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Subunit interface mutants of rabbit muscle aldolase form active dimers

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Abstract

We report the construction of subunit interface mutants of rabbit muscle aldolase A with altered quaternary structure. A mutation has been described that causes nonspherocytic hemolytic anemia and produces a thermolabile aldolase (Kishi H et al., 1987, *Proc Natl Acad Sci USA 84*:8623-8627). The disease arises from substitution of Gly for Asp-128, a residue at the subunit interface of human aldolase A. To elucidate the role of this residue in the highly homologous rabbit aldolase A, site-directed mutagenesis is used to replace Asp-128 with Gly, Ala, Asn, Gln, or Val. Rabbit aldolase D128G purified from *Escherichia coli* is found to be similar to human D128G by kinetic analysis, CD, and thermal inactivation assays. All of the mutant rabbit aldolases are similar to the wild-type rabbit enzyme in secondary structure and kinetic properties. In contrast, whereas the wild-type enzyme is a tetramer, chemical crosslinking and gel filtration indicate that a new dimeric species exists for the mutants. In sedimentation velocity experiments, the mutant enzymes exist as mixtures of dimer and tetramer at 4 °C. Sedimentation at 20 °C shows that the mutant enzymes are >99.5% dimeric and, in the presence of substrate, that the dimeric species is active. Differential scanning calorimetry demonstrates that T_m values of the mutant enzymes are decreased by 12 °C compared to wild-type enzyme. The results indicate that Asp-128 is important for interface stability and suggest that 1 role of the quaternary structure of aldolase is to provide thermostability.

Keywords: aldolase; quaternary structure; site-directed mutagenesis; subunit interface; thermolability

Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) is a highly conserved glycolytic enzyme that catalyzes the reversible aldol condensation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate into fructose-1,6-bisphosphate. Aldolases have been divided into 2 classes based on their mechanism (Rutter, 1964). Class I aldolases are cofactor-independent, whereas class II enzymes require a divalent metal ion for catalysis. Class I aldolases are found in most eukaryotes and some prokaryotes. The eukaryotic class I aldolases are invariably tetrameric (Penhoet et al., 1967; Lebherz, 1972), whereas the prokaryotic class I enzymes are variable in their quaternary structure (Baldwin & Perham, 1978; Rudolph et al., 1992). Class II aldolases are found in most prokaryotes, green algae, and fungi, usually existing as dimers (Baldwin et al., 1978).

In vertebrates, aldolase exists as 3 isozymes, A, B, and C, with different catalytic properties (Penhoet et al., 1969), tissue distribution (Lebherz & Rutter, 1969), and developmental expression (Weber, 1965). Vertebrate isozymes are thought to operate through a common mechanism involving Schiff base formation between the ε-amino group of Lys-229 and the carbonyl carbon of the ketose (Horecker et al., 1972). Aldolase A from muscle exists as an isologous homotetramer, with a subunit molecular mass of 40 kDa (Penhoet et al., 1967). Rabbit aldolase A is well characterized both functionally (Horecker et al., 1972; Berthiaume et al., 1993; Morris & Tolan, 1993) and structurally; a 2.7-Å structure has been reported (Sygusch et al., 1987), and it is now resolved to 1.9 Å (J. Sygusch, pers. comm.). This structure shows known catalytic residues near the center of each subunit. The vertebrate aldolase A tetramer is highly stable. Free subunits are undetectable at high dilution, and subunit exchange between tetramers is undetectable under nondenaturing conditions (Lebherz, 1975).

The role of quaternary structure of enzymes is not well understood, especially for homo-oligomeric enzymes such as aldolase. Possible roles of quaternary structure include formation of the active site, increased solubility, increased stability, decreased osmotic pressure in the cell, and the requirement of quaternary structure for the active conformation of the monomer (Eisenstein & Schachman, 1989). The latter proposed role of quaternary structure is analogous to quaternary constraint de-

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Abbreviations: ADH, alcohol dehydrogenase; β-ME, β-mercaptoethanol; DMS, dimethyl suberimidate; DSC, differential scanning calorimetry; DTT, dithiothreitol; Fru-1,6-P_2, fructose-1,6-bisphosphate; K_d, dissociation constant; TEA, triethanolamine; TIM, triosephosphate isomerase; $T_{0.5}$, half-maximal activity in thermal inactivation assays; T_m, melting temperature in calorimetric assays.

scribed for hemoglobin (Ackers et al., 1992). Quaternary structure of aldolase isozymes is highly conserved yet does not appear to play a functional role. Aldolase subunits from species as divergent as chicken and wheat form active hybrid tetramers (Swain & Lebherz, 1986), and rabbit isozymes form hybrids that are kinetically indistinguishable from a mixture of homotetramers (Penhoet & Rutter, 1971). The role of quaternary structure in aldolase can be investigated by disruption of subunit interfaces using site-directed mutagenesis.

A mutation in human aldolase A that causes hereditary hemolytic anemia has been described (Kishi et al., 1987). The mutation replaced Asp-128 with Gly, abbreviated D128G. The mutant cDNA was cloned and expressed in *Escherichia coli*, and additional mutants, D128E and D128S, were constructed using site-directed mutagenesis (Takasaki et al., 1990). The mutant enzymes' specific activity ranged from 15% to 92% of wild-type activity. All of these mutants were thermolabile, although glycerol stabilized the activity of D128G at higher temperature. These proteins were altered in their CD spectra and were more susceptible to tryptic digestion than wild-type aldolase. These observations indicated that Asp-128 may play a structural role in aldolase.

