Re-assessment of ammonium-ion affinities of NADP-specific glutamate dehydrogenases

Activation of the *Neurospora crassa* enzyme by ammonium and rubidium ions

John C. WOOTTON

Department of Genetics, University of Leeds, Leeds LS2 9JT, U.K.

(Received 6 September 1982/Accepted 2 November 1982)

The NADP-specific glutamate dehydrogenase of *Neurospora crassa* shows complex interactions with NH_4^+ ions, characterized by biphasic downwardly convex double-reciprocal plots. These kinetics are explained by the action of NH_4^+ both as a substrate and, acting at a separate cation-binding site, as an activator. Rb^+ ions, and to a smaller extent other univalent cations, also activate by acting as analogues of NH_4^+ . Previous failure to recognize this effect, which probably also occurs in homologous enzymes from some other species, has led to significant overestimates in published reports of the K_m for NH_4^+ of some NADP-specific glutamate dehydrogenases.

Glutamate dehydrogenases (EC 1.4.1.2, EC 1.4.1.3 and EC 1.4.1.4) occur in almost all micro-organisms, plants and animals. It is established that NADP-specific glutamate dehydrogenase (EC 1.4.1.4) functions as a major pathway of NH_4^+ assimilation in several prokaryotic and eukaryotic micro-organisms, but the roles of glutamate dehydrogenase in most organisms are not yet clear (see reviews by Smith *et al.*, 1975; Tyler, 1978; Stewart *et al.*, 1980; Mifflin & Lea, 1980). Although several authors have argued for or against the possible involvement of glutamate dehydrogenases in NH_4^+ assimilation or in L-glutamate deamination on the basis of K_m values for NH_4^+ , there have been remarkably few detailed studies of the interaction of any of these enzymes with NH_4^+ ions. Almost all published estimates of K_m (references cited in the above reviews) have been based on small numbers of enzyme assays with relatively limited ranges of NH_4^+ concentration.

I now report the results of a more extensive study that reveal biphasic effects of NH_4^+ on the NADP-specific glutamate dehydrogenase of the fungus *Neurospora crassa*. This enzyme, which is very well characterized physicochemically (complete sequence in Holder *et al.*, 1975; references to other properties in Smith *et al.*, 1975; Stewart *et al.*, 1980; Ashby *et al.*, 1981), functions as the predominant pathway of NH_4^+ assimilation in mycelium growing on non-limiting concentrations of NO_3^- or NH_4^+ . There are conflicting published estimates of the K_m for NH_4^+ of this enzyme [1.1 mM (Fincham & Bond, 1960); 10 mM (Sanwal & Lata, 1961); 0.7 mM

(Wootton *et al.*, 1976)]. The observations reported below resolve this conflict and suggest that some previous work has significantly underestimated the affinity for NH_4^+ of not only this enzyme but also some NADP-specific glutamate dehydrogenases from other species. I also report that Rb^+ appears to act as an analogue of NH_4^+ in its activating effects on this enzyme.

Materials and methods

Materials and methods for the purification and storage of NADP-specific glutamate dehydrogenase from *N. crassa* wild-type strain STA4, and criteria of purity, were as previously described (Ashby *et al.*, 1974, 1981; Watson & Wootton, 1977). Reagents for enzyme assays were as described by Ashby *et al.* (1974). Solutions of RbCl ('99% pure' grade; Sigma Chemical Co.) were passed through a Millipore filter (HA; 0.45 μm) before use to remove traces of insoluble impurities. Other inorganic chemicals, from BDH Chemicals, were of analytical grade and were used without further purification.

Enzyme assays were spectrophotometric measurements of the velocity of NADPH oxidation during reductive amination of 2-oxoglutarate at 37°C, performed essentially as previously described (Watson & Wootton, 1977). Assays at pH 8.5 used 0.1 M-Tris/HCl buffer containing 1 mM-EDTA; assays at pH 7.4 used 0.05 M-sodium phosphate buffer containing 1 mM-EDTA. An effectively saturating NADPH concentration, 0.17 mM, was used unless otherwise stated, with 2-oxoglutarate

and NH_4Cl concentrations as described for different experiments. All velocities reported are steady-state values measured from the linear range within which the activities are independent of decreasing NADPH concentration. Apparent K_m values were determined from linear unweighted least-squares regressions of the specified section of double-reciprocal ($1/v$ against $1/[S]$) plots. Control assays showed no significant background NADPH oxidation in the absence of enzyme or other substrates.

