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The *Neurospora am* gene and NADP-specific glutamate dehydrogenase: mutational sequence changes and functional effects – more mutants and a summary

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Summary

A further series of mutant *am* alleles, encoding potentially active NADP-specific glutamate dehydrogenase (GDH) and capable of complementation in heterocaryons, have been characterized with respect to both GDH properties and DNA sequence changes. Several mutants previously studied, and some of their same-site or second-site revertants, have also been sequenced for the first time. We present a summary of what is known of the properties of all *am* mutants that have been defined at the sequence level.

1. Introduction

The *Neurospora am* gene encodes the hexameric NADP-specific glutamate dehydrogenase (GDH), which catalyses the reversible reductive amination of α-ketoglutarate to form glutamate:

$$\text{Glutamate} + \text{NADP}^+ \rightleftharpoons \alpha\text{-Ketoglutarate} + \text{NH}_3 + \text{NADPH} + \text{H}^+$$

Mutations in the *am* gene have been studied from various points of view over a period of more than 40 years (reviewed briefly by Fincham, 1977, 1988). Sites of mutation within the gene have been mapped both by the unconventional criterion of conversion polarity (Fincham, 1967) and by the more certain analysis of deletion overlaps (Rambosek & Kinsey, 1983). The gene has been cloned and sequenced (Kinnaird & Fincham, 1983). A virtually unlimited number of *am* mutants became available following the discovery of an automatic method for selecting them (Kinsey, 1977). Several amino acid replacements in mutants have been identified by peptide analysis (Brett *et al.*, 1976; Kinsey *et al.*, 1980). A major point of interest is that several of these replacements, while eliminating GDH activity *in vivo*, still permit activity under special conditions (usually high concentration of glutamate or succinate) *in vitro*. These replacements appear to displace the normal allosterically controlled equilibrium between active and inactive forms of the enzyme (Ashby *et al.*, 1981). They are all complemented in heterocaryons by mutants which are GDH-negative for other reasons: failure to bind NADP (*am*^{1}, Ashby *et al.*, 1974) or presumed failure to form stable hexamers (*am*^{14}, Fincham & Baron, 1977). This interallelic complementation appears to be due to conformational normalization within hybrid hexamers (Coddington *et al.*, 1966, Watson & Wootton, 1978). Thus conformationally abnormal mutant forms of GDH can be more or less normalized either by cooperative binding of the substrate glutamate or its analogue succinate, or by complementation in hybrid hexamers.

Now that DNA sequencing has become routine, it has seemed worthwhile to extend the sequence analysis to all interesting *am* mutants, and to screen a further batch from the Kinsey collection. A further reason for renewed interest is that the three-dimensional structure of the hexameric *Neurospora* NADP-specific GDH has recently been solved (T. Stillman, D. W. Rice, *et al.*, in preparation).

This paper presents, in summary form, the molecular and enzymological information available on the whole set of *am* mutants, both old and new.

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2. Methods

(i) *Mutants*

The various origins of the mutants first studied, *am*^{1} to *am*^{11}, are given in Fincham (1959). All except *am*^{3} and *am*^{4}, which were induced by 20-methylcholanthrene and β-propiolactone respectively, were induced by ultraviolet irradiation. The series *am*^{14} to *am*^{19} were isolated by Stadler (Fincham & Stadler, 1965) following mutagenesis with nitrous acid. Other mutants are all from the Kinsey collection (Kinsey & Hung, 1981, and unpublished). Mutants 122, 130, 131 and 143 were previously selected for special study because of their complementation with *am*^{1} (Kinsey *et al.*, 1980; Kinsey & Hung, 1981), and those newly reported on here were picked virtually at random. Those with numbers prefixed by 23 or 73 were induced by diepoxybutane, and the others, numbered 889 to 924, were all induced by nitrous acid.

(ii) *Preparation of mycelial extracts and enzyme assays*

Mycelia were grown without shaking in 50 ml lots of Vogel's medium, supplemented with 0·5 mg/ml monosodium glutamate, at 27 °C for 45–47 h, harvested by filtration, washed with distilled water and blotted as dry as possible. Approximately 0·3 g of damp mycelium was ground with an equal weight of sand in 2·5 ml of 0·05 M sodium phosphate, 0·001 M EDTA, pH 7·4 and the homogenate centrifuged at 10000 rpm (SS-34 rotor) for 15 min. Supernatants generally contained 3–4 mg protein per millilitre. Samples of 20–40 μl were assayed spectrophotometrically for GDH activity at 35 °C in a system containing 0·1 M Tris-HCl pH 8·5, $1{\cdot}7 \times 10^{-4}$ M NADP and a range of glutamate concentrations.

In some experiments, extracts were incubated at pH 8·5 and 35 °C with 0·15 M succinate, a pretreatment which had been found to activate the rather stably inactive GDH of *am*^{19} ('system S' of Coddington *et al.*, 1966). Activity in the direction of glutamate synthesis was assayed in a system ('system A') containing the same Tris buffer, 10 mM disodium α-ketoglutarate, 33 mM NH_4Cl and 7.0×10^{-5} M NADPH.