In this report, the structural role of Asp-128 in the highly homologous rabbit aldolase A (Tolan et al., 1984; Sakakibara et al., 1985) is described. Examination of the crystal structure of rabbit aldolase A (Sygusch et al., 1987) shows that Asp-128 is in proximity with the peptide backbone of the adjacent subunit. The carboxylate oxygens of Asp-128 have 4 potential hydrogen bonding donors; all are backbone amide hydrogen atoms, 3 of which are on the adjacent subunit. Comparison of the characteristics of rabbit aldolase D128G with those of human D128G showed that mutations in both species had the same effect. Additional mutants were constructed to represent uncharged and hydrophobic amino acid side chains of different sizes. All mutant enzymes possessed only slightly altered specific activity, indicating that substitution at this site did not drastically affect function. However, any substitution at Asp-128 produced a thermolabile enzyme. Furthermore, these mutants had altered quaternary structure, forming dimers under certain conditions. This evidence suggests that the native quaternary structure of aldolase is not essential for catalysis but is, however, crucial for stability.

Results

Comparison of rabbit and human D128G enzymes

Mutant aldolases were constructed by site-directed mutagenesis (Taylor et al., 1985) and were expressed in and purified from *E. coli*. Purified recombinant rabbit D128G enzyme was analyzed structurally and functionally and compared to the previously described human D128G enzyme (Kishi et al., 1987; Takasaki et al., 1990). Rabbit D128G had a V_{max} of 13 U/mg at 30 °C, which was similar to 15 U/mg for the wild-type rabbit enzyme (Table 1). Human enzymes were reported to have specific activities of 6 U/mg for D128G and 17 U/mg for wild-type using the same assay (Takasaki et al., 1990). Because Asp-128 mutants were inactivated approximately 50% faster than wild-type enzyme in dilute solutions at 4 °C (data not shown), the apparent difference between kinetic constants of the mutant enzymes may have arisen from slight differences in methods. In our work, we tried to minimize this inactivation by diluting the enzyme immediately prior to each assay.

Table 1. *Kinetic constants of rabbit aldolase Asp-128 variants*

Source	V_{max} (U/mg)	K_m (μM)
Rabbit muscle	11.9 ± 1.0	17.2 ± 2.1
pPB14 (wild type)	14.8 ± 1.3	17.7 ± 2.2
pD128G	12.7 ± 0.4	11.7 ± 0.7
pD128A	7.4 ± 0.9	18.0 ± 2.9
pD128N	13.3 ± 1.1	15.4 ± 2.0
pD128Q	18.8 ± 0.5	11.0 ± 0.3
pD128V	11.5 ± 0.5	13.0 ± 1.0

The rabbit D128G enzyme was subjected to CD and thermal inactivation assays, similar to those reported for the human enzyme. The CD spectrum of aldolase D128G from rabbit showed slightly lower intensity from 185 to 240 nm than the wild-type enzyme (Fig. 1). A slightly greater difference between the mutant and wild-type human enzymes was reported (Takasaki et al., 1990). In thermal inactivation assays, the irreversible inactivation of wild-type and mutant enzymes was measured from 30 °C to 45 °C. Rabbit D128G retained 50% of its activity ($T_{0.5}$) after preincubation at 40 °C, compared to 42 °C reported for human D128G (Takasaki et al., 1990). The experiments described above validated the use of recombinant rabbit Asp-128 mutants to examine the defect that causes hereditary hemolytic anemia in humans.

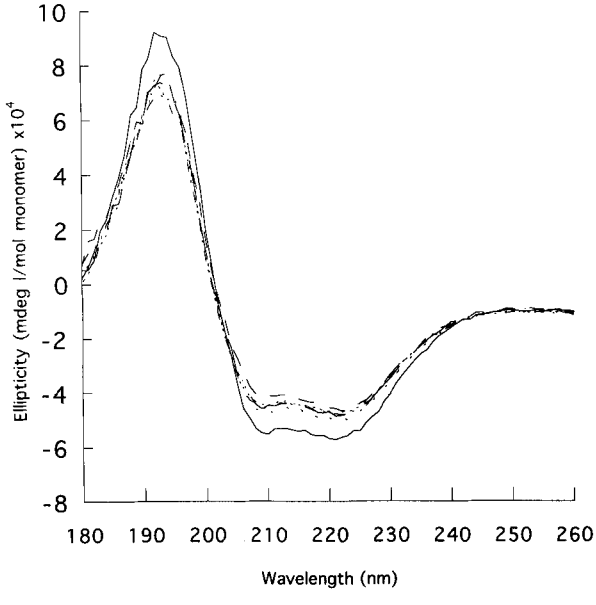

Fig. 1. CD spectra of wild-type and mutant rabbit aldolases. Spectra were determined at 1.0 mg/mL protein at 20 °C and data normalized to mdeg·L/mol monomer. Wild-type (——) and D128G (—— ——), D128A (- - -), D128N (·····), D128Q (· · · ·), and D128V (— · —) are shown.

Characterization of rabbit mutant enzymes

Changes at position 128 that might stabilize the enzyme relative to D128G were made by substitution of Ala, Asn, Gln, or Val. The Ala substitution might increase stability if the entropy of the unfolded state were important (Hecht et al., 1986). The Asn and Gln substitutions might retain possible hydrogen bonding capacity. The Gln and Val substitutions were made to assess if packing or hydrophobic interactions, respectively, might be important. The K_m values of the mutants ranged from 11 to 18 μM (Table 1) and were slightly less than wild type. The V_{max} values of these mutants ranged from 7 to 19 U/mg and were not significantly different from those measured for normal aldolase A or from those previously reported (Penhoet & Rutter, 1971; Morris & Tolan, 1993). The largest difference was with the D128A enzyme, which was 50% less active than wild-type.