Results and discussion

Evidence for substrate activation by NH_4^+

Fig. 1(a) shows double-reciprocal plots ($1/v$ versus $1/[\text{NH}_4\text{Cl}]$) covering the NH_4Cl concentration range 2–100 mM. There are clear downwardly convex deviations from linearity, more marked at lower concentrations of 2-oxoglutarate. Representative data in Fig. 1(b) extend these plots through the NH_4Cl concentration range 0.1–2 mM and show that the plots are linear over the 50-fold range 0.1–5 mM- NH_4Cl .

These effects of NH_4^+ are distinct from the well-established slow allosteric conformational changes between inactive I and active A states described by Ashby *et al.* (1974, 1981). The data (Fig. 1) were obtained after incubation of the enzyme with 2-oxoglutarate and therefore apply to the A conformation stabilized by this substrate. Similar assays made without preincubation, before the slow (2–5 min) transient phase of A–I-state equilibration, and also assays made at several different concentrations, 0.04–0.17 mM, of NADPH (which stabilizes the I conformation) gave downwardly convex double-reciprocal plots for NH_4^+ very similar to those in Fig. 1. Therefore the NH_4^+ effects reflect a rapidly equilibrating process and are unaffected by the A–I-state equilibrium position.

The downwardly convex plots might result from (a) heterogeneity in affinity of sites binding NH_4^+ as substrate, (b) negative co-operativity in NH_4^+ binding, (c) action of NH_4^+ as both substrate and activator ('substrate activation'), or (d) artifacts such as background non-enzymic activity (ruled out by the control assays described in the Materials and methods section) or enzyme heterogeneity. The last explanation is ruled out by previous physicochemical studies on the purity of the enzyme (Ashby *et al.*, 1974, 1981; Brett *et al.*, 1976). The enzyme is a hexamer of identical polypeptide chains, and experiments with hybrid hexamers constructed from purified polypeptides of active and inactive mutational variants have demonstrated that at effectively saturating substrate concentrations all six subunits are active and equivalent in catalytic-centre activity (Watson & Wootton, 1978). Although this earlier work does not rule out possible negative co-

Fig. 1. Effect of NH_4^+ concentration on activity of *N. crassa* and *C. pyrenoidosa* NADP-specific glutamate dehydrogenases: double-reciprocal plots

For the *N. crassa* enzyme velocities (pH 8.5 assays) were determined as described in the Materials and methods section, and control assays were used to correct for small differences in enzyme concentration between different experiments and to normalize all velocities to the same units. (a) NH_4^+ concentration range 2–100 mM. $\circ$, $\triangle$, $\bullet$ and $\blacktriangle$, *N. crassa* enzyme assayed at 2-oxoglutarate concentrations 0.5 mM ($\circ$), 1.0 mM ($\triangle$), 2.5 mM ($\bullet$) and 10.0 mM ($\blacktriangle$). $\blacksquare$, *Chlorella pyrenoidosa* enzyme, data replotted from Fig. 7 (a Hill plot) of Shatilov & Kretovich (1977), with calculation of velocities on the basis of an arbitrary value of V (1.61 units) chosen to bring them into the range observed in the experiments with the *N. crassa* enzyme. (b) NH_4^+ concentration range 0.1–2 mM. *N. crassa* enzyme assayed at 2-oxoglutarate concentrations 0.5 mM ($\circ$) and 2.5 mM ($\bullet$). Broken lines indicate the continuations of the same plots shown in (a).

operativity or site heterogeneity at subsaturating NH_4^+ concentrations, the effects of cations other than NH_4^+ , described below, support instead the hypothesis of substrate activation.

Effects of Rb^+ and other cations

Na^+ , K^+ , Rb^+ , Cs^+ , Mg^{2+} , Ca^{2+} and MeNH_3^+ were tested as possible analogues of NH_4^+ , by additions of their Cl^- salts to assays of purified *N. crassa* NADP-specific glutamate dehydrogenase at various NH_4Cl concentrations. All these salts, at concentrations greater than 50 mM, showed similar non-specific inhibitory effects, probably due to the Cl^- anions, consistent with other non-specific effects of ions previously described (Watson & Wootton, 1977). None of the ions tested acted as an inhibitor competitive with NH_4^+ as a substrate, indicating that the substrate-binding site is highly selective.