(iii) *Complementation tests*

Mutants were made heterocaryon-compatible with previously studied mutant strains and with each other by crossing to the standard wild-type 74*A*; *am* progeny were screened for efficient complementation with a standard arginine auxotroph (*arg-1*, *46004*, *a*) which had been highly inbred with 74*A*. To test for complementation, spots of dry conidia or drops of conidial suspensions were superimposed on plates of agar medium containing 0·02 M glycine to suppress the 'leaky' growth of *am* mutants, and 1% sorbose (together with 0·2% sucrose) to induce compact mycelial growth. Complementation to produce active GDH in heterocaryons was signalled by the appearance of strong growth in 2–3 days at 25 °C.

(iv) *DNA sequencing*

Genomic DNAs were isolated from mutant mycelia by the ethanolic perchlorate method of Stevens & Metzenberg (1982). Mutant *am* coding sequences were amplified by the polymerase chain reaction (PCR), using the primers 5′-TCTCTTGCTCATCC-ATCTTGCC-3′ and 5′-ATATCACGGTCACTAG-GAATGCG-3′ for 30 cycles at 92 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min with AmpliTaq (Perkin–Elmer, UK). For each mutant, six separate amplifications were performed; these PCR products were then pooled before sequencing with a series of 12 custom primers using an ABI 373-Stretch automated DNA sequencer employing dye-terminator chemistry.

3. Results and discussion

(i) *More mutants with conformationally disturbed GDH*

A total of 49 previously unexamined mutants were screened for potential GDH activity; 21 from the series 889 to 923, 14 from the series designated 23 and 73, and 14 from a series induced by hycanthone. As expected, none was active in the glutamate synthesis assay, but a total of 8 showed activity with 0·15 M glutamate, stimulated in two cases (*am*$^{23\text{-}61}$ and *am*$^{23\text{-}73}$) by preincubation at 35 °C for 30 min with 0·15 M succinate (system S of Coddington *et al.*, 1966). None of the hycanthone-induced mutants showed this potential activity. The proportion, 8 from 49, of mutants with inactive *in vivo* but capable of activation, is in broad agreement with that found among the *am* mutants previously studied.

Kinsey *et al.* (1980) ordered the potentially active mutant enzymes then known on the basis of their graded responses to increasingly activating conditions: a combination of increasing pH and increasing succinate concentration. The order in terms of ease of activation was found to be $131 > 130 = 3 > 2 > 122$; *am*^{19} GDH was a special case in that its activation was so slow. The present study, in which the newly studied mutants have been compared with the old ones for activity at pH 8·5 in a range of glutamate concentrations, glutamate and succinate being more or less equivalent as allosteric activators, shows that this linear seriation conceals additional complexities.

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Table 1. *Mutant forms of GDH-specific activities at different glutamate concentrations*

	Glutamate concentration (mM)					Ratios	
Allele	33	75	150	250	500	150/75	150/33
am^{+}	22[a]	37	44	45	—	1·2	2·0
am^{2}	—	12*	25*	43*	63*	2·2	—
$am^{73\text{-}24}$	—	16*	32*	55*	76*	2·0	—
am^{3}	—	87*	145*	151	—	1·7	—
am^{904}	31*	83*	101*	158	—	1·4	3·6
am^{908}	—	53*	92*	129*	—	1·5	—
am^{122}	—	6	43	108	140	7·1	—
am^{130}	21	66**	87	97	—	1·3	4·0
am^{131}	36*	72*	105	103	—	1·5	2·9
am^{48}	48	72	94	87	—	1·3	2·0
am^{923}	—	110**	149	161	—	1·4	—
$am^{23\text{-}61}$	—	—	1	—	4* → 83[b]	—	—
$am^{23\text{-}73}$	—	—	3	8*	39* → 70	—	—
am^{19}	—	—	—	12*	51* → 75*	—	—

—, test not done.
*Lag of at least 15 s before maximum rate attained.
**Reaction rate reduced by about 80% after 2 min.
[a] Specific activities are expressed as $100 \times \Delta OD_{340}$/min/mg protein in 3 ml assays. Assayed samples each contained 80–100 μg protein. The generally higher specific activities of fully activated mutant extracts compared with the wild-type may at least partly be due to derepression, in the glutamate-containing growth medium, of GDH synthesis in the auxotrophs.
[b] The arrowed activity values shown in the last three lines are those attained after 10 min preincubation in 0·5 M glutamate before the addition of NADP.

Table 1 and Fig. 1 summarize the results of one series of experiments.

In terms of conditions required for activation, the newly investigated mutant enzymes fit into the previously established series as follows. The GDH varieties encoded by $am^{23\text{-}61}$ and $am^{23\text{-}73}$ (am23-61p and am23-73p in the convention we adopt here) required very high levels of glutamate for activity and even so gained activity only slowly. The time course of activation of these proteins has not been determined beyond the demonstration that it extends over 5 min or more. Of the more readily activated mutant GDHs, am73-24p is quite similar to am2p; am898p, am904p and am908p are not very different from am3p; and am916p and am923p are even more readily activated than am131p. Indeed, at pH 8·5, am923p and am916p (Fig. 1*e*, *h*) hardly show any convincing requirement for allosteric activation, although these mutant enzymes do require high glutamate to maintain their activity as the reaction proceeds. However, in another series of tests, at pH 7·5, am916p did show a sigmoidal relationship, much more pronounced than in the wild-type, between initial rate and glutamate concentration (Fig. 1*i*); at this pH, am923p lost activity so quickly at lower glutamate concentrations that initial rates were difficult to measure (data not shown).