The CD spectra for wild-type and mutant aldolases showed that secondary structure was not dramatically changed in any of the mutant enzymes. The CD spectra of all mutants closely resembled wild-type (Fig. 1), with slightly lower intensity from 185 to 240 nm. The D128A enzyme was the most altered in its CD spectrum, although it was very similar to the other mutant enzymes. It is uncertain whether this effect was greater at the much lower protein concentration used for kinetic determinations and resulted in the lower V_{max} of D128A.

In thermal inactivation assays, all mutant enzymes behaved similarly to D128G, where activity dropped sharply with increased preincubation temperature. Although the wild-type enzyme remained fully active after preincubation at 45 °C, the mutants were inactivated following preincubation above 35 °C, having $T_{0.5}$ values between 40 °C and 42 °C (data not shown).

A more precise determination of the thermal instability of these mutant enzymes was done using DSC. The shapes of the unfolding transitions were different for the wild-type and mutant enzymes (Fig. 2), and deconvolution of the data indicated 3 overlapping transitions for the wild-type and 2 for the mutant enzymes. For these transitions, the wild-type enzyme exhibited T_m values of 55.7 °C, 58.7 °C, and 61.2 °C, whereas the mutant enzymes had T_m values of 43.9–45.9 °C and 46.0–47.1 °C (Table 2). Wild type had a calorimetric enthalpy change (ΔH_{total}) of 214 kcal/mol monomer, whereas the mutant enzymes had ΔH_{total} values from 140 to 169 kcal/mol monomer. Although heating above the T_m was irreversible for both wild-type and mutant enzymes, heating to 35 °C, just below the T_m of the mutants, showed a reversible change in heat capacity (data not shown). In summary, there was no significant correlation between the type of substitution at Asp-128 and kinetic constants, secondary structure, inactivation, or thermal melting.

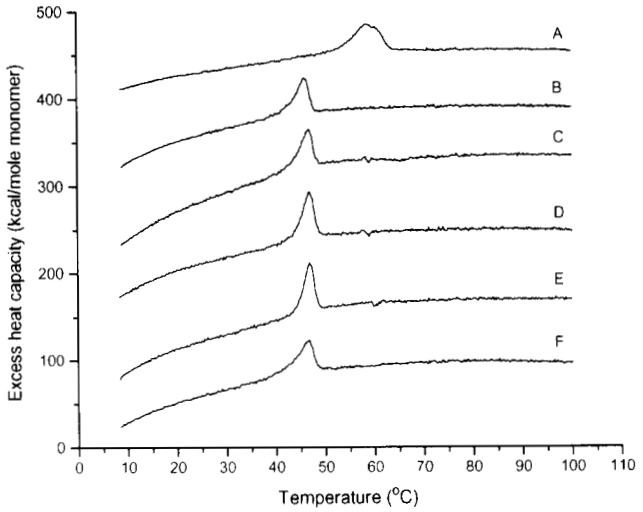

Fig. 2. Excess heat capacity versus temperature for wild-type and mutant aldolases. Experiments were performed at 2 mg/mL protein at a scan rate of 45 °C/h. Calorimetric profiles were normalized to kcal/mol monomer and were offset on the ordinate for clarity. A, Wild type; B, D128G; C, D128A; D, D128N; E, D128Q; F, D128V.

Quaternary structure

Because the amino acid at position 128 is near the subunit interface, we performed additional structural assays to assess if any of the effects described above were attributable to changes in quaternary structure. Crosslinking of subunits with DMS at 4 °C and 0.1 mg/mL protein indicated that all of the mutants were principally dimeric under these conditions (Fig. 3). Following SDS-PAGE, wild-type enzyme yielded a characteristic pattern of 4 bands corresponding to crosslinked monomer, dimer, trimer, and tetramer (Fig. 3, lane 2), whereas an uncrosslinked control (i.e., without DMS treatment; lane 1) gave a single band corresponding to monomer. Mutant enzymes (Fig. 3, lanes 3–7)

Table 2. *Energetics of unfolding transitions for rabbit aldolase Asp-128 variants*

Source	T_m1[a,b]	$\Delta H1$[c]	T_m2	$\Delta H2$	T_m3	$\Delta H3$	ΔH_{total}
pPB14	55.7 ± 1.0	61 ± 27	58.7 ± 0.08	119 ± 33	61.2 ± 0.1	34 ± 8	214 ± 43
pD128G	44.0 ± 0.3	67 ± 12	46.0 ± 0.04	73 ± 10	—[d]	—	140 ± 16
pD128A	45.0 ± 0.3	75 ± 11	46.8 ± 0.03	71 ± 10	—	—	146 ± 15
pD128N	45.6 ± 0.3	80 ± 15	47.0 ± 0.04	83 ± 15	—	—	162 ± 21
pD128Q	45.9 ± 0.3	67 ± 17	47.1 ± 0.05	77 ± 16	—	—	143 ± 23
pD128V	43.9 ± 0.2	91 ± 9	46.5 ± 0.03	78 ± 7	—	—	169 ± 11

[a] T_m values given in °C.
[b] Transition numbers assigned from lowest to highest T_m; transition numbers do not imply similar transitions for wild-type and mutant enzymes.
[c] ΔH values in units of kcal/mol monomer.
[d] Mutant enzymes unfolded with 2 transitions versus 3 for the wild-type enzyme.