These cations, in the concentration range 1–50 mM, showed differential activating effects. Rb^+ was the most effective activator, giving approx. 10% activation at 1 mM- and 25% at 10 mM- RbCl (Fig. 2), at NH_4^+ concentrations up to 4 mM. Higher RbCl concentrations up to 50 mM gave similar results to 10 mM (Fig. 2), which is apparently almost saturating for this effect. The other cations tested were considerably less effective, giving at most approx. 10% activation (with 50 mM- KCl or $-\text{CsCl}$). Rb^+ activation decreases with increasing NH_4^+ concentration in the range 4–100 mM- NH_4Cl , over which the double-reciprocal plots curve downwards (Fig. 2).

Very probably these activating effects of Rb^+ and other cations and the downwardly convex double-reciprocal plots for NH_4^+ reflect the same underlying property of the enzyme, namely the existence of an activator site that binds NH_4^+ and other univalent cations and is distinct from the NH_4^+ substrate site. Rb^+ is presumed to act as an analogue of NH_4^+ by binding at this site, other univalent cations either binding less strongly or acting as less effective activators of enzymic activity. Thus the change, with increasing NH_4^+ concentration greater than 5 mM, from activation by Rb^+ to activation by NH_4^+ (Fig. 2) is explained by competitive binding. That double-reciprocal plots for NH_4^+ are downward curving both in the presence and in the absence of Rb^+ (Fig. 2) suggests that NH_4^+ causes greater enhancement of catalytic activity than does Rb^+ , although Rb^+ might bind more strongly than NH_4^+ , giving significant activation at 1 mM- RbCl (Fig. 2). Several formal schemes are consistent with these results, and a detailed kinetic analysis, which would be complicated by non-specific ionic effects, has not been attempted. It is not necessary to postulate more complex models, involving for example separate effects of cation activation and negatively co-

Fig. 2. Effect of Rb^+ on double-reciprocal plots for *N. crassa* NADP-specific glutamate dehydrogenase and NH_4^+ .

All assays (pH 8.5 system) were at 2.5 mM-2-oxo-glutarate. For details see the Materials and methods section. ●, No RbCl present; ▲, 1 mM- RbCl ; ■, 10 mM- RbCl .

operative NH_4^+ binding, although such models are not formally disproved.

Rb^+ and NH_4^+ ions are very similar in some physical properties (Mellor's *Comprehensive Treatise on Inorganic and Theoretical Chemistry*, 1963, 1964), especially in ionic radii measured from electron-density maps of their salt crystals (NH_4^+ , 0.143 nm; Rb^+ , 0.148 nm; compare Cs^+ , 0.169 nm; K^+ , 0.133 nm; and the considerably smaller Na^+ , Ca^{2+} and Mg^{2+}). The existence of a cation-binding site preferentially binding Rb^+ and NH_4^+ , and less selective for other cations, is therefore very plausible. More widespread studies of the effect of Rb^+ on other NH_4^+ -binding biological systems might be fruitful, to supplement the established biological effects of Rb^+ (reviewed, for example, in Putney, 1978) as an Li^+ antagonist and as an analogue of K^+ in transport systems.

Affinity of NADP-specific glutamate dehydrogenases for NH_4^+

The occurrence of activation by NH_4^+ , as strongly supported by these results, implies that the affinity for NH_4^+ as a substrate is reflected by K_m values determined only from NH_4^+ concentrations between 0.1 mM (or less) and 5 mM. In this concentration range activation does not cause significant curvature of double-reciprocal plots. This is also the physiologically relevant range of NH_4^+ concentrations under many growth conditions in *N. crassa* (J. C. Wootton, unpublished work) and other

Table 1. Values of apparent K_m of *N. crassa* NADP-specific glutamate dehydrogenase for NH_4^+ measured from different parts of biphasic double-reciprocal plots. Values of apparent K_m , determined as described in the Materials and methods section, are means of estimates made from the data in Fig. 1 and other similar experiments (two or three experiments for each value) with the pH values and 2-oxoglutarate concentrations stated. N.M., Not measured.

