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Allosteric NADP-glutamate dehydrogenase from aspergilli: purification, characterization and implications for metabolic regulation at the carbon–nitrogen interface

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NADP-dependent glutamate dehydrogenase (NADP-GDH) mediates fungal ammonium assimilation through reductive synthesis of glutamate from 2-oxoglutarate. By virtue of its position at the interface of carbon and nitrogen metabolism, biosynthetic NADP-GDH is a potential candidate for metabolic control. In order to facilitate characterization, a new and effective dye-affinity method was devised to purify NADP-GDH from two aspergilli, *Aspergillus niger* and *Aspergillus nidulans*. The *A. niger* NADP-GDH was characterized at length and its kinetic interaction constants with glutamate (K_m 34·7 mM) and ammonium (K_m 1·05 mM; K_i 0·4 mM) were consistent with an anabolic role. Isophthalate, 2-methyleneglutarate and 2,4-pyridinedicarboxylate were significant inhibitors, with respective K_i values of 6·9, 9·2 and 202·0 μM. The *A. niger* enzyme showed allosteric properties and a sigmoid response ($n_H = 2·5$) towards 2-oxoglutarate saturation. The co-operative behaviour was a feature common to NADP-GDH from *Aspergillus awamori*, *A. nidulans* and *Aspergillus oryzae*. NADP-GDH may therefore be a crucial determinant in adjusting 2-oxoglutarate flux between the tricarboxylic acid cycle and glutamate biosynthesis in aspergilli.

INTRODUCTION

Aspergilli constitute a group of industrially important filamentous fungi (Bennett, 1998) with remarkable abilities to produce useful metabolites like organic acids (Ruijter *et al.*, 2002; Willeke & Vorlop, 2001) and extracellular enzymes (Bennett, 1998; Cardoza *et al.*, 1998; Pedersen *et al.*, 1999). *Aspergillus niger* is used extensively to produce citric acid. While the carbon flux exits the tricarboxylic acid (TCA) cycle at citrate, an enhanced flux to glutamate and the γ-aminobutyrate (GABA) shunt occurs during acidogenesis by *A. niger* (Kumar *et al.*, 2000). It thus appears that fungal carbon and nitrogen metabolism are closely interlinked, and the regulation of NADP-dependent glutamate dehydrogenase (NADP-GDH) at this interface may be critical.

Fungal ammonium assimilation occurs via its incorporation into glutamate and glutamine. Some progress has been made in understanding the integrated regulation of the carbon–nitrogen interface of metabolism (Mora, 1990; Marzluf, 1997; Cooper, 2004). Besides the glutamine synthetase/glutamate synthase route, the reaction catalysed by NADP-GDH (EC 1 . 4 . 1 . 4) mediates the reductive amination of 2-oxoglutarate to form glutamate. This places NADP-GDH at the crossroads of carbon and nitrogen metabolism, making this enzyme a candidate for metabolic control. It is argued that co-ordinated regulation of the two NADP-GDH isozymes in *Saccharomyces cerevisiae* enables a balanced utilization of 2-oxoglutarate for glutamate synthesis during diauxic growth (DeLuna *et al.*, 2001). Although transcription of the aspergillus NADP-GDH gene responds to carbon and nitrogen availability (Hawkins *et al.*, 1989; Cardoza *et al.*, 1998), knowledge of its regulation at the level of enzyme activity is lacking. Factors that govern NADP-GDH activity are important inputs in engineering carbon metabolic flux at this branch point.

GDHs from a variety of organisms have received considerable attention (Hudson & Daniel, 1993), but the biochemical characterization of NADP-GDH from filamentous fungi is limited (Blumenthal & Smith, 1973; Botton & Msatef, 1983; Martin *et al.*, 1983; Baars *et al.*, 1995; Bogati *et al.*, 1996). So far, only yeast NADP-GDHs are known to show a co-operative response towards 2-oxoglutarate (Holmes *et al.*, 1989; Perysinakis *et al.*, 1994; DeLuna *et al.*, 2001). As a step towards understanding the role of NADP-GDH in metabolic regulation at the carbon–nitrogen interface we have (i) devised an effective method to purify

Abbreviations: GDH, glutamate dehydrogenase; TCA, tricarboxylic acid.

Details of the gel-filtration results and inhibitor studies are available as supplementary material with the online version of this paper.

this enzyme from aspergilli and (ii) identified three NADP-GDH inhibitors that are potentially of value in metabolic studies. On biochemical characterization, the *A. niger* NADP-GDH exhibited sigmoid saturation kinetics with 2-oxoglutarate, suggesting its importance in carbon flux distribution between the TCA cycle and nitrogen metabolism.

METHODS

Materials. The following chemicals were purchased from Sigma: $NADP^+$, NADPH, L-glutamate, L-glutamine, 2-oxoglutarate, L-α-hydroxyglutaric acid, PMSF, 2-mercaptoethanol, acrylamide, glycine, Tween 20, Coomassie brilliant blue R-250, Coomassie brilliant blue G-250, SDS, *N,N′*-methylene-*bis*-acrylamide, an SDS-PAGE molecular mass marker kit and MES. For immunoblotting, the rabbit IgG antiserum, alkaline phosphatase substrates and nylon membrane were also obtained from Sigma. 3,5-Pyridinedicarboxylic acid, 2,6-pyridinedicarboxylic acid, 2,4-pyridinedicarboxylic acid, 3,5-pyrazoledicarboxylic acid monohydrate, Cibacron blue F3G-A, Procion yellow H-E3G and Procion red (Reactive red 120) were also purchased from Sigma. Cibacron red LS-B was from local textile dye supplier and also from CIBA Specialty Chemicals.

Chemicals required for gel electrophoresis and Sephadex G-25, Sephadex G-200, Sephacryl S-200, Sepharose CL-2B, Sepharose CL-4B and diethylaminoethyl (DEAE)-Sephacel were purchased from USB Chemicals and Amersham Biosciences. All other chemicals and media components used were of analytical reagent grade/of highest purity, locally available from Merck and other local suppliers.

2-Methyleneglutarate synthesis was accomplished by hydrolysis of 2-methylene-glutaronitrile (obtained from Sigma). 2-Methyleneglutaronitrile was heated in aqueous ethanol containing NaOH. The product was acidified with H_2SO_4 and extracted in ethyl acetate. 2-Methyleneglutarate, recrystallized from ethyl acetate, was characterized by its melting point (129–130 °C) and by TLC on silica gel (80 % ethyl acetate in petroleum ether). Finally, the identity of 2-methyleneglutarate was established by elemental analysis and proton NMR.