Thus the mutant GDHs which are inactive *in vivo* but potentially active *in vitro* can be placed in the following order with respect to ease of activation: 916 = 923 > 131 > 898 = 904 = 130 = 3 = 908 > 73-23 = 2 > 122 > 19 = 23-73 = 23-61, with the equals signs indicating only approximate equivalence, and the relationship between the last three being the most problematical. However, this linear seriation leaves out of account two other variables: the rate of the transition between active and inactive conformations, and the degree of inactivation by reduced NADP.

With respect to rapidity of activation, three broad categories may be distinguished. At one extreme there are the very slowly activating am19p, am23-61p and am23-73p. Among the mutant enzymes which become more or less fully active within the period of the usual 2 min assay there is a clear distinction between those that become maximally active for a given level of glutamate virtually instantaneously (am130p, am122p, am916p, am923p; Fig. 1*d*, *e*, *h*) and those which show an initial 15–45 s period of acceleration, most pronounced at lower glutamate concentrations (am2p, am73-24p, am3p, am131p, am898p, am904p, am908p, am23-73p; Fig. 1*b*, *c*, *g*). The same mutant enzymes that show this lag in glutamate oxidation display some persistence of the active state after removal of the activating conditions, as shown by their ability to catalyse glutamate synthesis. When activated with succinate and then transferred to the α-ketoglutarate–NADPH–NH_4^+ system, these enzyme

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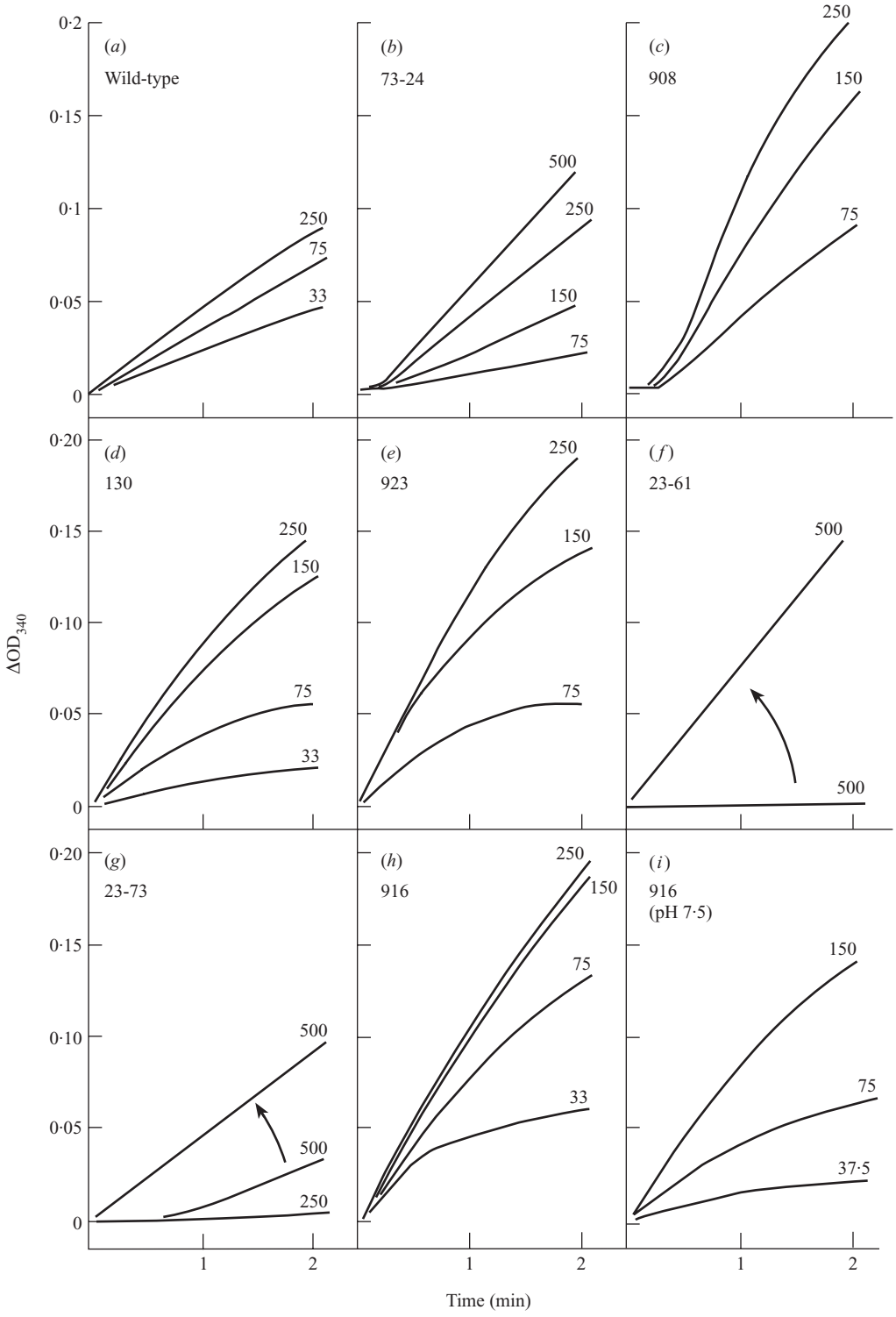

Fig. 1. GDH assays, as recorded on the spectrophotometer, from the series summarized in Table 1. Assay mixtures each contained 2·8 ml 0·1 M Tris–HCl, pH 8·5 (or in one case pH 7·5) containing different concentrations of sodium L-glutamate, 0·2 ml 0·2 % NADP; temperature 35 °C. The numbers on the curves are the final concentrations (mM) of monosodium glutamate. The reaction was started in each case by the addition, with simultaneous mixing, of 30 μl mycelial extract containing 90–120 μg protein: (*a*) wild-type, (*b*) *am*$^{73\text{-}24}$, (*c*) *am*^{908}, (*d*) *am*^{130}, (*e*) *am*^{923}, (*f*) *am*$^{23\text{-}61}$, (*g*) *am*$^{23\text{-}73}$, (*h*) and (*i*) *am*^{916} (in (*i*) the Tris was pH 7·5). Curved arrows in (*f*) and (*g*) show the effect of preincubation of the extract for 10 min before addition of the NADP.

