# STEREOSPECIFICITY AND NADH–$H_2O$ HYDROGEN EXCHANGE OF NADH-DEPENDENT GLUTAMATE SYNTHASE FROM LUPIN NODULES

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## 1. Introduction

NADH-dependent glutamate synthase has been implicated in ammonia metabolism in nitrogen-fixing legume nodules as the major source of de novo amino acids [1,2]. The enzyme from *Lupinus angustifolius* nodules has been purified to homogeneity [3] and the kinetic mechanism is known [4].

NADPH-dependent glutamate synthases have been purified from a number of bacterial sources [5–7] and have some common properties, such as containing two different types of subunits and catalysing an ammonia-dependent glutamate dehydrogenase reaction, which are not shared by the NADH-dependent eukaryotic enzyme. Both types of enzyme contain a flavin cofactor.

It has been shown [8] that prokaryotic glutamate synthases are specific for the B (*pro-S*) hydrogen of the dihydronicotinamide ring, and that this hydrogen appears in water when glutamine is the nitrogen donor, but in the product glutamate when ammonia is used. The glutamine-dependent activity has also been shown to depend on the presence of the flavin cofactor while the ammonia-dependent reaction can be catalysed by the apoenzyme.

For the NADH-dependent eukaryotic enzyme it was therefore of interest to determine the stereospecificity of the reaction, and whether the hydrogen from the nicotinamide nucleotide is transferred to the substrate or exchanges with water. We show here that this enzyme is B-side specific, and that hydrogen exchange with water occurs. The eukaryotic glutamate synthase is thus similar to the bacterial enzymes.

## 2. Materials and methods

Glutamate synthase was prepared as in [3]. Horse liver alcohol dehydrogenose (Boehringer) was passed through Sephadex G-25 before use to remove ethanol. Nucleotides and other enzymes were from Sigma, Omnifluor and Triton from New England Nuclear, and Dowex 1 ($Cl^-$ form) (AG 1-X8 200–400 mesh) was from Bio-Rad.

[1-$^3$H]Ethanol and [2-$^3$H]glycerol were synthesised by reduction of acetaldehyde (BDH) and dihydroxyacetone (Sigma), respectively, with $NaB^3H_4$ (Radiochemical Centre, Amersham, 555 Ci/mol).

A-side and B-side labelled [$^3$H]NADH were prepared by reduction of NAD with alcohol dehydrogenase and glycerol 3-phosphate dehydrogenase (rabbit muscle), respectively [9], using the appropriate substrates according to standard procedures [10,11] and were isolated from the reaction mixtures by modification of the method in [12]. Reaction mixtures (10 ml) were diluted with water (10 ml) and applied to DEAE Sephadex A-25 columns (20 × 40 mm). The columns were washed with 50 ml 10 mM imidazole–HCl buffer (pH 7.5)–50 mM NaCl and NADH was eluted with 0.2 M NaCl in the same buffer. 3 ml fractions were collected. NADH appeared after ~25 ml, and in each case a symmetrical peak of constant specific activity (cpm/$E_{340}$) was obtained. Specific activities found were: A-side labelled, $7.8 \times 10^7$ cpm/μmol; B-side labelled, $9.2 \times 10^7$ cpm/μmol.

Charcoal columns (5 × 40 mm) were made by layering 2% charcoal (Hopkin and Williams, 300 mesh,

for chromatography)–4% cellulose powder (Whatman, CF 11) in 0.1 M HCl over a small amount of Celite 545 and a glass-wool plug. They were washed with ethanol–ammonia–water (60:1:39, by vol.) and water before use.

Glutamate synthase was incubated separately with A-side and B-side labelled NADH. Reaction mixtures contained 2 ml NADH solution, prepared as above, 10 mM glutamine, 1 mM 2-oxoglutarate and enzyme solution (final conc. ~$10^{-8}$ M) in 3 ml total vol. Reactions were monitored at 340 nm. When reactions were complete, 10 μl samples were counted and 0.5 ml aliquots were applied to charcoal columns to adsorb nucleotides. The columns were washed with 10 mM imidazole–HCl (pH 7.5) until 5 ml eluate had been collected and 100 μl samples were counted. An aliquot (0.5 ml) of the charcoal column eluate from the B-side labelled reaction mixture was applied to a column of Dowex 1 (5 × 30 mm) to adsorb glutamate. This column was similarly washed with imidazole–HCl buffer until 5 ml eluate had been collected and 100 μl was counted.

To investigate NADH–$H_2O$ exchange with B-side labelled NADH, concentrations of reactants (except NADH) were the same as above, except that various substrates and enzyme were omitted from reaction mixtures (see table 2 for details), and the total volume was 1 ml. Reaction in the presence of all substrates was complete after 1 min, and after 5 min each mixture was applied to a charcoal column. The columns were washed as above and 100 μl of eluate was counted.

Radioactivity was determined by liquid scintillation counting in Omnifluor:Triton:Toluene .012:1:1 (w:v:v) using a 0.5–18 keV window.

## 3. Results and discussion

The data in table 1 show that the label from the reaction mixture containing A-side labelled NADH was retained by the charcoal column, and that the label from the reaction mixture containing B-side labelled NADH was eluted from both charcoal and Dowex. Control experiments showed that >95% of each labelled NADH was retained by charcoal, and that [$^{14}$C]glutamic acid was quantitatively retained by Dowex. Thus the A-side tritium was retained in the nucleotide during the reaction, while the B-side tritium was lost from the nucleotide, and exchanged with water during the reaction.

Table 1
Distribution of label in glutamate synthase-catalysed reaction with [$^3$H]NADH

| | % of counts | |
|---|---|---|
| | A-side label[a] | B-side label[b] |
| Reaction mixture | 100 | 100 |
| Eluate from charcoal | 0.7 | 92 |
| Eluate from charcoal and Dowex | – | 92 |

[a] 0.17 μmol NADH/reaction
[b] 0.29 μmol NADH/reaction

The data in table 2 show that the enzyme catalyses NADH–$H_2O$ exchange in the absence of other substrates. (Some decomposition of NADH had occurred since the experiment in table 1, and a greater proportion was not retained by charcoal.) This suggests an exchange mechanism via the flavin groups similar to that of the bacterial enzymes [8]. It therefore seems likely that these groups are involved in the transfer of electrons from NADH during the reaction. These results also support the observation [4] that NADH is the first substrate to bind to the enzyme.

Table 2
NADH–$H_2O$ hydrogen exchange catalysed by glutamate synthase

| | % counts eluted from charcoal |
|---|---|
| NADH[a] + enzyme + glutamine + 2-oxoglutarate | 94 |
| NADH + enzyme | 92 |
| NADH + enzyme + glutamine | 96 |
| NADH + enzyme + 2-oxoglutarate | 95 |
| NADH only | 16 |

[a] 0.024 μmol NADH/reaction

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