Strains and growth conditions. All aspergillus cultures, namely *A. niger* NCIM 565 (citric acid producer), *Aspergillus oryzae* NCIM 553, *Aspergillus terreus* NCIM 656, *Aspergillus awamori* NCIM 1225 and *Aspergillus nidulans* NCIM 1211, were obtained from National Chemical Laboratories, Pune, India, and stock cultures were maintained on potato dextrose agar (PDA) plates. The inoculum for liquid cultures was generated using spores from such PDA plates. The spore suspension was prepared in a sterile solution containing 0·005 % Tween 80. All species of aspergilli were grown on normal minimal medium (Kumar *et al.*, 2000), except that *A. terreus* medium contained a higher concentration (2·0 %) of glucose. Fungal mycelia for NADP-GDH purification or activity assay were routinely grown on medium containing sodium nitrate (50 mM) as the sole source of nitrogen. The fungal strains were grown as a surface culture in 1 l Erlenmeyer flasks containing 100 ml of the appropriate medium at 37 °C for 30 h (maximal growth phase). However, *A. terreus* was grown on a shaker at 30 °C for 34 h. The mycelia were stored at −20 °C until use.

Yeast strains, namely *S. cerevisiae* Y05499 (BY4741; *MAT***a** *his3*Δ *leu2*Δ *met15Δ0 ura3Δ0 YPR030w*::*kanMX4*), *S. cerevisiae* Y10345 (BY4739; *MATα leu2Δ0 lys2Δ0 ura3Δ0 YAL062w*::*kanMX4*) and *S. cerevisiae* Y11672 (BY4742; *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YOR375c*::*kanMX4*), were obtained from Euroscarf. While strain Y05499 possessed both the Gdh isozymes, Y10345 and Y11672 were deleted in *GDH3* and *GDH1*, respectively. All three strains were grown on yeast minimal medium (DeLuna *et al.*, 2001).

Preparation of affinity matrices. The reactive dyes Cibacron red LS-B, Cibacron blue F3G-A, Procion yellow H-E3G and Procion red were coupled directly to various matrices (Sepharose CL-2B, Sepharose CL-4B, Sephacryl S-200 and Sephadex G-200) by a standard procedure (Bohme *et al.*, 1972). The dye-coupled matrices were washed sequentially and extensively with double-distilled water, 3·0 M NaCl and buffers of pH 8·0 (0·1 M Tris/HCl) and pH 4·5 (0·1 M sodium acetate) to remove the unbound dye.

Enzyme extraction. All enzyme extraction and purification steps were performed at 4 °C. Frozen fungal mycelia were crushed with an equal amount (w/w) of acid-washed sand in a pre-cooled mortar and pestle, for about 15–20 min. The slurry was extracted with 5 vols (mycelial wet weight : volume) of ice-cold NADP-GDH extraction buffer, containing 100 mM potassium phosphate, 1·0 mM 2-mercaptoethanol, 1·0 mM EDTA and 1·0 mM PMSF (freshly added from a 100 mM stock in 2-propanol) at a final pH of 7·5. The extract was passed through two layers of cheesecloth and the filtrate was centrifuged at 15 000 ***g*** for 20 min in a refrigerated centrifuge. The final supernatant was designated and used as the 'crude enzyme extract'.

Purification of NADP-GDH from *A. niger* and *A. nidulans*

Identical purification protocols were employed for the isolation of NADP-GDH from *A. nidulans* and *A. niger*. A typical procedure for the *A. niger* enzyme is described below.

Ammonium sulfate fractionation. The enzyme was extracted as described above. The protein fraction precipitating between 35 and 70 % saturation of ammonium sulfate was collected.

Dye-affinity chromatography. The active ammonium sulfate pellet (35–70 % fraction) of the crude extract was dissolved in Buffer A (containing 20 mM potassium phosphate, 4·0 mM 2-mercaptoethanol and 1·0 mM EDTA at a final pH of 7·5) and loaded onto Cibacron red LS-B Sephadex G-200 (100 ml) matrix in a beaker. The affinity matrix was incubated with the protein for 3 h at 4 °C, with occasional swirling. The affinity matrix was conveniently used in a batch binding–elution mode. At each step of binding/washing/elution, the affinity matrix was separated from the supernatant by gentle centrifugation (3000 ***g*** for 10 min at 4 °C). The affinity matrix was then sequentially washed with Buffer A followed by 50 mM KCl in the same buffer. Elution of bound proteins was achieved with 0·2 M KCl in three batches of 50 ml each. The enzyme thus eluted from the affinity matrix was then saturated to 80 % with ammonium sulfate to precipitate the enzyme protein.

DEAE-Sephacel chromatography. The enzyme eluted from the dye-affinity matrix was subsequently resuspended in Buffer A (10 × dilution) and loaded on to a DEAE-Sephacel column (bed volume 20 ml) at a flow rate of 15 ml h^{-1}. Bound proteins were eluted at a flow rate of 18 ml h^{-1}, using a gradient of 0–0·5 M KCl (total volume 160 ml), and collected as 3 ml fractions. The fractions were assayed for enzyme activity and protein content.

Molecular mass determination. The native molecular mass of *A. niger* NADP-GDH was determined on a Superdex G-75 prep-grade gel-filtration column (bed volume 320 ml) equilibrated with Buffer A. The column was calibrated with molecular mass standards (66–443 kDa) from Sigma. The dye-affinity matrix-enriched enzyme was resuspended in the same buffer, loaded onto the column and eluted at a flow rate of 120 ml h^{-1}. Molecular mass was determined from a plot of log(molecular mass) against elution volume.

The subunit molecular mass of *A. niger* NADP-GDH was determined

by SDS-PAGE with molecular mass standards (29–205 kDa) from Sigma. MALDI-TOF analysis was performed on a Kratos PC Axima CFR machine.

Electrophoretic procedures and activity staining. Both SDS-PAGE and native PAGE were performed with 7·5 % gels. Proteins on polyacrylamide gels were visualized with Coomassie blue R-250. Activity staining for NADP-GDH was performed according to established methods (Gabriel & Gersten, 1992) but with some modifications. After electrophoresis (native PAGE run at 4 °C), the gels were transferred to clean Petri dishes. In order to ensure uniform pH along their entire length, gels were immersed in ice-cold 100 mM Tris/HCl, pH 7·5, for 15 min prior to staining. These pH-equilibrated gels were immersed in the staining solution and incubated at room temperature. The staining solution (10 ml) contained 100 mM L-glutamate (1·0 ml), 0·4 mM $NADP^+$ (0·5 ml), 100 mM Tris/HCl (pH 7·5, 2·0 ml), 1·0 mg nitroblue tetrazolium ml^{-1} (2·5 ml), 1·0 mg phenazine methosulfate ml^{-1} (2·5 ml) and distilled water (1·5 ml). For substrate controls, L-glutamate was omitted from the staining solution. During the entire process, the gels were protected from light. After incubation, the gels were washed sequentially in double-distilled water and 7·5 % acetic acid.

