Short Communications

Hybridization *in vitro* between Wild-Type and Mutant Forms of Glutamate Dehydrogenase from *Neurospora crassa*

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Markert (1963) has demonstrated that the five electrophoretically distinct forms of lactate dehydrogenase from ox tissues (L-lactate-NAD oxidoreductase, EC 1.1.1.27) are made up of all the possible tetramers of two basic sub-units A and B. The two extreme forms A_4 and B_4 show a large difference in electrophoretic mobility on starch gels at pH 8.6. On freezing and thawing a 1:1 mixture of A_4 and B_4 in the presence of M -NaCl-0.1M-phosphate buffer, pH 7.0, there was a dissociation into sub-units and a random re-association to give the five possible species, A_4 , A_3B , A_2B_2 , AB_3 and B_4 in the binomial proportions 1:4:6:4:1. The three hybrid species had electrophoretic mobilities intermediate between those of A_4 and B_4 . This work was amplified by Chilson, Costello & Kaplan (1965), who defined more closely the conditions required for random hybridization.

Inter-allelic complementation between several pairs of amination-deficient (*am*) mutants of *Neurospora crassa* has also been attributed to sub-unit interchange (Fincham & Coddington, 1963*a,b*; Coddington & Fincham, 1965). Of the primary mutants studied, six produce either an inactive or an activatable form of glutamate dehydrogenase [L-glutamate-NADP oxidoreductase (deaminating), EC 1.4.1.4] that is structurally similar to the wild-type enzyme. The wild-type and mutant enzymes each have a molecular weight of about 270 000 and are composed of eight to ten identical sub-units. Hybridization *in vitro* between certain pairs of purified mutant enzymes has previously been achieved by acidifying the enzyme mixture (in phosphate buffer, pH 7.4) to pH 5.8 and then readjusting to 7.4 after 0.5 hr. The product had enzyme activity similar to the wild-type enzyme and, in the am^1 plus am^3 pair, it was possible to separate it and to show that it contained material from each parent enzyme (Coddington & Fincham, 1965). It also resembled the starting material in

its sedimentation characteristics on a sucrose density gradient. Judged by the amount of hybrid material that could be isolated the extent of hybridization was small and it was concluded that a random rearrangement of sub-units could not be taking place.

When Markert's (1963) freezing-and-thawing technique was applied to mixtures of purified enzymes from pairs of *am* mutants the yield of activity was much greater than with the acidification technique and was consistent with the possibility that random hybridization was indeed occurring. Thus in the case involving purified enzymes from two mutants am^1 and am^3 , the am^1 enzyme is completely inactive but the am^3 enzyme can be activated by high concentrations of glutamate to catalyse the deamination of L-glutamate, i.e. the non-physiological reaction. The wild-type enzyme, on the other hand, catalyses the reaction in both directions. Thus it is useful to have two assay systems: *A*, which measures the synthesis of L-glutamate from α -oxoglutarate, NADPH₂ and NH₃, and *C* which measures the deamination of L-glutamate in the presence of NADP after prior activation with 0.15M-glutamate. The production of activity in system *A* by a mixture of mutant enzymes is taken to be due to stabilization of the potentially active component in the active form as a result of hybridization between them. The ratio *A* activity/*C* activity can be regarded as an index of the conformational normality of the hybrid product by comparing its value with that found for wild-type enzyme under similar assay conditions. With a mixture of purified am^1 and am^3 enzymes in 0.05-0.10M-phosphate buffer, pH 7.4, the optimum production of *A* activity occurred at a NaCl concentration between 0.05 and 0.15M. Either the freezing or the thawing step had to be slow, as was found by Chilson *et al.* (1965) for lactate dehydrogenase. The effect of varying the input ratio of am^1 to am^3 enzyme is shown in Table 1. When the am^3 component is kept constant and am^1 increased, there is an increase in both the *A* and the *C* activities with the *A* increasing more

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Table 1. *Enzyme activities obtained by freezing and thawing various mixtures of wild-type and mutant forms of glutamate dehydrogenase from N. crassa*

The hybridization procedure was as follows. Enzyme mixtures in a total volume of 0.30 ml. of 0.1M-phosphate buffer, pH 7.4, made 0.1M with respect to NaCl were frozen overnight in the deep freeze. Each solution contained about 25 μ g. of the minor component. After thawing at room temperature, enzyme activities were determined as follows: system *A*, 0.15 ml. of 0.2M-disodium α -oxoglutarate, 0.20 ml. of 0.1% NADPH₂, 0.10 ml. of M-NH₄Cl and 2.55 ml. of 0.05M-tris-HCl buffer, pH 8.45; enzyme solution was added at zero time. System *C*, 2.80 ml. of 0.15M-monosodium L-glutamate in 0.05M-tris-HCl buffer, pH 8.45; the enzyme solution was added at minus 2 min. and 0.20 ml. of 0.2% NADP at zero time. The temperature was 37° in each case. The change in E_{340} was followed on a Beckman model DB automatic recording spectrophotometer. The activity unit is a change in E_{340} of 0.001/min.

