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Complementation relationships of *Neurospora am* mutants in relation to their formation of abnormal varieties of glutamate dehydrogenase

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1. INTRODUCTION

Allelic mutants of *Neurospora crassa* of the *am* series, mapping within a short segment of linkage group V, are unable to form the NADP-linked glutamate dehydrogenase (GDH), or else they form defective varieties of it (Fincham, 1961). Of the mutants hitherto known, two pairs, $am^1 + am^2$ and $am^1 + am^3$, show complementation in heterocaryons to form active but abnormally heat-labile GDH (Fincham, 1958). These three complementing mutants each form an abnormal variety of the GDH protein; in the case of am^1 this protein has no known enzyme activity, but the proteins formed by am^2 and am^3 can be induced to show activity under appropriate conditions (Fincham, 1961). Complementation between mutants appears to be due to an interaction between mutant GDH varieties (Fincham & Coddington, 1963*a*) with the formation of enzymically active 'hybrid' protein. The wild-type enzyme is an aggregate of probably identical sub-units (Coddington & Fincham, in preparation).

Two generalizations have been suggested by previous work on inter-allelic complementation in this and other systems. The first is that complementing alleles form defective forms of the normal protein rather than none at all. This has seemed to be usually true, though there are apparent exceptions (Garen & Garen, 1963; Suyama & Bonner, 1964). The second is that at least one of each complementing pair of mutants forms a protein variety which has potential enzyme activity in itself (Fincham & Coddington, 1963*b*). This is not necessarily true, since complementation might result in the formation of a 'synthetic' active centre, with some groups contributed by one type of sub-unit and some by another, but our knowledge of the properties of the am^1, am^2 and am^3 protein varieties suggests that the generalization may be valid at least for the *am* mutants.

This paper describes an extension of our work on the *am* series made possible by the isolation of six new mutants. A preliminary mention of some of the results has already been made elsewhere (Fincham & Coddington, 1963*b*).

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2. MATERIALS AND METHODS

(i) Neurospora *strains*

All our recent work has been based on three strains: wild-type 74*A* (often referred to as St Lawrence *A*), B317–9–9 *a arg*-10 and 46004–10–1 *a arg*-1. The last two are non-allelic arginine-requiring mutants, originally supplied by Dr Dorothy Newmeyer, which are highly inbred with 74*A* and readily heterocaryon-compatible with each other. The entire series of *am* mutants, both those previously isolated and those obtained during the present work, have been crossed to one or other of these three reference strains, and re-isolates of *a* mating type have been tested for ability to form prototrophic heterocaryons with one or other of the arginine-requiring stocks. Since all the derived strains were heterocaryon-compatible with a reference strain they were assumed all to be heterocaryon-compatible with each other. Such an assumption has recently been questioned by De Serres (1962), but the chance of its being misleading is considered to be slight. The original derivations of mutants am^1–am^{11} have been given previously (Fincham, 1958); am^{12} and am^{13} appear to be recurrences of am^3 and am^7 respectively (Fincham, 1962; genetic data personally communicated by J. A. Pateman).

(ii) *Complementation tests*

Loops of conidial suspensions, or small spots of conidia carried on the tip of a moistened needle, were superimposed on the surface of plates containing minimal agar medium N (Vogel, 1958) with 0·4% sucrose and 0·9% sorbose to induce compact growth, and 0·02 M glycine to inhibit the adaptive growth of *am* mutants (Fincham, 1950). On this medium singly inoculated *am* mutants give little or no growth for 1½–2 weeks or more; consistently positive growth was shown by complementary combinations of mutants within a week and within 3 days in the cases of the stronger combinations (cf. Table 1).

(iii) *Crude enzyme extracts*

Cultures were grown from heavy conidial inocula in 50-ml. lots of liquid medium Ni n 250-ml. Erlenmeyer flasks, supplemented for *am* mutants with 0·005 M L-glutamate, for about 2 days without agitation. The washed and blotted mycelial pads were ground in 25 times their weight of 0·05 M sodium phosphate buffer, pH 8·0, in a mortar with powdered glass, and clarified by filtration through 2 g. of 'Celite' filter aid on a 5 cm. diameter Buchner funnel. Protein determinations were made by the procedure of Lowry, Rosebrough, Farr & Randall (1951).

