# AMINO ACID REPLACEMENTS RESULTING FROM SUPPRESSION AND MISSENSE REVERSION OF A CHAIN-TERMINATOR MUTATION IN NEUROSPORA

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Manuscript received October 11, 1976
Revised copy received November 29, 1976

ABSTRACT

The *Neurospora crassa* super-suppressor mutation, *ssu-1*, suppresses the auxotrophic phenotype of the mutant *am*(17) by inserting tyrosine at residue 313 of NADP-specific glutamate dehydrogenase, a position occupied in the wild type by glutamate. Two classes of *am*(17) revertants due to further mutation within the *am* gene have, respectively, tyrosine and leucine at residue 313. These replacements are consistent with a chain-terminating codon in *am*(17) of either the amber (UAG) or the ochre type (UAA), but are inconsistent with UGA. The $Leu^{313}$ and $Tyr^{313}$ variants of the enzyme have effective activity but are grossly different from the wild type in Michaelis constants (especially for ammonium) and heat stabilities at two different pH values. They show smaller but significant differences in these respects from each other.

OF the mutants in the structural gene (*am*) for *Neurospora crassa* NADP-specific glutamate dehydrogenase (GDH), *am*(17), which was isolated following nitrous acid treatment, produces no detectable enzyme protein and complements none of the other mutants of the series (FINCHAM and STADLER 1965). STADLER (1966) obtained three different classes of revertants from *am*(17) by ultraviolet mutagenesis; one class appeared to produce standard wild-type GDH, and the two others were distinguishable from each other and from the wild type on the basis of thermostability tests on extracts. STADLER's revertants all appeared to be due to further mutation within the *am* gene. SEALE (1968), however, identified one class of revertants from *am*(17) in which restored GDH activity was due to suppressor mutation in a gene or genes unlinked to *am*. CASE and GILES (1968) showed that the first suppressor (*ssu-1*) isolated by SEALE would also suppress several pleiotropic mutants in the *arom* cluster and subsequent work (summarized by SEALE, CASE and BARRATT 1969; SEALE 1972) identified seven different suppressor genes, designated *ssu-1* to *ssu-7*, mutants of which would suppress a range of specific alleles in *am*, *arom*, *his-3*, *trp-1* and *trp-2*. Many but not all of these alleles are suppressed by each of the suppressors (CHALMERS and SEALE 1971; SEALE 1976). It has been supposed that the Neurospora super-suppressible alleles are chain-terminator mutations and that the

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super-suppressor mutants have altered tRNA anticodons which permit the insertion of an amino acid in response to a chain-terminating codon. Evidence for such an interpretation has been provided in the analogous situation in yeast, where SHERMAN *et al.* (1969) have shown by reversion analysis that a super-suppressible mutant in the *cyc1* (cytochrome c) gene of *Saccharomyces cerevisiae* is "ochre" (UAA) and that one class of super-suppressor mutations inserts tyrosine in response to this codon (GILMORE, STEWART and SHERMAN 1968; SHERMAN *et al.* 1973). Direct confirmation of the involvement of tRNA in nonsense suppression in yeast recently has been reported by CAPECCHI, HUGHES and WAHL (1975) and GESTELAND *et al.* (1976). This paper reports a first investigation, by amino acid replacement analysis, of the molecular nature of *am*(17) and the action of one of its suppressors.

## MATERIALS AND METHODS

*Strains*: The origin of *am*(17) *ssu-1* (originally called *am*(17)RN33) and of the missense revertant RN35 is described by SEALE (1968). Both were isolated following nitrous acid treatment of *am*(17) and purified by backcrosses to *am*(17). The missense revertants RU4 and RU9 were similarly isolated and purified following ultraviolet mutagenesis (SEALE, unpublished). On the basis of thermostability tests on crude extracts, they were clearly different from RN35 and thought to be slightly different from each other. The strains of *am* mutants, combined with *sp* (spray) or *inos* (inositol-requiring), used for mapping *am*(17) were either the same as used previously (FINCHAM 1967) or derived similarly by appropriate crosses between strains, each of which was derived after at least six successive backcrosses to the standard wild type STA4.

*Growth of mycelium and purification of GDH* were carried out essentially as described by ASHBY, WOOTTON and FINCHAM (1974). Except in the case of the suppressed strain, *ssu-1 am*(17), over 100 mg of purified enzyme was obtained from 1.0–1.5 kg of damp mycelium.

*Carboxymethylation and digestion of GDH; fractionation, analysis and sequencing of peptides*: The methods used were as described by WOOTTON *et al.* (1975), except that, in the case of the GDH isolated from *ssu-1 am* (17), carboxymethylation was carried out using 2-$^{14}$C-iodoacetate (specific activity 160 $\mu$Ci/mmole). In the fractionation of tryptic peptides of carboxymethylated GDH on Dowex AG50X2, peptide T27 and its mutational derivatives were eluted from the column somewhat later than shown in Figure 1 of WOOTTON *et al.* (1975); it was recovered slightly later than T31 and in the same fractions that contained free asparagine (T38). The very early position of T27 recorded in the previous paper seems to have been exceptional.