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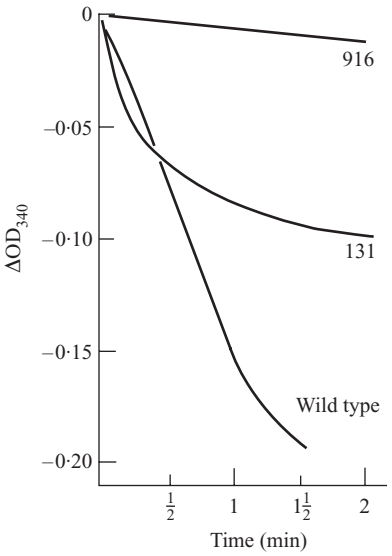

Fig. 2. Test for retention of GDH activity by mutant enzymes following preincubation for 3–4 min at pH 8·5 with 0·15 M sodium succinate. At time zero the enzyme–Tris–succinate mixture was diluted tenfold into the glutamate synthesis assay system. Note that the pretreated *am*^{131} extract showed high initial activity but lost activity rapidly, whereas similarly treated *am*^{916} extract retains virtually no activity. Wild-type extract was assayed for comparison without succinate pretreatment; note the slight but perceptible initial acceleration in activity consequent on the transfer of the pH 7·4 extract to the pH 8·5 reaction mixture. The very slow oxidation of NADPH seen in the *am*^{916} assay was only doubtfully in excess of that in a control without α-ketoglutarate and indistinguishable from that seen with either *am*^{916} or *am*^{131} extract without pretreatment. Approximately 160 μg of crude extract protein was used in each assay.

variants catalyse the oxidation of NADPH vigorously for 30–60 s, after which time the reaction, though still far from completion, slows dramatically (Fig. 2 shows this result with am131p). Following succinate treatment, and dilution of the succinate, these same enzyme varieties also retain enhanced activity on lower concentrations of glutamate. The am19p, which activates only very slowly in high glutamate or succinate, is an extreme example of the class, retaining its activity after succinate treatment for long enough to take the glutamate synthesis reaction nearly to completion (data not shown). Of the other very slowly activating forms, at least am23-61p retains some activity for glutamate synthesis, but am23-73p apparently does not. The capacity for glutamate-synthesizing activity in am2p and am3p has already been shown (Fincham, 1962).

In contrast to the group of mutant GDHs just considered, am130p, am122p, am916p and am923p, none of which show any perceptible lag in glutamate oxidation, show no persistence of any activation by succinate, either for glutamate oxidation or for glutamate synthesis, after the succinate has been diluted out (Fig. 2 shows the results for am916p). Their activity in glutamate synthesis has always been virtually zero, whatever the pretreatment. Although these mutant proteins, and especially am122p (Kinsey *et al*., 1980), respond strikingly to succinate as an allosteric activator in the reaction mixture, their enhanced activity in glutamate oxidation does not persist after dilution of the succinate. Thus, in general (with am23-73p still a problem), the rate of an allosteric transition in one direction is correlated with its rate in the other.

West *et al*. (1967) observed a temporary inactivation of wild-type GDH by NADPH. Prior exposure of the enzyme to this substrate caused a pronounced lag in the glutamate synthesis reaction at pH 7·4, with activity initially zero, although preincubation with NADPH and α-ketoglutarate together before addition of the ammonium salt had a pronounced activating effect. Fincham (1962) noted that glutamate oxidation catalysed by am2p or am3p tended to slow prematurely as compared with the progress to equilibrium catalysed by wild-type GDH, as though a reaction product, possibly NADPH, was having an inactivating effect on these mutant enzymes. This property applies generally to all the potentially active mutant GDHs, but to some more than others. Kinsey *et al*. (1980) noted in passing that am130p lost activity particularly rapidly as NADP reduction proceeded, and recent tests have confirmed this and demonstrated that the same applies to am923p and, to a lesser extent, am916p (Fig. 1*e*, *h*). The loss of activity takes effect most rapidly at lower glutamate concentrations. That the inactivating reaction product is NADPH has been confirmed in the cases of am923p and am916p. When these mutant enzymes were assayed in the alternative system in which NADPH is instantly reoxidized by diaphorase with coupled reduction (decolorization) of dichorophenol–indolphenol, the reaction proceeded almost linearly to completion, as it did with wild-type GDH (data not shown). A parallel experiment with am3p showed similar progress to completion but with an initial phase of acceleration as the enzyme became activated.

(ii) *Complementation tests*

The results of complementation tests on *am* mutants previously studied can be summarized briefly as follows. Mutants *am*^{1} (producing a conformationally near-normal but non-NADP-binding form of GDH) and *am*^{14} (producing an osmotically reparable GDH thought to be defective in stable oligomerization), complement each other, and either will complement any of the mutants producing allosterically disturbed enzyme. One other mutant, *am*^{7}, less well understood, complements *am*^{14} but not *am*^{1}.