(iv) *Purification of GDH and mutant proteins*

GDH was purified from large-scale cultures as previously described (Fincham & Coddington, 1963*a*). In the case of *am* mutants lacking GDH activity the same procedure was followed except that the second and third batches of calcium phosphate gel, added to the ammonium sulphate fraction to adsorb the protein, were eluted separately and the two eluates were concentrated and further fractionated on two separate DEAE-cellulose columns. The column fractions were examined for

the presence of GDH-like protein by disc electrophoresis in columns of polyacrylamide gel; GDH was easily recognized at this stage as a major band in a characteristic position. Fractions containing GDH-like protein as a major component were combined and purified by elution with a gradient of increasing phosphate concentration from a further DEAE column as previously described. The protein eluted as a single major peak, the best fractions of which appeared substantially homogeneous electrophoretically.

(v) *Disc electrophoresis*

The procedure of Ornstein & Davis (1961) was used, with only minor modifications. Samples of protein, 20 or 30 μl., in the concentration range 0·1 to 0·5 mg./ml., for purified proteins, or 1 to 5 mg./ml. for crude extracts, and with the addition of 5% sucrose, were layered-on to polyacrylamide columns 55 mm. long and 5 mm. in diameter. A voltage of 180–200 V. was applied for 40–60 min. The pH of the polyacrylamide gel was about 8·7. Staining for protein was with amido black, as described by Ornstein & Davis. Staining for enzyme activity was carried out by transferring the gels after electrophoresis into small test-tubes each containing 5 ml. of the following mixture (based on Markert & Møller, 1959): 50 ml. 0·05 M Tris-HCl buffer, pH 8·0; 850 mg. monosodium L-glutamate; 10 mg. NADP; 15 mg. neotetrazolium chloride; 5 mg. phenazine methosulphate; 5 ml. 0·5 M hydrazine, adjusted to pH 7·5 with HCl. The gel was incubated at 25° C. in the dark; a sharp band of deposition of red formazan dye on the surface of the gel marked the position of any NADP-linked GDH activity, and usually appeared within 10–20 min.

(vi) *Enzyme assays*

The enzyme assay systems, involving measuring the rate of either NADP reduction with glutamate, or NADPH oxidation with α-oxoglutarate and ammonium salt, were as previously described (Fincham & Coddington, 1963*a*).

(vii) *Nitrous acid induction of* am *mutants*

The method adopted was a slight modification of that used by Siddiqi (1962). Conidia from 9-day slant cultures of 74*A* were suspended in 0·1 M acetate buffer, pH 4·4, and filtered through gauze. Of the resulting suspension, 11·6 ml. was brought to 37° C. and 0·4 ml. of fresh 0·5 M $NaNO_2$ was added. After 12 min. the treatment was stopped by addition of 12 ml. of 0·1 M Na_2HPO_4 at room temperature. Dilutions of conidia, plated before and after treatment, showed there was 22% survival. The conidia were centrifuged down and resuspended in 240 ml. of the liquid minimal medium of Westergaard & Mitchell (1947). with the addition of 0·02 M glycine. The suspension was incubated with magnetic stirring at 25° C., filtered every 12 hours for 2 days and then once a day for 8 more days. Then the remaining conidia were centrifuged down and plated on 100 plates of minimal sorbose medium supplemented with alanine (to support the growth of any *am* mutants) and incubated for 24 hours at 33° C. 1368 colonies from these plates were tested and six proved to be *am* mutants. These were designated am^{14} to am^{19}.

3. RESULTS

(i) *Formation of GDH-like proteins by the* am *mutants*

The isolation and some of the properties of the protein varieties produced by am^1, am^2 and am^3 have been previously described (Fincham, 1962; Fincham & Coddington, 1963*a*, *b*). Each of these proteins is found in about the same amount per unit weight of mycelium as is wild-type GDH, and each is electrophoretically identical to wild-type GDH, or very nearly so.

Attempts to isolate mutant proteins from am^4, am^7, am^{14} and am^{19} were made, and were successful except in the case of am^{14}. Two separate fractionations of am^{14} extracts showed an absence of any major protein component resembling GDH in any of the fractions eluted from calcium phosphate gel and concentrated by DEAE-cellulose chromatography. The other three mutants, on the other hand, each yielded, at the first attempt, a quantity of GDH-like protein in a yield within the range found with GDH from wild-type: that is to say 15–25 mg. of purified protein from 250 g. moist weight of mycelium. The presence of GDH-like protein in am^4, am^7 and am^{19}, and its apparent absence in am^{14}, is in complete agreement with the recent immunochemical results of Roberts & Pateman (1964).

