, and one sample volume of acetonitrile was added to stop the reaction. The mixture separated by HPLC (HP3300) on a RP18 reversed phase column with acetonitrile–water–1% formic acid mobile phase. Retention time of α-ketoglutarate (UV detection, 260 nm) was 6.8 min.

3. Results

3.1. Characterization of GluDH

First, we attempted to utilize L-gluDH from bovine liver for the task of oxidizing monosodium glutamate (MSG) to α-ketoglutarate. However, we found the activity level in the direction of oxidative deamination to be insufficient and the pH profile of the enzyme to be unfavorable. In contrast to the specified 104 U/mg in the reductive amination direction, we measured only a specific activity level of 1.2 U/mg in 0.1 M $NaHCO_3$-buffer at 30 °C and pH 9.0, a pH value favoring oxidative deamination. Even at pH 8.0, close to the reported pH optimum of 8.3,[14] we found a low specific activity of just 1.5 U/mg. Such a low activity level was deemed to be too low to sustain a coupled system with cofactor regeneration.

We then proceeded to test the L-gluDH from *C. symbiosum* at the same conditions. At pH 8.0, the enzyme's pH optimum, with 250 mM MSG and 4 mM NAD^+, we measured a specific v_{max} of 12.5 U/mg, suitable for coupled cofactor regeneration with NADH oxidase. The K_M values were found to be 22.5 mM with respect to L-glutamate (MSG) and 0.39 mM with respect to NAD^+. Both values indicate fairly weak binding of the native substrates L-glutamate and NAD^+ to L-gluDH. In comparison, the K_M value for NADH on NADH oxidase is 6.1 μM.[5]

In accordance with the literature,[7,8,15,16] we found L-gluDH to be inhibited by all three products of the oxidative deamination reaction, ammonium ion,[15,16] α-ketoglutarate, and NADH.

3.1.1. Inhibition by ammonia. When comparing $NaHCO_3/Na_2CO_3$- and $(NH_4)HCO_3/(NH_4)_2CO_3$-containing buffers at a concentration level of 0.1 M, the specific activity of the former was about twice as high as the latter. As L-glutamate advantageously is added as monosodium glutamate (MSG), the only potential disadvantage of Na^+ is its more difficult isolation in comparison with NH_4^+.

3.1.2. Inhibition by α-ketoglutarate. α-Ketoglutarate inhibits L-gluDH nearly competitively (Fig. 2) with a K_{IP}-value of 2.8 mM; with the K_M value of 22.5 mM for MSG, the inhibition ratio K_M/K_{IP} equals 8.0.

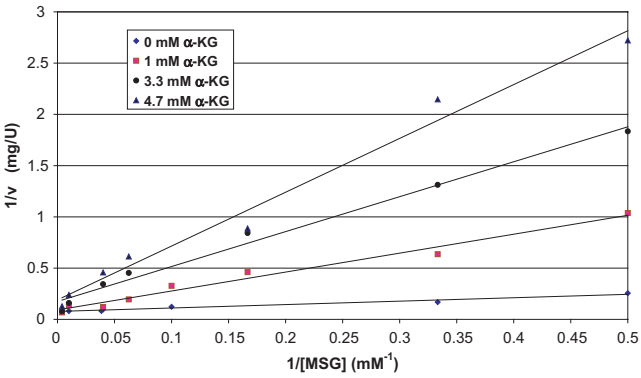

Figure 2. Competitive inhibition of L-gluDH by α-ketoglutarate (Lineweaver–Burk plot, K_{IP} = 2.8 mM).

3.1.3. Inhibition by NADH. Within an NADH concentration range of 0–0.5 mM, the Lineweaver–Burk plot provides evidence that NADH also inhibits L-gluDH competitively (Fig. 3), as does α-ketoglutarate, but with a K_{IP} value of 8.5 μM; the inhibition ratio K_M/K_{IP} equals 45.6, given the K_M value of 0.39 mM.

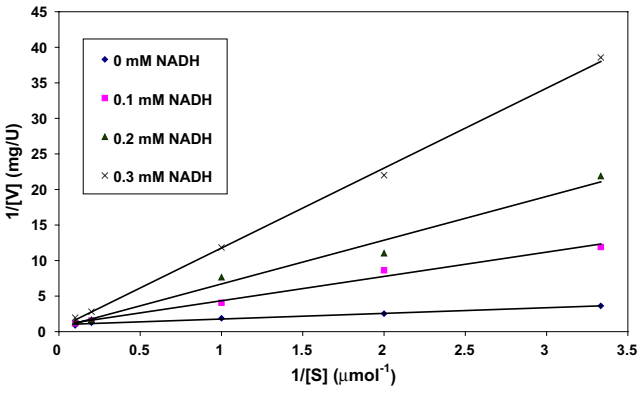

Figure 3. Competitive inhibition of L-gluDH by NADH (Lineweaver–Burk plot, K_{IP} = 8.5 μM).

