Discontinuities in the topography of alcohol dehydrogenase–sodium dodecyl sulphate complexes revealed by mutagenesis and proteolysis

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Experiments utilizing proteolytic mapping of maize *Alcohol dehydrogenase-1* protein (EC 1.1.1.1; ADH) showed that, in the presence of sodium dodecyl sulphate (SDS), two discrete areas of the protein molecule were hypersensitive to digestion with *Staphylococcus aureus* V8 proteinase. These areas were mapped to points 11 and 27 kDa along the 41 kDa polypeptide. Furthermore, ADH1 proteins isolated from the ethyl methanesulphonate-induced mutants *U725* and *W586* showed both a change in electrophoretic mobility in SDS gels, and an altered V8 proteinase digestion pattern. Protein from *U725* migrated in SDS gels as though it was 2 kDa smaller than wild-type ADH protein and lacked the 11 kDa cleavage site. Protein from *W586* lacked the 30 kDa cleavage site and migrated as if it was 3 kDa smaller than wild type. The possible relationships between proteinase cleavage sites, mutations and SDS gel mobilities are discussed.

Maize alcohol dehydrogenase-1 has been under intense genetic and biochemical study for quite some time, and a number of experiments have attempted to assign a molecular mass to the protein. Freeling & Schwartz (1973) assign a monomer molecular mass of 40 kDa based on column chromatography of the active dimer. However, Laemmli (1970) SDS gel analysis of purified ADH1 suggests a molecular mass ≥ 43 kDa (Ferl *et al.*, 1983). Some resolution to this dilemma comes from the discovery of mutants of *Adh1* that cause increased migration of the ADH1 protein in SDS gels (Ferl *et al.*, 1979, 1983).

In general, mutations that alter the mobility of proteins in SDS gels without causing a change in the number of amino acid residues fall into three categories: those that alter conformational properties (DeJong *et al.*, 1978), those that alter hydrophobicity (DeJong *et al.*, 1978), and those that influence the charge on the protein (Ferl *et al.*, 1983; Noel *et al.*, 1979). Two mutations of maize alcohol dehydrogenase, *U725* and *W586*, affect both the charge and SDS gel mobility of the maize ADH1 protein (Ferl *et al.*, 1979, 1983). Furthermore it appears that when a mutation in ADH does cause an altered SDS gel mobility, changes to a more basic isoenzyme form correlate with an

increase in the SDS gel mobility. However, it must be remembered that not all charge change mutations will cause SDS gel mobility changes.

These data are consistent with the physico-chemical data on protein–SDS interactions as described by Mattice and his coworkers. They find that glycyl, arginyl, histidyl and lysyl residues play a critical role in determining the secondary structure of proteins in the presence of SDS (Mattice *et al.*, 1976; Robinson *et al.*, 1982). The key role of the basic amino acids in protein–SDS complexes offers one explanation for the mechanism by which mutations affecting the charge character of a protein may also influence the hydrodynamic character of its protein–SDS complex.

The present paper details recent efforts to define precisely the topography of the ADH protein–SDS complex by analysis of the nature of the *U725* and *W586* mutations and the effects of these mutations on the ADH protein–SDS complexes. Partial proteolysis was used to probe for prominent topographical features of the complex.

Methods

Purification of maize alcohol dehydrogenase

Purification of ADH proteins was from dry kernels as previously described (Ferl, 1980; Ferl *et al.*, 1983). ADH preparations were $>95\%$ pure as

Abbreviations used: ADH, alcohol dehydrogenase; SDS, sodium dodecyl sulphate.

determined by relative staining of bands on SDS gels. A minor contaminant appears in the W586 (and to a lesser extent in the U725) preparation, but does not interfere with this analysis.

Proteolytic mapping and gel electrophoresis

Partial proteolytic mapping was performed essentially according to Cleveland *et al.* (1977) in SDS gels according to Laemmli (1970). Purified ADH proteins were boiled in a buffer containing 0.05M-Tris/HCl, pH 6.8, 2.3% (w/v) SDS, 5% (w/v) 2-mercaptoethanol and 10% (v/v) glycerol. *Staphylococcus aureus* V8 proteinase was added in various concentrations, the samples were loaded onto a Laemmli SDS gel and run to the stacking gel/separating gel interface. The current was then turned off for 27 min to allow digestion of the ADH protein to occur. The gel was then run to completion.

Isoelectric points of the ADH variants were determined by using 8M-urea isoelectric focusing slab gels [the first dimension of the system of O'Farrell (1975)].

Results

Nature of the mutations in U725 and W586

The naturally occurring *Adh1* alleles *F* and *S* encode ADH1 proteins that differ by four amino acid substitutions, only one of which involves a charge change (Dennis *et al.*, 1984). *F* has negatively charged amino acid (aspartic acid) substituted for a neutral amino acid (asparagine) in *S*. This results in a 1-unit charge change between the *F* and *S* proteins.