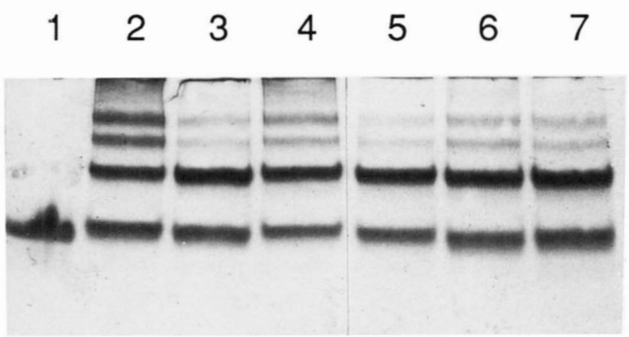

Fig. 3. SDS-PAGE of crosslinked wild-type and mutant aldolases. Lane 1, uncrosslinked wild type; lane 2, wild type; lane 3, D128A; lane 4, D128G; lane 5, D128N; lane 6, D128Q; lane 7, D128V. Crosslinking was performed at 0.1 mg/mL protein, 2 mg/mL DMS, and incubation was at 4 °C for 3 h. Either 10 μg (lane 1) or 20 μg (lanes 2–7) were loaded.

showed a much higher proportion of dimer product and correspondingly less trimer and tetramer product. Although the ratio of dimer to tetramer increased when crosslinking was performed at a higher temperature or lower protein concentration (data not shown), some tetramer was detected in all experiments. This may indicate interoligomeric crosslinking or a mixture of oligomeric species.

The molecular mass of the mutant enzymes was estimated by gel filtration, to verify that the crosslinking pattern truly reflected a decrease in tetramer stability and was not due to a conformational change restricting the access of DMS to certain lysine residues. In experiments at 20 °C with wild-type enzyme, aldolase activity eluted at a volume corresponding to 155–180 kDa, whereas the mutants D128G and D128Q eluted at a volume corresponding to 68–83 kDa, which approximates the predicted molecular mass of a dimer. After gel filtration of D128G at 4 °C, both putative dimer and tetramer peaks were detected (data not shown), suggesting that the smaller species was indeed a dimer and not a tetramer with an altered conformation.

Sedimentation velocity experiments confirmed the sizes of the species observed in gel filtration. At 4 °C, the ratio of dimer to tetramer could be accurately determined and varied for different mutant enzymes at the same concentration, 1.0 mg/mL (Fig. 4). An extrapolated sedimentation coefficient ($s_{20,w}$) of 7.0S was calculated for the tetramer, in agreement with a previously reported value of 7.35S (Taylor & Lowry, 1956), whereas the dimer sedimented with a velocity of 4.8S. The detection of distinct peaks representing dimer and tetramer indicated that these species were not in rapid equilibrium. The tetramer was most stable in the Gln mutant and the least stable in the Ala and Val mutants. This suggested that hydrogen bonds may be important in stabilizing the tetramer, as predicted from the crystal structure.

Sedimentation velocity experiments showed that the dimer/tetramer ratio was dependent on temperature. Experiments at 20 °C showed a dramatic shift of the dimer/tetramer ratio toward dissociation (Fig. 5A). For example, D128Q is 31% dimer at 4 °C, whereas at 20 °C it is >99.5% dimer. A similar shift was observed for all Asp-128 mutant enzymes (data not shown). This temperature-dependent dissociation was reversible by preincubation at 20 °C prior to sedimentation at 4 °C (data not shown).

The assays for quaternary structure were performed under different conditions than those used to determine the enzymatic activity and thermal stability. To ensure that the behavior of the mutant enzymes was not different than expected under these different conditions, active enzyme sedimentation experiments were performed in the presence of substrate under conditions similar to those used for activity measurements. Sedimentation was at 20 °C, with an enzyme concentration of 5 μg/mL, and the product of the reaction was monitored at 240 nm (Dische & Landsberg, 1960). These experiments showed that the enzymatic activities of wild-type and mutant enzymes sedimented at different velocities (Fig. 5B). The hydrazone product was detected up to a position in the gradient that corresponded to the position to which wild-type and D128Q enzymes sedimented in similar gradients in the absence of substrate (Fig. 5A). The shape of the curve for D128Q was distinctly different than wild type, showing a slow decrease in the amount of product up to the position of the dimer, and forming much less product than wild type. These observations suggested that the mutant enzyme was inactivated during preincubation and sedimentation. All of the mutant enzymes gave a pattern similar to D128Q (data not shown). These assays indicated that the activity of the mutant enzymes detected in the kinetic and thermal inactivation assays was that of the dimer. The fact that wild-type and mutant enzymes showed virtually identical activity in kinetic assays indicated that the dimer was approximately as active as the tetramer.