Electrophoretic comparison between each of the new mutant GDH varieties and am^1 protein were made. The am^1 protein can itself be regarded as a marker for the electrophoretic position of the wild-type enzyme. The results are shown in Plate I, Fig. 1. The proteins of am^1 and am^7 are inseparable from each other, and thus both closely similar to wild-type GDH electrophoretically. On the other hand both am^4 and am^{19} proteins are faster, and thus, presumably, more negatively charged at pH 8·7, than the am^1 protein. The am^{19} protein is about 10–11% faster and the am^4 protein about 4% faster than am^1; am^4 and am^{19} proteins are separable from each other (Fig. 1B). A difference of the same magnitude between the am^1 and am^{19} proteins also appeared when a mixture of them was fractionated by electrophoresis at pH 8·4 on cellulose acetate strips; thus the difference seems likely to be a true charge difference, rather than due to the sieving effect of the gel, which might achieve a fractionation on the basis of small differences in size or shape.

The isolated mutant proteins were assayed for GDH activity. The am^{19} protein showed a slight activity in an assay system, previously found effective for activating am^2 and am^3 proteins, containing TPN and 0·14 M glutamate at pH 8·7. The specific activity in this system was calculated to be 900; wild-type or am^3 GDH (cf. Fincham, 1962; Fincham & Coddington, 1963*a*), would have given a specific activity of 100,000 or more in the same system. No enzyme activity was detected in either the am^4 or the am^7 protein.

(ii) *Complementation relationships*

Complementation tests among the whole series of mutants revealed a number of new complementary pairs. In addition to *1* + *2* and *1* + *3*, which had been previously known to be complementary, *19* was found to complement *1*, while *14* complemented *1*, *2*, *3*, *7* and *19*. The combinations *1* + *14*, *1* + *19* and *3* + *14* gave comparatively