Immunochemical procedures. Preimmune serum was collected before immunizing a white male New Zealand rabbit. Immunization was initiated by injecting 270 μg purified *A. niger* NADP-GDH protein emulsified in Freund's complete adjuvant. A second injection of 270 μg protein mixed with Freund's incomplete adjuvant was given a week later followed by two more injections (130 μg protein each time) at weekly intervals. Bleedings were taken from the marginal ear vein after 1 month and the serum obtained was decomplemented.

Proteins resolved on native PAGE were electroblotted onto nylon membrane (Hybond-N+) at constant voltage (200 V) for 3–4 h. After transfer, the membrane was blocked with blocking solution (1·5 % casein and 1·5 % gelatin in 50 mM phosphate buffer, pH 7·2) for 2–3 h. Subsequently, the antiserum (1 : 100 dilution) was added and the membrane incubated for 1 h. After adequate washing, the nylon membrane was incubated for 1 h with rabbit IgG antiserum (1 : 10 000) labelled with alkaline phosphatase. Immunoreactive bands were visualized by incubation with the substrate.

Enzyme assays and protein determination. All enzyme activity measurements were made at the ambient temperature of 28 °C. NADP-GDH activity was measured by following the change in absorbance at 340 nm. Unless otherwise mentioned, the following standard assay conditions were used. Reductive amination was determined with an appropriate amount of enzyme protein in a reaction mixture (1·0 ml) containing 100 mM Tris/HCl, pH 8·0, 10 mM NH_4Cl, 10 mM 2-oxoglutarate and 0·1 mM NADPH. The reaction was routinely started by the addition of NADPH. Oxidative deamination activity was determined with an appropriate amount of enzyme protein in a reaction mixture (1·0 ml) containing 100 mM Tris/HCl, pH 9·3, 0·4 mM $NADP^+$ and 100 mM L-glutamate. This reaction was started by the addition of $NADP^+$.

One unit of activity is defined as the amount of enzyme required to reduce/oxidize 1 μmol $NADP^+$/NADPH min^{-1}. Specific activity is defined as U $(mg\ protein)^{-1}$. Protein was estimated (Bradford, 1976) using BSA as a reference.

Procedures for determination of NADP-GDH kinetics. Pure *A. niger* NADP-GDH was used for the kinetic analysis. In all the kinetic measurements, sufficient enzyme was added to give a change in A_{340} of 0·1–0·2 min^{-1}. The reaction was carried out for 2–3 min and rates were obtained from the initial linear portion of the curves. Initiating the forward reaction of the *A. niger* NADP-GDH with different components of the assay resulted in different initial velocities. The initial velocities were always higher when the *A. niger* enzyme was exposed to either NADPH or NADPH plus 2-oxoglutarate before starting the reaction. In the reverse reaction, however, the initial velocity was the same irrespective of how the reaction was initiated. To overcome complications due to these variations, all enzyme kinetic assays were initiated by the addition of either NADPH (forward) or $NADP^+$ (reverse). In substrate saturation experiments, standard assay conditions were employed except for the concentration of the substrate being varied. These changes are mentioned at appropriate places.

The various potential inhibitors were incubated with the enzyme in the standard forward assay and the reaction was subsequently initiated by the addition of NADPH. For experiments in which pH was varied, 25 mM acetic acid, 25 mM MES, 50 mM Tris was used as buffer to minimize the change of ionic strength with pH (Ellis & Morrison, 1982).

Statistical treatment and analysis of data. Enzyme kinetic data presented are typical reproductions of at least three independent experiments and the data were analysed and plotted using the enzyme kinetics software SigmaPlot (version 8.0). A correlation coefficient (r) of 0·95 or higher was always obtained for these data. Experimentally determined values are presented as points, while the lines represent best-fits of these data.

RESULTS

Being at the junction of carbon and nitrogen metabolism of aspergilli, it was anticipated that NADP-GDH activity might be regulated at the enzyme level (DeLuna *et al.*, 2001). A convenient purification method, a prerequisite in elucidating such features, was therefore first established. The specific activity of NADP-GDH is often influenced by the nature of the growth medium (Kinghorn & Pateman, 1973; Cardoza *et al.*, 1998; Pedersen *et al.*, 1999). The *A. niger* NADP-GDH activity profile, when resolved on DEAE-Sephacel matrix, was qualitatively identical irrespective of the nature of the nitrogen source used for cell growth (data not shown). Enzyme levels in *A. niger* were maximally induced by nitrate (2·80 U mg^{-1}) and therefore nitrate-grown mycelia were routinely used for the purpose of enzyme purification.

Purification of NADP-GDH from aspergilli

Several reactive dyes are reported to be useful in the purification of GDHs (Hudson & Daniel, 1993). The *A. niger* NADP-GDH activity was inhibited by the four reactive dyes tested, Cibacron red LS-B, Cibacron blue F3G-A, Procion yellow H-E3G and Procion red. Cibacron red LS-B inhibited the enzyme maximally and the enzyme bound most efficiently to Cibacron red LS-B-coupled Sephadex G-200 matrix. Therefore, Cibacron red LS-B was also coupled to Sepharose CL-2B, Sepharose CL-4B and Sephacryl S-200 using standard methods (Bohme *et al.*, 1972). All these matrices bound the *A. niger* NADP-GDH and the enzyme activity eluted efficiently using a KCl gradient (not shown). The Cibacron red LS-B dye-affinity purification (with a step KCl gradient elution) enriched the *A. niger* NADP-GDH by more than sevenfold and provided

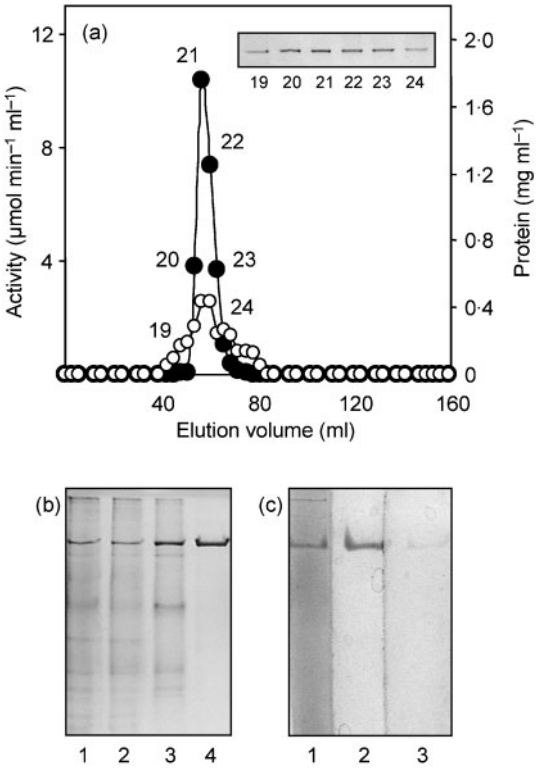

Fig. 1. Purification of *A. niger* NADP-GDH. (a) The dye-affinity-enriched enzyme was bound to DEAE-Sephacel matrix and eluted by a 0–0·5 M KCl gradient. Activity (●) and total protein (○) are plotted. The electrophoretic pattern of relevant fractions around the peak is shown in the inset. (b) Native PAGE of NADP-GDH from *A. niger* at different stages of purification. Lanes: 1, crude extract; 2, 35–70 % ammonium sulfate fraction; 3, dye-affinity-purified enzyme; 4, DEAE-Sephacel peak fraction. (c) Activity staining of *A. niger* NADP-GDH on native PAGE. The enzyme was visualized by activity staining with (lane 2) or without (lane 3) 100 mM glutamate. Coomassie blue staining of the same sample is shown in lane 1.