Lineweaver–Burk and Eadie–Hofstee plots yielded similar K_M and K_I values, usually within a 10% range.

3.2. Characterization of NADH oxidase

Characterization of NADH oxidase from *L. sanfranciscensis* has been described in the literature.[5,6] The enzyme is active between pH 7.0 and 8.5. The specific

activity according to an improved purification protocol is 221 U/mg (at pH 7.0).

We tested two features that may influence performance in a coupled system of cofactor regeneration with L-gluDH, inhibition patterns by any compound present in such a system, and operational stability of NADH oxidase at the reaction conditions of such a coupled system.

3.2.1. Inhibition patterns. We tested NADH oxidase for inhibition in the presence of NADH and NAD^+ as well as MSG and α-ketoglutarate. No inhibition was found up to 0.25 mM NADH, 0.5 mM NAD^+, or 250 mM MSG and 25 mM α-ketoglutarate.

3.2.2. Operational stability of NADH oxidase. When testing the operational stability of NADH oxidase, we found that the enzyme under reaction conditions (30 °C, pH 7.5, 0.2 mM NADH) is not deactivated by temperature but is limited by catalytic turnover. We tested the degree of conversion, as calculated from the decrease of absorption of NADH at 340 nm, with various concentrations of NADH oxidase. Especially at low concentrations of NADH oxidase, up to 40 nM, we often observed the reaction come to a standstill with incomplete conversion of NADH (Fig. 4). We verified inactivation of the enzyme by spiking again with NADH, but no further conversion of NADH occurred. As concentration of NADH oxidase and degree of conversion of NADH seem to be related, we calculated the number of turnovers of the enzyme at each of its concentration levels: we divided the number of moles of converted NADH substrate by the molar concentration of active sites at various enzyme concentration levels. Given one site per subunit of 48.8 kDa[5] and an NADH oxidase purity level of 95%,[6] we found a total turnover number of 5000 ± 1500.

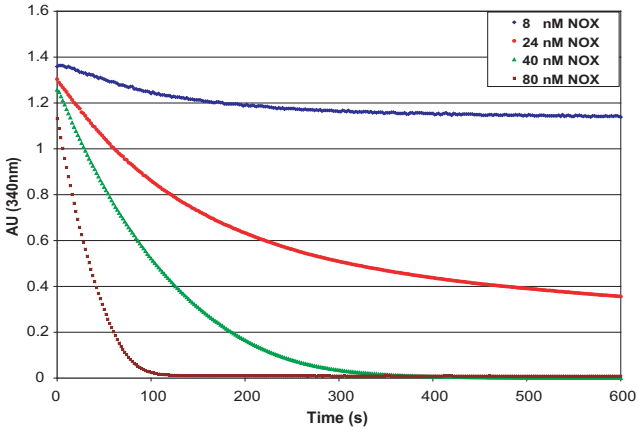

Figure 4. Turnover-limited stability of NADH oxidase from *L. sanfranciscensis*; calculation: (A340/ε)/[*E*], 1 active site/subunit.

The data above were obtained in the absence of any thiol-protecting agent such as β-mercaptoethanol or dithiothreitol (DTT). We also found that in presence of 5 mM DTT, the number of turnovers increased more than 20-fold to >112,500; the enzyme was so stable that we could not observe complete deactivation after more than 24 h.

3.3. Coupled GluDH/NADH oxidase experiments

The high value of 45.6 for the inhibition ratio, K_M/K_{IP}, of L-gluDH with respect to NAD^+/NADH causes a marked progressive decrease in reaction rate at high degrees of conversion as product accumulates.[17] We attempted to avoid the accumulation of NADH in the coupled reaction of L-gluDH and NADH oxidase by supplying sufficiently high levels of the latter, such as to keep the steady-state level of NADH low. To check the steady-state level of NADH, we measured the time course of absorption at 340 nm over the course of the coupled reaction run. The data in Figure 5 represent runs with different amounts and different ratios of the two enzymes; they were collected under the conditions as in the coupled system. The top curve, however, was measured with 50 mM MSG rather than 5 mM. Figure 5 demonstrates that the steady-state level of NADH indeed is low: the absorption of 0.004 units corresponds to [NADH] of 0.7 μM, that is, about 0.15% of the total cofactor concentration of 0.5 mM.