*Peptide maps* ("fingerprints") were prepared and stained by conventional methods as described by BRETT *et al.* (1976).

## RESULTS

### *Mapping of* am*(17)*

The position in the gene map of *am*(17) has already been reported by SMYTH (1973), who placed it between *am*(5) and *am*(1), but closer to the latter. SMYTH's crosses were all homozygous for *rec-3*, an unlinked recessive which increases recombination frequency within *am* by a factor of nearly ten. Our own crosses in a *rec-3*$^+$ background show *am*(17) to be close to *am*(5) (25 *am*$^+$ recombinants among $8.4 \times 10^6$; germinated ascospores; frequency $3.0 \times 10^{-6}$) and even closer to *am*(1) (4 *am*$^+$ among $11.5 \times 10^6$; frequency $0.4 \times 10^{-6}$). No clear indication of order was obtained from flanking marker distribution in our

rather sparse data, but they are consistent with SMYTH's conclusion that the *am*(17) site is a little to the left of the *am*(1) site. Identification of the amino acid replacements associated with *am*(2), *am*(19), *am*(7), *am*(1) and *am*(3) (BRETT *et al.* 1976) establishes the "left" and "right" ends in the map as corresponding, respectively, to the N- and C-termini of the polypeptide chain. Since the *am*(1) replacement is at residue 336 (Ser → Phe) in the N-terminal region of the tryptic peptide T28, a plausible position for the mutation in *am*(17) would, on the basis of the map, be somewhere within T27, which includes residues 304 to 330.

*The missense revertants*

*RN35*: A two-dimensional fingerprint of trypitic peptides from carboxymethylated GDH isolated from RN35 showed only one apparent difference from the wild-type pattern. The sulphur-containing peptide T27 was moved to a slightly higher $R_F$ value in the chromatographic dimension and, unusually, gave a positive stain for tyrosine (Figure 1). The fingerprint difference was not regarded as entirely convincing because of the streaky character of the apparently altered peptide; nevertheless, the subsequent analysis showed that the fingerprint's indication of an altered residue in T27 was correct. Since the position of *am*(17) in the gene map also suggested T27 as likely to contain an altered residue, this peptide was looked for in a tryptic digest of 120 mg of RN35 GDH. After a first fractionation on a column of Dowex AG50X4, a pool of fractions (pool 4) eluted from the column early in the gradient of increasing pH was identified as probably containing T27 on the basis of its high content of S-carboxymethyl cysteine. Refractionation of this pool on a column of Sephadex G50 resulted in the recov-

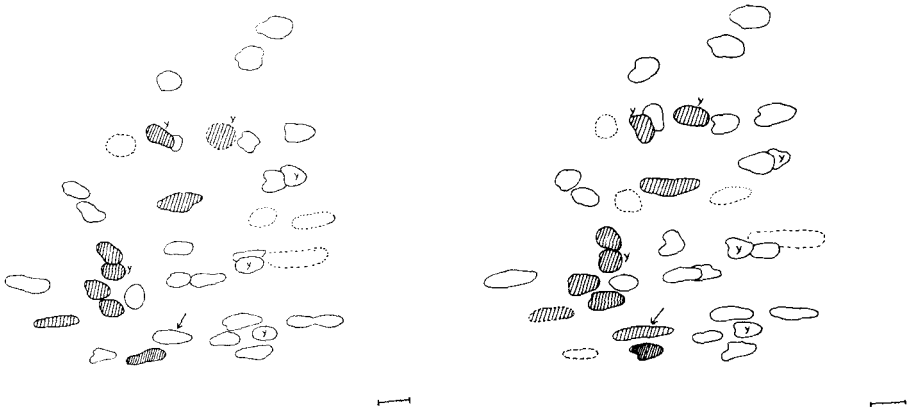

FIGURE 1.—Fingerprints (prepared in parallel at the same time) of tryptic peptides of carbomethylated GHD from (a) (left) *am*(3)-R17 and (b) (right) *am*(17) RN35. The revertant *am*(3)-R17 fingerprint is not detectably different from that of the wild type. Separation in the vertical direction was by electrophoresis at pH 3.5 and in the horizontal direction by chromatography using butanol-acetic acid-water-pyridine (details of methods in BRETT *et al.* 1976). Peptide spots staining for tyrosine are cross-hatched. Arrows indicate peptide T27 and its altered version in RN35. γ indicates yellow with cadmiumninhydrin reagent.