Discussion

The tetrameric structure of rabbit muscle aldolase has been well described (Penhoet et al., 1967; Horecker et al., 1972). The stability (Lebherz, 1972) and conservation (Lebherz & Rutter, 1969) of this tetramer was well established, although the role of this structure has remained unknown. Some previous work had indicated that the tetrameric structure of aldolase was not essential for catalysis (Chan, 1970; Chan & Mawer, 1972; Chan et al., 1973). Matrix-bound subunits of aldolase were generated by covalently binding enzyme to a Sepharose matrix, denaturing with 8 M urea, then washing with a renaturing buffer. Renatured subunits were found to have partial activity that was more sensitive to increased pH, temperature, and denaturant concentration than wild-type activity (Chan & Mawer, 1972). Evidence from kinetics of refolding also indicated that 1 or more intermediates in the folding pathway were active (Chan et al., 1973). Although these previous studies provided evidence that the native structure was not essential for catalysis, these experiments depended on indirect means of assaying for the presence of subunits. Our work using recombinant mutant aldolases clearly showed that the tetrameric structure was not required for catalysis.

Although active subunits of many enzymes are difficult to isolate, in some cases it is possible. NADP-dependent malate dehydrogenase was reported to be catalytically active as tetramer, dimer, and monomer, with a K_d dependent on pH and ionic strength (Iglesias & Andreo, 1990). These 3 forms exhibited different kinetic properties, with tetramer being the most active and monomer the least active. Carbamoyl-phosphate synthetase was shown to be active as dimer and monomer, with its equilibrium dependent on concentration (Lusty, 1981). Human $\beta_1\beta_1$ ADH dimers and monomers were reported with different kinetic properties using a freeze–thaw procedure in liquid nitrogen to disrupt the native structure (Ehrig et al., 1993). More recently,

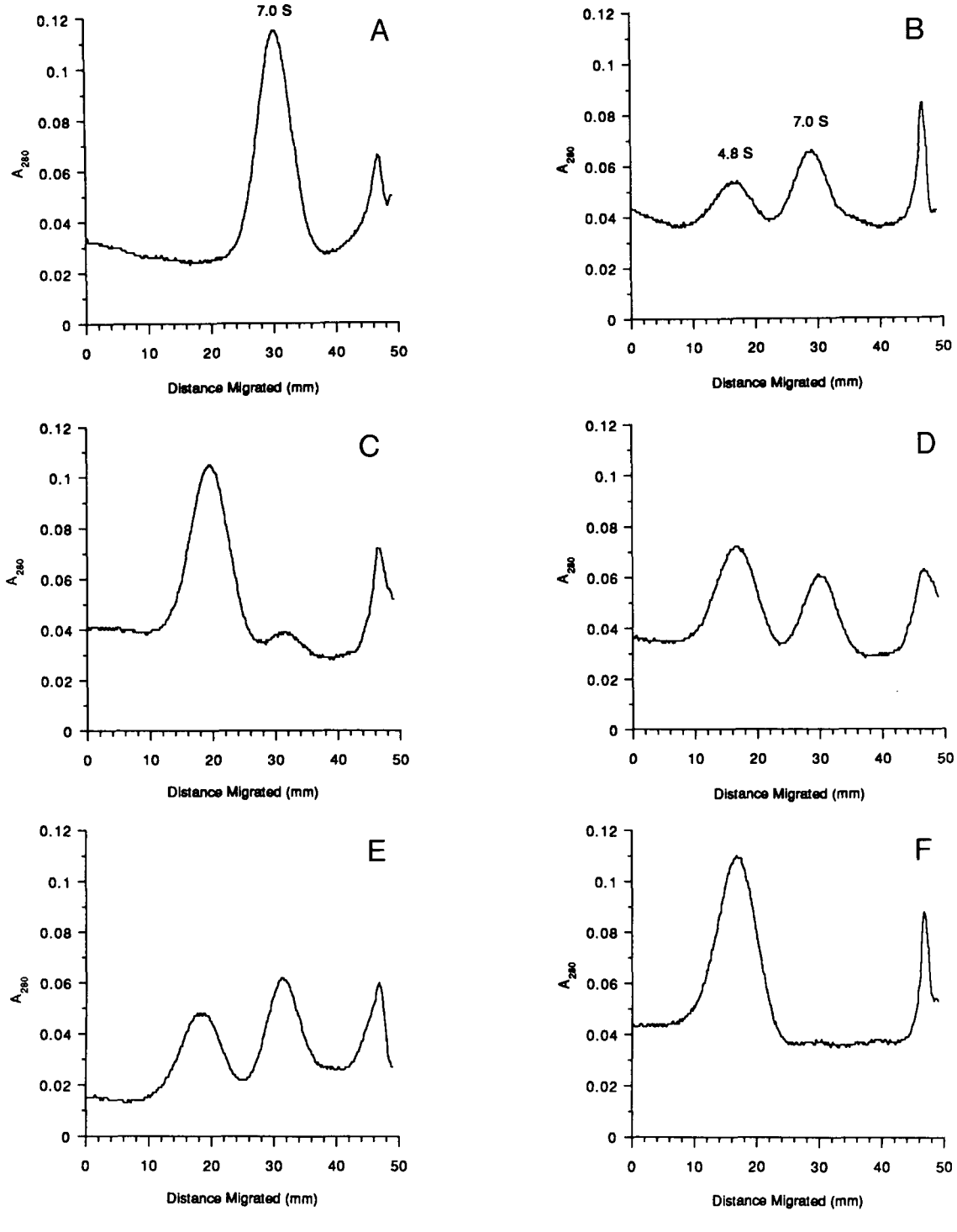

Fig. 4. Sucrose density gradient profiles of wild-type and mutant aldolases at 4 °C. **A:** Wild type. **B:** D128G. **C:** D128A. **D:** D128N. **E:** D128Q. **F:** D128V. Sedimentation coefficients are indicated above the dimer (4.8S) and tetramer (7.0S) peaks. Aliquots (100 μL) of protein (1.0 mg/mL) were layered on 5–20% sucrose gradients and centrifugation was at 38,000 RPM for 20 h. Protein was detected by absorbance at 280 nm in a flow cell. Sedimentation is depicted from left to right.

site-directed mutagenesis has been used to generate subunits of enzymes. A subunit interface mutant, H147A, of the trypanosomal TIM dimer dissociated into monomers upon dilution, which results in almost complete inactivation (Borchert et al., 1993). Also, a mutant, N78D, of yeast TIM dissociated into inactive monomers under high ionic strength (Casal et al., 1987).

