Re-investigation of the Effects of L-Glutamine and L-Asparagine on the *Neurospora crassa* NADP-Specific Glutamate Dehydrogenase

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L-Glutamine, when purified free of traces of NH_4^+ present in solution, does not act as an alternative substrate to NH_4^+ for the NADP-specific glutamate dehydrogenase of *Neurospora*. L-Glutamine interferes with detection of small quantities of NH_4^+ by Nessler's reagent. L-Asparagine is not an alternative substrate to NH_4^+ for this enzyme.

Blumenthal & Smith (1975) claimed that the amide group of L-glutamine acts as an alternative nitrogen donor to NH_4^+ in the reaction catalysed by the NADP-specific glutamate dehydrogenase (EC 1.4.1.4) of *Neurospora crassa* and by the bovine liver enzyme (EC 1.4.1.3), and that L-asparagine functions similarly for only the *Neurospora* NADP-specific enzyme. If true this claim would be of considerable interest from both mechanistic and evolutionary viewpoints, as discussed by Blumenthal & Smith (1975). We have re-investigated this claim both because of its potential interest and because of suspicions, which were also raised by our prior experiments on effects of L-glutamine on the *Neurospora* enzyme, that enzyme activity observed with L-glutamine and L-asparagine might be a spurious result caused by contaminating NH_4^+ .

Our earlier experiments (J. C. Wootton & M. G. Lovett, unpublished work) showed two effects of L-glutamine on this *Neurospora* enzyme. The first was a unidirectional inhibition of the catalysis by the enzyme of the oxidative deamination of L-glutamate. Approx. 70–85% inhibition of the standard assay system at pH 8.5 and 37°C was observed in the presence of 100 mM L-glutamine. This inhibition was unidirectional: L-glutamine added to the full assay system in the direction of reductive amination of 2-oxoglutarate had very little effect on activity. Secondly, L-glutamine solutions substituted for NH_4^+ as a substrate in the reductive-amination assay, 100 mM L-glutamine giving approx. 40–60% of the activity obtained with 33 mM NH_4Cl at pH 8.5 and 37°C, as found by Blumenthal & Smith (1975). An apparent K_m for L-glutamine of approx. 65 mM was determined. A problem with these experiments was that different batches of L-glutamine gave quantitatively different results, and consistent results could be obtained only by using solutions made up freshly from the same batch. This suggested that small quantities of NH_4^+ present in the L-glutamine solutions were possibly responsible for both types of

effect by acting respectively as product inhibitor and substrate. Considering the high affinity of this enzyme for NH_4^+ (apparent $K_m = 0.7$ mM, and K_i as a product inhibitor of the oxidative-deamination reaction = 0.4 mM, both constants measured in 0.1 M Tris/HCl buffer, pH 8.5 at 37°C), the results with L-glutamine solutions could be accounted for quantitatively if the concentration of NH_4^+ was approx. 0.5–1.5% of that of L-glutamine. Another suggestive result was that the mutationally modified variant of this enzyme from mutant *am*^{3a}, which has an approx. 10-fold higher K_m for NH_4^+ (Fincham & Bond, 1960), showed a similarly raised apparent K_m for solutions of L-glutamine.

Blumenthal & Smith (1975) monitored their solutions of L-glutamine and L-asparagine for contaminating NH_4^+ by reaction with Nessler's reagent, and claimed that negligible amounts, less than 10 μM , were present. This result is surprising, because it might be expected that small but significant amounts of NH_4^+ and L-2-pyrrolidone-5-carboxylate, and perhaps also L-glutamate, would be present in solutions of L-glutamine as a consequence of hydrolytic deamidation. Accordingly we have also investigated the effect of L-glutamine on the reaction of NH_4^+ with Nessler's reagent.

Materials and Methods

Reagents for enzyme purification and assay were as described in Ashby *et al.* (1974). L-Glutamine and L-asparagine were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Nessler's reagent, described by the suppliers as 'containing about 3.6% w/v $\text{HgI}_2 \cdot \text{KI} \cdot 1\frac{1}{2}\text{H}_2\text{O}$ and about 9% w/v Na_2O ', was obtained from BDH Chemicals, Poole, Dorset, U.K., as were other chemicals, of analytical grade.

Enzyme purification, criteria of purity and assay methods were as described by Ashby *et al.* (1974). For assays testing possible substitutes for NH_4^+ , the

NH_4Cl component was omitted and replaced by the appropriate test solution.