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Table 2. *Nucleotide replacements in* am *mutants and amino acid replacements in NADP-specific GDH*

Mutant no.	Nucleotide[a]	Amino acid	Effect on GDH or other comment
6	Delete T342 or C343	—	N-terminal frameshift
6R27	Delete T342 or C343* Insert C 354–355*	SNLPS to MLTFP 1–5	N-terminal double frameshift Reduced GDH thermostability
126	Duplication 437–470, overlap intron–exon	—	No GDH protein; unstable, reverting to wild-type
14	T466 to A	L20 to H	Osmotically reparable GDH
14R1	T466 to A C465 to T	 H20 to Y	 14 defect largely remedied
14R5	T466 to A C673 to T	L20 to H L82 to F*	Increased K_m values for glutamate and NADP
15	Delete C575*	—	Frameshift
15R11	Delete C575* Insert G565-568*	DDN to GRQ 54–56	Double frameshift Reduced GDH thermostability
130	C652 to T*	P75 to S	Activated by glutamate** Strongly inhibited by NADPH
8	TA785, 786 to GT	Altered 3′ splice site	No GDH protein
4	G803 to A	G112 to D*	Inactive, thermolabile
143	G797 to A + 3 changes in intron 2	G110 to D*	Inactive
23-61	G811 to T	G115 to C*	Slow activation by high glutamate
73-24	C883 to T	L139 to F*	Activation by high glutamate
19	A890 to T*	K141 to M	Slow activation by high glutamate Electrophoretic shift
19/R24	A890 to T A1640 to G	K141 to M Q391 to R	*In vivo* activity restored; residual GDH abnormalities
2	C894 to G*	H142 to Q	Activation by high glutamate
2/2*l*	C894 to G G904 to A + 3 changes in introns	H142 to Q* D146 to N*	Restored activity, cold-sensitive GDH
23-73	G899 to A	G144 to D*	Slow activation by high glutamate
908	T970 to C	Y168 to H*	Activation by glutamate
131	G1016 to A	G183 to D*	Activation by glutamate
5	TC to CTT 1021–1022	Frameshift	No GDH protein
923	A1049 to T	E194 to V*	Strongly inhibited by NADPH
H	C1179 to T	No change	New *Hind*III site
17	C1405 to T*	Q313 to amber	Chain termination
17/RN35	C1405 to T* G1407 to Py*	Q313 to Y	High K_m for NH_4^+; altered thermostability
17/RU4	C1405 to T* A1406 to T*	Q313 to L	High K_m for NH_4^+; altered thermostability
1	C1475 to T*	S336 to F	Inactive; fails to bind NADP
7	G1582 to T*	G372 to S	Inactive
916	G1609 to A	A381 to T*	Activated by glutamate

In the present study, all the newly investigated mutants with potentially active GDH were tested for complementation with each other and with the mutants previously characterized. They showed no complementation among themselves. The mutants $am^{23\text{-}61}$ and $am^{23\text{-}73}$, which produce GDH varieties which are particularly hard to activate, complemented only am^{1}. The other new mutants fitted into the established pattern in that they complemented both am^{1} and am^{14}. In addition, am^{923}, which produces one of the most readily activated of the mutant GDH varieties, showed some relatively weak complementation for growth with am^{7}.

Kinsey & Hung (1981) reported complementation between am^{1} and am^{143}, which now seems surprising, since neither of these mutants has shown evidence of having potential GDH activity. Our recent tests have confirmed that these two mutants do indeed complement one another, though the complementation growth takes longer to become established (about 4 days rather than 3) than is the case with the other complementing pairs. The mechanism of complementation in this case would be interesting to investigate. Kinsey and Hung also found a number of complementing mutant pairs in which one partner was a temperature-sensitive mutant. This is not surprising,

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Table 2 (*cont.*)

Mutant no.	Nucleotide[a]	Amino acid	Effect on GDH or other comment
122	C1624 to T	R386 to C*	Activated by high glutamate
3	A1646 to G*	E393 to G	Activation by glutamate
3/3a	A1646 to G	E393 to G	*In vivo* activity restored; high K_m values NH_4^+, glutamate
	G1161 to T	Q231 to H*	
	A1163 to C+7 'silent' in introns or third codon positions	Y232 to S*	
3/3b	A1646 to G	E393 to G	*In vivo* activity restored; allosterically still abnormal
	T685 to C	F93 to S*	
3/3-18	A1646 to G	E393 to G	*In vivo* activity restored; needs EDTA in extracts
	G997 to T+3 changes in intron 2	G177 to C*	
9	CAT to AC 1670–1672	−1 frameshift	No GDH protein
S	G1810 to A	D448 to N*	Active but thermolabile

