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# STABILIZATION OF THE PREDOMINANT DISEASE-CAUSING ALDOLASE VARIANT (A149P) WITH ZWITTERIONIC OSMOLYTES<sup>†</sup>

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## Abstract

Hereditary fructose intolerance (HFI) is a disease of carbohydrate metabolism that can result in hyperuricemia, hypoglycemia, liver and kidney failure, coma, and death. Currently, the only treatment for HFI is a strict fructose-free diet. HFI arises from aldolase B deficiency, and the most predominant HFI mutation is an alanine to proline substitution at position 149 (A149P). The resulting aldolase B with the A149P substitution (AP-aldolase) has activity <100-fold that of wild type. The X-ray crystal structure of AP-aldolase at both 4 and 18 °C reveals disordered adjacent loops of the ( $\alpha/\beta$ )<sub>8</sub> fold centered around the substitution, which leads to a dimeric structure as opposed to the wild-type tetramer. The effects of osmolytes were tested for restoration of structure and function. An initial screen of osmolytes (glycerol, sucrose, polyethylene glycol, 2,4-methylpentane diol, glutamic acid, arginine, glycine, proline, betaine, sarcosine, trimethylamine N-oxide) reveals that glycine, and similarly structured compounds, betaine and sarcosine, protects AP-aldolase structure and activity from thermal inactivation. The concentration and functional moieties required for thermal protection show a zwitterion requirement. The effect of osmolytes in restoring structure and function of AP-aldolase are described. Testing zwitterionic osmolytes of increasing size and decreasing fractional polar surface area suggests that osmolyte-mediated AP-aldolase stabilization is neither primarily through excluded volume effects nor through transfer free energy effects. These data suggest that AP-aldolase is stabilized by binding to the native structure and they provide a foundation for developing stabilizing compounds for potential therapeutics for HFI.

Hereditary fructose intolerance (HFI)<sup>1</sup> is an inborn error of metabolism caused by autosomal recessive mutations in the human aldolase B gene (1,2). Aldolase B is one isozyme of fructose-1,6-bisphosphate aldolase (EC 4.1.2.13), which is found in the liver, kidney, and small intestines (3), and the enzyme is crucial for cleavage of the metabolic intermediate fructose 1-phosphate (Fru 1-P) to dihydroxyacetone phosphate (DHAP) and glyceraldehyde in fructose metabolism (4). Upon ingestion of fructose, a deficiency in aldolase B activity results in a buildup of Fru 1-P, and leads to hypoglycemia, abdominal

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<sup>1</sup>ABBREVIATIONS AND FOOTNOTES: The abbreviations used are: AP-aldolase, aldolase B with the Ala-149 substituted by Pro; BSA, bovine serum albumin; CD, circular dichroism; DHAP, dihydroxyacetone phosphate; DTT, dithiothreitol; Fru 1-P, fructose 1-phosphate; Fru 1,6-P<sub>2</sub>, fructose 1,6-bisphosphate; G3PDH,  $\alpha$ -glycerophosphate dehydrogenase; GAH, glyceraldehyde; GSH, glutathione; HFI, Hereditary Fructose Intolerance; IPTG,  $\beta$ -D-1-thiogalactopyranoside; PBS, phosphate buffered saline; PEG, polyethylene glycol; TEA, triethanolamine; TIM, triosephosphate isomerase.

pain, diarrhea, and vomiting (2). Persistent ingestion of the sugar can progress to liver and kidney failure, seizures, growth retardation, coma, and possibly death (5). Symptoms are only present upon ingestion of fructose, and currently the only viable treatment option for HFI is a strict fructose-free diet (6). Given the changes in the Western diet (7), this is increasingly difficult. Although there are dozens of mutations in *aldoB* known to cause HFI, the most prevalent is a missense mutation resulting in a proline substitution at alanine-149 of aldolase B (A149P). This A149P variant is commonly referred to as AP-aldolase, and it occurs in approximately 57% of HFI alleles worldwide (8).

The AP-aldolase crystal structure shows structural disorder at the site of the A149P substitution that is propagated to adjacent loop regions including those at one dimer-dimer interface causing a loss of quaternary structure (9). This substitution results in a partially active aldolase enzyme that is very sensitive to temperature (10). The specific activity toward both cleavage substrates, fructose 1,6-bisphosphate (Fru 1,6-P<sub>2</sub>) and Fru 1-P, decreases from 16% of wild-type levels at 10 °C to 0.5% of wild-type levels at 30 °C. The substitution causes a lowered stability of both the secondary (10 °C decrease of T<sub>1/2</sub>) and tertiary structure (5 °C decrease of T<sub>1/2</sub>) of the enzyme. The loss of quaternary structure may be the root of the observed loss of thermal stability and activity, and it agrees with the general thought that the tetrameric structure plays a role in overall protein stability(11).

The AP-aldolase offers a potential therapeutic target for HFI given that 82% of HFI patients inherit at least one copy of this allele (8). The first question is whether the instability of AP-aldolase can be reversed. *In vivo*, a class of small organic molecule compounds referred to as osmolytes are produced to counteract stresses that lead to protein destabilization (12). These stresses include osmotic changes, extremes of temperature, high salinity, high pressure, extreme pH, or the presence of protein denaturants such as urea. Osmolytes have been shown to stabilize proteins against all these stresses (13–16). Concentrations of osmolytes are tightly regulated, either through diet or enzymatic processes, and there are several different osmolytes present in a cell. These osmolytes are generally of low molecular weight and have a high solubility in water, in some cases reaching molar concentrations in the cell (13,17,18). Osmolytes can be broken down into three general categories, methylamines, polyols, and certain amino acids (13). The current mechanism(s) thought to explain how osmolytes exert their effects on protein structure, which have largely considered reversibly folding proteins, are based on two thermodynamic effects: (i) transfer free energy, through destabilization of the denatured state, and (ii) excluded volume effects (19–23).

The transfer free energy model for osmolyte-mediated protein stabilization involves an increase in free energy when protein backbone unfavorably interacts with osmolyte. This unfavorable interaction destabilizes the denatured state of a protein shifting the equilibrium of protein folding toward the native state in the presence of protecting osmolytes (21). On the other hand, in the presence of protein denaturants, such as urea or guanidine (deprotecting osmolytes), the denatured state is stabilized. In this model, the presence of protecting osmolytes results in unfavorable interactions of the solvent with the protein backbone (19,21), thus the overly exposed protein backbone in the denatured state is destabilized relative to the folded protein.

The excluded volume model for osmolyte-mediated protein stabilization is an indirect effect. The model considers the volume occupied by the high concentration of osmolyte in solution, which results in a crowded environment (22). In this environment, a macromolecule will favor the folded state, which has less volume than the denatured state (24). According to this model, the larger the osmolyte molecule the greater the occupied volume, and therefore the native state would be more favored in solution (23,25).

This study investigated whether a small molecule could counteract the temperature-dependent instability caused by the A149P substitution. After a screen of osmolytes in the three general categories, zwitterionic compounds, in particular the amino acid glycine, fully restored secondary and tertiary structure stability of AP-aldolase to wild-type melting temperatures, staving off the start of the irreversible unfolding reaction. In addition, glycine stabilized the aldolase tetrameric quaternary structure, and increased activity over ten fold, to ~4% of wild type. Different sized zwitterionic osmolytes with different fractional polar surface areas (FPSA) were tested, and the results indicated that the mechanisms of osmolyte-mediated stabilization for reversibly folding proteins do not completely explain the data for AP-aldolase. These results could potentially lead to further improvement in stabilization of AP-aldolase by its interaction of small molecules.

## EXPERIMENTAL PROCEDURES

### Materials

The enzymes  $\alpha$ -glycerophosphate dehydrogenase (G3PDH), triosephosphate isomerase (TIM), lysozyme, and thrombin were from Sigma (St. Louis, MO). The plasmid pGEX-2T, molecular weight standards, and glutathione Sepharose 4B were purchased from GE Healthcare Life Sciences. Bacto yeast extract and tryptone peptone were purchased from VWR. Triton X-100 was obtained from Eastman Kodak (Rochester, NY). Fru 1,6-P<sub>2</sub>, Fru 1-P, isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG), glutathione (GSH), and other chemicals were from Sigma.