an almost homogeneous enzyme. However, a few minor contaminating protein bands were still found in this preparation. A subsequent DEAE-Sephacel gradient purification (Fig. 1a) yielded electrophoretically homogeneous protein (Fig. 1b) with a recovery of over 30 %. Identity of the purified protein as *A. niger* NADP-GDH was confirmed by activity staining (Fig. 1c). The results of the entire purification protocol for obtaining pure *A. niger* NADP-GDH are summarized in Table 1.

It was of interest to compare and extrapolate the above purification protocol to isolate NADP-GDH from other aspergilli. The same dye-affinity matrix and identical purification steps (as described in Methods) served well to purify *A. nidulans* NADP-GDH. The purification results for the *A. nidulans* enzyme are also detailed in Table 1. This enzyme protein was enriched 17-fold with a modest recovery of 4 %; considerable loss of enzyme activity occurred during the purification. Inclusion of stabilizing agents could possibly overcome such low recoveries. Although the DEAE-Sephacel elution profile for the *A. nidulans* NADP-GDH (Fig. 2a) was similar to that of the *A. niger* enzyme (Fig. 1a), the two NADP-GDHs clearly differed in their mobility on native PAGE (Fig. 2b).

Characterization of *A. niger* NADP-GDH

The simple purification protocol ensured that milligram quantities of electrophoretically homogeneous *A. niger* NADP-GDH were routinely available. This purified enzyme was characterized for its molecular, kinetic and regulatory features. Wherever relevant, the properties of the *A. nidulans* enzyme were also compared.

Molecular properties. The pure *A. niger* NADP-GDH retained full activity at 4 °C for a week, while the *A. nidulans* enzyme lost more than 50 % activity within 3 days. The UV spectrum of the homogeneous *A. niger* NADP-GDH protein showed a strong shoulder around 280 nm

Table 1. Summary of protocols employed to purify NADP-GDH from *A. niger* and *A. nidulans*

Step	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Recovery (%)
A. niger				
Crude extract	464	2·17	1·0	100·0
35–70 % ammonium sulfate fraction	345	2·50	1·1	74·4
Dye-affinity chromatography	169	16·00	7·3	36·3
DEAE-Sephacel chromatography	145	59·40	27·4	31·3
A. nidulans				
Crude extract	140	0·37	1·0	100·0
35–70 % ammonium sulfate fraction	95	0·90	2·4	68·0
Dye-affinity chromatography	110	3·50	9·5	79·0
DEAE-Sephacel chromatography	6·0	7·00	17·3	4·3

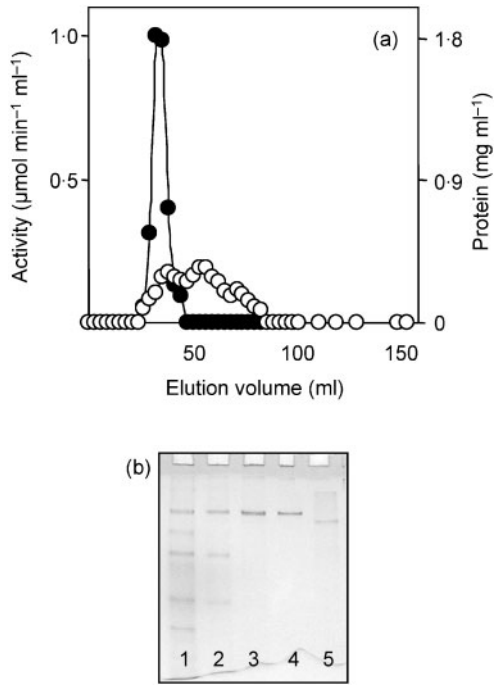

Fig. 2. Purification of *A. nidulans* NADP-GDH. (a) The dye-affinity-enriched enzyme was bound to DEAE-Sephacel matrix and eluted by a 0–0·5 M KCl gradient. Activity (●) and total protein (○) are plotted. (b) Native PAGE of NADP-GDH from *A. nidulans* at different stages of purification. Lanes: 1, crude extract; 2, 35–70 % ammonium sulfate fraction; 3, dye-affinity-purified enzyme; 4, DEAE-Sephacel peak fraction; 5, pure *A. niger* NADP-GDH.

and a 1·0 mg ml^{-1} solution of the pure enzyme protein gave an A_{280} of 1·45. The native molecular mass of pure *A. niger* enzyme was determined to be 350 kDa by FPLC on a calibrated Superdex G-75 prep-grade gel-filtration column. Details of the gel-filtration results are available as Supplementary Fig. S1 with the online version of this paper. The subunit molecular mass of *A. niger* NADP-GDH as determined by SDS-PAGE was 50 kDa. On MALDI-TOF analysis, the pure protein showed a single sharp peak with M_r 49 153. From a comparison of its native and subunit molecular masses, the *A. niger* NADP-GDH appeared to be a hexamer. Sequencing attempts suggested that the protein had a blocked N terminus.

Polyclonal antibodies raised against the purified *A. niger* NADP-GDH were used to immunoblot NADP-GDHs from other aspergilli, namely *A. nidulans*, *A. oryzae* and *A. terreus*, and three *S. cerevisiae* strains [Y05449, Y10345 (*GDH3*Δ) and Y11672 (*GDH1*Δ)]. Cross-reacting protein bands were observed on Western blots in lanes corresponding to *A. niger*, *A. nidulans*, *A. oryzae* and *A. terreus* crude extracts (Fig. 3). However the antiserum did not cross-react with either the Gdh1 or Gdh3 protein of *S. cerevisiae*.