In Fig. 1, the denaturing isoelectric focusing points of several ADH1 variants were determined. The unit charge change between *F* and *S* was readily apparent. Also, the *U* variants showed isoelectric focusing points indicating a 2-unit charge change from the *F* protein, and the *W* variants showed a 2-unit charge change from the *S* protein.

Proteolytic mapping of *F* and *S* protein-SDS complexes

At low proteinase concentrations very few sites on the ADH molecule were sensitive to cleavage. This resulted in a relatively simple gel pattern (Fig. 2). *S. aureus* V8 proteinase cleaves mainly at the carboxy side of glutamic acid residues (Houmar & Drapeau, 1972). The derived amino acid sequence shows that ADH *F* contains 28 glutamic acid residues (see Fig. 4a) (Dennis *et al.*, 1984). Clearly, at low proteinase concentrations, very few of the glutamyl residues in the ADH-SDS complex were available for proteinase recognition and/or cleavage since the observed cleavage events were localized to specific hypersensitive sites along the

Fig. 1. Diagrammatic representation of the denaturing isoelectric focusing points of the ADH1 variants

Note the intrinsic charge relationship between the variants. The pH curve of the gel was determined as described by O'Farrell (1975).

ADH protein chain. Increasing the proteinase concentration (or the length of digestion time; results not shown) resulted in an increasing amount of degradation. The digest bands in Fig. 2 are labelled according to their true molecular mass as determined by the sequence evidence presented below rather than by comparison with markers.

Fig. 3 shows a graphic representation of the change in digestion pattern with increasing proteinase concentration. Initial cleavage of the ADH *F* protein yielded four bands, at 30, 27, 14 and 11 kDa. Upon further digestion, the 27 kDa and 30 kDa bands disappeared, the 11 kDa and 14 kDa bands became more intense, and a 16 kDa band became apparent.

As part of a separate study to confirm the DNA sequence data of *Adh1-F* cDNA clones, the 16 kDa and 14 kDa peptide bands have been subjected to amino acid sequence analysis (Dennis *et al.*, 1984). Results confirmed that the proteinase is indeed cleaving the ADH protein at glutamyl residues, and allowed the assignment of the precise location of the cleavage sites (Fig. 4a) and the ordering of the proteolytic fragments into the physical map presented in Fig. 4(b).

It was also observed, however, that the 14 kDa band is actually a doublet of two bands with slightly different proteinase-generated *N*-terminal ends. The sequence data in Fig. 4(a) indicate that there are two glutamyl residues separated by three amino acids in that region. Both of these glutamyl residues were apparently available to proteinase attack. At the 11 kDa site there are glutamyl residues separated by only two amino acids.

Fig. 2. V8 proteolysis of ADH1-F demonstrating the early preferential cleavage pattern

There is 5 μ g of ADH/lane and the amount of V8 proteinase in each lane is: 1, 0 ng; 2, 1.0 ng; 3, 2.5 ng; 4, 5 ng; 5, 10 ng; 6, 25 ng; 7, 50 ng; 8, 100 ng. The gel is 10% acrylamide. At this concentration of acrylamide, the 27 kDa and 30 kDa bands are well resolved, but the 14 kDa and 16 kDa bands are barely separated. Lane M contains markers (Bio-Rad): phosphorylase *b* (93 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soya bean trypsin inhibitor (21 kDa) and lysozyme (14 kDa).

Fig. 3. Graph of the appearance and disappearance of proteolytic bands

The experiment in Fig. 2 was repeated and run on a 15% polyacrylamide gel to allow maximum resolution of the 11, 14 and 16 kDa fragments. The gel was stained with Coomassie Blue and scanned with an E-C 910 densitometer. Raw peak area values were corrected for the size of the fragment. The data are presented as the relative number of molecules present as each of the fragments, arbitrarily assuming 90 molecules of intact ADH protein at the start. On 15% gels, the 27 and 30 kDa fragments essentially comigrate, but the 14 and 16 kDa fragments are well separated.

However, only one seems to be open to attack since amino acid sequencing of the 16 kDa band shows no evidence of multiple proteinase-generated *N*-terminal ends.

Proteolytic mapping of U725 and W586

When the U725 and W586 proteins were subjected to proteolysis (Fig. 5), the resulting patterns were simpler than those of the F and S proteins. The U725 protein lacked the 11 kDa proteinase-hypersensitive site and thus showed only 27 kDa and 14 kDa fragments. Furthermore, the 27 kDa fragment migrated slightly faster than its F counterpart in the SDS gel at a rate more appropriate for its size as deduced from the derived amino acid sequence (Dennis *et al.*, 1984). The change in migration of the U725 27 kDa fragment reflected the change in SDS mobility initially observed in the intact U725 protein (Ferl *et al.*, 1979, 1983).