ery of a large peptide, eluting close to the void volume of the column; the analysis of this peptide is shown in Table 1, and was consistent with standard T27 except that there was too little glutamate, one equivalent of tyrosine and too much glycine. The extra glycine was evidently due to contamination because two other separately recovered fractions of T27 which eluted at either edge of main peak had one less equivalent of glutamate and one more equivalent of tyrosine, but were otherwise identical to wild-type T27. A subdigest of the main peak showed that the extra glycine was a contaminant. The peptide was subdigested with chymotrypsin, and the products were fractionated on a column of Biogel P4 equilibrated with 0.05 M $NH_4HCO_3$. Two barely resolved peptides (C5 and C6 in order of elution) were recovered from just behind the void volume of the column. The analysis, shown in Table 1, of C5 was strongly suggestive of the first 10 residues of T27 with tyrosine replacing the usual $Glu^{313}$ acid at the C-terminus, while that of the other main product, C6, was consistent with residues of 11–22 of the normal sequence of T27, terminating at $Leu^{324}$. Sequence determination of C5 and C6 confirmed these interpretations and showed unequivocally the replacement of $Glu^{313}$ by Tyr (Table 2).

*RU4*: By essentially the same methods as used in the case of RN35 (fractionation on AG50X4 followed by subfractionation on Sephadex G50) a peptide equivalent to T27 was recovered from a tryptic digest of 150 mg of carboxymethylated GDH from revertant RU4. The analysis of this peptide is shown in Table 1, line 4 and showed no tyrosine. It differed significantly from the analysis of wild-type T27 only in having about one equivalent less of glutamate and nearly one equivalent more of leucine. Since, on the basis of the RN35 sequence, it was expected that the altered residue would be only the tenth from the N-terminus of the peptide, sequencing was performed directly without prior chymo-

TABLE 1

*Amino acid compositions of tryptic peptides equivalent to T27 from* am*(17) revertants*

| Source of peptide | CM‡ Cys | Amino acid composition* Asp | Thr | Ser | Glu | Pro | Gly | Ala | Val | Ile | Leu | Tyr | Phe | Lys |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Wild type† | 2 | 2 | 1 | 1 | 5 | 1 | 2 | 5 | 2 | 1 | 3 | 0 | – | 2 |
| RN35 | 1.5 | 2.0 | 1.0 | 1.1 | 3.6 | 1.0 | 3.7 | 4.9 | 1.6 | 1.1 | 2.3 | 0.9 | – | 1.8 |
| Chymotrypsin C5 subdigestion | 0.5 | 1.4 | 1.0 | 0.6 | 0.9 | c.1§ | 0.8 | 2.4 | 1.0 | 0.9 | 1.2 | 0.9 | – | – |
| products C6‖ | – | 1.2 | 0.4 | 1.3 | 3.7 | – | 1.6 | 1.5 | 0.9 | – | 1.6 | – | – | – |
| RU4 | 1.5 | 2.0 | 1.0 | 0.95 | 3.8 | c.1§ | 2.5 | 4.6 | 1.8 | 0.9 | 3.65 | – | – | 1.9 |
| RU9 (uncorrected) | 1.5 | 2.4 | 0.9 | 1.3 | 4.1 | 0.8 | 2.4 | 3.9 | 2.3 | 1.1 | 2.9 | – | 0.5 | 1.5 |
| (correcting for T28 as 40% contaminant) | 1.8 | 2.2 | 1.0 | 1.5 | 4.3 | 1.1 | 2.7 | 4.8 | 2.5 | 1.1 | 3.9 | – | – | 2.2 |
| *am(17)-ssu-1* | 0.7 | 2.0 | 0.9 | 0.8 | 4.3 | 1.1 | 2.1 | 4.8 | 2.1 | 1.0 | 2.8 | 1.0 | – | 2.3 |

* Molar ratios. Residues not recorded were 0.2 or less.
† From unknown sequence of wild type (Wootton *et al.* 1975; Holder *et al.* 1975).
‡ Analyses of S-carboxymethylcysteine are often low, probably because of partial oxidation.
§ Peak height insufficient for accurate integration.
‖ Analysis apparently somewhat contaminated with C5.

