THE MOLECULAR BASIS FOR ALLELIC COMPLEMENTATION OF ALCOHOL DEHYDROGENASE MUTANTS OF MAIZE

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ABSTRACT

Studies on interallelic complementation with two temperature-sensitive alcohol dehydrogenase mutants in maize are described. The data suggest that the phenomenon results from instability or abnormal maturation of the mutant homodimer rather than correction of configurational defects in mutant heterodimers.

IT has been clearly established that interallelic complementation involves interaction between subunits in polymeric proteins (CATCHESIDE and OVERTON 1958; BRENNER 1959; WOODWARD 1959; FINCHAM 1959). However, the molecular basis for this complementation remains ambiguous. CRICK and ORGEL (1964) have proposed a correction mechanism. Complementing mutations are visualized as causing localized misfolding of protein protomers, and in heteropolymers the configurational defect in one subunit is corrected by association with the homologous but correctly folded region in a second subunit specified by the other mutant allele. While it is certainly to be expected that close association of polypeptides in a heteropolymer could lead to configurational alterations, the hypothesis requires that the interaction must cause a highly specific change leading to correction of the defect. The purpose of this paper is to present an alternative mechanism for intragenic complementation. Instead of involving activation in a mutant *heteropolymer,* I propose that the complementation phenomenon results from misfolding, or defective maturation of the mutant *homopolymer.* Rather than have misfolded monomers form a correctly folded heteropolymer, I propose that the mutant polypeptides have the normal protomer configuration, but that the misfolding occurs during maturation of the mutant homopolymer. The advantage of this alternative scheme is that it eliminates the requirement for activation of mutant enzymes with altered amino acid sequences. Instability of the homopolymers is sufficient to account for the phenomenon, and correction becomes superfluous.

Homopolymer "denaturation" or instability is the basis for the experimental distinction between the two hypotheses. According to the correction hypothesis, monomers arising from in vitro dissociation of mutant homopolymers should still be capable of reassociating with other allelic mutant monomers to form active heteropolymers, as the hypothesis does not require a defective structural alteration of the subunit during homopolymerization. However, such alteration in

tertiary structure of the polypeptides in a mutant homopolymer is the basis for the phenomenon, according to the alternative hypothesis. If the configurational changes are irreversible, the dissociated monomers would be incapable of re-associating with other monomers to form active enzyme. This appears to be the case for two complementing alcohol dehydrogenase (ADH; EC 1.1.1.1) mutants in maize.

METHODS AND MATERIALS

The complementing mutants used in this study, *Adh-S1108* (SCHWARTZ 1971) and *Adh-F430* were both induced by ethyl methanesulfonate (EMS) treatment. Both mutants are temperature-sensitive and show much reduced activity in homozygotes. Heterodimers containing a mutant and a wild-type subunit are as active as wild-type homodimers. Kernels homozygous for the *Adh-S* or *Adh-F* alleles were immersed in 0.08 M EMS at room temperature for 10 hours and washed thoroughly before planting as described by BRIGGS, AMANO and SMITH (1965). The treated seeds were planted in the field and pollinated by *Adh-F* and *Adh-S* pollen respectively, so that the F_1 kernels would be heterozygous for two *Adh* alleles. Mutants arise as sectors in the F_1 ears, and 12 seeds from each ear were tested electrophoretically in the screening for *Adh* mutants. The starch gel electrophoretic procedures and techniques for developing the ADH zymograms have been described previously (SCHWARTZ and ENDO 1966).

The procedure of HART (1971) was followed for the dissociation of the ADH dimers. Kernels of the appropriate genotypes were grounded in a Wiley Mill through a 20-mesh screen and the meal extracted for 15 minutes at room temperature with dissociation buffer composed of 0.1 M sodium phosphate buffer, pH 7.0; 1.0 M sodium chloride; 0.4 M sucrose; and 0.1 M B-mercaptoethanol. After centrifugation for 15 minutes at 39,000 $\times$ g, the supernatant was frozen in 2-ml aliquots at $-20°$. Samples were kept at $4°$ for non-dissociated controls. The mercaptoethanol was omitted during the extraction of *Adh-S1108*. The *Adh-F430* enzyme was also extracted without the reducing agent, but after extraction, mercaptoethanol was added to the supernatant prior to the freezing step to a final concentration of 0.1 M. Reassociation was accomplished by simply incubating the frozen sample for 2 hours in a $30°$ water bath. Reassociation readily proceeds in the presence of the high salt concentration, and restoration of activity is completed by 2 hours (FISCHER and SCHWARTZ 1973). This procedure gave the best recovery of activity for mutant as well as wild-type enzyme. Other procedures tried involved reassociation with zinc supplementation during dialysis at room temperature and dialysis in the cold followed by incubation at $30°$. Enzyme activity was measured by following the reduction of NAD at 340 nm as previously described (EFRON and SCHWARTZ 1968). A unit of activity represents a change of OD of 0.001 per minute.