### Expression and purification of recombinant aldolase B and AP-aldolase

Wild-type aldolase B and AP-aldolase were produced by heterologous expression in *Escherichia coli* DH5 $\alpha$  as previously described (10). AP-aldolase was purified as a glutathione-S-transferase-fusion protein and subsequently was cleaved by thrombin, and elution in 137 mM NaCl, 2.7 mM KCl, 12 mM phosphate, pH 7.4 (PBS), yielded the full-length protein (10). Aldolase B was purified by affinity elution ion-exchange chromatography as previously described (26). Both enzymes were stored in 70% saturated ammonium sulfate at 4 °C.

### Determination of protein concentration

Protein concentration was determined by dye-binding (27) using bovine serum albumin (BSA) as a standard or by absorbance at 280 nm ( $E_{280}^{0.1\%} = 0.85 \text{ M}^{-1}\text{cm}^{-1}$ ) (28).

### Circular dichroism spectrophotometry

The far-UV CD spectra (250–185 nm) were recorded at 4 °C using the Aviv 62DS spectrometer with a 0.1-mm-pathlength quartz cuvette. Data were collected in 1-nm increments with a 15-s averaging time. The effect of temperature on protein secondary structure was monitored at 222 nm in 2 °C increments (30-s equilibration time) from 10 to 80 °C, with a 15-s averaging time. The protein concentration was 1.0 mg/mL in PBS containing 0.5 mM DTT. Near-UV CD spectra (250–320 nm) were recorded similarly in a 1-mm pathlength quartz cuvette. The temperature dependence of the ellipticity at 263 nm was monitored in 2 °C increments from 20 to 70 °C, with a 15-s averaging time.

### Gel filtration chromatography

Prior to the experiment, purified aldolase in ammonium sulfate was dialyzed twice at 4 °C against approximately 2500 volumes of PBS containing 0.5 mM DTT. Protein samples were loaded onto a Superdex-200 FPLC column (Pharmacia) at 4 °C. Absorbance of eluate at 0.5 mL/min was monitored at 280 nm. The molecular masses of the eluting species were

calculated from a standard curve consisting of thyroglobulin (669 kD); ferritin (440 kD); aldolase (159 kD); conalbumin (75 kD), and ovalbumin (43 kD).

### Aldolase activity

Aldolase cleavage kinetics toward Fru 1,6-P<sub>2</sub> and Fru 1-P were measured in coupled assays containing NADH, G3PDH, and TIM, as previously described (29,30).

### Thermal inactivation

The effect of osmolyte on thermal inactivation of aldolase activity was monitored by first diluting enzyme to 0.4 mg/mL with the desired concentration of osmolyte in 50 mM triethanolamine (TEA), pH 7.4. An aliquot was assayed for Fru-1,6-P<sub>2</sub>-cleavage activity prior to thermal incubation. The enzyme-osmolyte solution was then incubated at 30 °C for five min, and then at 10 °C for two min. The solution was centrifuged at 25 °C at 2000g for one min to remove any aggregated material. Another aliquot was assayed for Fru-1,6-P<sub>2</sub>-cleavage activity at 25 °C to measure the protection from thermal inactivation. All activity values were normalized to a no osmolyte pre-incubation activity.

### Molecular Dynamics

Molecular construction and manipulations were carried out using the InsightII and Discovery suites from Accelrys Inc. Simulations were performed using the CHARMM force field (release 30) (31) on an IBM p655. The molecular series from 2-aminoacetic acid to 7-aminoheptanoic acid was built *ab initio*, the total charge set to zero, and optimized. A short, 50 ps molecular dynamics run *in vacuo* yielded compact conformers, in the cis-orientation for the longer chains. These were soaked in ~1200 molecules of water and dynamics continued for 3 ns. In the case of 6-amino hexanoic acid and 7-aminoheptanoic acid, the backbone dihedrals were then rotated to the trans-orientation, and an ensemble of 7 randomly placed copies each of the cis- and trans-forms, in ~1200 molecules of water. Molecular volume, radius of gyration, and surface area were calculated as defined by InsightII and Discovery suites using the equations of Stanton and Jurs (32). The relative hydrophobic surface area (RHSA) and the FPSA were measured on 140 structures selected from 10 equally spaced intervals from the last 1 ns of the simulations.

## RESULTS

### Osmolyte Screening

Measuring enzymatic activity as a function of temperature provides a very sensitive way to appraise the effects of a potential stabilizing osmolyte on an enzyme and allows quantification of perturbations in protein structure. A thermal inactivation assay was developed for potential stabilizing osmolytes on the HFI-causing AP-aldolase enzyme. AP-aldolase has maximal activity at temperatures below 15 °C, but gradually loses activity at increasing temperatures, having negligible activity at 37 °C (<200 fold that of wild type) (10). For the initial screen of osmolytes, an inactivating temperature of 30 °C was chosen. Five minutes at 30 °C results in an 80% loss of AP-aldolase activity. Several osmolytes from each osmolyte class (methylamines, polyols, and amino acids) were tested using concentrations that have been effective for other proteins (33). AP-aldolase was diluted to 0.4 mg/mL in TEA buffer containing each osmolyte and subjected to the thermal inactivation assay. The pre-incubation assay served to control for any non-temperature dependent effects on AP-aldolase. In addition, a negative control of AP-aldolase in 1 M urea yielded a decreased but still measurable amount of activity after incubation. The results of this initial screen are shown in Table 1. All pre- and post-incubation values were normalized

to the pre-incubation assay without osmolyte. The osmolytes most effective at stabilizing AP-aldolase activity at 30 °C were betaine, sarcosine, and glycine.

### Optimal Osmolyte Concentration Determination

The optimal concentration of glycine necessary for stabilizing AP-aldolase in the thermal inactivation assay was determined by testing concentrations of glycine between 0.125 – 2.0 M. Below 2 M glycine, the complete stabilization effect on AP-aldolase was essentially lost, although at 1 M and 0.5 M some significant protection against thermal inactivation remained (Fig 1). At 0.25 M glycine the protective effect on thermal inactivation was lost. Glycine must be present in 2 M concentrations to fully stabilize AP-aldolase activity at 30 °C *in vitro*.

### Determination of Osmolyte Functional Moiety Necessary for Stabilization

The three most effective osmolytes were glycine, sarcosine, and betaine. These three osmolytes are structurally related, each being zwitterionic at physiological pH and differing as primary, secondary, and quaternary amines, respectively. Exploration of the structural element(s) important for stabilizing AP-aldolase used a group of molecules that separated the structural element(s) of these osmolytes. Alanine,  $\beta$ -alanine, ethanolamine, ethylamine, propionic acid, and acetic acid were used in the thermal inactivation assay (Table 2). Alanine and  $\beta$ -alanine, an alanine isomer, were closely related in size and structure to glycine. Both ethylamine and ethanolamine maintained a positively charged amino group at pH 7.4, but lacked the negatively charged carboxyl group, although ethanolamine maintained a polar end. Propionic acid and acetic acid were used to probe the role of the carboxyl group alone. Lastly, equal amounts of triethylamine and acetic acid were tested, thus separating the charges completely. Compounds other than the amino acids, including the triethylamine-acetic acid mixture did not result in any thermal protection, and destroyed enzyme activity. Thermal inactivation assays using these compounds showed that both charges were necessary on the same molecule for stabilizing AP-aldolase (Table 2).

### Analyzing the Effects of Glycine on Secondary and Tertiary Structure

The effects of glycine on the perturbed secondary and tertiary structures of AP-aldolase were investigated by CD spectrophotometry. A melting curve was generated by monitoring the CD absorbance of AP-aldolase at 222 nm from 10–80 °C (Fig 2). In the presence of glycine, AP-aldolase showed a melting curve similar to that of wild-type aldolase B. The presence of glycine shifted the  $T_{1/2}$  from 45 °C to approximately 55 °C, about 3 °C less than wild-type aldolase B. The overall secondary structure of AP-aldolase was not greatly affected by the A149P substitution when compared to the overall secondary structure of the wild-type protein (10), and the presence of glycine did not change the far-UV spectrum of AP-aldolase (data not shown).