TABLE 2

*Amino acid sequences of peptides equivalent to T27* (cf. *Table 1*)

| Source of peptide | Sequence |
|---|---|
| Wild type | Val Asp Ile Ala Leu Pro Cys Ala Thr Glu Asp Glu Val Ser Lys Glu Glu Ala Glu Gly Leu Leu Ala Gly Cys Lys |
| RN35 C5 | Val Asx Ile Ala Leu Pro Cys Ala Thr Tyr |
| RN35 C6 | Asx Glx Val Ser Lys Glx Glx Ala Glx Gly Leu |
| RU4 | Val Asx Ile Ala Leu Pro Cys Ala Thr Leu Asx . . . . |
| RU9 | (Phe) (Ala Glx Gly) (Gly)* Val Asx Ile Ala Leu Pro Cys Ala Thr Leu Asx . . . . |
| *am*(17) *ssu-1* | Val Asx Ile Ala Leu Pro Cys**Ala Thr Tyr Asx Glx Val Ser(Lys)†Glx Glx Ala Glx Leu Leu Ala Gly Cys**(Lys) |

Notes: The wild type sequence is taken from HOLDER *et al.* (1975).
— indicates residue identified as dansyl derivative after the relevant step of the Edman degradation.
— indicates Edman degradation without clear identification of the dansylated end group.
** indicates $^{14}$C-carboxylmethylcysteine identified by radioactive counts in butyl acetate extracted phenylthiohydantoin derivative, even though dansyl derivative not clearly identified.
† PTC-Lysine probably seen but not certainly identified.
* The bracketed residues recorded for RU9 were recorded as minor dansyl spots and were as expected for contaminating T28.

trypsin subdigestion. The sequence, carried to the eleventh residue, is shown in Table 2. The tenth residue (number 313 in the whole chain) was unequivocally leucine. Thus, in RU4, $Glu^{313}$ is replaced by Leu.

*RU9*: The analsyis of RU9, which started with 150 mg of carboxymethylated GDH, was performed almost exactly as for RU4 but, in this instance, the peptide recovered as equivalent to T27 gave an analysis suggestive of contamination to the extent of about 25 to 30 percent with T28 in the form lacking the N-terminal Lys (cf., WOOTTON *et al.* 1975). Allowing for this inferred contamination, the residual analysis agreed reasonably well with T27, but again with about one equivalent less of Glu. Sequencing of the contaminated peptide to residue 11 showed, on the basis of the major dansylated endgroup detected after each round, the normal sequence of T27 except that Leu unequivocally replaced Glu at residue 10. In the earlier rounds of the degradation (but not by the time residue 10 was reached) the normal sequence of T28 was detected as a series of lower-yield end groups. Thus RU9 appears to be identical to RU4 ($Glu^{313} \rightarrow$ Leu), a conclusion in agreement with the comparative tests on enzymic properties described below.

*The amino acid inserted by* ssu-1

The analysis of the GDH made by *am*(17) *ssu-1* was a special problem because *ssu-1*, though probably the most efficient of the known super-suppressors, restores only about 20 percent of the normal level of gene product under the conditions employed in these experiments. It was necessary to combine the GDH purified from two successive 1.0 to 1.5 kg batches of the suppressed mutant mycelium and, even so, only 23 mg of the purified enzyme was obtained. In isolating the peptide equivalent to T27 from this rather small quantity of protein, use was made of the fact that this peptide contains two of the six cysteine residues present in the entire chain. These cysteine residues were labelled by carrying out the S-carboxymethylation using 2-$^{14}$C-iodoacetic acid, and the radioactivity in small samples of alternate fractions from the AG50X4 column was determined by liquid scintillation counting. The detection of the cysteine-containing peptides by radioactivity used much less material than required for our usual detection by paper chromatography and allowed the smaller amount of peptide to be recovered with minimal loss. Two major and relatively early peaks of radioactivity, the second about twice the magnitude of the first, were resolved, and were taken to correspond to T31 and T27 respectively (the first with one carboxymethylated cysteine and the second with two.) The fractions corresponding to the second peak were pooled, concentrated, and refractionated on Sephadex G50 as for RN35, RU4 and RU9. A single radioactive peak proved to contain a peptide, in about 35 nmole yield, corresponding closely in its analysis to the corresponding peptide from RN35( with Tyr replacing one of the Glu residues present in standard T27 (Table 1).

About 30 nmol of the peptide was taken for sequencing and it was possible to verify the entire sequence up to the penultimate residue $Cys^{329}$ (Table 2). (This successful sequencing of 26 residues illustrates the power and sensitivity

of the normal dansyl-Edman technique when the peptide is adequately pure, as was the case here.) As in RN35, $Glu^{312}$ was clearly replaced by Tyr.

*Comparative properties of the $Glu^{313}$, $Leu^{313}$ and $Tyr^{313}$ GDH varieties*

Small batches (3 g of freeze-dried mycelium in each case) of wild type. RN35, RU4, RU9 and two different isolates of *ssu-1 am*(17) were purified in parallel up to and including the first DEAE-Sephadex (A50) column fractionation (ASHBY, WOOTTON and FINCHAM 1975). At this stage a check of purity by polyacrylamide gel electrophoresis showed GDH as the strongest single protein component in the wild type and missense revertant preparations, but a considerably weaker band (comparable in intensity to several of the contaminants) in the same position in the *ssu-1 am*(17) preparations. To judge from the relative intensities of the array of contaminating proteins, all the preparations were at a very similar stage of purification. The specific activity of the wild-type preparation corresponded to about 20 percent purity.