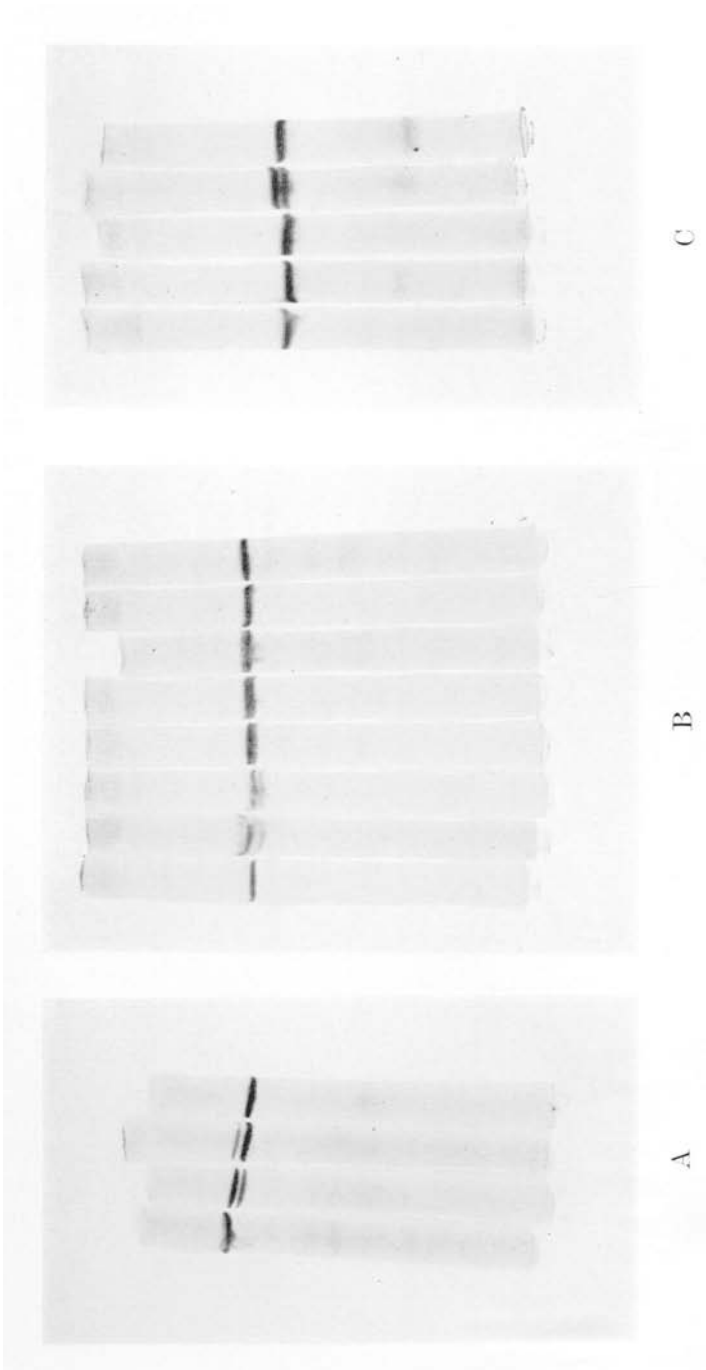

Fig. 1. Polyacrylamide electrophoretic gels, stained with amido black, to show the difference between am^4, am^{19} and am^1 proteins and the electrophoretic similarity of am^7 and am^1 proteins. The top (spacer) gel is seen still attached at the upper (negative) end of each gel; 20 μl. of each protein solution was applied at the top and the proteins have migrated downwards towards the anode.

A. Left to right: am^1, am^1 and am^{19} in 4:1 mixture; am^1 and am^{19} in 1:4 mixture; am^{19}.

B. Left to right: am^{19}, $am^{19}+am^1$ (1:1 by vol.), am^4+am^{19} (1:1 by vol.), am^1+am^4 (2:1), am^1+am^4 (1:1), am^1+am^4 (1:3), am^1, am^4.

C. Left to right: am^{19}, am^4, am^1+am^7, am^4+am^7, am^7. The protein concentrations of the various solutions were all in the range 0·25–0·45 mg./ml.

rapid positive responses and vigorous growth, while the other combinations were apparently weaker, especially *14* + *19* and *14* + *2*, which grew on the sorbose-glycine minimal plates only after about a week. The results are summarized in Table 1. They can be represented by the complementation map shown in Fig. 2.

Table 1. *Results of complementation tests involving new* am *mutants*

	1	2	3	4	5	6	7	8	9	11	14	15	16	17	18
19	++	—	—	—	—	—	—	—	—	—	±	—	—	—	—
18	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
17	—	—	—	—	—	—	—	—	—	—	—	—	—		
16	—	—	—	—	—	—	—	—	—	—	—	—			
15	—	—	—	—	—	—	—	—	—	—	—				
14	++	±	++	—	—	—	+	—	—	—					
3	++	—	—	—	—	—	—	—	—						
2	++	—	—	—	—	—	—	—							

The lowest two lines show complementation reactions previously reported and repeatedly confirmed. No. 10 was a sterile non-complementing mutant; 12 and 13 seem to be identical with 3 and 7 respectively.

Key: ++ = strong complementation; growth in 2–4 days.
+ = growth usually in 3–6 days.
± = weak complementation; growth usually only after 6–7 days.

(iii) *Properties of GDH varieties formed by complementation*

It was previously reported (Fincham, 1959) that the heterocaryons $am^1 + am^3$ and $am^1 + am^2$ produced about 20% and 10–15% respectively of the wild-type level of GDH activity. The new complementary combinations also show relatively low activity, not exceeding 5% of typical wild-type levels when measured in the assay

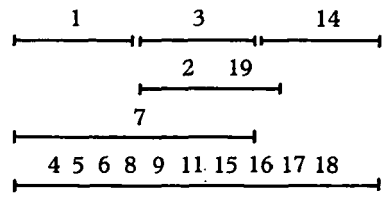

Fig. 2. Complementation map of the *am* mutants. The mutants *2* and *19* are represented as partially overlapping *14* on the basis of their weak complementation with this mutant; the more usual convention of representing all positive complementation tests, of whatever strength, in the same way would place *2* and *19* in the same segment as *3*.

system A (see Table 2) which measures the physiologically important glutamate synthesizing ability.