[2-Oxoglutarate] (mm)	Apparent K_m for NH_4^+ (mm)			
	[NH_4^+] range 0.1–2 mm		[NH_4^+] range 5–100 mm	
	pH 7.4	pH 8.5	pH 7.4	pH 8.5
0.5	0.60	0.55	5.3	11.9
0.7	0.73	N.M.	4.3	N.M.
1.0	0.81	0.58	3.0	10.1
2.0	1.09	N.M.	N.M.	N.M.
2.5	N.M.	0.63	N.M.	5.8
5.0	1.08	0.80	N.M.	N.M.
10.0	N.M.	1.02	N.M.	3.9
20.0	1.11	N.M.	N.M.	N.M.

organisms (Stewart *et al.*, 1980). Table 1 summarizes measurements of apparent K_m determined from different ranges of double-reciprocal plots of velocities at different pH values and 2-oxoglutarate concentrations. These show that the correct values of apparent K_m for NH_4^+ range from 0.5 mm to 1.1 mm, extrapolating to a K_m of 1.1 mm at infinite 2-oxoglutarate concentration. Further evidence that the lower range of apparent K_m values is correct comes from similar tests with NH_4^+ as a product inhibitor of the oxidative deamination of L-glutamate. These experiments gave complex non-linear plots of $1/v$ versus $[\text{NH}_4^+]$, but approximate estimates of K_i for NH_4^+ as product inhibitor from different sections of the plots by the method of Dixon (1953) were in the range 0.2–1.5 mm. A previous 10-fold overestimate of K_m (Sanwal & Lata, 1961) is explained because these authors used an inappropriately high range of NH_4^+ concentration (10–80 mm) at low 2-oxoglutarate concentration.

A survey of published reports (cited in reviews; see the introduction) of K_m values for NH_4^+ of NADP-specific glutamate dehydrogenases, which quote widely differing values mostly greater than 5 mm, reveals that biphasic interactions with NH_4^+ might apply to such enzymes of other organisms. The great majority of authors used only NH_4^+ concentrations greater than 5 mm, or did not quote the range of concentrations used, but two reports give data relevant to the effects described in the present paper. First, the data of Shatilov &

Kretovich (1977) on the NH_4^+ -inducible NADP-specific glutamate dehydrogenase of *Chlorella pyrenoidosa* Pringsheim 82T, when replotted as a double-reciprocal plot (Fig. 1a), show biphasic interactions with NH_4^+ indistinguishable from those of the *N. crassa* enzyme. These authors postulated negative co-operativity to explain this effect, but they also reported marked activation of the *C. pyrenoidosa* enzyme by Li^+ , Na^+ , K^+ and Cs^+ (Rb^+ was not tested), which strongly supports the alternative explanation of cation activation as argued in the present paper for the *N. crassa* enzyme. This re-interpretation of their results indicates that the apparent K_m of *C. pyrenoidosa* NADP-specific glutamate dehydrogenase for NH_4^+ is approx. 0.6 mm, rather than the 9 mm [for $(\text{NH}_4)_2\text{HPO}_4$] calculated by Shatilov & Kretovich (1977). Secondly, the very thorough experiments of Coulton & Kapoor (1973) on the NADP-specific glutamate dehydrogenase of *Salmonella typhimurium* used the NH_4^+ concentration range 0.05–2 mm and clearly established a low K_m of 0.29 mm. These authors did not use higher NH_4^+ concentrations, and so would not have observed biphasic effects if present.

Clearly re-investigations are required of the interactions with NH_4^+ of NADP-specific glutamate dehydrogenases from a range of bacteria, fungi, algae and plants. Recent molecular studies have demonstrated very strong sequence conservation between such enzymes of widely divergent prokaryotic and eukaryotic organisms, shown by DNA sequences and heterologous probing of Southern blots at low stringency (Mattaj *et al.*, 1982; J. C. Wootton & B. Hohn, unpublished work). Enzymological properties are also strongly conserved, as shown, for example, by the very similar slow allosteric A–I-state conformational transitions in the *N. crassa*, *Escherichia coli* and *C. pyrenoidosa* NADP-specific glutamate dehydrogenases (Ashby *et al.*, 1974, 1981; Shatilov & Kretovich, 1977; J. C. Wootton, unpublished work).