*Electrophoretic mobility*: Mixtures of the wild-type preparation with each of the revertant preparations were subjected to polyacrylamide gel electrophoresis. No separation of the wild-type and revertant GDH varieties could be discerned in spite of the fact that the latter might be expected to have lost a negative charge. It thus appears that $Glu^{313}$ does not contribute effectively to the net surface charge of the native enzyme.

*Effects of substrate concentration*: The most striking effect of replacing $Glu^{313}$ by either Leu or Tyr is on the Michaelis constant for ammonium ion in the amination reaction. Figure 2 shows the double-reciprocal plots. The downward-curving property of the wild-type plot is characteristic and becomes more pronounced at lower $\alpha$-oxoglutarate concentration. Extrapolating from the main linear part of the curve one obtains an apparent $K_m$ of about 2mM (at pH 7.8—values of around 1mM have been obtained at pH 8.5). In contrast, the plots for the RU9, RU4, RN35 and *ssu-1 am*(17) enzyme preparations do not depart significantly from linearity over the entire range of ammonium ion concentrations and extrapolate to $K_m$ values of 23 and 43mM for the $Tyr^{313}$ [*i.e.*, RN35 and *ssu-1 am*(17)] and $Leu^{313}$ [*i.e.*, RU4 and RU9]enzyme varieties, respectively.

The $K_m$ values with respect to NADPH and $\alpha$-oxoglutarate were not found to be clearly different from the wild-type values for either mutant enzyme variety.

In the glutamate oxidation (deamination) reaction, differences in response to varying substrate concentration were observed with respect both to NADP and to glutamate. In the case of NADP, although the values were not determined with great precision, $Tyr^{313}$ and $Leu^{313}$ enzyme varieties appeared to have $K_m$ values of about $10^{-4}$ M and $2 \times 10^{-4}$ M respectively, as compared with about $3 \times 10^{-5}$ M for the wild type (Figure 3). At an NADP concentration of $2 \times 10^{-5}$ M (not shown in Figure 3), the two mutant enzyme varieties had activities considerably lower than predicted from extrapolation from the approximately linear part of the double-reciprocal plot, and it seems that a minimum NADP concentration is needed for activation of these enzymes.

$K_m$ values for glutamate could not be calculated for the mutant enzyme prep-

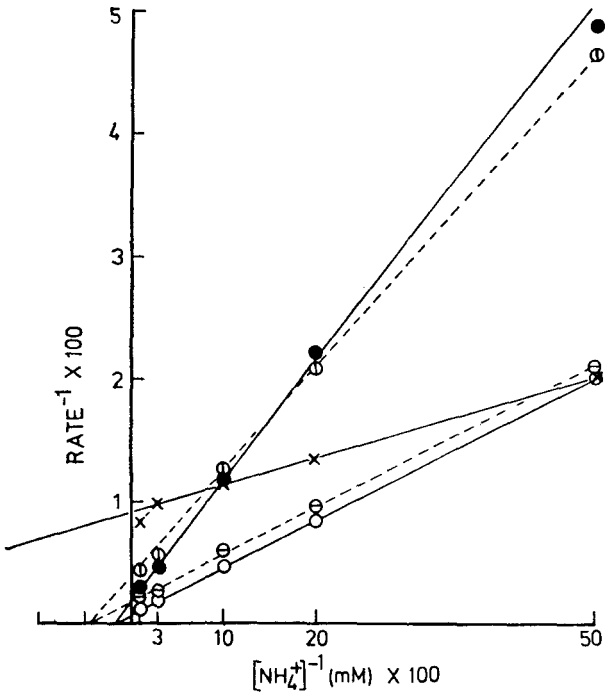


FIGURE 2.—Double reciprocal plots for the determination of $K_m$ values for ammonium. The assay system (3 ml, 35°) contained 0.085-M Tris-HCl pH 8.5, 10mM disodium $\alpha$-oxoglutarate, $8.5 \times 10^{-5}$ $NADPH_2$, wild-type (x). RU4 (○), RU9 (●), RN35 (⊖) or *am*(17) *ssu-1* (⊕) protein and ammonium chloride to the concentrations indicated as final addition to the system in 0.1 ml. Quantities of protein, measured by O.D. at 280 nm, were approximately 1.4 μg (wild type), 5.8 μg (RU4), 4.2 μg (RU9), 2.8 μg (RN35) and 10.1 μg [(*am*(17)*ssu-1*)].

arations since they all showed unduly low activities at the lower glutamate concentrations, as if high glutamate was a necessary prerequisite for their full activity (Figure 4). Activation by glutamate is a well-known feature of other mutant GDH varieties, for example that of *am*(3) (FINCHAM 1962). In the case of the $Leu^{313}$ and $Glu^{313}$ enzymes, unlike that of *am*(3) ($Glu^{393} \rightarrow Gly^{393}$), the level of activity associated with a given level of glutamate is evidently quickly attained since there were no indications of time-dependent increases in activity, reflected in accelerating reaction rates or strong effects of preincubation with glutamate.