An amino acid substitution in rabbit aldolase A, D128G, had a similar effect as the analogous substitution in human aldol-

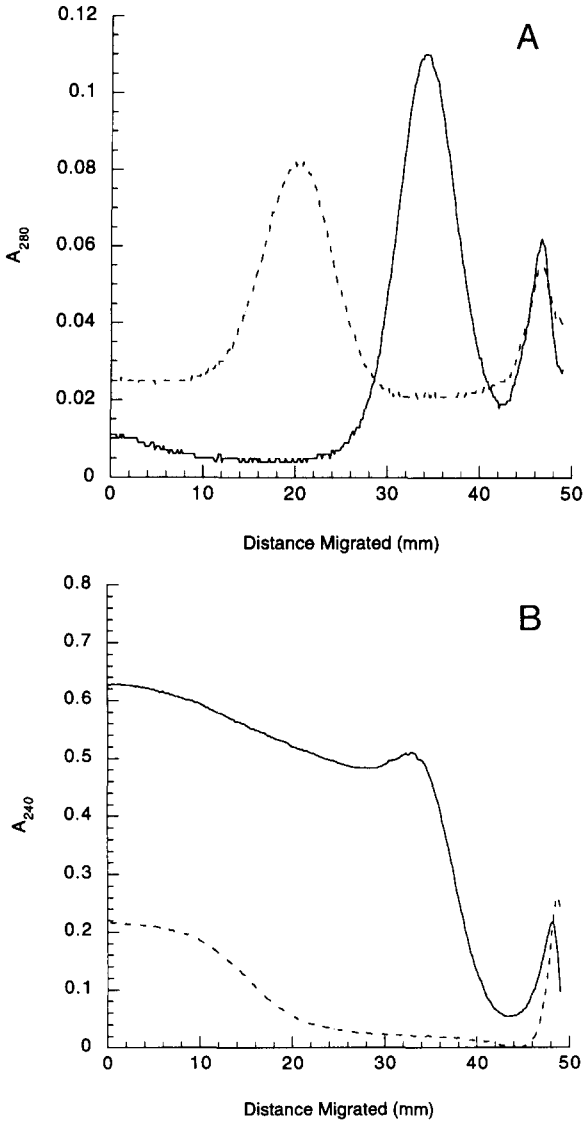

Fig. 5. Sucrose density gradient profiles at 20 °C. Wild type (—) and D128Q (· · · · · · ·) are shown. Sucrose gradients (5–20%) were centrifuged at 38,000 RPM for 14.5 h. Sedimentation is depicted from left to right. **A:** Sedimentation at 1.0 mg/mL protein, with protein detected by absorbance at 280 nm. **B:** Active enzyme sedimentation at 5 μg/mL protein, 2 mM Fru-1,6-P_2, 2 mM hydrazine sulfate, with product detected by absorbance at 240 nm.

ase A, which causes hereditary nonspherocytic hemolytic anemia. Because there are only 3 amino acid differences between rabbit and human aldolase A (Tolan et al., 1984; Sakakibara et al., 1985), this behavior was not unexpected.

Extension of these studies using rabbit aldolase A with different substitutions at Asp-128 resulted in enzymes in which the secondary structure and kinetic properties were largely intact, but in which the quaternary structure was disrupted. The slight difference in CD spectra between wild-type and mutant enzymes may have resulted from quaternary, not secondary, structural changes. Although not directly assayed, tertiary structure was apparently not significantly altered in the mutants, as judged from the values of kinetic constants and the ability to be eluted with substrate from Cm-Sepharose. The wide range in dimer/tetramer ratio among wild-type and Asp-128 mutant enzymes suggests that certain properties of the amino acid side chain determine the strength of the subunit interaction.

Although different assays were done under different conditions of protein concentration and temperature, we attempted to use conditions that allowed correlation of results between assays. For example, CD was performed at the same protein concentration and temperature (1 mg/mL, 20 °C) as sedimentation experiments, indicating that the secondary structure of the dimer was being examined for the mutant enzymes. More importantly, to demonstrate that the oligomeric state of the mutant enzymes in kinetic assays (1 μg/mL, 30 °C) was dimeric, sedimentation in the presence of substrate was performed (5 μg/mL, 20 °C). The inactivation of mutant enzyme during sedimentation at a low protein concentration prevented accurate determination of the sedimentation coefficient of the active species. However, similar results were obtained with centrifugation at a much higher velocity, suggesting that the activity profile generated was not an artifact of the duration of the experiment (data not shown). Moreover, sedimentation at the same temperature, but at a higher protein concentration (1 mg/mL, 20 °C), showed the mutant enzymes sedimented predominantly as dimer. It seems unlikely that a lower protein concentration would produce more of the tetrameric species, and this is inconsistent with our results showing increased dissociation of D128G with lowered concentration (data not shown).