Kinetic properties. The pH optima for the *A. niger* NADP-GDH reaction in the reductive amination and oxidative deamination reactions were respectively 8·50 and 9·75. The ability of *A. niger* NADP-GDH to accept a number of substrate-/product-like molecules as potential substrates was evaluated. The enzyme was strictly specific for NADP(H) and no activity was detected with NAD(H). No reaction was observed with D-glutamate, L-glutamine or DL-2-hydroxyglutarate (as possible substrates in place of L-glutamate), and methylamine was unable to replace ammonium in the biosynthetic reaction; none of these inhibited the *A. niger* NADP-GDH either. Substrate saturation curves for the forward (reductive amination) and reverse (oxidative deamination) reactions of *A. niger* NADP-GDH were obtained. Respective K_m values for these substrates, evaluated from Lineweaver–Burk plots, are summarized in Table 2. An interesting kinetic characteristic of the *A. niger* enzyme is the sigmoidal dependence of its reaction velocity on 2-oxoglutarate concentration, and this aspect will be elaborated on below.

While ammonium is a substrate in the forward (biosynthetic) reaction, it is a product for the reverse reaction of NADP-GDH. Therefore, the effect of ammonium on the enzyme-catalysed oxidative deamination of L-glutamate was assessed. Ammonium inhibited the *A. niger* enzyme, with a K_i value of 0·40 mM, and this inhibition was competitive with L-glutamate (not shown).

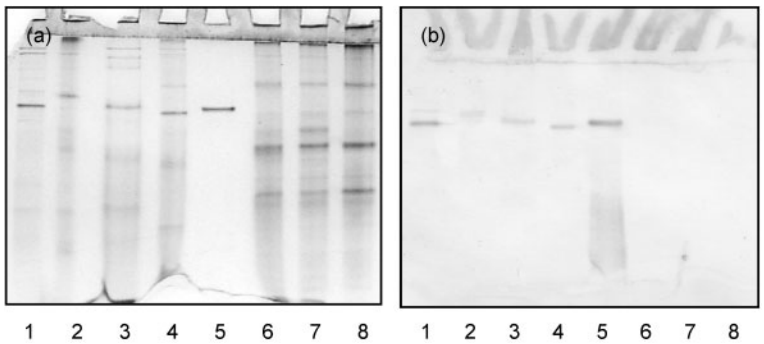

Fig. 3. Comparison of aspergillus NADP-GDHs on Western blots. (a) Coomassie blue-stained native PAGE of crude extracts (5·5 μg protein in each lane) from aspergilli and yeasts. Lanes 1, 2, 3 and 4 correspond to *A. niger*, *A. nidulans*, *A. terreus* and *A. oryzae*, respectively, while lanes 6, 7 and 8 correspond to crude extracts from the yeast strains Y05449 (wild-type), Y10345 (*GDH3*Δ) and Y11672 (*GDH1*Δ), respectively. Lane 5 contained the pure *A. niger* NADP-GDH. (b) Western blot of the same gel probed in parallel using *A. niger* NADP-GDH antiserum.

Table 2. Michaelis constants for the various substrates of *A. niger* NADP-GDH

Substrate	K_m (mM)
Ammonium	1·05
2-Oxoglutarate	4·78*
NADPH	0·011
L-Glutamate	34·70
$NADP^+$	0·017

*Shows sigmoidal behaviour; $S_{0·5}$ is reported.

2-Oxoglutarate saturation and co-operativity

The pure *A. niger* NADP-GDH showed sigmoidal saturation behaviour with respect to 2-oxoglutarate (Fig. 4). These saturation data, when fitted to the Hill equation, gave $S_{0·5}$ and n_H values of 4·8 mM and 2·50, respectively (Fig. 4a, b). When tested, the purified enzyme from *A. nidulans* also showed co-operative 2-oxoglutarate saturation kinetics, albeit with a lower n_H value of 1·5 (Fig. 4c, d). It was of interest to explore whether the observed co-operative behaviour was (i) an artefact of purification and (ii) a common feature of other aspergilli. The NADP-GDH in the crude mycelial extracts of four different aspergilli showed sigmoid 2-oxoglutarate kinetics (Fig. 5). However, their individual n_H values were different.

Yeast NADP-GDH (the Gdh3p isozyme) was recently shown to exhibit a pH-dependent change in its co-operative behaviour towards varying concentrations of 2-oxoglutarate (DeLuna *et al.*, 2001). This property was implicated in metabolic alterations during the diauxic shift. The 2-oxoglutarate saturation of *A. niger* NADP-GDH, performed at two different pH values, was similar, however (not shown). These data on Hill plot analysis yielded identical n_H values (2·5) at both pH 6·0 and pH 8·0.

Reductive amination assays for *A. niger* NADP-GDH were invariably initiated by the addition of NADPH (see Methods). The enzyme activity was stimulated by prior incubation with $NADP^+$ (the product), especially at lower 2-oxoglutarate concentrations. On further analysis, $NADP^+$ showed a biphasic effect; while it stimulated the enzyme at lower 2-oxoglutarate levels, the activity was slightly inhibited at higher concentrations (Fig. 6).

NADP-GDH inhibitors: a search

While NADP-GDH is considered a key enzyme, the paucity of inhibitors (both *in vitro* and *in vivo*) to define its importance in nitrogen metabolism is obvious (Cunliffe *et al.*, 1983; Denton *et al.*, 2001). A number of compounds similar to 2-oxoglutarate and '2-iminoglutarate' (the proposed reaction intermediate; Srinivasan *et al.*, 1988) were evaluated for their ability to inhibit the biosynthetic reaction of the *A. niger* enzyme. The results of such a screen are shown in Table 3. Three molecules, isophthalate, 2,4-pyridinedicarboxylate and 2-methyleneglutarate, were significant inhibitors of *A. niger* NADP-GDH. All other molecules were either ineffective as inhibitors or showed marginal inhibition under the standard assay conditions used. Although not inhibitory, some of the dicarboxylic