*Specific activities*: Even at high substrate concentrations, the mutant enzyme varieties appear to have lower $V^{max}$ values than the wild-type enzyme, with the Leu replacement having (perhaps surprisingly) a greater depressing effect than the Tyr replacement (Table 3). The depression is much more marked in the oxidative deamination assay (Table 3), and it seems that the $Leu^{313}$ and $Tyr^{313}$ enzyme varieties, especially the former, are largely inactive in this system at moderate substrate concentrations and need high concentrations of both glutamate and NADP in order to maintain an active conformation.

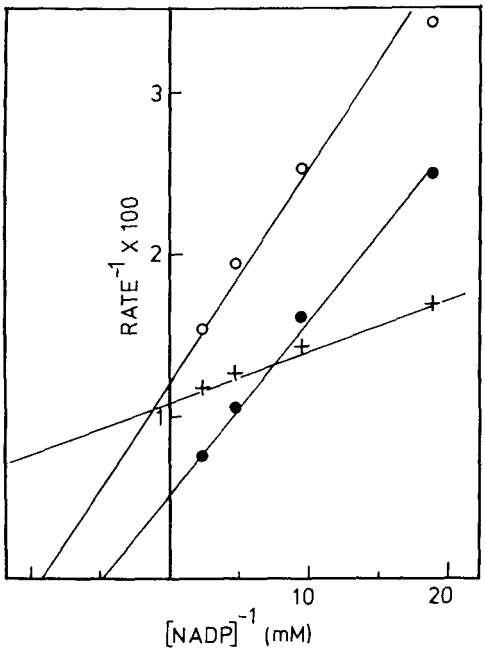


FIGURE 3.—Double reciprocal plots for the determination of $K_m$ values for NADP. The assay system contained, in 3 ml at 35°, 0.097-M Tris-HCl pH 8.5, 0.155-M sodium L-glutamate, wild type (+), RU4 (●) or RN35 (○) protein and NADP, to the concentrations indicated as final addition to the system in 0.1 ml. Quantities of protein, measured by O.D. at 280 nm, were approximately 2.5 μg (wild type), 9.8 μg (RN35) and 70 μg (RU4). Reaction rates are expressed as $\Delta O.D._{340}$/min. × 100. Apparent $K_m$'s are $3.5 \times 10^{-5}$, $10^{-4}$ and $2 \times 10^{-4}$ M for wild, RN35 and RU4 respectively.

TABLE 3

*Comparison of specific activities (preparations all the same stage of purification, see* METHODS)

| Assay system | Specific activities* Wild | RU4 | RU9 | RN35 | *am*(17) *ssu-1* |
|---|---|---|---|---|---|
| Reductive amination (standard system)† | 59,000 | 17,400 | 16,600 | 46,800 | 7,700 |
| (extrapolated to infinite $NH_4^+$ concentration)‡ | 77,800 | 33,000 | 33,000 | 65,000 | 10,000 |
| Oxidative deamination (standard system)§ | 23,000 | 1,400 | 1,400 | 6,000 | 820 |
| (extrapolated to infinite NADP concentration)‖ | 26,000 | 2,800 | 2,800 | 9,000 | 1,200 |

* Expressed as $100 \times \Delta O.D._{340}$/min/mg protein.

† 10 mM α-oxoglutarate, 33.3 mM $NH_4Cl$, ca. $5 \times 10^{-5}$ M NADPH, 0.08 M Tris HCl, pH 8.5, 35° (system A of CODDINGTON, FINCHAM and SUNDARAM 1966).

‡From unpublished experiment similar to that of Figure 2 but at pH 8.5.

§ 0.15 M Na L-glutamate, $1.7 \times 10^{-4}$ M NADP, 0.09 M-Tris HCl pH 8.5, 35° (system C of CODDINGTON, FINCHAM and SUNDARAM 1966).

‖ From the data shown in Figure 3.