The near-UV CD spectrophotometric spectra of the AP-aldolase and of wild-type aldolase B were different, and the presence of glycine was unable to change the AP-aldolase spectrum to resemble that of wild-type aldolase B at 10 °C (Fig 3A). However, much like the secondary structure, the tertiary structure of AP-aldolase was sensitive to temperature (10). CD spectrophotometry examined the effect of glycine on changes in the near-UV CD absorbance for AP-aldolase as a function of temperature at 263 nm from 10–70 °C (Fig 3B). In the presence of glycine, the melting curve of AP-aldolase resembled that of wild type. AP-aldolase in the absence of glycine had a  $T_{1/2}$  of 44 °C, in contrast to the  $T_{1/2}$  of the wild-type protein and AP-aldolase in the presence of glycine, which was 53 °C. This indicated that, although there remained overall differences of AP-aldolase tertiary structure, glycine was able to restore the stability of the tertiary structure.



## Effect of Glycine on Quaternary Structure

The disorder of several loops in AP-aldolase at the dimer-dimer interface perturbed the aldolase B dimer↔tetramer equilibrium (9,10). The loss of quaternary structure in aldolases is highly correlated with loss of thermal stability (11,34). The question arises whether the thermal stabilization afforded by glycine correlates with increased quaternary structure of AP-aldolase. Changes in quaternary structure with and without glycine were determined by size-exclusion chromatography. In the absence of glycine at 4 °C, the dominant species of AP-aldolase in solution was the dimeric (>2-fold over the tetramer) and the wild-type aldolase B was tetrameric, as has been previously published ((3,10)) (Fig 4A). In the presence of glycine at 4 °C, dominant species was tetrameric (Fig 4B). The correlation of regained tetrameric structure with thermal stability of the secondary and tertiary *structure* and the activity could be the basis for the effects of glycine on AP-aldolase.

## Effect of Glycine on Enzymatic Activity and Substrate Affinity

In addition to the A149P substitution perturbing the dimer-dimer interface, the active site is disturbed, which results in a 20-fold increase in the  $K_m$  value toward Fru 1-P (10). Should the increased structural stability observed in the presence of glycine result in the reformation of quaternary structure alone, a restoration of  $K_m$  values in the presence of glycine would not necessarily follow. Due to its instability and to ensure maximal retention of activity during purification, AP-aldolase was purified in the presence of 2 M glycine starting from cell lysis. The steady-state kinetic constants of AP-aldolase toward substrates, Fru 1,6-P<sub>2</sub> (Table 3) and Fru 1-P (Table 4) were examined at 10 °C and at 30 °C in both the presence and absence of 2 M glycine

At 10 °C, the effect of glycine on  $k_{cat}$  values toward Fru 1,6-P<sub>2</sub> was insignificant; however, at 30 °C, the presence of glycine resulted in a 10–13-fold improvement in activity when compared to the enzyme in the absence of glycine. The unchanged value of  $k_{cat}$  for AP-aldolase at 10 °C ±glycine indicated that at 10 °C AP-aldolase is at its most stable structure. In the presence of glycine, the  $K_m$  value toward Fru 1,6-P<sub>2</sub> at 10 °C unexpectedly increased when compared to the value in the absence of glycine, but this effect decreased at 30 °C.

A more striking improvement in the  $K_m$  value was found with Fru 1-P for AP-aldolase in the presence of glycine (Table 4). At both temperatures, the  $K_m$  values in the presence of glycine were within 1.6–3-fold that of wild type. This was a vast improvement compared with AP-aldolase in the absence of glycine, whose  $K_m$  values were approximately 20-fold greater than wild type. The  $k_{cat}$  values toward Fru 1-P were affected by glycine similarly as those toward Fru 1,6-P<sub>2</sub>; a larger effect was seen at 30 °C than at 10 °C, although it is notable that at 10 °C the activity of AP-aldolase toward Fru 1-P was within 30% of wild type. These results suggested that, although the AP-aldolase active site remained slightly different than that of wild type, the presence of 2 M glycine improved the catalytic efficiency ( $k_{cat}/K_m$ ) toward Fru 1-P by over 150- fold at 30 °C. Lastly, the changes in  $K_m$  values toward either substrate indicated that the effects of glycine at the active site might be unrelated to the effect of glycine on restoring quaternary or tertiary structural stability.

## Determination of Optimal Size of Zwitterionic Osmolyte

Much insight has been gained on the mechanism of osmolyte action, largely using small monomeric two-state folding proteins. Two major theories often used to explain these stabilizing effects are excluded volume and/or transfer free energy (20). According to excluded volume theory, larger osmolytes should increase any stabilizing effects, whereas according to transfer free energy theory a decrease in FPSA should increase any stabilization effects. Further exploration of the stabilization of AP-aldolase was tested with zwitterions of different sizes and different FPSAs (Fig 5A). The different sized zwitterions had increasing

numbers of methylene groups between the amino and carboxylate groups. Increasing the size of the osmolyte showed no significant increase in protection against thermal inactivation. As size increased, however, the larger zwitterions lost their effect. The  $\gamma$ -aminobutyric acid with three methylene groups was just as effective as glycine and  $\beta$ -alanine, but  $\delta$ -aminopentanoic acid was only able to protect the enzyme ~50%, and  $\epsilon$ -aminohexanoic acid was able to protect ~20%. For the case of 7-aminoheptanoic acid, the compound inhibited activity altogether. These data revealed that both positive and negative functional groups in a single molecule were required for effective stabilization of AP-aldolase, and that the zwitterion must be of a certain size.

The molecular volume, radius of gyration, solvent-accessible surface area, FPSA, and relative hydrophobic surface area (RHSA) were calculated from structures of these compounds after 3 ns of molecular dynamics simulations. There was a linear increase in molecular volume that demonstrated a 2-fold increase from glycine to  $\epsilon$ -aminohexanoate (data not shown). The larger osmolytes in this linear range were accompanied by a decrease in thermal stabilization rather than an increase, which excluded volume theory would predict. The radius of gyration, solvent-accessible surface area, and RHSA were not linearly increasing after  $\delta$ -aminopentanoic acid, and there was a striking correlation of RHSA to the loss of stabilization (Fig 5B). As the size of molecules increased, there was a linear increase in RHSA until  $\delta$ -aminopentanoic acid, the first compound unable to fully protect AP-aldolase from thermal inactivation (Fig 5A). The average structures after molecular dynamics simulation showed a tendency for more compaction as the charged groups tended to interact intramolecularly (Fig 5C). The largest compound, 7-aminoheptanoic acid, showed a detergent-like structure that perhaps explained its non-temperature dependent inhibition of activity.

According to the transfer free energy model, increased osmolyte-induced stabilization of reversibly folding proteins correlates with decreased FPSA (19). The FPSA of these zwitterions was calculated and plotted against their observed percentage protection of AP-aldolase from thermal inactivation (Fig 5D). What was observed was the opposite correlation. Increased osmolyte-induced stabilization of AP-aldolase correlated with increased FPSA, with perhaps the exception of GABA.

The lack of correlation of stability with decreasing FPSA and increase size of the osmolyte suggests that neither the free energy transfer nor the excluded volume model explains the mechanism of osmolyte-mediated protein stabilization of AP-aldolase. Another model is that the osmolytes interact with and stabilize the native state of the protein. Consistent with this model, the concentration dependence of glycine versus an indirect result of any binding, protection of AP-aldolase from thermal inactivation, was measured between 0 – 3 M. The data fit well to a sigmoidal binding curve (Fig 6A) with the best fit to such a curve ( $R^2=0.99$ ) showing saturation at ~30 M (Fig 6B), which is well beyond the 3.3 M solubility of glycine. The apparent  $K_d$  was  $6.6 \pm 0.4$  M, and the Hill plot gave a coefficient of  $2.06 \pm 0.11$ . Clearly, this theoretical binding has a very low affinity with multiple sites. These data suggest that glycine has binding sites that can stabilize AP-aldolase to thermal inactivation and they may offer another explanation for osmolyte-mediated protein stability in general.