The W586 protein lacked the 27 kDa proteinase-hypersensitive site, but retained the 11 kDa site. Its proteolytic pattern showed 30 kDa and 11 kDa fragments. Furthermore, the 30 kDa fragment migrated faster than its F and S counterparts in the

Fig. 4. (a) Derived amino acid sequence of maize ADH1-F (Dennis *et al.*, 1984) and (b) V8 proteolytic cleavage map of the maize ADH-SDS complex with the N-terminus to the left. Cleavage/mutation sites in (a) are highlighted.

SDS gel at a rate more consistent with the derived amino acid sequence (Dennis *et al.*, 1984). Like U725, this change in the apparent size of the 30 kDa fragment reflected the apparent change in size of the intact W586 protein (Ferl *et al.*, 1983). It should be noted at this point that other proteins of the U and W series that have SDS gel migrations identical to those of F and S show the standard F and S proteolytic digestion pattern (R. J. Ferl, unpublished work).

Discussion

Several important topographical features of the maize ADH-SDS protein complex have been identified in this study. Two aberrant regions in the complex map at 11 kDa and 27 kDa along the

protein molecule. Only in these regions of the protein-SDS complex is the ADH polypeptide chain available for digestion at low proteinase concentrations. It appears that these regions represent peculiar structural features of the ADH-SDS complex that are responsible for the hypersensitive cleavage and SDS gel migrations slower than that predicted from the actual sequence.

Analysis of mutant ADH proteins strengthens this conclusion. In several studies we have examined the SDS gel migration of ten ADH variants (Ferl *et al.*, 1983). Only two, U725 and W586, have shown migrations faster than F or S, migrations that more closely approach that expected from the true molecular mass of ADH. U725 was derived by ethyl methanesulphonate mutagenesis of F, while W586 was similarly derived from S (Schwartz,

Fig. 5. V8 proteolytic digestion of ADH variants

For each set of digests, the following amounts of V8 proteinase were used: 0, 1.0, 5, 25 and 100 ng. Note the altered digestion patterns of U725 and W586, as well as the similarity of F and S. Further experiments have shown that U and W variants other than U725 and W586 have patterns similar to those of F and S.

1969). The present study suggests that these proteins have 2-unit charge changes from the progenitor ADHs. The constraints of the genetic code dictate that U725 and W586 have a lysine (positively charged amino acid, coded by AAA or AAG) replacing a glutamic acid (negatively charged, coded by GAA or GAG) to account for the two-step change if ethyl methanesulphonate mutagenesis caused a single G-to-A transition in the *Adh* gene. While a single transition is the most likely ethyl methanesulphonate mutagenic event (Krieg, 1963), less likely events such as transversion, two mutations per codon and small rearrangements cannot be ruled out by the present data.

The mutations in U725 and W586 have simultaneously changed the SDS gel mobility of the intact ADH to a more 'correct' migration (Fertl *et al.*, 1983), and caused the loss of one of the proteinase-hypersensitive sites. The apparent change from a glutamyl to a lysyl residue in those two peculiar regions of the ADH molecule (11 kDa and 27 kDa) causes a simultaneous change in the ADH hydrodynamic character and V8 cleavage pattern. The simplest and most direct hypothesis is that the U725 and W586 mutations compensate the structural perturbation of the ADH-SDS complex which normally exists at the 11 kDa and 27 kDa sites.

When a glutamyl residue within one of the proteinase-hypersensitive areas is lost by muta-

tion, the recognition site for the proteinase would be lost automatically. However, the observed effect is more than simply the loss of the recognition sequence of the proteinase. First, the loss of cleavage site strictly coincides with a change in migration in SDS. Second, in W586, neither one of the two glutamyl residues normally open to V8 attack at the 27 kDa site are cleaved. Since both are normally open for attack, and there is no simple way that W586 could have lost both glutamyl residues in a single mutagenic event, it would appear that it is the change in character of the ADH-SDS complex in W586 and U725 that is responsible for both the altered migrations and loss of hypersensitive V8 cleavage sites.

One possible hypothesis to explain the change in the character of the ADH-SDS complex lies in altered SDS binding. For a protein the size of ADH, the 5% mobility change in the case of U725 would require a change in binding of a large number of SDS molecules. Such large-scale changes in SDS binding would at first seem impossible to accomplish via a single amino acid substitution, since only one SDS molecule is bound for every two amino acids (Pitt-Rivers & Impiombato, 1968). SDS binding may still be a possible explanation due to the co-operative nature of SDS binding changes (Robinson & Tanford, 1975), especially in the varying salt concentrations of the Laemmli gel system while undergoing discontinuous electrophoresis.

An alternative, and perhaps more likely, explanation for the alterations in SDS gel mobilities comes from an examination of the secondary structures of protein-SDS complexes. The present data are consistent with the described important role of lysyl residues in determining the secondary structure of protein-SDS complexes by strongly contributing to helical formations (Robinson *et al.*, 1982). The 11 kDa and 27 kDa sites then are areas of distinctive ADH-SDS secondary structure that serve both as impediments to gel migration and as substrates for V8 proteinase. It is these secondary structures that are removed by the mutations in U725 and W586.

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