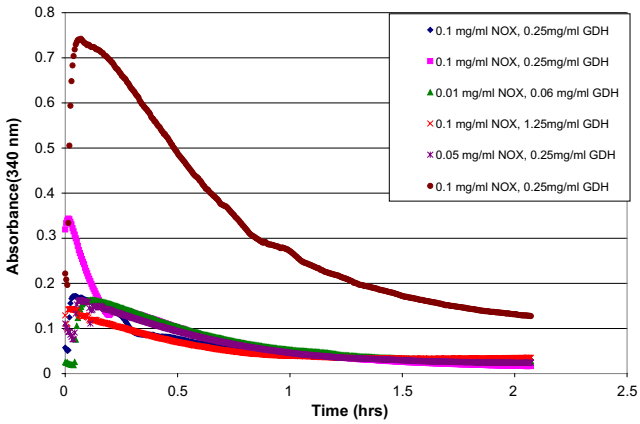

Figure 5. Approach of NADH absorption to steady-state level.

After demonstrating that a concentration level of 0.5 mM of cofactor should not unduly inhibit the coupled L-gluDH/NADH oxidase reaction, we conducted a batch experiment to recycle the cofactor NAD(H) while oxidizing MSG to α-ketoglutarate with concomitant reduction of molecular oxygen to water. Figure 6 depicts the conversion–time course of L-glutamate oxidation to α-ketoglutarate; we measured formation of α-ketoglutarate product via HPLC.

From the slope of the conversion–time plot, we calculate an initial rate of 0.33 mmol/(L·h) and an average rate of 0.26 mmol/(L·h). Given the duration of run with 18 h and the molecular mass of MSG of 169.1 g/mol, we obtain an average volumetric productivity (space–time–yield) of 1.07 g/(L·d). However, in a coupled reaction run at 25 mM MSG, conversion after 18 h was just beyond 25% instead of near 100% as at 5 mM MSG. This

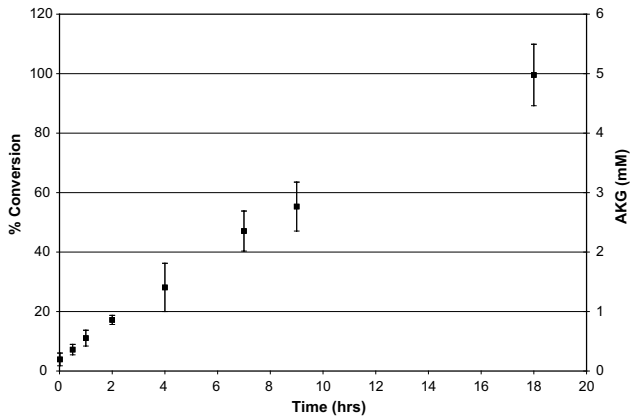

Figure 6. Conversion–time course of α-ketoglutarate formation from MSG catalyzed by L-gluDH/NADH oxidase (Conditions: 5 mM MSG, 0.5 mM NAD^+, 3.2 U/mL L-gluDH, 22 U/mL NADH oxidase, 30 °C, pH 8.0, 0.1 M Tris buffer).

finding is consistent with inhibition of L-gluDH by α-ketoglutarate, as found by our inhibition studies (Figure 2).

4. Discussion

4.1. Kinetics of L-gluDH

Inhibition results on L-gluDH strongly depend on the origin of the enzyme. In addition, no clear picture emerges from the literature about the mechanism of L-gluDH.[7,8,15,16] The mechanism of L-glutamate oxidation by bacterial L-gluDH has been found to be best represented by a random mechanism,[7] and not to depend on allosteric activation by compounds such as ADP.[15,16] As we employed concentrations of NAD^+ >40 μM, our observations of linear Lineweaver–Burk plots is consistent with the literature.[18]

4.2. Stability of NADH oxidase

Stability limitations caused by catalytic turnover are frequently encountered in enzymes processing molecular oxygen. Proteins possessing two neighboring cysteines instead of one, as does NADH oxidase, are prone to similar limitations.[19]

4.3. Coupled enzyme system L-gluDH/NADH oxidase

When conducting the coupled enzyme reaction of L-gluDH/NADH oxidase with regeneration of NADH cofactor back to NAD^+, the conversion–time profile of MSG to α-ketoglutarate is consistent with predictions from inhibition studies. The observation that we could achieve complete conversion justifies our treatment of the α-ketoglutarate and NADH inhibitions on L-gluDH as competitive.

5. Conclusion

In conclusion, we demonstrated the feasibility of an enzymatic process to α-ketoglutarate from monosodium glutamate (MSG) with catalysis by a coupled system of L-gluDH and NADH oxidase. However, before implementation, the space–time–yield of the system has to be improved. The main challenge seems to be inhibitions of L-gluDH by the products α-ketoglutarate and NADH. Provided DTT or another thiol-protecting agent is present, stability of NADH oxidase against turnover-dependent deactivation seems sufficient.

Acknowledgements

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