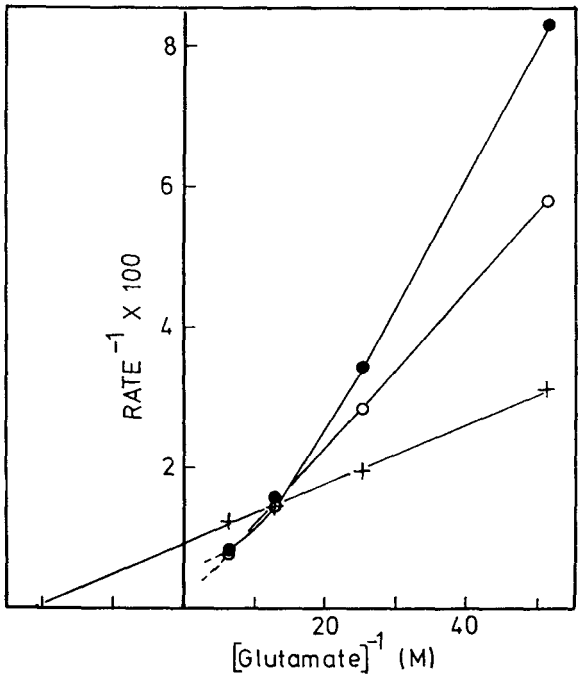


FIGURE 4.—Double reciprocal plots for glutamate. The assay system was as described in the caption for Figure 3 but with NADP constant at $4.2 \times 10^{-4}$ M and varying glutamate concentration. Quantities of protein and symbols as for Figure 3. The reciprocal rates for RN35 were halved for convenience of plotting.

*Thermostabilities:* The replacements at residue 313 have large, pH-dependent effects on thermostability. At pH 6.5 (Figure 5a) the $Tyr^{313}$ replacement (both in RN35 and in *ssu-1 am*(17) considerably *increases* the stability, while the $Leu^{313}$ replacement (both in RU4 and RU9) makes little difference. At pH 8.0 (Figure 5b), both replacements render the enzyme markedly more *labile* than the wild type, with the $Leu^{313}$ replacement having a somewhat more extreme effect.

In all the comparisons made with regard to enzymic properties, RU4 and RU9 appear to be identical, as do RN35 and *ssu-1am*(17) (apart from the purely quantitative depression of enzyme activity in the latter).

## DISCUSSION

*Neurospora crassa* follows the precedent set by both *Escherichia coli* (GOODMAN *et al.* 1968) and *Saccharomyces cerevisiae* (GILMORE, STEWART and SHERMAN 1971; SHERMAN *et al.* 1973; BRUENN and JACOBSON 1972) in that a chain-termination suppressor inserts tyrosine in response to the chain-terminating codon. In the first *E. coli* case, the chain-terminating codon concerned was shown to be UAG ("amber") by the reversion analysis of WEIGERT and GAREN (1965) and WEIGERT, LANKA and GAREN (1967). Similar reasoning, based on

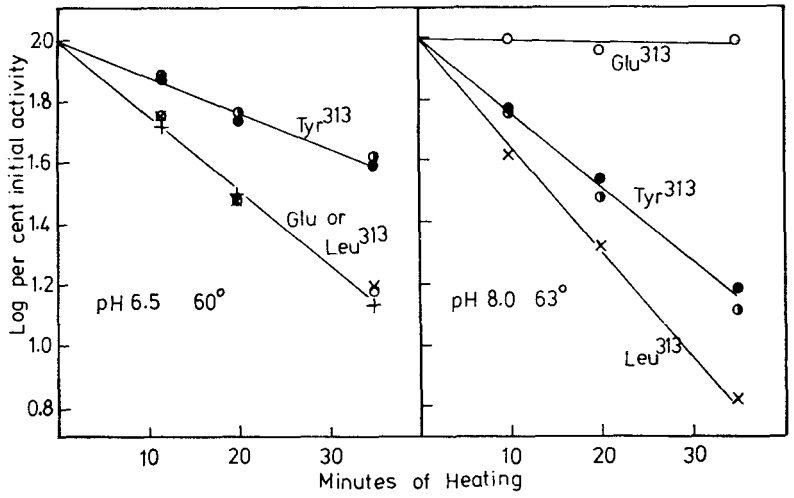


FIGURE 5.—Heat stabilities at two pH values of GHD from wild type (○), RU4 (×), RU9 (+), RN35 (●) and *am*(17) *ssu-1* (○). Appropriate amounts of protein 0.15 M phosphate pH 7.4 were diluted into 10 volumes of 0.1-M phosphate ($Na_2HPO_4$—$NaH_2PO_4$)—0.001-M EDTA at either pH 8.0 or pH 6.5, heated at 63° or 60° for the times indicated, cooled rapidly in ice and assayed in the direction of glutamate synthesis (*cf.* Figure 2).