The dramatic effects on the dimer/tetramer ratio suggested that the overall contribution of Asp-128 to the strength of the subunit interaction is large. The observation that the Asn and Gln substitution formed significantly more tetramer than the Ala and Val substitutions supports the hypothesis that hydrogen bonds at position 128 are important for interface stability. It is not certain why the Gly substitution yields a lower dimer/tetramer ratio than the Ala or Val substitution, but interactions involving water may be important. These observations are also consistent with analysis of the crystal structure (Sygusch et al., 1987), which shows 4 backbone amide hydrogen atoms as potential hydrogen bond donors, 3 of which are on the adjacent subunit.

Quaternary structure appeared to be important for the thermal stability of aldolase. Because thermal inactivation assays were done under conditions where the mutants were overwhelmingly dimer, this assay measured the dimer to unfolded transition and not the tetramer to dimer transition. Therefore, the $T_{0.5}$ values of the mutants indicated that the stability of the dimer was similar among mutant enzymes. Furthermore, DSC showed that the mutant enzymes undergo unfolding transitions with similar T_m and ΔH values. The D128N substitution, which should retain the ability to form the 1 proposed intramolecular hydrogen bond, failed to increase the T_m or ΔH substantially compared to D128G, consistent with the idea that loss of quaternary, not tertiary, interactions is responsible for loss of thermostability. This suggested that Asp-128 is primarily involved in intersubunit interactions and the aldolase dimer is intrinsically less stable than the tetramer. This hypothesis is supported by the thermolability of matrix-bound subunits observed by Chan (1970). DSC was not able to detect the expected dissociation of tetramer to dimer occurring between 4 °C and 20 °C. This indicated that the change in heat capacity for this transition is small or occurs very close to 4 °C, where it would be difficult to detect.

Calculations of the solvent exposure of Asp-128 were performed using the 2.3-Å rabbit aldolase A structure (J. Sygusch, pers. comm.). Asp-128 in the tetramer was calculated to be 6% solvated versus 49% in the dimer. These values suggested that this residue is largely buried in the tetramer but considerably exposed in the dimer. This is consistent with our results that show that replacing Asp-128 affects the dimer/tetramer ratio and not the stability of the tertiary structure. Proposed intersubunit interactions confer on the native tetramer additional stability that is not inherent in the monomer.

By secondary structural and kinetic analysis, substitution at Asp-128 does not significantly alter the properties of aldolase A. Yet, substitution at this position dramatically affects the strength of the subunit interaction at this interface, favoring formation of dimers. The specific activity of the resulting aldolase dimer is approximately equal to that of the tetramer. Furthermore, replacement of Asp-128 produces a thermolabile enzyme. These studies indicate that quaternary structure, at least on this interface, confers thermal stability on this critical glycolytic enzyme. Interactions at the other interface may contribute different properties to the structure or function of this enzyme.

Materials and methods

Materials

Restriction endonucleases, T4 DNA ligase, exonuclease III, and DNA polymerase I were from New England Biolabs. DNA polymerase I (Klenow fragment), calf intestine alkaline phosphatase, and glycerol-3-phosphate dehydrogenase/TIM were from Boehringer Mannheim. Deoxynucleoside triphosphates, Cm-Sepharose® CL-6B Fast Flow and Sephadex® G-150 were from Pharmacia LKB Biotechnology, Inc. [α^{35}S]Deoxynucleoside triphosphates were from Amersham Corp. Nitrocellulose filters were from Sartorius. Oligonucleotides used for site-directed mutagenesis and sequencing were synthesized on Milligen/Biosearch DNA synthesizers using phosphoramidite chemistry and the manufacturer's protocols. Ultrapure sucrose was from Gibco. Crystalline rabbit muscle aldolase, Fru-1,6-P_2, DMS, hydrazine sulfate, TEA, and other chemicals were from Sigma.

Strains

E. coli DH5α (Sambrook et al., 1989) and JM83 (Yanisch-Perron et al., 1985) were used for cloning and expression. TG1 (Sambrook et al., 1989) was used for mutagenesis.

Site-directed mutagenesis

Site-directed mutagenesis (Taylor et al., 1985) was performed on the M13-based cDNA clone, AM1 (Morris & Tolan, 1993). The Asp-128 codon (GAT) was changed to Gly, Ala, Asn, Gln, or Val using the oligodeoxyribonucleotides 5′-CAAGGGTTGN NNGGGCTGTCC-3′, where NNN was GGT, GCT, AAT, CAA, or GTT, respectively. The potential mutants were screened by DNA sequence determination using dideoxy termination (Sanger et al., 1977) employing 7-deaza dGuo-triphosphate (Barr et al., 1986). The *Eco*R I–*Hin*d III fragments containing the mutant genes were cloned into the high-copy plasmid pPB1 (Beernink & Tolan, 1992) to create pD128G, pD128A, pD128N, pD128Q, and pD128V. These plasmids were transformed into JM83 and expressed proteins with Asp-128 replaced by Gly, Ala, Asn, Gln, and Val, respectively. The DNA sequence of the aldolase coding regions was determined to confirm that only the desired substitutions were present. Restriction enzyme digestions, ligation reactions, and transformations were performed as described (Sambrook et al., 1989).

Expression and purification of recombinant aldolase

A single colony of JM83 carrying the appropriate expression plasmid was used to inoculate a liter of 2× YT medium (Sambrook et al., 1989) containing 75 μg/mL ampicillin. Aldolase was purified as described (Morris & Tolan, 1993), except that the Cm-Sepharose CL-6B fast flow column was washed with a buffer at pH 7.6 prior to substrate elution.