*Inferred.
**Activation by glutamate, here and elsewhere, can equally well be effected by succinate.
[a] Nucleotides are numbered from the upstream end of the *Bam*H1 fragment containing the *am* gene as in Kinnaird & Fincham (1983).
References: am^{6}, $am^{6R\,27}$, Siddig *et al.* (1980); am^{126}, Rambosek & Kinsey (1984); am^{14}, Fincham & Baron (1977); am^{15} and revertants, Burns *et al.* (1986), am^{130}, am^{122}, am^{131}, Kinsey *et al.* (1980); am^{2}, am^{3}, Fincham (1962); am^{3} revertants, Fincham & Bond (1960), Pateman & Fincham (1964); am^{21}, Fincham (1957); am^{4}, am^{8}, Kinnaird *et al.* (1992); am^{19}, Coddington *et al.* (1966); am^{19R24}, Sundaram & Fincham (1964); am^{17} and revertants, Seale *et al.* (1977); amino acid replacements in am^{1}, am^{2}, am^{3}, am^{7} and am^{19}, Brett *et al.* (1976); am^{H}, am^{S}, Fincham (1990). The nucleotide replacement in am^{143} was initially determined by Craig Murray, and the replacements in am^{5}, am^{122} and am^{131} by Lance Graham, both Honours undergraduates in Genetics in the University of Edinburgh.

since thermolabile GDH monomers would be expected to be stabilized by association in hybrid hexamers with any mutant monomer, even if inactive, that could confer greater stability.

(iii) *Sequence information*

The *am* alleles of all the newly investigated mutants, and several previously unsequenced mutants and their revertants, have been sequenced following PCR amplification. The determinations of nucleotide or amino acid replacements (sometimes only one determined and the other inferred) are shown in Table 2, which includes data from both recent and previously published studies. Mutants am^{898} and am^{904} turned out to have the same nucleotide replacement as am^{3}. Mutants am^{2}, am^{3} and am^{19} were previously investigated by peptide, not DNA analysis, but some of their second-site revertants have now had their DNA sequenced, and the predicted base-pair substitutions at the primary mutant sites have been confirmed in each case. The tentative conclusion that am122p had lost a tryptophan residue (Kinsey *et al.*, 1980) is not confirmed, and the disappearance of a tryptophan-containing spot from the tryptic peptide map can be accounted for by the abolition of a site of cleavage by trypsin, moving the tryptophan into a larger and less soluble peptide. In general, the amino acid replacements that eliminate GDH activity unconditionally affect residues that are highly conserved between species (for comparisons see Britton *et al.*, 1992).

The majority of the DNA changes affect single base-pairs, though the mutation that disrupts the 3′-splice site in am^{8} involves changes in two adjacent bases, and the frameshifts in am^{9} and am^{5} involve local sequence rearrangements: CAT to AC and TC to CTT respectively. The most surprising result was for the am^{3} revertant am^{3a}, where there not only two non-adjacent nucleotide replacements affecting adjacent codons but also seven 'silent' replacements in introns or third-base positions in codons. Multiple silent changes are also seen in $am^{3\text{-}18}$, am^{21} and am^{143}. We have not ruled out the possibility that the silent changes in am^{21} and am^{143} could have been present in the different wild-type strains from which the mutants were long ago isolated, but this explanation cannot apply to am^{3a} and $am^{3\text{-}18}$, since these are revertants from the same mutant strain and their silent changes are different in the two cases and are not present in a third am^{3} revertant, am^{3b}.

(iv) *Explaining the enzyme phenotypes*

A key to the understanding of the potentially active mutant GDH varieties must be the observation (West *et al.*, 1967; Ashby *et al.*, 1974) that the wild-type enzyme exists in a pH-dependent equilibrium between active and inactive forms. Our hypothesis is that the potentially active mutant enzymes are stabilized to a

greater or lesser extent in a conformation similar to that of the inactive form of the wild-type. This is made the more plausible by the fact that the conditions that tend to activate the mutant enzymes (higher pH, allosteric effector in the form of glutamate or succinate) are more extreme forms of those that activate the wild-type. The effect of substrate, or substrate analogue, on the conformational state of the hexameric enzyme is clearly cooperative, with operation of quaternary constraints between monomers. The same cooperativity can explain much of the interallelic complementation, where an irreparably inactive but conformationally near-normal mutant monomer can assist a potentially active one towards the active form in a mixed hexamer.

The best information about the likely nature of the conformational shift comes not from the *Neurospora* enzyme but from the NAD-specific GDH of *Clostridium symbiosum*, which is nearly 50% identical to the NADP-specific *Neurospora* enzyme in amino acid sequence and very similar to it in its hexameric three-dimensional structure (Baker *et al*., 1992, and unpublished information from the same group communicated by D. W. Rice). In the *Clostridium* enzyme, the cleft between the lobes in the bilobed structure of the monomer has been shown to close on glutamate binding (Stillman *et al*., 1993) and the closed form has been shown to exist even in the absence of substrate (Stillman *et al*., 1998). It seems likely that a further, less dramatic conformational shift is associated with the formation of the ternary complex.

Assuming that the same kind of interconversion of open and closed forms occurs in *Neurospora* GDH, one can speculate as follows. The closed form of the enzyme is likely to be the inactive form, stabilized in the conformational mutants as compared with the wild-type. The reason for its inactivity is that closure of the cleft without substrate excludes substrate molecules from their binding pockets, effectively locking them out, as it were. There may appear to be a contradiction between the supposition that the open form is the active one and the fact that actual activity must involve closure. But one can imagine that initiation of activity, starting with the closed form without substrate, takes place in two steps. First, the opening of the cleft may be facilitated by cooperative glutamate (or succinate) binding, with the opening of each monomer of the hexamer facilitating the opening of the others. Then, when most or all of the six substrate-binding sites are occupied by glutamate, the six monomers may undergo a concerted closure to initiate glutamate oxidation. The binding of the NADP could follow the binding of glutamate or occur concurrently with it.

