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Single Gene Heterosis for Alcohol Dehydrogenase in Maize: the Nature of the Subunit Interaction[1]

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Summary. The products of the Adh_1^F allele which specifies an active enzyme, and the Adh_1^{Cm} allele which specifies a stable enzyme, interact in the heterodimer to give an active stable alcohol dehydrogenase. Investigations on the nature of the heterotic interaction are presented including comparisons of *in vivo* and *in vitro* synthesized heterodimers.

The first evidence for the presence of hybrid enzymes in heterozygotes was presented in 1960 in a report of investigations of the E_1 esterase in maize (Schwartz, 1960). The electrophoretic analysis clearly indicated that the homologous subunits which comprise multimeric enzymes can be specified by different alleles. It was proposed in that paper that hybrid enzyme formation might be a basis for the hybrid vigor or heterosis observed in heterozygotes, i.e., an enzyme molecule composed of polypeptides specified by two different alleles, AA', might be more active than the corresponding homomultimers AA or $A'A'$. This could readily be detected in electrophoretograms since the enzymatic activity of the heteromultimeric isozyme band would be higher than would be predicted from the relative intensities of the homomultimeric isozyme bands. However, no well established cases of such interactions have been reported except in intragenic complementation studies with allelic mutants (Schlesinger and Levinthal, 1963; Coddington, Fincham, and Sundaram, 1966).

Single gene heterosis need not be limited to increased specific activity of an enzyme and should include any allelic interactions which lead to the formation of a "better" enzyme. A few years ago we reported evidence of single gene heterosis for alcohol dehydrogenase (*ADH*) in maize (Schwartz and Laughner, 1969). The interaction was observed in heterozygotes which contained an Adh_1^F allele which specifies an active but somewhat labile enzyme, and an Adh_1^{Cm} allele which produces a relatively inactive but more stable form of the enzyme. Since the enzyme is a dimer, three *ADH* isozymes are formed in heterozygotes — FF with only the active subunits, $C^m C^m$ with only stable subunits, and the heterodimer $F C^m$ which contains one active and one stable subunit. The heterodimer is both active and stable. The F subunit in the heterodimer maintains its full activity and this dimer with only one active subunit is half as active as the FF homodimer with two active subunits (Schwartz and Endo, 1966). The stable subunit in some way confers its stability to the active subunit in the heterodimer leading to heterosis. Heterozygous plants contain the stable-active FC^m enzyme whereas each homozygote has either the stable-inactive C^mC^m, or the active-labile FF enzyme. This paper is concerned with the nature of the heterotic interaction between the subunits.

[1] Dedicated to Dr. M. M. Rhoades on the occasion of this 70th birthday, with gratitude, admiration, and appreciation.

Methods and Materials

Enzyme analyses were conducted on crude extracts from dry kernels. The kernels were ground in a Wiley Mill through a 20 mesh screen, the meal steeped in 0.005 M sodium phosphate buffer, pH 7.5, for 15 minutes and the slurry centrifuged at 39,000 × g for 15 minutes. The pellet was discarded. Enzyme activity was measured by following the reduction of NAD by optical density measurements at 340 nm as previously described (Efron and Schwartz, 1968). A unit of activity represents a change of absorbancy of 0.001 per minute.

The enzyme was dissociated and reassociated according to the method of Hart (1971) as modified by Meir Fischer (personal communication), a graduate student in this laboratory. The extraction procedure is as described above except that the extraction was made with a solution containing 0.1 M sodium phosphate buffer, pH 7.5, 0.4 M sucrose, 1.0 M NaCl, and 0.1 M β-mercaptoethanol. The extract was frozen overnight at −20 °C, then thawed rapidly by immersion in warm water. Extracts from different genotypes can be mixed either before freezing or after thawing, that is, before or after dissociation. The reassociation modification introduced by Fischer is to allow the thawed sample to stand at room temperature for $1^1/_2$ hours after thawing rather than immediate dialysis. The *ADH* subunits of maize reassociate readily at room temperature even in the high salt concentration. Dimerization is usually completed by $1^1/_2$ hours and as high as 90% of initial enzyme activity can be recovered. When the samples are to be run in electrophoresis they are subsequently dialyzed in the cold for 3 hours to remove the NaCl. Dialysis buffer is the same as the extraction buffer except that the NaCl is omitted. In experiments designed to test for the degree of dissociation, it was necessary to prevent the recombination of the dissociated monomers to reform active dimers. This was accomplished by adding EDTA to the frozen sample prior to thawing. Sufficient EDTA was added such that upon liquification the concentration of chelating agent was 0.01 M, and the