a comprehensive analysis of amino acid replacements in revertants, showed that *cyc1* mutants in Saccharomyces responding to tyrosine-inserting suppressors could be either "amber," UAG (STEWART and SHERMAN, 1972, 1973), or "ochre," UAA (STEWART *et al.* 1972). Our analysis, in which only leucine and tyrosine have been identified as missense revertant residues, is compatible with either UAG or UAA as the terminating codon, but is incompatible with the third terminator, UGA. Although *ssu-1* may be the most effective of the known Neurospora super-suppressors (up to 20 percent suppression in terms of enzyme product under the condition we employed), this level of gene product restoration does not allow one to distinguish between ochre and amber suppressors. In yeast, as contrasted to bacteria, certain high-level ochre suppressors (40–50 percent) occur which do not appreciably affect growth. The distinction between UAG and UAA depends on the occurrence or not of a tryptophan (UGG) class among the revertants. So far 22 revertants have been screened for different GHD properties, and none clearly distinct from the $Tyr^{313}$ and $Leu^{313}$ classes has been found. This could mean either that the chain-terminating codon is after all UAA, or that tryptophan at residue 313 is incompatible with enzyme activity, or simply that we have been unlucky in our sample. Obviously the analysis will have to be pursued further either with *am*(17) or with another chain terminating *am* mutant that may lead to a different spectrum of acceptable revertant residues.

In *Saccharomyces cerevisiae*, super-suppressors at eight distinct chromosomal loci, and suppressing the same spectrum of mutants in different genes, all insert tyrosine and presumably represent a family of genes coding for a set of iso-accepting tRNA's. It will obviously be of great interest to find out whether the

series of Neurospora super-suppressors, *ssu-1* to *ssu-7*, (SEALE 1972), which likewise share much the same specificity of action, are also alike in inserting tyrosine. The action spectrum of these suppressors suggests that they are not identical (SEALE 1976). Tests of GDH properties in crude extracts have suggested that at least some of these super-suppressors insert other amino acids (SEALE and KINNIBURGH, submitted for publication), but direct identification of the inserted residue in each case is really necessary. The purification of the low levels of GDH restored by the less efficient suppressors is likely to be laborious.

The unusual properties of the $Leu^{313}$ and $Tyr^{313}$ GDH varieties show that residue 313 has a significant role in maintaining the normal activity of the enzyme. Replacement of $Glu^{313}$ by tyrosine (in contrast to leucine) renders the enzyme more stable to heat at pH 6.5, but less stable to heat at pH 8.0. These opposite effects at the two pH values may be connected with the fact that the enzyme normally undergoes a pH-dependent conformation shift in the range 7.0–7.5 (ASHBY, WOOTTON and FINCHAM, 1974). One interpretation of the effect of a hydrophobic-for-hydrophilic replacement at residues 313 is that it stabilizes the low pH (inactive) form and destabilizes the high pH (active) form of the enzyme. The responses of the mutant GDH varieties to variation in glutamate and NADP concentrations also indicate some sort of stabilization of an inactive state. The more than first order dependence of activity on concentration of both of these substrates cannot be wholly explained by decreased substrate affinity of a single form of the enzyme; a conformational change dependent on both NADP and glutamate must also be invoked. The nonlinear nature of the double-reciprocal plots with respect to glutamate (Figure 4) makes extrapolation uncertain, but it seems possible that at extremely high glutamate concentrations (if they could be achieved) combine with high NADP, the variant enzymes might approach high type in specific activity. The full potential activity of these enzyme varieties seem to be approached more nearly in the $\alpha$-oxoglutarate-NADPH-ammonium system. The relation of this effect to the conformational abnormality in other mutants such as *am*(3) ($Glu^{393} \rightarrow Gly$) (BRETT *et al.* 1976; ASHBY, WOOTTON and BRETT, in preparation) remains to be explored. The substrate-dependence properties of the $Leu^{313}$ and $Tyr^{313}$ enzymes, including the very greatly decreased affinity for ammonium ion (Figure 3), are strikingly similar to those of the GDH variety produced by a revertant *am*(3a), derived from *am*(3) (FINCHAM and BOND 1960). It is hoped to analyze this revertant to see whether it also has a replacement in or close to residue 313.

Whether the other replacements for Glu which might have been expected as a result of mutation from either UAG or UAA, namely serine, glutamine or lysine, are consistent with normal enzyme properties can at present only be guessed. It seems not unlikely that among the common revertant class with enzyme properties similar or identical to wild type one could find some or all of these other expected missense replacements. Either glutamine or serine, as neutral hydrophilic residues of small to moderate size, might substitute adequately for glutamate; the acceptability of lysine would be more surprising.

We are glad to thank MRS. BARBARA MATTHEWS for help with the mapping of *am*(17), MR. A. A. JACKSON for making the fingerprints and MR. J. C. WOOTTON for discussion and advice. The visit of T.W.S. to Leeds, during which much of the work was done, was made possible by a Fellowship from the Science Research Council as part of a Project Grant supporting our work with glutamate dehydrogenase.

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Corresponding editor: R. L. METZENBERG