Determination of protein concentration

Protein concentration was determined by reading the absorbance at 280 nm of a diluted sample (25–100 μg/mL), using an extinction coefficient, $E_{280}(0.1\%)$, of 0.91 $cm^2\ mg^{-1}$ (Baranowski & Niederland, 1949).

Kinetic analysis

The Fru-1,6-P_2 cleavage rate was measured at 30 °C by decrease in absorbance at 340 nm in an assay coupled to glycerol-3-phosphate dehydrogenase (Racker, 1952). Substrate concentration ranged from 2.5 to 80 μM and enzyme concentration was 0.3–0.9 μg/mL.

CD spectrum determination

CD spectra were determined using a protein concentration of 1.0 mg/mL in 1 mM Tris-HCl, 1 mM DTT, pH 7.5, at 20 °C. Spectra were read on an AVIV 62DS spectrometer with a 0.1-mm-pathlength cuvette from 260 to 180 nm, with readings averaged for 5 s at each nanometer.

Thermal inactivation assays

Irreversible thermal inactivation was assayed by preincubating 0.5 μg enzyme in 50 mM TEA-HCl, pH 7.4, 10 mM EDTA, 0.16 mM NADH in the cuvette at the desired temperature for 10 min. After equilibration at 30 °C for 2 min, the assay was initiated by adding Fru-1,6-P_2 and a mixture of glycerol-3-phosphate dehydrogenase/TIM to a final concentration of 2.0 mM and 10 μg/mL, respectively. Mutant enzymes lost 10–50% of their activity during the 2-min incubation at 30 °C.

Crosslinking with DMS

Prior to crosslinking, protein was dialyzed in 0.2 M TEA-HCl, 0.5 mM DTT, pH 8.5 (600-fold excess). Crosslinking was performed at 0.1 mg/mL protein and 2 mg/mL DMS in 0.2 M TEA-HCl, pH 8.5. Reactions were preincubated for 30 min at 4 °C before adding DMS. Reactions were quenched with 0.15 volumes 0.5 M glycine and concentrated under vacuum. Protein was denatured in 1% SDS, 1% β-ME at 90 °C for 30 min. SDS-PAGE was performed as described (Davies & Stark, 1970) using slab gels instead of tube gels. Gels were stained in 0.1%

Coomassie Blue R-250 in water/methanol/acetic acid (5:5:1) and destained in the same solvent.

DSC

Thermal unfolding was analyzed using a MicroCal MC-2 differential scanning calorimeter. Protein was dialyzed against 10 mM sodium phosphate buffer, pH 7.4, 0.02% NaN_3 (600-fold excess), and centrifuged 3 min at 10,000 × *g* to remove aggregates. The concentration was determined in triplicate (see above). The clarified dialysate was degassed ≥30 min at 4 °C. Protein (2 mg/mL) was scanned at 45 °C/h from 4 to 100 °C. Calorimetric data were normalized using the monomer molecular weight. T_m and ΔH values were obtained by fitting the data to a non-2-state model using Origin software (MicroCal, Inc.).

Sephadex G-150 filtration

Purified aldolase (2 U) and M_r markers (ferritin, 440 kDa; catalase, 220 kDa; lactate dehydrogenase, 146 kDa; TIM, 53 kDa; myoglobin, 17.3 kDa) were separated at 4 °C or 25 °C on a 70 × 1.6-cm column of Sephadex G-150 in 50 mM TEA-HCl, 50 mM NaCl, 1 mM DTT at a flow rate of 0.15 mL/min. Enzyme was preincubated at 25 °C or 37 °C for 30 min prior to chromatography.

Sucrose density gradient sedimentation

Sedimentation velocity was determined using a modified version of the method described by Martin and Ames (1961). Linear gradients of 5–20% sucrose in 20 mM TEA-HCl, pH 7.5, were equilibrated at the appropriate temperature for 2–4 h prior to use. Protein was diluted in the same buffer to the desired concentration (0.05–0.40 mg in 100 μL), preincubated for 30 min at the temperature of centrifugation, then layered on the gradients using a 20-gauge needle. Gradients were centrifuged between 38,000 and 55,000 RPM for 7–20 h in a Beckman SW55 Ti rotor. Gradients were scanned at 0.4 mL/min using a flow cell (1 cm pathlength) at 280 nm, taking readings at 2-s intervals. A change in absorbance at the bottom of every gradient was a consequence of refracted light and aggregated protein. Percent dimer values were calculated from the average of 4 experiments done at 4 °C, using a DuPont Model 310 Curve Resolver to estimate relative areas of dimer and tetramer peaks from plots of absorbance at 280 nm versus distance migrated. The detection level was estimated to be 0.5%. The protein concentration was confirmed by calculating the area under dimer and tetramer peaks. Sedimentation coefficients were calculated from the peak protein position from the same plots. Plots of sucrose density and viscosity versus sucrose concentration were used to obtain equations describing them. These formulas were used to calculate *s* using the trapezoidal approximation (Martin & Ames, 1961).

Active enzyme sedimentation was performed under the same conditions, except the gradient contained 2 mM Fru-1,6-P_2 and 2 mM hydrazine sulfate (Dische & Landsberg, 1960). Protein at 5 μg/mL was preincubated at 20 °C for 30 min, and centrifugation was performed for 14.5 h at 20 °C. Absorbance at 240 nm was measured at 2-s intervals and plotted versus distance migrated.

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