Several of the new complementary combinations of alleles also resemble those previously studied in producing varieties of GDH which are less stable than the wild-type enzyme. Crude extracts of the wild-type retained 90% of their GDH

activity after heating to 61° C. for 20 min. An extract of the heterocaryon $am^1 + am^{14}$ was very nearly or quite as stable, retaining 87% of its original activity after similar heating, but an extract of $am^7 + am^{14}$ lost 80% of its activity in the same test. The

Table 2. *Glutamate dehydrogenase activity given by complementation between pairs of* am *mutants*

The three assay systems contained in 3 ml. and at 35° C in each case: (A) 30 μmoles disodium α-oxoglutarate, 100 μmoles ammonium chloride, 10 μg. NADPH and 125 μmoles Tris (+HCl), pH 8·5; (B) 100 μmoles sodium L-glutamate, 40 μg. NADP, 125 μmoles Tris (+HCl), pH 8·5; (C) 400 μmoles sodium L-glutamate, 40 μg. NADP, 135 μmoles Tris (+HCl), pH 8·5. In A and B the enzyme (100 μl. of crude extract) was added as the final addition to the reaction mixture; in C it was pre-incubated with the glutamate and Tris in 2·8 ml. for 2 min. before addition of the TPN. Specific activities expressed as 100 × change in O.D. at 340 mμ/mg. protein.

	Specific activity		
Strain	A	B	C
Wild (74*A*)	4500	1400	2000
Heterocaryons:			
$am^1 + am^{14}$	170	45	65
$am^1 + am^{19}$	200	60	100
$am^2 + am^{14}$	45	55*	90*
$am^3 + am^{14}$	60	120*	500*
$am^7 + am^{14}$	135	50	100
$am^{14} + am^{19}$	40	20	60

* Much of the activity here can be attributed to the am^2 or am^3 protein alone.

GDH formed by $am^3 + am^{14}$ was less stable still, having lost all detectable activity after as little as 5 min. at 61°C. All these heat-stability tests were performed with extracts in 0·05 M phosphate, pH 8·0, at a protein concentration of about 0·4 mg./ml.

In another respect the GDH formed by complementation seemed closely similar to the wild-type enzyme. Samples of crude extracts of several of the complementing heterocaryons were fractionated by electrophoresis on polyacrylamide gels both alone and in mixture with amounts of wild-type extract containing comparable numbers of enzyme activity units. The gels were stained for enzyme activity. The allelic combinations *1 + 14*, *3 + 14*, *7 + 14* and *1 + 19*, which were the only ones which formed enough and sufficiently stable enzyme activity to be easily detected in gels, formed enzyme which failed to separate from the wild-type. This can be taken as an indication that the complementation product in each of these cases is very similar in size, as well as in charge, to wild-type GDH, since the strong sieving effect of the gel should certainly result in fractionation according to molecular size in the size range we are concerned with here (the enzyme has a molecular weight of 260,000—Fincham & Coddington, 1963*b*). The fact that the *1 + 19* product is electrophoretically normal, or nearly so, is remarkable in view of the electrophoretic abnormality of the am^{19} protein. We shall return to this point in the Discussion section.

4. DISCUSSION

Interallelic complementation has been shown in several experimental systems to involve interaction between differentially defective mutant proteins. In at least one case (Schlesinger & Levinthal, 1963) it has been shown beyond doubt that the active complementation product is a hybrid dimer containing one sub-unit of each of two different mutant varieties. The *Neurospora* NADP-linked GDH is a more complex polymer probably containing 8 or 10 identical polypeptide chains; in the case of the $am^1 + am^3$ interaction it has been possible to demonstrate that the enzymically active product is a hybrid polymer containing close to equal amounts of am^1 and am^3 protein (Coddington & Fincham, in preparation). We can assume that a similar molecular hybridization is involved in the other cases of complementation between *am* mutants.

There are two features of the present data which are not, at first sight, readily explicable. Firstly, it is unexpected to find that the mutant, am^{14}, which complements in more combinations than any other, should fail to produce a detectable variety of the GDH protein. It seems easiest to assume that it does produce the polypeptide sub-unit of the protein, but altered in such a way that it cannot form aggregates recognizable as related to GDH. If this is true its very inability to form stable homopolymers might make more sub-units available for mixed polymerization with the mutant varieties. Kaplan, Ensign, Bonner & Mills (1964) have recently obtained evidence that *tryp-3* mutants of *Neurospora*, which produced no protein related to tryptophan synthetase detectable by their usual immunochemical tests, but some of which nevertheless complemented other mutants, did, in fact, produce rather drastically altered polypeptide sub-units of the enzyme which could be detected by complement fixation tests. The question of the polypeptide product of am^{14} will have to be further investigated.