The relatively high affinities of NADP-specific glutamate dehydrogenases for NH_4^+ as a substrate presumably reflect a physiological role in NH_4^+ assimilation, although this role has been established in relatively few organisms, and wider investigations are needed. For several species, particularly plants, there is strong evidence for the operation of the pathway of NH_4^+ assimilation involving glutamine synthetase (which in most cases has a higher affinity for NH_4^+ than does NADP-specific glutamate dehydrogenase) and glutamate synthase (Mifflin & Lea, 1980; Stewart *et al.*, 1980). Interestingly, biphasic effects of NH_4^+ on *E. coli* glutamine synthetase have been reported (Meek & Villafranca, 1980), and were attributed by these authors to negative co-operativity, although NH_4^+ activation is an equally plausible explanation. Possibly the ac-

tivation of NADP-specific glutamate dehydrogenase by high NH_4^+ concentrations, as reported in the present paper, might increase the rate at which potentially toxic intracellular NH_4^+ concentrations are decreased.

I thank Mr. Andrew Baron for assistance with enzyme purifications and some of the assays. Financial support was from a Science Research Council project grant.

References

- Ashby, B., Wootton, J. C. & Fincham, J. R. S. (1974) *Biochem. J.* **143**, 317–329
- Ashby, B., Nixon, J. S. & Wootton, J. C. (1981) *J. Mol. Biol.* **149**, 521–540
- Brett, M., Chambers, G. K., Holder, A. A., Fincham, J. R. S. & Wootton, J. C. (1976) *J. Mol. Biol.* **106**, 1–22
- Coulton, J. W. & Kapoor, M. (1973) *Can. J. Microbiol.* **19**, 439–450
- Dixon, M. (1953) *Biochem. J.* **55**, 161–171
- Fincham, J. R. S. & Bond, P. A. (1960) *Biochem. J.* **77**, 96–105
- Holder, A. A., Wootton, J. C., Baron, A. J., Chambers, G. K. & Fincham, J. R. S. (1975) *Biochem. J.* **149**, 757–773
- Mattaj, I. W., McPherson, M. J. & Wootton, J. C. (1982) *FEBS Lett.* **147**, 21–25
- Meek, T. D. & Villafranca, J. J. (1980) *Biochemistry* **19**, 5513–5519
- Mellor's *Comprehensive Treatise on Inorganic and Theoretical Chemistry* (1963) *The Alkali Metals (Part 2)*, vol. 2, suppl. 3, Longmans, London
- Mellor's *Comprehensive Treatise on Inorganic and Theoretical Chemistry* (1964) *Nitrogen (Part 1)*, vol. 8, suppl. 1, Longmans, London
- Mifflin, B. J. & Lea, P. J. (1980) in *The Biochemistry of Plants: a Comprehensive Treatise: Volume 5, Amino Acids and Derivatives* (Mifflin, B. J., ed.), pp. 169–202, Academic Press, New York and London
- Putney, J. W. (1978) *Pharmacol. Rev.* **30**, 209–245
- Sanwal, B. D. & Lata, M. (1961) *Can. J. Microbiol.* **7**, 319–328
- Shatilov, V. R. & Kretovich, W. L. (1977) *Mol. Cell. Biochem.* **15**, 201–212
- Smith, E. L., Austen, B. M., Blumenthal, K. M. & Nyc, J. F. (1975) *Enzymes 3rd Ed.* **11**, 293–367
- Stewart, G. R., Mann, A. F. & Fentem, P. A. (1980) in *The Biochemistry of Plants: a Comprehensive Treatise: Volume 5, Amino Acids and Derivatives* (Mifflin, B. J., ed.), pp. 271–327, Academic Press, New York and London
- Tyler, B. (1978) *Annu. Rev. Biochem.* **47**, 1127–1162
- Watson, D. H. & Wootton, J. C. (1977) *Biochem. J.* **167**, 95–108
- Watson, D. H. & Wootton, J. C. (1978) *Biochem. J.* **175**, 1125–1133
- Wootton, J. C., Kavanagh, J. P., Baron, A. J. & Lovett, M. G. (1976) *Biochem. J.* **159**, 803–806