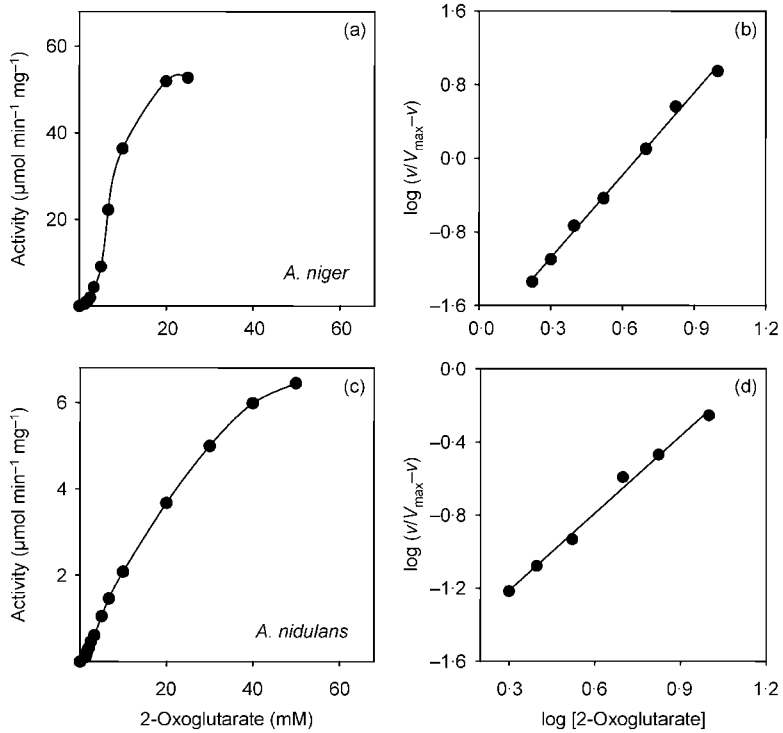

Fig. 4. Kinetic analysis of NADP-GDH saturation by 2-oxoglutarate. The pure enzymes from *A. niger* (0·50–10 μg protein) (a) and *A. nidulans* (1·34–13·40 μg protein) (c) were assayed in the reductive amination direction at pH 8·0 with 100 μM NADPH and 10 mM ammonium as the fixed substrates. The corresponding Hill plots for these data are also shown (b, d).

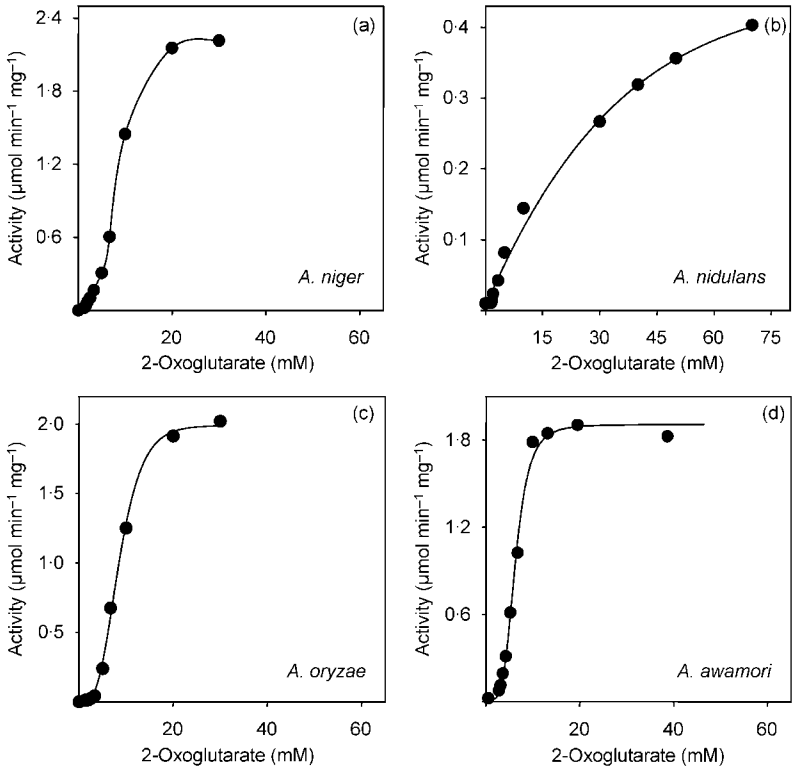

Fig. 5. Comparative kinetic analysis of NADP-GDH from mycelial extracts of four different aspergilli. 2-Oxoglutarate saturation of NADP-GDH in crude enzyme extracts from *A. niger* (5–50 μg protein) (a), *A. nidulans* (28–138 μg protein) (b), *A. oryzae* (5–160 μg protein) (c) and *A. awamori* (7–56 μg protein) (d) was performed in the standard reductive amination direction at pH 8·0 and the reaction was initiated with 100 μM NADPH (see Methods).

acid compounds activated *A. niger* NADP-GDH at the lower 2-oxoglutarate concentration (Table 3). These structural mimics include glutarate, DL-2-hydroxyglutarate, oxalylglycine, itaconate, 3,5-pyridinedicarboxylate and 3,5-pyrazoledicarboxylate. Interestingly, itaconate activated the enzyme significantly even in the presence of 10 mM 2-oxoglutarate.

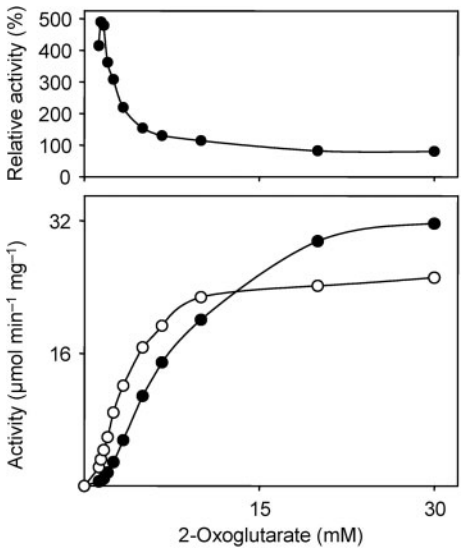

Fig. 6. 2-Oxoglutarate saturation of *A. niger* NADP-GDH shows a biphasic response to $NADP^+$. The lower plot shows the dependence of reductive amination on the concentration of 2-oxoglutarate, in the presence (○) or absence (●) of 0·1 mM $NADP^+$. Relative enzyme activity as a percentage of that without $NADP^+$ is also shown.

The enzyme inhibition by isophthalate, 2,4-pyridinedicarboxylate and 2-methyleneglutarate was further characterized. While these three molecules inhibited the *A. niger* NADP-GDH reaction in either direction, the deamination reaction was always more sensitive to the inhibitors. The sigmoid 2-oxoglutarate saturation also complicated the kinetics and, hence, the deamination reaction was preferred for the inhibition analysis. Isophthalate, 2,4-pyridinedicarboxylate and 2-methyleneglutarate competed with L-glutamate for the *A. niger* enzyme and were linear inhibitors. Structures of the various inhibitors are shown in Fig. 7, and details of the inhibition studies are shown in Supplementary Fig. S2 with the online version of this paper. The K_i values for isophthalate, 2,4-pyridinedicarboxylate and 2-methyleneglutarate, calculated from corresponding replots of the data were respectively 6·9, 202·0 and 9·2 μM.

DISCUSSION

NADP-GDH is at the crossroads of fungal carbon and nitrogen metabolism. This reaction complements the glutamine synthetase/glutamate synthase pathway in fully defining fungal ammonium assimilation (Mora, 1990; ter Schure *et al.*, 2000) and contributes biosynthetic flux to the GABA shunt (Kumar *et al.*, 2000). While enzyme expression is regulated at the mRNA level in response to nitrogen availability (Hawkins *et al.*, 1989; Cardoza *et al.*, 1998; Diez

Table 3. Screening for potential inhibitors of *A. niger* NADP-GDH

NADP-GDH activity was measured in the standard forward reaction (see Methods); ND, not determined.