sample was dialyzed in the cold with 0.01 M EDTA in the buffer (Fischer, personal communication). The block to reassociation to form active enzymes can be overcome by the addition of zinc chloride. This concentration of EDTA has no effect on the activity of the undissociated dimer enzyme. The procedures for starch gel electrophoresis and development of the ADH zymograms have been previously described (Schwartz and Endo, 1966).

Results and Discussion

Previous results have indicated that an *F* subunit is more stable to treatment with high temperature and pH above 10 when in a dimer with a C^m subunit than when it is in an *FF* homodimer. This has been interpreted as evidence for stabilization of *F* by C^m. However, it could be argued that the treatment alters the structure of the *F* polypeptide regardless of its partner in the dimer, but that C^m interacts with altered *F* subunit such as to render it active, i.e., complementation between the subunits. This hypothesis was tested by the following experiments. Kernels of the genotype Adh_1^F/Adh_1^{Cm} were extracted in 0.005 M phosphate buffer and heated at 65 °C for 30 minutes. This treatment is sufficient to inactivate the *FF* dimer but the FC^m remains active (Schwartz and Laughner, 1969). An equal volume of double strength solution of the dissociation buffer was added to bring the extract to the usual concentration of the dissociation buffer. The sample was subjected to the dissociation-reassociation procedure, dialyzed to remove the NaCl, and subjected to electrophoresis in starch gel. After dissociation the subunits recombine randomly so that some of the *F* subunits in the reassociated *FF* dimer had been derived from an *FF* dimer and some were present in an FC^m dimer prior to dissociation. According to the complementation hypothesis, only the reassociated FC^m dimer should be active and hence only the FC^m isozyme band should develop in the zymogram. On the other hand, if *F* is stabilized by its C^m partner in the dimer, some of the dissociated *F* subunits should be unaltered and could reassociate to form active *FF* as well as active FC^m dimers. The results are shown in Fig. 1. Both

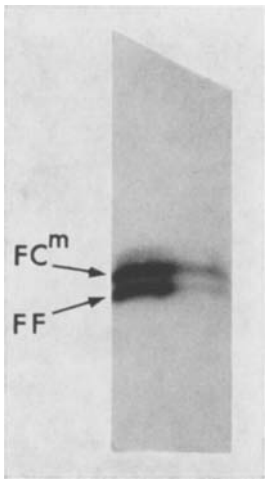

Fig. 1. Test of complementation hypothesis with Adh_1^F/Adh_1^{Cm} extracts. Left: control sample showing *FF* and FC^m *ADH* isozyme bands of equal intensity. Extract in dissociation buffer kept at 4 °C overnight then dialyzed to remove the NaCl. Right: treated sample heated 65 °C for 30 minutes, dissociated, reassociated, and dialyzed. Note both the *FF* and FC^m bands are observed in the treated sample. The two isozyme bands are not equal in intensity since the *F* and C^m subunits were unequal in concentration for reassociation

FF and FC^m active enzyme bands are observed. As an additional test the experiment was performed in the reverse manner. An *FF* extract was heated to complete inactivation, mixed with the C^mC^m extract, dissociated, reassociated, dialyzed, and subjected to electrophoresis. According to the complementation hypothesis activity would be expected at the FC^m position but not at the *FF*. However, neither an FC^m nor an *FF* band was observed. These results eliminate complementation, or activation of an inactivated *F* by the C^m subunit, as the mechanism responsible for the FC^m interaction. The *F* subunit in an FC^m dimer is stabilized and the question arises as to how C^m confers its stability to *F*. A number of schemes have been considered and tested.