Cation-exchange chromatography was at room temperature (approx. 25°C) on a column ($15\text{cm} \times 0.9\text{cm}$ diameter) of the resin AG50 X4 (Bio-Rad, Richmond, CA, U.S.A.). Before each run the column was eluted with a few bed volumes of 0.2M - NaOH , then equilibrated with 0.2M -trisodium citrate/ HCl buffer, $\text{pH} 3.25$, until the pH of the effluent was 3.25 . Samples were applied dissolved in 2ml of 0.2M -trisodium citrate/ HCl buffer, $\text{pH} 2.2$, and washed in with 0.5ml of the same buffer. L -Glutamine, L -asparagine and L -glutamate were eluted with the above $\text{pH} 3.25$ buffer, then any retarded NH_4^+ was eluted with 0.35M -trisodium citrate/ HCl buffer, $\text{pH} 5.28$. Flow rate was 65ml/h , maintained with a peristaltic pump; 2ml fractions were collected, of which 0.1ml portions were taken for the manual ninhydrin reaction (ninhydrin/ SnCl_2 reagent was made by the recipe of Hill & Delany, 1967).

Reactions with Nessler's reagent were carried out as follows. To 2.7ml of test solution in 0.1M -Tris/ HCl buffer, $\text{pH} 8.5$, was added 0.3ml of Nessler's reagent, and the solutions were mixed on a vortex mixer for a few seconds in as standard a manner as possible. The development of colour was observed during 20min at room temperature. For the data in Fig. 2, the E_{480} was read spectrophotometrically after 10min .

Results and Discussion

The crucial evidence that traces of NH_4^+ account completely for the nitrogen-donating ability of solutions of L -glutamine comes from experiments in which L -glutamine was resolved from the contaminating NH_4^+ by cation-exchange chromatography. The batch of L -glutamine used in these experiments was a typical one, giving quantitatively similar results, as an alternative substrate to NH_4^+ , to that used by Blumenthal & Smith (1975). Fig. 1(a) shows that a clear small NH_4^+ peak (compare the separate run of authentic NH_4Cl ; Fig. 1c) was obtained from a 2ml sample of a freshly dissolved 0.3M solution of L -glutamine. At this concentration the L -glutamine greatly overloaded the resin and appeared in the $\text{pH} 3.25$ eluate as a large breakthrough peak and a small, broader, retarded peak. The NH_4^+ peak is derived from the L -glutamine sample and not from the buffer solutions, as shown by the fact that chromatography of 2ml samples of 0.2M - L -asparagine (Fig. 1d) or 0.2M - L -glutamate under the same conditions in the same buffer solutions gave no detectable ninhydrin-positive material in the NH_4^+ -peak position.

Samples of fractions from the same run as in Fig. 1(a) were tested as nitrogen donors for the reductive-amination assay in place of the usual 33.3mM - NH_4Cl .

Fig. 1. Cation-exchange chromatography of solutions of L -glutamine, L -asparagine and NH_4Cl

Experimental procedures are described in the Materials and Methods section. The samples, volume 2ml , were in (a) 0.3M - L -glutamine, in (c) 0.01M - NH_4Cl , in (d) 0.2M - L -asparagine. Elution was initially with 64ml of $\text{pH} 3.25$ buffer (see the Materials and Methods section), changing to $\text{pH} 5.28$ buffer at the arrow. In (a), (c) and (d) the colour yield in the ninhydrin reaction was measured at 570nm ($\bullet$). In (b) 0.1ml portions of fractions obtained from chromatography of L -glutamine [same run as in (a)] were used instead of NH_4Cl in otherwise standard assays for the reductive-amination activity (Δ) of glutamate dehydrogenase. Each assay mixture contained $1\mu\text{l}$ of a sample of purified enzyme of high specific activity. One arbitrary unit equals $0.159\mu\text{mol}$ of NADPH oxidized/ min .

The citrate buffer in these samples lowered the pH of the assay mixture by not more than 0.05pH unit, and

controls in which citrate buffer was added to the complete assay containing NH_4Cl showed that neither the buffer components nor the pH difference significantly affected enzyme activity. The results (Fig. 1b) show clearly that only the fractions containing NH_4^+ were able to act as a substrate for the enzyme. The concentration of NH_4^+ in the fractions of the main NH_4^+ peak (effluent volume 72–82 ml) was estimated by comparison with standard assays that used the same amount of enzyme and known concentrations of NH_4Cl . The total amount summed over the peak, by using interpolated estimates for the fractions not assayed, corresponds to a concentration of approx. 2.6 mM-NH_4^+ in the 0.3 M-L-glutamine solution before chromatography. This is very close to the value of 2.8 mM-NH_4^+ required to account completely for the nitrogen-donating activity given by this unchromatographed solution of L-glutamine.