Inhibitor	NADP-GDH activity (U mg^{-1})			
2-Oxoglutarate concentration…	2·5 mM		10 mM	
Inhibitor concentration…	5·0 mM	10·0 mM	5·0 mM	10·0 mM
None	1·88	1·88	12·08	12·08
Glutarate	3·96	4·48	11·91	11·88
2-Hydroxyglutarate	3·79	3·93	11·03	9·90
2-Methyleneglutarate	**0·44**	**0·24**	**1·15**	**0·60**
Oxalylglycine	2·77	3·35	12·22	11·25
Itaconate	2·77	3·79	21·28	24·06
Terephthalate	1·82	1·67	11·61	11·87
Phthalate	2·19	2·53	12·64	13·08
Isophthalate	**0·86**	**0·69**	**1·98**	**1·51**
2,4-Pyridinedicarboxylate	**1·42**	**0·93**	**4·84**	**3·46**
2,6-Pyridinedicarboxylate	2·24	1·97	8·00	6·16
3,5-Pyridinedicarboxylate	3·08	3·76	12·62	13·08
3,5-Pyrazoledicarboxylate	3·44	ND	13·08	13·93

et al., 1999), the enzyme activity itself is responsive to the concentration of 2-oxoglutarate, a TCA cycle metabolite (DeLuna *et al.*, 2001). Although aspergilli constitute an industrially important group, the NADP-GDH protein from these organisms is poorly characterized. Therefore, the enzyme from *A. niger* was considered for this study.

A. niger mycelia grown on nitrate as the sole source of nitrogen exhibited maximum NADP-GDH specific activity, a feature common to other aspergilli (Cardoza *et al.*, 1998; Pedersen *et al.*, 1999). Nitrate-grown *A. niger* mycelia were therefore suitable starting material in the purification of NADP-GDH activity. Dye-affinity chromatography has been exploited to purify NADP-GDH from a few organisms (Watson *et al.*, 1978; Agrawal & Rao, 1983; Aguirre & Hansberg, 1988; Syed *et al.*, 1991). Keeping this in mind, four different reactive dyes were explored and the Cibacron red LS-B-coupled matrix was most effective in purifying the *A. niger* NADP-GDH (Fig. 1b). An added advantage of this novel dye-affinity purification is a simple and convenient elution by KCl, while, in all the earlier methods, NADP(H) was required for elution. It is thus possible to obtain milligram quantities (Table 1) of electrophoretically pure *A. niger* NADP-GDH. The general applicability of this procedure to NADP-GDH from other aspergilli was obvious, as we could purify the *A. nidulans* enzyme to homogeneity (Fig. 2 and Table 1) by the same method.

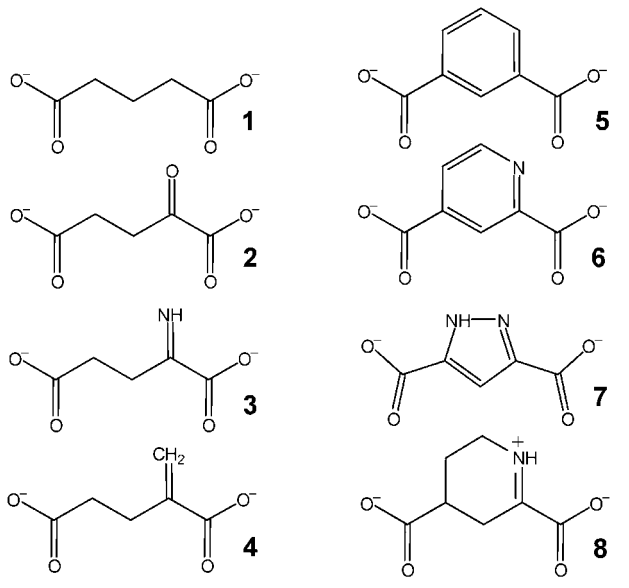

Fig. 7. Structures resembling 2-oxoglutarate and the 'imino-glutarate' intermediate of GDH. 1, 2-Oxoglutarate; 2, glutarate; 3, '2-iminoglutarate'; 4, 2-methyleneglutarate; 5, isophthalate; 6, 2,4-pyridinedicarboxylate; 7, 3,5-pyrazoledicarboxylate; 8, Δ^1-piperidine-2,4-dicarboxylate.

Unlike the NADP-GDH from *Neurospora crassa* and *Aspergillus ochraceus* (Blumenthal & Smith, 1973; Agrawal & Rao, 1983), the *A. niger* NADP-GDH was unusually stable. In contrast, the *A. nidulans* enzyme was very labile, leading to poor recoveries during purification (Table 1). This loss could not be attributed to proteolytic degradation, since the stored (1-week-old) inactive protein moved as a single band on native PAGE. Based on native and subunit molecular masses, the *A. niger* enzyme appeared to be a hexamer, like the other NADP-GDHs reported (Blumenthal & Smith, 1973; Botton & Msatef, 1983; Martin *et al.*, 1983; Hudson & Daniel, 1993; Baars *et al.*, 1995; Bogati *et al.*, 1996). Since its N terminus appears to be blocked, the ready availability of milligram quantities of the *A. niger* protein should now permit access to internal peptide fragments for sequencing.

The only report of a partial protein sequence is for the *Penicillium chrysogenum* NADP-GDH (Bogati *et al.*, 1996; Diez *et al.*, 1999). While *S. cerevisiae* contains two distinct NADP-GDH genes, filamentous fungi including *N. crassa*, *P. chrysogenum* and various aspergilli contain a single copy of the NADP-GDH gene (DeLuna *et al.*, 2001). We compared the known fungal NADP-GDH sequences (deduced amino acid sequences) using CLUSTAL W. Among filamentous fungi, NADP-GDHs from *A. awamori* (Cardoza *et al.*, 1998), *A. nidulans* (Hawkins *et al.*, 1989) and *Aspergillus fumigatus* (http://www.tigr.org) form a cluster. Pairwise identities (generated using BLAST) also indicated greater than 80 % identity between these three deduced protein sequences. While it would be useful to have the *A. niger* NADP-GDH protein sequence in hand, antibodies provide yet another tool for comparison. NADP-GDH proteins from all the aspergilli tested cross-reacted with the antiserum raised against pure *A. niger* NADP-GDH (Fig. 3), indicating their antigenic relatedness and similarity. The yeast isozymes (DeLuna *et al.*, 2001) are clearly distinct, as they did not cross-react with these antibodies.