Whereas a high concentration of a reducing agent such as mercaptoethanol is normally required for dissociation of maize ADH, dissociation of dimers which contain a mutant S-1108 subunit occurs in the absence of the reducing agent. This is true for both S-1108 homodimers and heterodimers which contain a mutant and a wild-type subunit (SCHWARTZ, unpublished). In fact, the S-1108 monomer is very sensitive to the high mercaptoethanol concentration and is irreversibly altered such that it cannot undergo subsequent reassociation. The S-1108 homo- and heterodimers probably differ from wild-type enzyme in that they lack an interpolypeptide disulfide bond.

RESULTS AND DISCUSSION

Kernels homozygous for the *Adh-S1108* allele contain only about 20% of the ADH activity of their wild-type siblings. The mutant complements with other ADH alleles and mutant wild-type heterodimers are fully active (SCHWARTZ 1971). The S-1108 mutant aso complements with other inactive *Adh* mutants, and in heterozygotes only a single band of ADH activity (the heterodimer isozyme) is observed.

Experiments on dissociation and reassociation of the mutant enzyme preparations have led to the interesting observation that while the percent of activity recovered with wild-type enzyme is very high, around 90%, only a small fraction, 10–20%, of the initial activity, is recovered after dissociation and reassociation of an extract from *Adh-S1108* homozygotes. This is not due to instability of the dissociated S1108 monomers. These monomers are stable and fully capable of reassociation. In a typical experiment, 11.9% of control activity (35/295 units per ml) was recovered following dissociation and reassociation of an *Adh-S1108/Adh-S1108* extract. However, high recovery of S1108 activity occurred when the reassociation was in the presence of an excess of other kinds of dissociated monomers. This is indicated by the high recovery of ADH activity (710/830 units per ml = 86%) in dissociation reassociation of wild-type mutant heterozygotes (*Adh-Ct/Adh-S1108*), without mercaptoethanol. The *Adh-S1108* allele produces the normal amount of protomers in heterozygotes since the Ct-Ct homodimers and Ct-S1108 heterodimers occur in a 1:2 ratio. However the concentration of the mutant homodimers is only about 20% of the wild-type homodimers. Thus, in the heterozygote, two-thirds of the activity is contributed by the heterodimer but the recovered activity is high. A stronger indication that dissociated S1108 monomers are fully capable of reassociation comes from experiments with heterozygotes of *Adh-S1108* with *Adh-C70-6*, a CRM⁺, EMS-induced mutant which specifies a polypeptide that is enzymatically inactive but dimerizes normally with itself and all other ADH protomers. A C70-60 wild-type heterodimer has half of the activity of a wild-type homodimer (SCHWARZ, unpublished). Extracts from *Adh-S1108/Adh-C70-6* kernels were subjected to the dissociation reassociation procedure without mercaptoethanol. The reassociated sample contained 51% of the activity in the non-dissociated control (115/305 units per ml). As will be shown below, this is the level of activity expected for almost complete reassociation of S1108 monomers.

The following experiment was performed to critically test this point. Extracts were made from *Adh-S1108* homozygous kernels without mercaptoethanol in the dissociation buffer. Some aliquots were frozen and others kept at 4° for control. Kernels homozygous for the CRM⁺ mutant, *Adh-C70-6*, and for *Adh-0-5657*, were extracted with dissociation buffer containing 0.1 M mercaptoethanol and similarly treated. *Adh-0-5657* is an EMS-induced null mutant which produces no product capable of dimerizing other ADH protomers. After 16 hours, the frozen samples were thawed, mixed, and incubated at 30° for 2 hours. In the 1:1 mixture of S1108 with the CRM⁺ inactive C70-6 extract, 62% of the S1108 activity was recovered after reassociation (85/137.8 units per ml). However, in the mixture with the *Adh-0-5657* null extract, only 7.3% of the S1108 activity was recovered (10/137.8 units per ml). Other CRM⁺ inactive mutants give the same results as *Adh-C70-6*. These experiments clearly establish that the S1108 monomer is stable and fully capable of reassociation. Thus, the poor recovery of S1108 activity by reassociation in the absence of other ADH monomers must be due to the instability of the S1108 homodimer. If dissociated monomers of the inactive C70-6 enzyme are added to a dissociated S1108 sample which was preincubated for two hours, no increase in activity occurs upon further incuba-

tion of the mixture. Since recovery of activity in dissociated extracts is completed by two hours of incubation at 30°, this result indicates that after the preincubation the S1108 sample had no monomers remaining which are incapable of self-dimerization, but still capable of associating with C70-6 monomers.