## DISCUSSION

### Effects of the A149P Substitution on Aldolase Structure and Activity

Previous reports on AP-aldolase have hypothesized that the structural perturbation at Pro-149 causes two separate and perhaps unrelated problems, one affecting structural stability and temperature sensitivity through loss of quaternary structure, and another affecting the maximal activity of the enzyme by perturbations at the active site (9,10). This



idea arose from the observation that the loss of activity of AP-aldolase occurs well before any detectable loss in secondary or tertiary structure. Consistent with this, the crystal structure of AP-aldolase shows that Arg-148, which typically forms a salt-bridge with Glu-189 (forming a wall of the active site cleft), was in a salt-bridge with Glu-187, a critical residue involved in acid/base catalysis (35), thus potentially altering the electrostatics at the active site(9). In addition, the nearby essential catalytic residue Lys-146 (36) had a slightly altered orientation due to the adjacent proline substitution. Finally, Arg-303, a well-known C1-phosphate binding site (37), was not in the wild-type conformation, which could explain the large increases in  $K_m$  values toward Fru 1-P at both 10 and 30 °C (10). Two molar concentrations of glycine completely restored wild-type stability in the secondary and tertiary structure of AP-aldolase, and furthermore, stabilized wild-type tetrameric structure, although not to full wild-type levels. In addition to this structural stabilization, glycine restored activity toward Fru 1-P to 30% of wild type. Although AP-aldolase did not regain wild-type activity in the presence of glycine compared to the absence of glycine, there was >10-fold improvement in the degree of activity retained at 30 °C. That glycine had increased both structural stability and activity indicated that the two problems with AP-aldolase might be intertwined. Similar losses of enzyme activity without observable structural changes have been reported for other enzymes (38,39), and it would be interesting if glycine had similar effects.

### Mechanism of Osmolyte-Mediated Protein Stabilization

In other studies on osmolyte action, excluded volume effects have been shown to play a major role (20,40). For example, using various osmolytes of the polyol class of increasing size ranging from glycerol to the trisaccharide, melezitose, to study the folding of yeast iso-1-ferricytochrome c, a correlation between an increase in protein stability and osmolyte size was shown (23). This correlation strongly supported the excluded volume model of osmolyte-mediated protein stabilization for this reversibly folding protein.

The transfer free energy model focuses on the thermodynamic interaction between a reversibly folding protein and solvent, either with or without osmolyte. Numerous studies have shown that osmolytes affect interactions between protein backbone (as opposed to the R-groups) and solvent (41–44). The protein backbone, which needs to be highly solvated in order for a protein to fully denature, has an unfavorable interaction with osmolyte-containing solutions. This osmolyte-backbone interaction has a much higher transfer free energy than the water-backbone interaction. So, when transitioning from water as the solvent to a solution with a high concentration of osmolyte, the free energy of the denatured state increases, thus shifting the folding equilibria in favor of the native state. Furthermore, it has been shown that this transfer free energy, when used as a measure of osmolyte-mediated protein stabilization, correlates with an individual osmolyte's relatively smaller FPSA. This specific characteristic of an osmolyte molecule might be the source of its ability to stabilize protein structure (19). Although the mechanism of osmolyte action for most proteins remains unclear, major contributions involving both these effects are likely involved (20).

In this study, charged zwitterionic osmolytes were important in the osmolyte-mediated stabilization of AP-aldolase. If glycine were acting *via* the transfer free energy model, the polarity of these molecules would play a crucial role in the stabilizing effects toward AP-aldolase. Consistent with this, smaller methylamine osmolytes, such as betaine and sarcosine, have a smaller FPSA compared to polyol osmolytes such as sucrose and glycerol, and these smaller osmolytes had an increased capacity for stabilization of AP-aldolase (Table 1). On the other hand, if a relatively low FPSA were crucial for the osmolyte-mediated stabilization of AP-aldolase, as has been predicted computationally for similar compounds (19), then increasing the size of the zwitterion without adding any further polarity should lower the FPSA and further stabilize AP-aldolase. This was not observed

and in fact, the opposite correlation was observed (Fig 5D). Furthermore, it was not simply the charges of the osmolyte that were required, but that these charges were on the same molecule and not too distant from each other. Should the charges be all that were necessary, then equal amounts of positively and negatively charged compounds should have stabilized AP-aldolase as well as glycine, which was not the case. Perhaps if overall polarity were maintained with the larger zwitterions, their effectiveness in stabilizing AP-aldolase would increase and include favorable excluded volume effects, as seen with larger osmolytes such as sucrose (23).

Should the excluded volume model of osmolyte action be the major driving force for the stabilization effects on AP-aldolase, then simply increasing the size of the zwitterion should decrease the available volume and force the N $\leftrightarrow$ D equilibrium toward the N-state, which has a smaller radius. This was not observed. Taken together, these results do not support a model that these small zwitterionic osmolytes stabilize AP-aldolase through either a transfer free energy or an excluded volume mechanism. Although, it is possible that excluded volume plays a minor role in stabilizing AP-aldolase in response to temperature (GABA seemed to increase the stabilization slightly over the smaller  $\beta$ -alanine (Fig 5A)). There are at least two very complex reactions at play here, namely, relative reaction rates of folding intermediates and the differential interactions of these intermediates with the osmolytes; however, this lack of consistency with these models of osmolyte action indicated another mode of action. It is possible that these zwitterionic osmolytes are directly interacting with the native state of the protein, forcing the protein to adopt a more stable conformation than in a solution without osmolyte present. Several of these osmolytes increased enzyme activity before any thermal inactivation (see 10 °C data in Tables 1 and 2 ), indicating a stabilizing effect before significant denaturation. Moreover, unlike most of the reversibly folding proteins used for testing models of osmolyte action, AP-aldolase is not able to reversibly unfold in the assay used here. In particular, the transfer free energy model is based on the osmolyte's ability to shift the equilibrium between the native and denatured states, and if there is no equilibrium or it is largely perturbed by aggregation, then these models would not readily apply. The thermal melting of AP-aldolase is not reversible above 20 °C (45). Given that the thermal stabilization assays was performed at 30 °C, AP-aldolase was not in equilibrium with its unfolded state, which was largely precluded by irreversible aggregation. Furthermore, the effects of glycine on AP-aldolase activity showed that during the five min incubation at 30 °C there was no change in secondary or tertiary structure as determined by CD (Figs 2 and 3), thus indicating little change in the N $\leftrightarrow$ D equilibrium. Although, parts of the protein, such as loops that are invisible to CD spectroscopy, could be in such a reversible transition. Overall, these observations of increased activity and stability of AP-aldolase at temperatures lower than those needed for significant structural perturbation suggest that the osmolyte-mediated protein stabilization of AP-aldolase is most likely affecting the native state of the protein; although, possible involvement of osmolyte-affected transfer free energy on partially denatured structures and minor excluded volume effects cannot be ruled out. Consistent with this model for direct binding of osmolytes to the native structure, such binding was observed for proline to an allosteric site on pyruvate kinase (46).

### Using Small Molecules as a Treatment for HFI

Currently, the only viable treatment option for patients afflicted with HFI is complete avoidance of the fructose in their dietary intake. The discovery herein of zwitterions useful in stabilizing AP-aldolase leads to the prospect of designing small molecules that could potentially lead to treatments. Most rational drug designs are for inhibitors, as opposed to compounds that stabilize proteins and allow for natural function. An intelligently designed drug, taking into account the observed effect of zwitterionic molecules on AP-aldolase, and using a molecule of similar structure to glycine as a starting point, would only need to

produce an additional 10-fold increase in activity to restore AP-aldolase to near wild-type activity. Thus, the idea that a small molecule can enforce structural stability of AP-aldolase at 37 °C and be useful as a therapeutic for HFI may have merit. Further studies are underway to examine any potential binding sites of glycine on aldolase, which may provide a potential stabilizing binding pocket, and would be useful in the intelligent design of a zwitterionic compound with increased affinity to the surface of AP-aldolase.