It is possible that C^m may confer its stability to the dimer as a whole rather than to the *F* subunit. *ADH* of maize is active only in dimer form; the monomers are completely inactive. The C^mC^m dimer is more stable than *FF* and one could propose that the C^m has exceptionally strong bonds for maintenance of the dimeric structure and therefore the FC^m dimer is more stable than the *FF* dimer. This was tested by comparing the degree of dissociation of the *FF* and FC^m dimers frozen at increasing salt concentrations. Maize *ADH* dissociates completely upon freezing in the dissociation buffer with 1.0 M NaCl. The dissociation is complete since all *ADH* activity is eliminated and after dissociation and reassociation of a mixture of homodimers the concentration of the heterodimer is twice that of either homodimer. *ADH* does not dissociate when frozen in 0.1 M NaCl. At this low salt concentration a similar mixing procedure yields only the homodimeric forms and no heterodimers are observed. If the bonds which hold the FC^m dimer intact are stronger than those in the *FF* one would expect the *FF* dimer to dissociate upon freezing at a lower salt concentration than is required to dissociate the FC^m dimer. This in fact was found to be the case for the temperature sensitive enzyme specified by the $Adh_1^{S\,1108}$ allele (Fischer, personal communication). A heterodimer containing one temperature sensitive and one standard subunit dissociates at a lower salt concentration than is required to dissociate a dimer composed of two standard subunits.

For this test Adh_1^F/Adh_1^{Cm} kernels were mealed and extracted in a series of solutions all of which contained 0.1 M sodium phosphate, 0.4 M sucrose, and 0.1 M β-mercaptoethanol but varied in the NaCl concentration ranging from 0.1 M to 1.0 M. The extracts were frozen at −20 °C overnight. To prevent reassociation of the dissociated monomers, EDTA was added to the sample prior to thawing, and the solution was dialyzed at 4 °C to remove the NaCl in preparation for electrophoresis. Since reassociation is prevented by chelation of the zinc with the EDTA, and active dimer enzymes are not affected by the EDTA, enzyme activity is a good measure of the degree of disso-

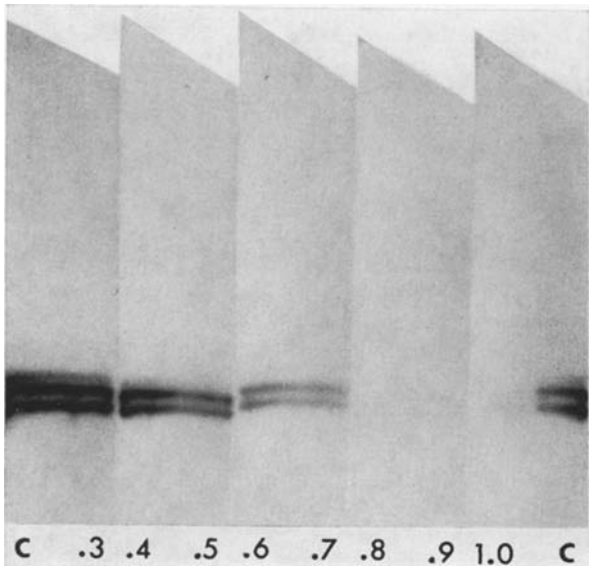

Fig. 2. Relative dissociation of FF and FC^m dimers at increasing NaCl concentrations. Two samples were run side by side on each gel. C = control sample kept in cold and not dissociated by freezing. The numbers indicate the NaCl concentrations in the samples subjected to the dissociation treatment

ciation. The greater the degree of dissociation, the lower the dimer concentration in the sample, and hence the lower the *ADH* activity. The relative dissociation of the FF and FC^m dimers can readily be compared by observing the relative intensities of the two isozyme bands in the gel following electrophoresis. If interdimeric bonds are weaker and FF dimers dissociate at a lower NaCl concentration than the FC^m dimers, then as the salt concentration in the extraction solution is increased the relative intensities of the FF and FC^m isozyme bands should deviate from the 1:1 ratio observed in the undissociated control extracts. The FF isozyme band should decrease in intensity relative to the FC^m band. However, as is clearly seen in fig. 2 the relative intensities of the FF and FC^m bands were constant in all NaCl concentrations. This result tends to eliminate differences in the strength of the interdimeric bonds as the factor responsible for the increased stability of the F subunit in the heterodimer.