Similar results are obtained with the *Adh-F430* mutant. Kernels homozygous for *Adh-F430* show approximately 40% of the activity of the wild-type sibs. The F430 polypeptide is fully active in heterodimers. In a typical experiment, only 38.5% of the control activity (260/675 units per ml) was recovered after dissociation and reassociation of the *Adh-F430* homozygous extract. However, the recovered activity increased to 65.1% (220/338) when the same extract was mixed with an equal volume of dissociated CRM^+ C70-6 monomers prior to being subjected to reassociation conditions. Recovery of activity is increased with the addition of excess C70-6 monomers.

These results suggest that during the process of homopolymerization the S1108 or F430 polypeptides can assume either an active or an inactive dimeric form. The probability that an S1108 homodimer will mature into an active configuration is less than 20%. The F430 homodimer has a greater tendency to fold into the active form. about 40%. In heterodimers with other subunits, these polypeptides always maintain the active configuration. The alternative to this proposition is that the S1108 and F430 mutant homodimers represent a homogeneous population, and all mutant homodimers are active but only to a much lesser degree than wild-type dimers, rather than a small percentage of the dimers being fully active and the remainder inactive. The alternative is rendered unlikely by the dissociation-reassociation results. If the mutant homodimers are a homogeneous population, with each dimer having 20% of the wild-type activity, full initial activity should be recovered following dissociation-reassociation, since mutant homodimers have been shown to be stable and capable of reassociation. The more likely hypothesis is less than 20% of the S1108 homodimers are in an active configuration, and these are fully active; the remainder are inactive and are "denatured" in the sense that they are irreversibly altered such that. even upon subsequent dissociation, they cannot reassociate with other monomers. If the probability of an S1108 homodimer's maturing into an active stable form is only 20%, the remaining 80% would be "removed from the pool". and after a second round of dissociation-reassociation. one should recover only 20% of the 20% dimers initially present in the active form. That this is the case is clearly shown by the double dissociation experiment.

Scutela from homozygous *Adh-S1108* one-day-soaked seeds were excised and macerated in a small volume of dissociation buffer without mercaptoethanol to obtain an extract with relatively high ADH activity. After centrifugation an aliquot was frozen overnight for dissociation and another kept at 4° as control. The frozen sample was thawed and incubated for 2 hours at 30° to allow reassociation to proceed. The sample was then refrozen overnight to dissociate the reassociated enzyme. After the second thawing, the sample was once again incubated at 30° for 2 hours to reassociate. The control 4° sample had 1600 units per ml. After dissociation and reassociation, the activity measured was 280

units per ml. or 17.5% recovery. After the second round of dissociation, recovery was only 60 units per ml., equivalent to 3.7% of the initial activity or 21% of the activity present after the first dissociation-reassociation. A similar double dissociation-reassociation experiment with wild-type enzyme gave 89% and 90% recovery of initial activity after the first and second rounds of reassociation. In order to show that after the second round of dissociation the S1108 monomers are still fully capable of reassociation to produce active dimers, the twice-dissociated S1108 extract was mixed with the CRM⁺ C70-6 dissociated monomers and incubated for two hours at 30° for reassociation. Seventy-five percent of the activity present after the first round of dissociation-reassociation was recovered, in contrast to the 21% recovery in the aliquot incubated without the C70-6 dissociated monomers.

Evidence for a maturation step in the activation of maize ADH has recently been presented (FISCHER and SCHWARTZ, 1974). Polypeptides first associate into inactive dimers which subsequently mature and assume the active configuration.

These results indicate that for the complementing mutants *Adh-S1108* and *Adh-F430*, the monomer can become an active subunit in either homo- or heterodimers. However, upon polymerization, mutant homodimers may undergo an alteration during enzyme maturation which renders them inactive, and the subunits are irreversibly altered such that upon subsequent dissociation, they cannot reassociate with other monomers to form active enzyme. Hence, in the case of the complementing *Adh* mutants, there is no need to postulate a correction mechanism between interacting subunits in the heterodimer to explain complementation. Defective maturation of the homodimer is sufficient to account for the phenomenon.

This explanation for allelic complementation could also apply to other cases reported in bacteria and fungi even where mutant haploids or homokaryons show zero enzyme activity. Formation of active heterodimers after dissociation and reassociation of mixtures of completely inactive mutant extracts would simply require that the mutant homodimers not be irreversibly altered.

The revised genetic nomenclature for maize as presented in volume 48 (1974) of the Maize Genetics Cooperation News Letter is being used in this paper.

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