## Acknowledgments

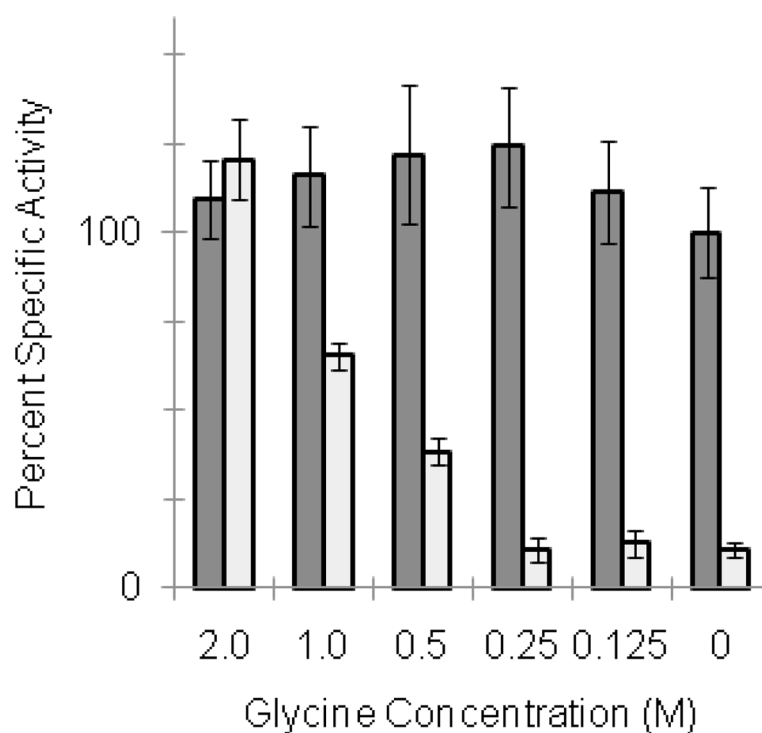
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## References

1. Hers HG, Joassin G. Anomalie de l'aldolase hepatique dans l'intolerance au fructose. *Enzymol Biol Clin.* 1961; 1:4–14.
2. Steinmann, B.; Gitzelmann, R.; Van den Berghe, G. Disorders of Fructose Metabolism. In: Scriver, C.; Beaudet, A.; Sly, W.; Valle, D., editors. *The Metabolic and Molecular Basis of Inherited Disease*. McGraw-Hill, Inc; New York: 2001. p. 1489-1520.
3. Leberherz HG, Rutter WJ. Distribution of fructose diphosphate aldolase variants in biological systems. *Biochemistry.* 1969; 8:109–121. [PubMed: 5777313]
4. Hers H, Kusaka T. Le Métabolisme du fructose 1-phosphate dans le foie. *Biochim Biophys Acta.* 1953; 11:427–432. [PubMed: 13093749]
5. Cox TM. Iatrogenic deaths in hereditary fructose intolerance. *Arch Dis Childhood.* 1993; 69:423–415. [PubMed: 8259871]
6. Froesch ER, Wolf HP, Baitsch H, Prader A, Labhart A. Hereditary fructose intolerance. An inborn defect of hepatic fructose-1-phosphate splitting aldolase. *Am J Med.* 1963; 34:151–167. [PubMed: 13959929]
7. Gaby AR. Adverse effects of dietary fructose. *Altern Med Rev.* 2005; 10:294–306. [PubMed: 16366738]
8. Coffee EM, Yerkes L, Ewen EP, Zee T, Tolan DR. Increased prevalence of mutant null alleles that cause hereditary fructose intolerance in the American population. *J Inherit Metab Dis.* 2009; 33:33–42. [PubMed: 20033295]
9. Malay AD, Allen KN, Tolan DR. Structure of the thermolabile mutant aldolase B, A149P: molecular basis of hereditary fructose intolerance. *J Mol Biol.* 2005; 347:135–144. [PubMed: 15733923]
10. Malay AD, Prociou SL, Tolan DR. The temperature dependence of activity and structure for the most prevalent mutant aldolase B associated with hereditary fructose intolerance. *Arch Biochem Biophys.* 2002; 408:295–304. [PubMed: 12464284]
11. Beernink PT, Tolan DR. Disruption of the aldolase A tetramer into catalytically active monomers. *Proc Natl Acad Sci USA.* 1996; 93:5374–5379. [PubMed: 8643582]
12. Bolen DW, Baskakov IV. The osmophobic effect: natural selection of a thermodynamic force in protein folding. *J Mol Biol.* 2001; 310:955–963. [PubMed: 11502004]
13. Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN. Living with water stress: evolution of osmolyte systems. *Science.* 1982; 217:1214–22. [PubMed: 7112124]
14. Yancey PH, Blake WR, Conley J. Unusual organic osmolytes in deep-sea animals: adaptations to hydrostatic pressure and other perturbants. *Comp Biochem Physiol.* 2002; 133:667–676.
15. Wang A, Bolen DW. A naturally occurring protective system in urea-rich cells: mechanism of osmolyte protection of proteins against urea denaturation. *Biochemistry.* 1997; 36:9101–8. [PubMed: 9230042]
16. Gillett MB, Suko JR, Santoso FO, Yancey PH. Elevated levels of trimethylamine oxide in muscles of deep-sea gadiform teleosts: a high-pressure adaptation? *J Exp Zool.* 1997; 279:386–391.
17. Burg MB, Ferraris JD. Intracellular organic osmolytes: function and regulation. *J Biol Chem.* 2008; 283:7309–7313. [PubMed: 18256030]

18. Burg MB, Kwon ED, Peters EM. Glycerophosphorylcholine and betaine counteract the effect of urea on pyruvate kinase. *Kidn Int.* 1996; 50:S100–S104.
19. Street TO, Bolen DW, Rose GD. A molecular mechanism for osmolyte-induced protein stability. *Proc Natl Acad Sci USA.* 2006; 103:13997–14002. [PubMed: 16968772]
20. Schellman JA. Protein stability in mixed solvents: a balance of contact interaction and excluded volume. *Biophys J.* 2003; 85:108–125. [PubMed: 12829469]
21. Auton M, Ferreón AC, Bolen DW. Metrics that differentiate the origins of osmolyte effects on protein stability: a test of the surface tension proposal. *J Mol Biol.* 2006; 361:983–992. [PubMed: 16889793]
22. Minton AP. Influence of excluded volume upon macromolecular structure and associations in ‘crowded’ media. *Curr Opin Biotechnol.* 1997; 8:65–69. [PubMed: 9013656]
23. Saunders AJ, Davis-Searles PR, Allen DL, Pielak GJ, Erie DA. Osmolyte-induced changes in protein conformational equilibria. *Biopoly.* 2000; 53:293–307.
24. Davis-Searles PR, Saunders AJ, Erie DA, Winzor DJ, Pielak GJ. Interpreting the effects of small uncharged solutes on protein-folding equilibria. *Annu Rev Biophys Biomol Struct.* 2001; 30:271–306. [PubMed: 11340061]
25. Ralston GB. Effects of “crowding” in protein solutions. *J Chem Edu.* 1990; 67:857–860.
26. Doyle SA, Tolan DR. Characterization of recombinant human aldolase B and purification by metal chelate chromatography. *Biochem Biophys Res Commun.* 1995; 206:902–908. [PubMed: 7832803]
27. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72:248–254. [PubMed: 942051]
28. Eagles PA, Iqbal M. A comparative study of aldolase from human muscle and liver. *Biochem J.* 1973; 133:429–439. [PubMed: 4733235]
29. Morris AJ, Tolan DR. Site-directed mutagenesis identifies aspartate 33 as a previously unidentified critical residue in the catalytic mechanism of rabbit aldolase A. *J Biol Chem.* 1993; 268:1095–1100. [PubMed: 8419316]
30. Racker E. Spectrophotometric measurement of hexokinase and phosphohexokinase activity. *J Biol Chem.* 1947; 167:843–854. [PubMed: 20287918]
31. Brooks BR, Brooks CL III, Mackerell AD, Nilsson L, Petrella RJ, Roux B, Won Y, Archontis G, Bartels C, Boresch S, Caflisch A, Caves L, Cui Q, Dinner AR, Feig M, Fischer S, Gao J, Hodoseck M, Im W, Kuczera K, Lazaridis T, Ma J, Ovchinnikov V, Paci E, Pastor RW, Post CB, Pu JZ, Schaefer M, Tidor B, Venable RM, Woodcock HL, Wu X, Yang WD, York M, Karplus M. CHARMM: The Biomolecular simulation Program. *J Comp Chem.* 1998; 30:1545–1615. [PubMed: 19444816]
32. Stanton DT, Jurs PC. Computer-assisted study of the relationship between molecular structure and surface tension of organic compounds. *J Chem Inf Comp Sci.* 1992; 32:109–115.
33. Bolen DW. Effects of naturally occurring osmolytes on protein stability and solubility: issues important in protein crystallization. *Meth.* 2004; 34:312–322.
34. Beermink PT, Tolan DR. Subunit interface mutants of rabbit muscle aldolase form active dimers. *Protein Sci.* 1994; 3:1383–1391. [PubMed: 7833800]
35. Choi KH, Lai V, Foster CE, Morris AJ, Tolan DR, Allen KN. New superfamily members identified for Schiff-base enzymes based on verification of catalytically essential residues. *Biochemistry.* 2006; 45:8546–8555. [PubMed: 16834328]
36. Morris AJ, Tolan DR. Lysine-146 of rabbit muscle aldolase is essential for cleavage and condensation of the C3-C4 bond of fructose 1,6-bis(phosphate). *Biochemistry.* 1994; 33:12291–12297. [PubMed: 7918450]
37. Choi KH, Shi J, Hopkins CE, Tolan DR, Allen KN. Snapshots of catalysis: the structure of fructose-1,6-(bis)phosphate aldolase covalently bound to the substrate dihydroxyacetone phosphate. *Biochemistry.* 2001; 40:13868–13875. [PubMed: 11705376]
38. Zhang YL, Zhou JM, Tsou CL. Inactivation precedes conformation change during thermal denaturation of adenylate kinase. *Biochim Biophys Acta.* 1993; 1164:61–67. [PubMed: 8518297]