The second oddity in our data is the fact that, although the am^{19} mutant protein is easily separable from wild-type GDH by electrophoresis, the active product formed by $am^{19} + am^1$ is electrophoretically normal. Our present hypothesis to account for this is that the electrophoretic abnormality in am^{19} is not due to a difference with respect to the content of charged amino acids but to a conformational difference, with a consequent alteration in the interaction between the charged groups and a shift in the pK values of some of them. Some plausibility is lent to this idea by the recent finding (Sundaram & Fincham, in the press) that a secondary mutant derivative of am^{19} produces a GDH variety which can be converted reversibly from one electrophoretic form to another by shifts in the ionic environment; here only the electrophoretically normal form is enzymically active. In the case of am^{19} itself we picture the restoration of the normal conformation as being achieved by hybridization with the conformationally normal am^1 protein.

All our data so far are consistent with the idea that the gain in enzyme activity on complementation between *am* mutants is due to the stabilization of the incipient activity of the protein sub-units produced by one of the mutants. On this hypothesis we could expect the hypothetical gene product of am^{14} to have all the essential

components of the catalytically active centre, and we know this is the case with *am*^{2}, *am*^{3} and *am*^{19} proteins because of the activity which can be obtained from each of them under suitable activating conditions. The *am*^{1} protein can be regarded as one which, though totally devoid of activity itself (presumably because it lacks an essential amino acid component of the active centre), is very stable conformationally and very good at stabilizing the active conformation of other mutant proteins. The situation in *am*^{2} + *am*^{14}, *am*^{3} + *am*^{14} and *am*^{19} + *am*^{14}, where we suppose that two conformationally unstable protein sub-units are probably correcting each other, might be expected to be a very unstable one, and it is no surprise to find that the activity formed by *am*^{3} + *am*^{14} is extremely unstable to heat.

These views on the nature of the defects in the various mutant proteins are consistent with recent results on the different kinds of prototrophic revertants obtainable from each of them (Stadler, in preparation). All revertants from *am*^{1} seem to be indistinguishable from wild-type, as if activity can be restored to the protein only by a reversal of the primary mutational change. However, those from *am*^{14} and *am*^{19}, like those from *am*^{3} (Pateman & Fincham, in preparation), include a range of different types producing enzyme varieties with abnormal properties, as if a number of different alterations in the conformation of the protein can restore activity in each case.

SUMMARY

Six new *am* mutants of *Neurospora*, *am*^{14}–*am*^{19}, with defective formation of NADP-linked glutamate dehydrogenase, were isolated following treatment of conidia with nitrous acid. This brings the number of genetically distinct and viable *am* mutants to sixteen. Heterocaryon-compatible isolates of each of the new mutants were tested for complementation with each of the mutants previously known. A number of new complementary combinations were found which add to the complexity of the complementation map which, however, remains linear.

The mutants which show complementation are *am*^{1}, *am*^{2}, *am*^{3}, *am*^{7}, *am*^{14} and *am*^{19}. It has proved possible to isolate an altered form of glutamate dehydrogenase from each of these except *am*^{14}, which appears to produce no protein fractionating like the normal enzyme. Among the non-complementing mutants, *am*^{4} produces an easily-isolated inactive form of glutamate dehydrogenase. These results agree with the immunochemical findings of Roberts & Pateman (1964).

Of the mutationally altered forms of the enzyme, the *am*^{1}, *am*^{2}, *am*^{3} and *am*^{7} varieties are electrophoretically identical or closely similar to the normal protein, while the *am*^{4} and *am*^{19} varieties move faster towards the anode at pH 8·5, *am*^{19} being the faster of the two. The *am*^{19} protein, like the *am*^{2} and *am*^{3} proteins previously reported on, can be activated to give some enzyme activity, but the activity obtained is much lower for this mutant than for *am*^{2} or *am*^{3}.*

* Note added in proof: It has been recently found that *am*^{19} protein can be activated to a much greater extent by high concentrations of succinate (Sundaram & Fincham, in the press).

All the complementary pairs of mutants, including those involving am^{19}, form active enzyme varieties which have very close to normal electrophoretic properties. In several cases the complementation enzyme is less stable to heat than the wild-type enzyme.

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