Combination of proteins	Input ratio	10 ⁻³ × Specific activity (units/mg.)		<i>A/C</i> activity ratio
		<i>A</i>	<i>C</i>	
<i>am</i> ¹ plus <i>am</i> ³	0:1	0	51.2	—
	1:1	65.6	72.8	0.90
	3:1	163	96.1	1.70
	5:1	216	108	2.12
	10:1	268	110	2.46
<i>am</i> ³ plus <i>am</i> ¹	1:1	65.6	72.8	0.90
	3:1	94.5	244	0.39
	5:1	106	324	0.33
	10:1	114	624	0.18
Wild type	—	490	160	3.04
<i>am</i> ¹ plus wild type*	0:1	443	173	2.56
	1:1	362	150	2.41
	2:1	410	156	2.62
	2:1†	419	179	2.34
	4:1	428	167	2.56
<i>am</i> ² plus wild type*	1:1	200	185	1.08
	2:1	138	219	0.63
	2:1†	447	179	2.50
	4:1	91	259	0.35
<i>am</i> ³ plus wild type*	1:1	238	238	1.00
	2:1	162	357	0.46
	2:1†	452	300	1.51
	4:1	105	434	0.24
Wild type*	—	488	165	2.65

* *C* activity measured at pH 9.0.

† Control mixture not frozen.

rapidly than the *C*. At an input ratio 10:1 the *A/C* ratio is 81% of that found for the wild-type enzyme under identical conditions. However, the specific activity, with respect to the minor component, is only 55% of wild type. In the complementary experiment when *am*¹ is constant and the *am*³ component is increased, there is again an increase in both the *A* and *C* activities. The specific *A* activity with respect to the minor component for the 10:1 input ratio is 114 000 in comparison with 268 000 for the corresponding case when *am*¹ is in excess.

If, by analogy with the lactate-dehydrogenase system, one can postulate that the *A* activity is

due to hybrid formation between *am*¹ and *am*³ sub-units then these results support the mechanism of inter-allelic complementation put forward by Fincham & Coddington (1963*b*). In that paper it was postulated that the *am*³ sub-unit has an active site that is not very different from that of the wild type as evidenced by its being capable of activation. The *am*¹ sub-unit, on the other hand, is completely inactive enzymically but it possesses a tertiary structure very similar to wild type. When *am*¹ and *am*³ sub-units are present in the same molecule the *am*¹ sub-units are capable of holding the *am*³ sub-units in such a conformation that they will catalyse the reaction in both directions without

prior activation. Freezing and thawing a mixture of am^1 and am^3 enzymes where am^1 is in excess will produce a preponderance of am^1 -rich hybrids that are active in system *A*. A similar mixture of enzymes in which am^3 is in excess will produce hybrids rich in am^3 . Such hybrids would not be expected to be as active in system *A*. The experimental results quoted above tend to support this explanation.

Such an argument presupposes a similarity between wild-type and mutant sub-units and hybridization might be expected to occur between them. This is clearly shown between am^3 and wild type and am^2 and wild type (see Table 1). The enzyme from am^2 is similar to that from am^3 in that it can be activated by glutamate. In both cases increasing the proportion of mutant to wild-type enzyme in the starting mixture causes, after freezing and thawing, a progressive decrease in *A* activity. This must be due to the formation of increasing amounts of hybrids rich in mutant sub-units that are not as active in system *A* as was the wild-type enzyme alone. These hybrids do, however, approach wild-type activity in system *C*. It would seem that the am^2 and am^3 sub-units can impose their faulty conformations on to the wild-type sub-units with which they are hybridized.

With am^1 and wild-type mixtures there appeared to be no significant change in either the *A* or *C* specific activity after freezing and thawing. This could either be due to a complete lack of hybridization between the two or to the fact that the presence of the am^1 sub-unit together with that of wild type in a hybrid does not alter the conformation of the wild-type sub-unit in any way. The undoubted involvement of am^1 with am^3 (Coddington & Fincham, 1965) and with am^{19} (Coddington, Fincham & Sundaram, 1966) enzyme in hybrid formation suggests that the latter explanation is the correct one.

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The Linkage between Teichoic Acid and Glycosaminopeptide in the Walls of a Strain of *Staphylococcus lactis*

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One of the major unsolved problems in the chemistry of bacterial cell walls is the way in which the various component polymers are held together. The ribitol teichoic acid of *Staphylococcus aureus* has been shown to be held in the wall by linkage to the glycosaminopeptide through the terminal phosphate residue of the teichoic acid chain (Strominger & Ghuyssen, 1963; Ghuyssen, Tipper & Strominger, 1965). Moreover, the polysaccharide in walls of *Lactobacillus casei* N.I.R.D. RO 94 is believed to be held in the wall by a sugar 1-phosphate linkage between the reducing terminal amino sugar residue of the polysaccharide and the glycosaminopeptide (Knox & Hall, 1965); acid hydrolysis of the glycosaminopeptide gave an

organic phosphate, probably that of muramic acid, and it seems likely that the polysaccharide is attached to the glycosaminopeptide by a phosphodiester linkage between position 1 of the reducing terminal *N*-acetylglucosamine residue of the polysaccharide and a hydroxyl group of a muramic acid residue in the glycosaminopeptide (Hall & Knox, 1965).

Although suggestions have been made (Hay, Archibald & Baddiley, 1965; Archibald & Baddiley, 1965), the chemical nature of the linkage between teichoic acid and glycosaminopeptide has not been established so far, and in particular the identity of the residue in the mucopeptide involved in such a linkage is unknown. We now present evidence