The biosynthetic role of fungal NADP-GDH is well supported by genetic (Fincham, 1962; Kinghorn & Pateman, 1973; Fincham *et al.*, 2000), physiological (Martin *et al.*, 1988; Kusnan *et al.*, 1989; Schwartz *et al.*, 1991) and kinetic (Hudson & Daniel, 1993) data. The kinetic constants obtained for the various substrates of the *A. niger* NADP-GDH (Table 2) are consistent with its role in ammonium assimilation. Ammonium could also act as a product inhibitor in the reverse reaction (Baars *et al.*, 1995). A strong inhibition of the reverse reaction by ammonium (K_i of 0·40 mM) and a substantial difference in the *A. niger* NADP-GDH affinities for ammonium and L-glutamate (as reflected by their respective K_m values) favour the enzyme reaction towards glutamate formation *in vivo*. Absence of inhibition of the reductive amination reaction by glutamate (>100 mM; not shown) is also consistent with its assimilatory role.

There is a paucity of information on NADP-GDH inhibitors, with the literature being biased towards NAD-GDH (Caughey *et al.*, 1956; Veronese *et al.*, 1974; Stevens *et al.*, 1989). Attempts to generate more isophthalate derivatives as inhibitors and also to obtain useful conformationally restricted substrate analogues for GDHs have been made (Cunliffe *et al.*, 1983; Denton *et al.*, 2001). Specific NADP-GDH inhibitors, in addition to dissecting metabolic details (Kusnan *et al.*, 1987), could be valuable in mechanistic studies. Selected NAD-GDH inhibitors were included in our screening (Table 3) on two counts: (i) the extensive similarity in chemical mechanism of the NAD-GDH and NADP-GDH reactions (Srinivasan *et al.*, 1988; Hudson & Daniel, 1993; Brunhuber & Blanchard, 1994) and (ii) the largely identical active-site geometry for the two reactions and conserved structural layout of their active-site amino acid residues (Baker *et al.*, 1992). This is the first report of NADP-GDH inhibition by isophthalate (the most studied NAD-GDH inhibitor) and identifies 2-methyleneglutarate as an equally potent non-aromatic inhibitor.

Considering the proposed 'iminoglutarate' intermediate (Fig. 7; structure 3) and the observed potent isophthalate inhibition, other dicarboxylate compounds were evaluated for their inhibitory potential. As expected, inhibition of *A. niger* NADP-GDH by the three compounds (isophthalate, 2,4-pyridinedicarboxylate and 2-methyleneglutarate) was competitive with respect to L-glutamate (Supplementary Fig. S2). Preliminary data (unpublished) suggest their promise as *in vivo* inhibitors of *A. niger* NADP-GDH and fungal nitrogen metabolism. 2-Methyleneglutarate, owing to its close structural resemblance to 2-oxoglutarate and 'iminoglutarate' (Srinivasan *et al.*, 1988; Fisher *et al.*, 2001), was synthesized and tested as a potential NADP-GDH inhibitor. Although not a substrate, this molecule was an excellent inhibitor of the NADP-GDH reaction. Itaconate, a lower homologue of 2-methyleneglutarate, unexpectedly activated *A. niger* NADP-GDH, and this needs further investigation. Both 2,4-pyridinedicarboxylate and 3,5-pyrazoledicarboxylate were anticipated to mimic 'iminoglutarate'; only the former showed significant inhibition of *A. niger* NADP-GDH (Supplementary Fig. S2 and Table 3). As invoked in the case of isophthalate (Caughey *et al.*, 1956), the distance between the two negatively charged groups (an important determinant for binding) possibly renders 3,5-pyrazoledicarboxylate and the other pyridinedicarboxylate isomers less effective. An interesting proposition is that the nitrogen atom of 2,4-pyridinedicarboxylate, occupying a position similar to the one in 'iminoglutarate', may contribute to its potency. In fact, the synthesis and properties of related compounds (Δ^1-piperidine-2,4-dicarboxylate; Fig. 7, structure 8) have already been reported (Nielsen *et al.*, 2001) and are worth pursuing.

The homogeneous *A. niger* NADP-GDH exhibited a strong sigmoidal response (n_H of 2·5) towards varying concentrations of 2-oxoglutarate (Fig. 4). The *A. nidulans* enzyme also showed co-operative behaviour, albeit to a lesser degree. The sigmoid saturation was not an artefact of purification, but an inherent property of aspergillus NADP-GDH, as crude mycelial extracts also showed similar behaviour. This novel report of an allosteric 2-oxoglutarate interaction with NADP-GDH appears to be a regulatory feature common at least to the four aspergilli tested. Among the fungal enzymes studied so far, only yeast NADP-GDHs are reported to exhibit sigmoidal 2-oxoglutarate saturation (Holmes *et al.*, 1989; Perysinakis *et al.*, 1994; DeLuna *et al.*, 2001). The two *S. cerevisiae* isozymes (Gdh1p and Gdh3p) are implicated in fine-tuning yeast glutamate synthesis and carbon metabolism during the diauxic shift. While both exhibited co-operative behaviour towards varying concentrations of 2-oxoglutarate, only Gdh3p showed a pH dependence of its Hill coefficient (DeLuna *et al.*, 2001). *A. niger* NADP-GDH resembles yeast Gdh1p in that the n_H value (from 2-oxoglutarate saturation) was pH independent.

The observed activation at lower 2-oxoglutarate concentrations by some of the dicarboxylic acids (Table 3) and the biphasic response to $NADP^+$ (Fig. 6) may be manifestations of this sigmoidal behaviour of *A. niger* NADP-GDH. At subsaturating concentrations of 2-oxoglutarate (the substrate exhibiting sigmoid saturation), such ligands could stimulate the velocity by promoting the T↔R allosteric transition (Gerhart & Pardee, 1963; Segel, 1993; MacRae *et al.*, 2002). They act as pure competitive inhibitors at higher concentrations. Co-operative effects of substrate and substrate analogue (succinate) on *N. crassa* NADP-GDH conformational states are documented (Fincham *et al.*, 2000).

Attempts to engineer microbial metabolic fluxes through manipulations at the level of NADP-GDH (Marx *et al.*, 1999; Nissen *et al.*, 2000) clearly suggest the significance of this enzyme at the carbon–nitrogen interface of metabolism. It is interesting that, although NADP-GDH is central to aspergillus nitrogen metabolism (and ammonia assimilation), its activity is amenable to regulation by 2-oxoglutarate. The co-operative kinetics with 2-oxoglutarate suggest that the *A. niger* enzyme is responsive to and has the potential to set the carbon flux at the interface of carbon and nitrogen metabolism. Spare carbon flux, available due to a TCA cycle block after 2-oxoglutarate during acidogenesis, may thus account for the increased flux towards glutamate (Meixner-Monori *et al.*, 1985; Kumar *et al.*, 2000; Ruijter *et al.*, 2002). Selective enzyme inhibitors are useful in mechanistic studies and are potential metabolic tools to achieve pathway blocks *in vivo* (Kusnan *et al.*, 1987, 1989). The inhibitors identified and characterized in this study are expected to help to define the role of NADP-GDH further in fungal carbon metabolic flux at this branch point.

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