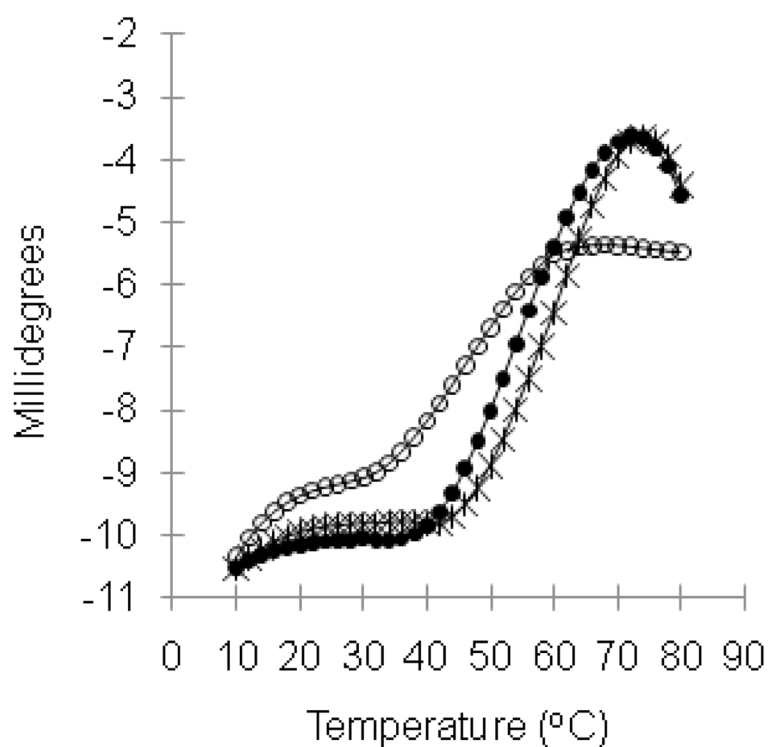
39. Tsou CL. Conformational flexibility of enzyme active sites. *Science*. 1993; 262:380–381. [PubMed: 8211158]
40. Patel CN, Noble SM, Weatherly GT, Tripathy A, Winzor DJ, Pielak GJ. Effects of molecular crowding by saccharides on alpha-chymotrypsin dimerization. *Protein Sci*. 2002; 11:997–1003. [PubMed: 11967357]
41. Bolen DW, Rose GD. Structure and energetics of the hydrogen-bonded backbone in protein folding. *Annu Rev Biochem*. 2008; 77:339–362. [PubMed: 18518824]
42. Auton M, Bolen DW. Additive transfer free energies of the peptide backbone unit that are independent of the model compound and the choice of concentration scale. *Biochemistry*. 2004; 43:1329–42. [PubMed: 14756570]
43. Auton M, Bolen DW. Predicting the energetics of osmolyte-induced protein folding/unfolding. *Proc Natl Acad Sci USA*. 2005; 102:15065–15068. [PubMed: 16214887]
44. Liu Y, Bolen DW. The peptide backbone plays a dominant role in protein stabilization by naturally occurring osmolytes. *Biochemistry*. 1995; 34:12884–91. [PubMed: 7548045]
45. Malay, AD. Boston University, Ph.D. 2004. The Structure and Function of the Most Prevalent Mutant Form of Aldolase B Associated with Hereditary Fructose Intolerance.
46. Fenton AW, Johnson TA, Holyoak T. The pyruvate kinase model system, a cautionary tale for the use of osmolyte perturbations to support conformational equilibria in allostery. *Protein Sci*. 2010; 19:1796–1800. [PubMed: 20629175]



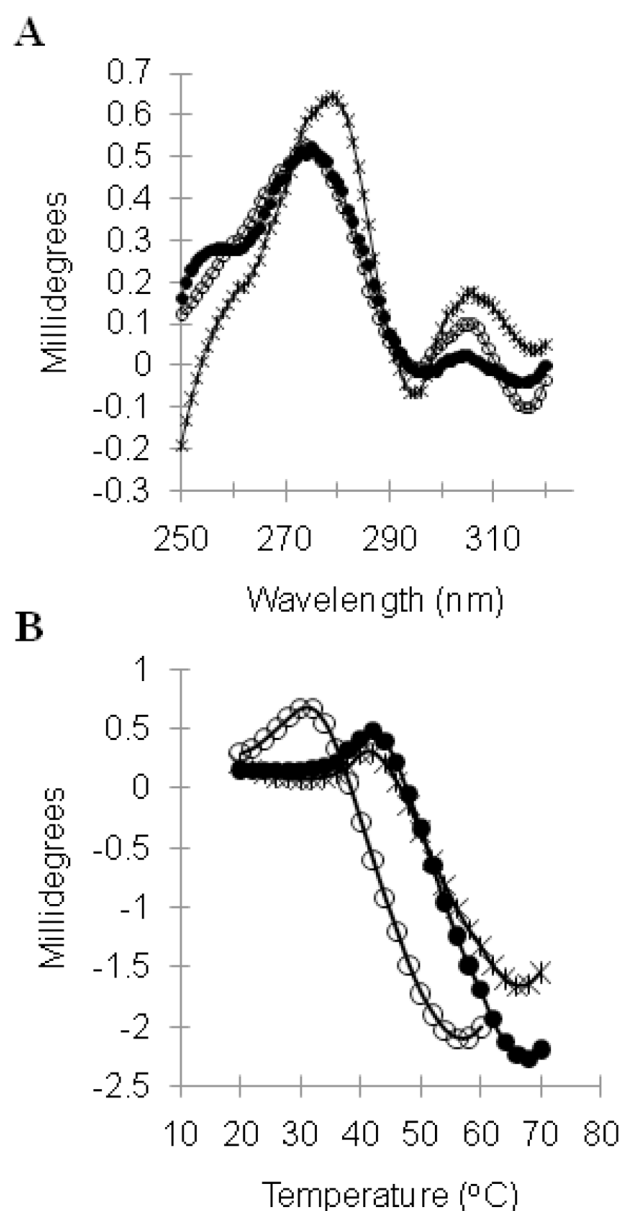
**Fig. 1. Two Molar Glycine Necessary for Desired Effect on Stability of AP-aldolase**