Two possible pathways for the association of polypeptide chains into active oligomeric enzymes have been proposed (see review by Paulus and Alpers, 1971). The subunit can be folded into its conformation while still in the monomeric form and then the subunits aggregate to the oligomer without changing configuration. Alternatively, subunits can first aggregate then undergo a conformation change to yield the thermodynamic stable structure. The latter is thermodynamically more attractive and raises a question as regards the stabilization of FC^m . Does the C^m subunit stabilize F by influencing the folding of the F subunit as it undergoes its conformational change in the FC^m dimer or is it simply the presence of the C^m subunit in the dimer which stabilizes F? Specifically, will an F subunit derived from an FF homodimer be stabilized when associated with a C^m subunit which has come from a C^mC^m homodimer? This was tested by utilizing the techniques of dimer dissociation and reassociation. FF and C^mC^m extracts were dissociated separately by freezing dissociation buffer extracts of Adh_1^F/Adh_1^F and Adh_1^{Cm}/Adh_1^{Cm} kernels. Immediately after thawing the two extracts were mixed to allow random reassociation of the F and C^m subunits according to the procedures outlined. As control, an extract from an Adh_1^F/Adh_1^{Cm} heterozygote was prepared in the same fashion but kept in the cold without freezing. Thus both the Adh_1^F/Adh_1^{Cm} control and the dissociated-reassociated mixture contained FC^m dimers, but differed in the source of the F subunit in the FC^m dimer. The samples were dialyzed for four hours against dissociation buffer minus NaCl and half of each sample was then dialyzed against a 0.1 M carbonate bicarbonate buffer, pH 10, overnight to compare the relative stability of the FF and FC^m dimers at the high pH. The results are presented in fig. 3. The difference in stability of the FF and FC^m dimers is still observed in both samples and hence the source of the F subunit in the heterodimer is immaterial. Simply the association of the F subunit with C^m in the dimer is sufficient to confer stability to F.

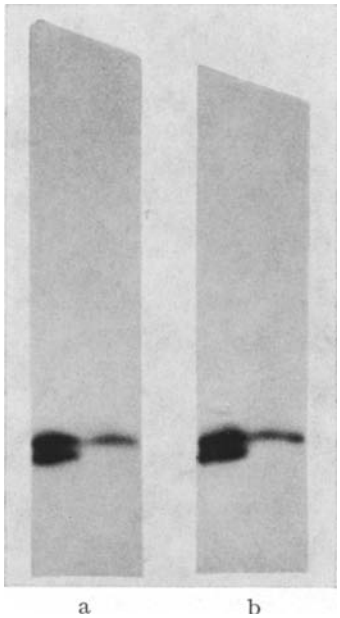

Fig. 3. Test of the influence of the source of the F subunit in the relative stability of the FF and FC^m dimer (see text). Left half of each gel: crude extract from Adh_1^F/Adh_1^{Cm} heterozygotes. Right half of each gel: pH 10 treatment. a. dissociated and reassociated mixture of Adh_1^F/Adh_1^F and Adh_1^{Cm}/Adh_1^{Cm} extracts. b. control extract from Adh_1^F/Adh_1^{Cm} heterozygote

The association with C^m may alter the conformation of F subunit in the heterodimer such that it is less sensitive to high temperature or pH. However, if such a configurational change occurs it does not alter the net surface charge of the F subunit since this would be reflected in an alteration in electrophoretic mobility. Furthermore, this is not the most attractive hypothesis since as was pointed out in the earlier paper (Schwartz and Laughner, 1969) high stability and high activity in *ADH* appeared to be mutually exclusive. If the F subunit can be influenced by C^m in the heterodimer to assume a configuration which is both active and stable one would expect selection forces to have operated to fix an Adh_1 allele which makes an active stable enzyme in the population. Instead, an Adh_1^{FCm} duplicate locus was fixed which insures the advantage of heterozygosity.

In view of the results described in this paper I favor the proposal that the two *ADH* subunits are very intimately associated and the mere presence of a stable subunit in the dimer is sufficient to stabilize the other subunit. If, as a result of an adverse treatment, bonds are ruptured in only one subunit in a dimer, the presence of the stable undenatured subunit can influence or direct the reformation of the ruptured bonds such that the polypeptide will reassume an active configuration.

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