The effects of NADPH could be explained on the following lines. The binding of this substrate by itself may tend to lock the monomers in the closed position so that, in the wild-type enzyme, glutamate or α-ketoglutarate can gain access only slowly. In the conformational mutants, because the closed form is excessively stabilized, the locking effect of NADPH may be much stronger, so that glutamate has more difficulty in gaining access and α-ketoglutarate is excluded altogether. This will explain not only the failure of these mutant enzymes to catalyse glutamate synthesis but also their premature loss of activity during glutamate oxidation.

To explain the short-term ability of the mutant enzymes in one set of mutants to catalyse glutamate synthesis after exposure of the GDH to glutamate or succinate, one may suppose that the open form of the enzyme, as glutamate or succinate is released, persists for long enough in these mutants, but not in others, for α-ketoglutarate to gain access before it is locked out by closure of the cleft.

The properties of the potentially active mutant enzymes can be seen as mainly governed by two thermodynamic parameters: firstly the overall free energy change associated with the conformational transition, reflected in the allosteric equilibrium constant; and secondly the energy barrier (required activation energy) that has to be surmounted for the transition to take place. These parameters, which can vary independently, are reflected in the different requirements for activation at allosteric equilibrium, and the different rates, immeasurably rapid or relatively slow, at which equilibrium is attained. A third relevant parameter must be the NADPH binding constant. The greater effect of NADPH seen in am130p and am923p is presumably due to its tighter binding in these mutant enzymes.

Unfortunately, the information on the molecular nature of the *am* mutations helps to explain their effects only to a very limited extent. It is not surprising that replacement of the very highly conserved glycine residues flanking the α-ketoglutarate-binding lysine at residue 113 should have dramatic effects on enzyme activity. The reason why am3-18p needs to be activated by EDTA may be that its extra cysteine residue makes it more susceptible to binding of a metal, perhaps zinc, which is at relatively high concentration in the growth medium and is known to stabilize the wild-type enzyme in an inactive form (unpublished). Now that the three-dimensional structure of the open form of the *Neurospora* enzyme is known, it is easy to understand that the introduction of phenylalanine for serine in the NADP-binding pocket must block the access of this substrate. The replacements in am130p and am923p, both thought to enhance NADPH binding, at least fall within the substrate-binding cleft, even though their possible interactions with NADPH have not been worked out. In the case of am^{14}, the osmotically reparable mutant previously thought likely to be unable to form stable

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hexamers, the replacement of the hydrophobic leucine-20 by the ionic histidine at a position close to the inter-monomer interface seems a good explanation for destabilization of the hexameric enzyme structure, and it seems reasonable to suppose that the further replacement of the histidine by tyrosine in the revertant R1 could have a restabilizing effect. But it is less easy to see why the replacement in R5 of the hydrophobic leucine-80 by the bulkier hydrophobe phenylalanine, at a position not obviously close to an interface, should have a similar remedial effect. One can only invoke some kind of 'knock-on' effect.

With regard to the mutant enzymes capable of allosteric activation, too little is known at present about the conformational shift, and the side-chain interactions that facilitate or impede it, to permit explanation of most of the enzyme phenotypes in terms of their amino acid substitutions. It is striking that very similar effects can be induced by sequence changes in quite widely separated parts of the enzyme structure, and also that changes in revertants are not usually particularly close to the primary changes for which they are compensating. The intramolecular interactions that govern the behaviour of allosteric enzymes are indeed many and complex.

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References

Ashby, B., Wootton, J. C. & Fincham, J. R. S. (1974). Slow conformational changes of a *Neurospora* glutamate dehydrogenase studied by protein fluorescence. *Biochemical Journal* **143**, 317–329.

Ashby, B., Nixon, J. S. & Wootton, J. C. (1981). Mutational variants of *Neurospora crassa* NADP-specific glutamate dehydrogenase altered in a conformational equilibrium. *Journal of Molecular Biology* **149**, 521–540.

Baker, P. J., Britton, K. L., Engel, P. C., Farrants, G. V., Willey, K. S., Rice, D. W. & Stillman, T. J. (1992). Subunit assembly and active site location in the structure of glutamate dehydrogenase. *Proteins: Structure, Function, Genetics* **12**, 75–86.

Brett, M., Chambers, G. K., Holder, A. A., Fincham, J. R. S. & Wootton, J. C. (1976). Mutational amino acid replacements in *Neurospora crassa* NADP-specific glutamate dehydrogenase. *Journal of Molecular Biology* **6**, 1–22.

Britton, K. L., Baker, P. J., Rice, D. W. & Stillman, T. J. (1992). Structural relationships between the hexameric and tetrameric families of glutamate dehydrogenase. *European Journal of Biochemistry* **209**, 851–859.

Burns, P. A., Kinnaird, J. H., Kinsey, B. J. & Fincham, J. R. S. (1986). Sequencing studies of IC-170 mutagenic specificity in the *am* (NADP-specific glutamate dehydrogenase) gene of *Neurospora crassa*. *Genetics* **113**, 45–51.

Coddington, A., Fincham, J. R. S. & Sundaram, T. K. (1966). Multiple active forms of *Neurospora* glutamate dehydrogenase formed by hybridisation between two inactive mutant proteins *in vivo* and *in vitro*. *Journal of Molecular Biology* **17**, 502–512.