A possibly significant but very slight activity appeared to be given by the peak fraction of L-glutamine (effluent volume 12 ml; Fig. 1b). This very probably reflects the release by deamidation of a very small trace of NH_4^+ (estimated from the activity observed as less than 0.1 mM-NH_4^+ in this fraction) during the few hours between elution of the fractions and the assays. More preliminary assays, carried out earlier in the experiment as soon as possible after the fractions were eluted, showed that no significant activity was given by using this fraction or others containing L-glutamine.

We were unable to find significant ability of our batch of L-asparagine to act as an alternative nitrogen donor to NH_4^+ of this enzyme. Chromatography of 2 ml of an 0.2 M solution showed no detectable NH_4^+ (Fig. 1d), and no fractions from this run gave any significant enzyme activity when used instead of NH_4^+ in assays.

Blumenthal & Smith (1975) observed a difference in the pH-dependence of the reductive-amination reaction of this enzyme using their standard concentration of NH_4^+ (pH optimum 7.8) and using $200\text{ mM-L-glutamine}$ (pH optimum 8.4). This may be explained as a consequence of the low concentration of NH_4^+ contaminating solutions of L-glutamine. We found that lowering the NH_4Cl concentration in our reductive-amination assay to 1 mM raised the pH optimum to approx. 8.3, as expected from the effect of pH on the affinity of the enzyme for NH_4^+ .

The data in Fig. 2 demonstrate that L-glutamine interferes with the colorimetric determination of NH_4^+ by Nessler's reagent. In these and in similar experiments we found that concentrations of NH_4^+ up to 2 mM gave no significant coloured product with Nessler's reagent in the presence of concentrations of L-glutamine of 150 mM or higher. The interference is probably caused by formation of a complex between the L-glutamine and Nessler's reagent, as suggested by the observation that addition of 2.7 ml

Fig. 2. Effect of L-glutamine on the reaction of NH_4^+ with Nessler's reagent

Different concentrations of $(\text{NH}_4)_2\text{SO}_4$ were made to react with Nessler's reagent, as described in the Materials and Methods section, in the absence of L-glutamine (○) and in the presence of (□) 50 mM- , (△) 100 mM- and (■) $200\text{ mM-L-glutamine}$. Each point represents an average of duplicate tests.

of 50 mM- or $100\text{ mM-L-glutamine}$, containing no added NH_4^+ , to 0.3 ml of Nessler's reagent resulted in slow development of an orange colour that formed a precipitate after approx. 12 min at 25°C . It proved possible to measure with Nessler's reagent the approximate concentration of contaminating NH_4^+ in L-glutamine solutions of 10 mM or less. Approx. 0.08 mM-NH_4^+ was measured in a fresh 10 mM solution of the batch used for chromatography (Figs. 1a and 1b), in agreement with the amount estimated from the enzyme assays. This method was also used to monitor over a 2-day period at 25°C the rate of generation of NH_4^+ by deamidation. A linear increase of approx. 0.8 mM per 24 h was observed in 75 mM , 100 mM and 150 mM solutions of L-glutamine in 0.1 M-Tris/HCl buffer, $\text{pH}8.5$.

It is expected that the NAD-specific glutamate dehydrogenase (EC 1.4.1.2) of *Neurospora* would contrast with the NADP-specific enzyme in showing little activity with solutions of L-glutamine because of its relatively high K_m for NH_4^+ (17 mM ; Sanwal & Lata, 1961). This was observed by Blumenthal & Smith (1975). The latter authors' results with the bovine liver enzyme are very probably explained by NH_4^+ contamination. Gross *et al.* (1976) suggested that these and other effects of L-glutamine on the latter enzyme are spurious. They found that, when traces of NH_4^+ were sequestered from solutions of L-glutamine by interaction with glutamine synthetase (EC 6.3.1.2), the solutions failed to act as alternative nitrogen donors to NH_4^+ . It is also likely that

the reported unidirectional inhibition by L-glutamine of the NADP-specific glutamate dehydrogenase of *Schizophyllum commune* (Dennen & Niederpruem, 1967) was caused by NH_4^+ acting as a product inhibitor. A further implication of our results is that care is needed in assaying for activity of 'glutamate synthase' in extracts that also contain glutamate dehydrogenase.

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