Thermal inactivation assays at 30 °C were performed on AP-aldolase (0.4 mg/mL) in the presence of glycine in concentrations varying from 0 – 2 M. Pre- (dark) and post-incubation (light) activity values are normalized to the pre-incubation, no-compound control value (~0.01 U/mg). All trials were performed at pH 7.4. Error bars are presented as 1σ of three separate trials



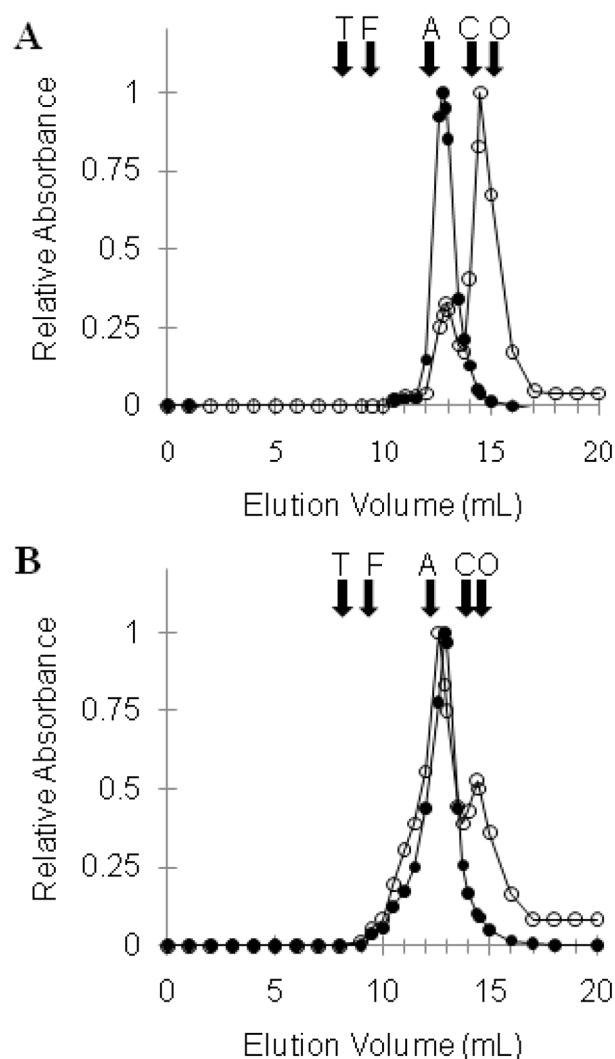


**Fig. 2. Secondary Structure of AP-aldolase Stabilized in the Presence of 2 M Glycine** Far UV-CD measurements were taken from 10 – 90 °C at 2 °C increments at 222 nm at a concentration of 1.0 mg/mL of AP-aldolase in the presence (closed circle) and absence (open circle) of 2 M glycine. Wild-type aldolase B in the absence of glycine was subjected to the same conditions and shown for comparison (crosses).

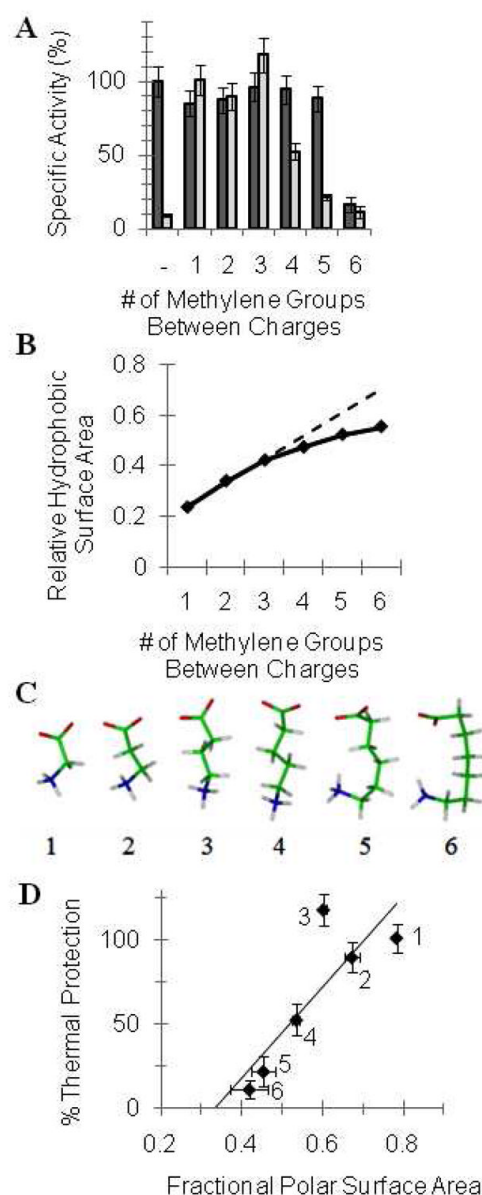


**Fig. 3. Tertiary Structure of AP-aldolase Stabilized in the Presence of 2 M Glycine**

*Panel A*, near-UV CD spectra were collected at 250–320 nm in a 0.1-cm pathlength quartz cuvette at a protein concentration of 1.0 mg/mL at 10 °C of AP-aldolase in the presence (closed circle) and absence (open circle) of 2 M glycine. Wild-type aldolase B in the absence of glycine was subjected to the same conditions and shown for comparison (crosses). Data were collected in 1-nm increments with a 10-s averaging time. *Panel B*, near UV-CD measurements were taken from 10–90 °C at 2 °C increments at 263 nm at a concentration of 1.0 mg/mL of AP-aldolase in the presence (closed circle) and absence (open circle) of 2 M glycine. Wild-type aldolase B in the absence of glycine was subjected to the same conditions and shown for comparison (crosses).



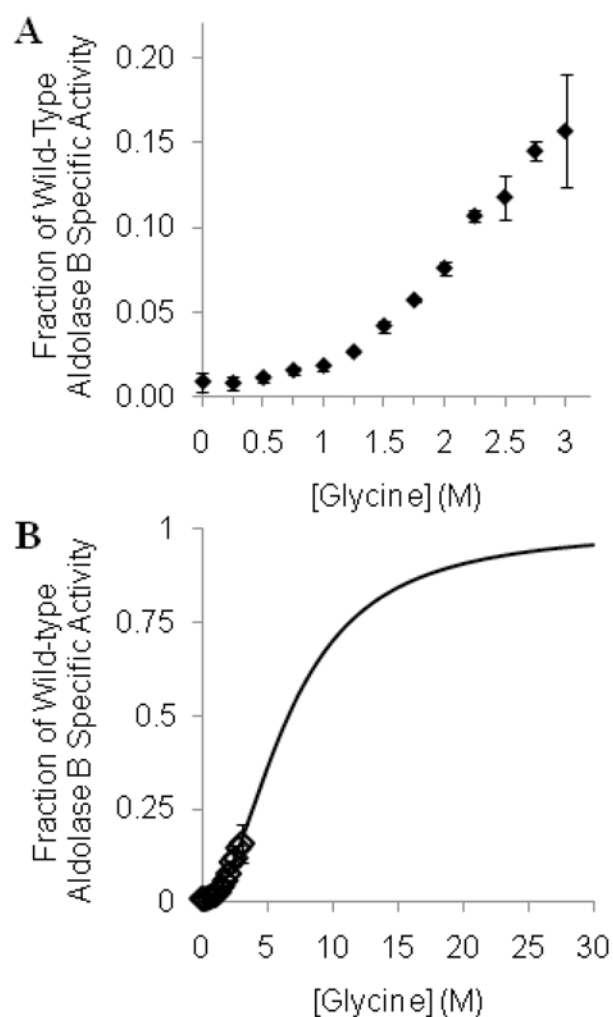
**Fig. 4. Quaternary Structure of AP-aldolase Stabilized in the Presence of 2 M Glycine**  
Gel filtration was performed on AP-aldolase (open circles) and Aldolase B (closed circles) at a concentration of 8 mg/mL using a Superdex-200 column in the absence (Panel A) and presence (Panel B) of 2 M glycine at 4 °C. Results are normalized to the peak fraction, 1 is set equal to 1.8 absorbance units at 280 nm. Arrows indicate the peak of elution for markers; T, thyroglobulin; F, ferritin; A, aldolase A; C, conalbumin; O, ovalbumin.