Fincham, J. R. S. (1957). A modified glutamic dehydrogenase as a result of gene mutation in *Neurospora crassa*. *Biochemical Journal* **65**, 721–728.

Fincham, J. R. S. (1959). The role of chromosomal loci in enzyme formation. In *Proceedings of the Tenth International Congress of Genetics* (Montreal), vol. 1, pp. 355–374.

Fincham, J. R. S. (1962). Genetically determined multiple form of glutamate dehydrogenase in *Neurospora crassa*. *Journal of Molecular Biology* **4**, 257–274.

Fincham, J. R. S. (1967). Recombination within the *am* gene of *Neurospora crassa*. *Genetical Research* **9**, 49–62.

Fincham, J. R. S. (1977). A gene-enzyme relationship in *Neurospora*. *Stadler Symposium* **9**, 169–178.

Fincham, J. R. S. (1988). The *Neurospora am* gene and allelic complementation. *BioEssays* **9**, 169–172.

Fincham, J. R. S. (1990). Generation of new functional mutant alleles by premeiotic disruption of the *Neurospora crassa am* gene. *Current Genetics* **18**, 441–445.

Fincham, J. R. S. & Baron, A. J. (1977). The molecular basis of an osmotically reparable mutant of *Neurospora crassa* producing unstable glutamate dehydrogenase. *Journal of Molecular Biology* **110**, 627–642.

Fincham, J. R. S. & Bond, P. A. (1960). A further genetic variety of glutamic acid dehydrogenase in *Neurospora crassa*. *Biochemical Journal* **77**, 96–105.

Fincham, J. R. S. & Stadler, D. R. (1965). Complementation relationships of *Neurospora* mutants in relation to their formation of abnormal forms of glutamate dehydrogenase. *Genetical Research* **6**, 121–129.

Kinnaird, J. H. & Fincham, J. R. S. (1983). The complete nucleotide sequence of the *Neurospora crassa am* (NADP-specific glutamate dehydrogenase) gene. *Gene* **26**, 253–260.

Kinnaird, J. H., Revel, D. F., Connerton, I. F., Hasleham, I. & Fincham, J. R. S. (1992). Alternative modes of mRNA processing in a 3′ splice site mutant of *Neurospora crassa*. *Current Genetics* **22**, 37–40.

Kinsey, J. A. (1977). Direct selective procedure for isolating *Neurospora* mutants defective in NADP-specific glutamate dehydrogenase. *Journal of Bacteriology* **132**, 751–756.

Kinsey, J. A. & Hung, R.-S. T. (1981). Mutation at the *am* locus of *Neurospora crassa*. *Genetics* **99**, 405–444.

Kinsey, J. A., Fincham, J. R. S., Siddig, M. A. M. & Keighren, M. (1980). New mutational varieties of *Neurospora* NADP-specific glutamate dehydrogenase. *Genetics* **93**, 305–316.

Pateman, J. A. & Fincham, J. R. S. (1964). Complementation and enzyme studies of revertants induced in an *am* mutant of *Neurospora crassa*. *Genetical Research* **6**, 419–432.

Rambosek, J. A. & Kinsey, J. A. (1983). Fine structure map of the *am* (GDH) locus of *Neurospora*. *Genetics* **105**, 293–307.

Rambosek, J. A. & Kinsey, J. A. (1984). An unstable mutant gene of the *am* locus of *Neurospora* results from a small duplication. *Gene* **27**, 101–107.

Seale, T. W., Brett, M., Baron, A. J. & Fincham, J. R. S. (1977). Amino acid replacements resulting from suppression and mis-sense reversion of a chain terminator mutation in *Neurospora*. *Genetics* **86**, 261–274.

Siddig, M. A. M., Kinsey, J. A., Fincham, J. R. S. &

Keighren, M. (1980). Frameshift mutations affecting the N-terminal sequence of *Neurospora* NADP-specific glutamate dehydrogenase. *Journal of Molecular Biology* **137**, 125–135.

Stevens, J. N. & Metzenberg, R. L. (1982). An easy method for preparing *Neurospora* DNA. *Neurospora Newsletter* **29**, 27–28.

Stillman, T. J., Baker, P. J., Britton, K. L. & Rice, D. W. (1993). Conformational flexibility in glutamate dehydrogenase: role of water in substrate recognition and catalysis. *Journal of Molecular Biology* **234**, 1131–1139.

Stillman, T. J., Miguels, A. G. B., Wang, X.-G., Baker, P. J., Britton, K. L., Engel, P. C. & Rice, D. W. (1998). Insights into the mechanism of domain closure and substrate specificity of glutamate dehydrogenase from *Clostridium symbiosum*. *Journal of Molecular Biology* **285**, 875–885.

Sundaram, T. K. & Fincham, J. R. S. (1964). A mutant enzyme of *Neurospora crass* interconvertible between electrophoretically distinct active and inactive forms. *Journal of Molecular Biology* **10**, 423–437.

Watson, D. H. & Wootton, J. C. (1978). Subunit ratios of separated hybrid hexamers of *Neurospora* NADP-specific glutamate dehydrogenase containing complementing mutationally modified monomers. *Biochemical Journal* **75**, 1125–1133.

West, D. J., Tuveson, R. W., Barratt, R. W. & Fincham, J. R. S. (1967). Allosteric effects in NADP-specific glutamate dehydrogenase from *Neurospora*. *Journal of Biochemistry* **242**, 2134–2138.