**Fig. 5. The Size of Zwitterionic Osmolyte Is Crucial for Stabilizing AP-aldolase**

*Panel A*, Thermal inactivation assays at 30 °C were performed on AP-aldolase (0.4 mg/mL) in 2 M of various compounds with increasing number of methylene groups between charged ends indicated at the bottom. Pre- (dark) and post-incubation (light) activity values normalized to the pre-incubation, no-compound control value (0.07 U/mg). All trials were performed at pH 7.4. Error bars are presented as 1 $\sigma$  of three separate trials. Reagents were 1, glycine; 2,  $\beta$ -alanine; 3,  $\gamma$ -aminobutyric acid (GABA); 4,  $\delta$ -aminopentanoic acid (DAPA); 5,  $\epsilon$ -aminohexanoic acid (EAHA); 6, 7-aminoheptanoic acid (7AHA). *Panel B*, Average RHSA calculated from molecular dynamics simulations of the molecular series of compounds is plotted versus the number of methylene groups between charged ends. The RHSA was calculated from structures obtained in the molecular dynamics simulations by first summing the solvent-accessible surface areas of atoms with absolute partial charge <0.2, and termed the total hydrophobic surface area (THSA). This was divided by molecular

solvent accessible surface area (SASA), which was calculated with the solvent represented as a probe of radius 1.4 Å. Partial charge assignments were done in the CHARMM force field and hydrophobic atoms defined as having partial charge <0.2. *Panel C*, Representative structures of the molecular series, chosen for RHSA values that approach the average values depicted in panel B *Panel D*, FPSA is plotted versus the percent thermal inactivation protection observed from panel A. The linear regression line (solid line) has a positive slope with a correlation coefficient of 0.72. The FPSA was calculated from structures obtained in the molecular dynamics simulations as described for Panel B using the sum of surface areas of atoms with absolute value of partial charges  $\geq 0.2$ , and termed the total polar surface area (TPSA), and divided by SASA.



**Fig. 6. Glycine Concentration versus Thermal Stabilization Fits a Sigmoidal Binding Curve**  
*Panel A* Thermal inactivation assays at 30 °C were performed on AP-aldolase (0.5 mg/mL) in the presence of glycine in concentrations varying from 0 – 3 M. Values of residual specific activity of AP-aldolase were normalized to that of wild-type Aldolase B at 25 °C (1 U/mg). All trials were performed at pH 7.4. Error bars are presented as  $3\sigma$  of three separate trials. *Panel B* The same data in Panel A was fit to a sigmoidal binding curve with equation  $Y = [S]^n / (K_d^n + [S]^n)$  using KaleidaGraph (Synergy Software) ( $R^2$  value of 0.9890).



**TABLE 1**

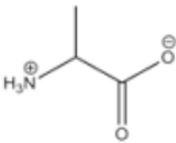
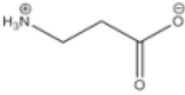

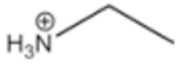
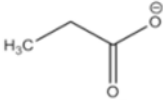
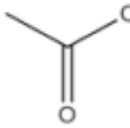
## Screen of Potential Stabilizing Osmolytes

CLASS	OSMOLYTE	CONC.	SPECIFIC ACTIVITY (%) <sup>a</sup>	
			PRE-INCUBATION	POST-INCUBATION
CONTROLS	None	-	100 ± 12	20 ± 7
	Urea	1 M	38 ± 8.7	7 ± 0.22
POLYOLS	Glycerol	4 M	126 ± 42	52 ± 3.8
	2,4-methylpentanediol	4 M	10 ± 1.0	17 ± 0.87
	PEG-8000	2% (w/v)	126 ± 10	27 ± 0.30
	Sucrose	1 M	124 ± 10	42 ± 0.95
METHYLAMINES	Trimethylamine N-oxide	1 M	66 ± 5.6	43 ± 2.5
	Betaine	4 M	103 ± 9.3	101 ± 10
	Sarcosine	1 M	106 ± 18	98 ± 10
AMINO ACIDS	Glycine	2 M	138 ± 10	127 ± 18
	Arginine	1 M	6 ± 0.45	1 ± 0.35
	Proline	3 M	122 ± 14	35 ± 0.38

<sup>a</sup> Calculated using enzyme coupled aldolase activity assay and normalizing to preincubation without osmolyte. (100% = 0.02 U/mg)

**TABLE 2**

Screen of Functional Groups Required for Stabilizing AP-aldolase Activity

COMPOUND <sup>b</sup>	STRUCTURE	SPECIFIC ACTIVITY (%) <sup>a</sup>	
		PRE-INCUBATION	POST-INCUBATION
Alanine		110 ± 28	160 ± 17
β-alanine		100 ± 17	110 ± 29
Ethanolamine		22 ± 10	19 ± 19
Ethylamine		21 ± 8	23 ± 15
Propionic Acid		82 ± 13	21 ± 5
Acetic Acid		16 ± 9	16 ± 5
Triethylamine + Acetic Acid		12 ± 3	18 ± 2

<sup>a</sup> Calculated using enzyme coupled aldolase activity assay and normalizing to preincubation without osmolyte. (100% = 0.03 U/mg).<sup>b</sup> all at 2 M.

Effect of 2 M Glycine on Kinetic Values of AP-aldolase at 10 °C and 30 °C with the substrate Fructose 1,6-bisphosphate.

TABLE 3

ENZYME	10 °C					30 °C		
	$k_{cat}$ (sec <sup>-1</sup> )	% <sup>a</sup>	$K_m$ (μM)	Ratio <sup>b</sup>	$k_{cat}$ (sec <sup>-1</sup> )	% <sup>a</sup>	$K_m$ (μM)	Ratio <sup>b</sup>
Aldolase B <sup>c</sup>	0.190 ± 0.002	-	1.7 ± 0.2	-	2.1 ± 0.02	-	2.4 ± 0.4	-
AP-aldolase	0.029 ± 0.006	15	2.8 ± 0.3	1.6	0.008 ± 0.00002	0.38	15.9 ± 0.01	6.6
AP-aldolase + glycine	0.028 ± 0.002	15	33 ± 4.0	20	0.076 ± 0.012	3.7	27.9 ± 5.6	12

<sup>a</sup> measured as a percentage of wild-type activity  
<sup>b</sup> measured as a ratio with the  $K_m$  value of wild-type aldolase  
<sup>c</sup> values reported in Malay *et al.* 2002 (10)

Effect of 2 M Glycine on Kinetic Values of AP-aldolase at 10 °C and 30 °C with the substrate Fructose 1-phosphate.

TABLE 4

ENZYME	10 °C					30 °C				
	$k_{cat}$ (sec <sup>-1</sup> )	% <sup>a</sup>	$K_m$ (μM)	Ratio <sup>b</sup>	$k_{cat}$ (sec <sup>-1</sup> )	% <sup>a</sup>	$K_m$ (μM)	Ratio <sup>b</sup>		
Aldolase B <sup>c</sup>	0.120 ± 0.004	-	240 ± 10	-	2.0 ± 0.2	-	880 ± 80	-		
AP-aldolase <sup>c</sup>	0.019 ± 0.006	16	4200 ± 460	20	0.008 ± 0.00004	0.4	16400 ± 980	20		
AP-aldolase + glycine	0.035 ± 0.002	30	640 ± 95	2.7	0.152 ± 0.005	8	2190 ± 290	2.5		

<sup>a</sup> measured as a percentage of wild-type activity

<sup>b</sup> measured as a ratio with the  $K_m$  value of wild-type aldolase

<sup>c</sup> values reported in Malay *et al.* 2002 (10)