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Glycine to Alanine Substitutions in Helices of Glyceraldehyde-3-phosphate Dehydrogenase: Effects on Stability†

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ABSTRACT: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from chicken was expressed in and purified from *Escherichia coli*. To investigate the physical basis of possible protein stabilization strategies, the effect of substitutions of glycine residues by alanine in helical regions was determined. One Gly to Ala substitution (G316A) located in the central core of the subunit was found to strongly stabilize the protein, while the other mutations are neutral or destabilize the protein. The effect seen for the stabilizing mutant in irreversible heat denaturation correlates with the first transition in folding equilibrium experiments that is observable by fluorescence, but not with the one detected by circular dichroism measurements or in dilution-induced dissociation experiments. The stabilizing effect of a Gly to Ala substitution therefore does not seem to be caused by an entropic effect on the unfolded state. Rather, an internal cavity is filled by the substitution G316A, probably stabilizing the native state. In large oligomeric proteins, imperfect packing may be a frequent cause of limited stability.

The resistance of proteins to denaturation is of great importance for life in extreme environments, and in biotechnology. The process of natural selection has lead to variants of many proteins that function even under extreme conditions such as high temperature, high salt, or extreme pH values (Jaenicke, 1981). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[1] is a protein which has been adapted by nature to work under extreme conditions (Harris & Waters, 1976). Thus, it should be possible to improve the stability of variants of this enzyme upon which selective pressure has not acted.

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[1] Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); EDTA, ethylenediaminetetraacetic acid; NAD, nicotinamide adenine dinucleotide, oxidized form; GAP, D-glyceraldehyde 3-phosphate.

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Sequences of this enzyme from various sources are known [for recent alignments, see, e.g., Hocking and Harris (1980), Branlant and Branlant (1985), and Fabry et al. (1989)]. The three-dimensional structures of the enzyme from lobster (Moras et al., 1975), human muscle (Mercer et al., 1976), and *Bacillus stearothermophilus* (Skarzynski et al., 1987; Skarzynski & Wonacott, 1988) have been solved, albeit at different resolution. Unfortunately, many mutations probably unrelated to changes in stability have accumulated throughout evolution and may obscure the essential stabilizing improvements. In most cases, direct sequence comparisons are therefore unlikely to unravel the physical basis of increased stability.

We have therefore decided to approach the problem analytically, starting from a well-characterized enzyme adapted to function at moderate temperature and investigate the effect of amino acid substitutions on the behavior of this variant. We chose the enzyme from chicken because of the availability of its gene (Arnold et al., 1982) and the very high homology to the crystallized enzyme from lobster.

The advent of gene technology has made it possible to investigate the effects of various stabilization strategies. It is important to stress the difference between the effects on rates and temperature dependence of irreversible denaturation reactions on the one hand and the effects on equilibrium constants seen in reversible denaturation–renaturation experiments on the other hand. Equilibrium effects on protein stability may be caused by enthalpic or entropic effects on either the folded or the unfolded state. Irreversible denaturation, on the other hand, is a kinetic phenomenon and usually at least a two-step process. The irreversible step may occur after a reversible unfolding step and may be due to an irreversible chemical inactivation [e.g., peptide bond cleavage, oxidation of cysteines or methionines, deamidation, racemization, and β-elimination of disulfide bonds [see, e.g., Ahern and Klibanov (1985)]] or mechanisms involving conformational trapping and adsorption. Amino acid substitutions may have an effect on either process. Thus, the effect of a particular mutation on stability depends on how the particular protein of interest is inactivated and cannot be generalized.

The aspects of the stability of the native protein conformation that have been scrutinized by site-directed mutagenesis include interactions of helix dipoles with point charges (Sali et al., 1988; Nicholson et al., 1988; Serrano & Fersht, 1989), hydrophobic packing (Matsumura et al., 1988; Kellis et al., 1988, 1989; Lim & Sauer, 1989; Sandberg & Terwilliger, 1989; Karpusas et al., 1989), H-bond patterns (Grütter et al., 1987), and engineered disulfide bonds (Pantoliano et al., 1987; Wetzel et al., 1988; Matsumura et al., 1989).

Matthews et al. (1987) have proposed that substitutions which decrease the chain entropy of the unfolded state might improve stability. These substitutions might shift the equilibrium to the folded state, provided that there are no enthalpic penalties for these changes in the folded state. An interesting aspect of this hypothesis is that it, if correct, might provide a general approach to the stabilization of a protein even if its three-dimensional structure is not known. Glycine residues would lose most conformational entropy upon folding. Relatively few positions would have to be tested to find those whose substitution results in a stabilizing effect. We investigate the mechanistic basis of this strategy in this paper with a GAPDH of known structure.

We report here an expression and purification system to obtain chicken GAPDH from *Escherichia coli* and the development of analytical techniques to characterize the irreversible denaturation, the urea-induced denaturation, the dilution-induced dissociation, and the enzymatic parameters of this recombinant protein. We then investigated the consequences of substitutions of glycine residues in α-helices with alanine or other residues.

Materials and Methods

General Methods. Bacterial growth and growth media were as described by Miller (1972). Recombinant DNA experiments were performed essentially according to Maniatis et al. (1982). DNA sequencing was carried out according to Sanger (1977) and site-directed mutagenesis according to Kunkel et al. (1987), Vieira and Messing (1987), and Geisselsoder et al. (1987). All mutations were verified by DNA sequencing. Synthetic oligonucleotides were synthesized with a 380A DNA synthesizer (Applied Biosystems). SDS–polyacrylamide electrophoresis was carried out according to Laemmli (1970) and Fling and Gregerson (1986). Protein concentration was determined by using the method of Bradford (1976) using a weighted amount of chicken GAPDH (Sigma) for preparing a standard solution.

Bacterial Strains. Initially, the expression of chicken GAPDH was investigated in the *gap*$^-$ *E. coli* strain DF221 (*gap-2*, *relA1*, *tonA22*, *T2*^{R}, *pit-10*, *spoT1*, *ompF627*), obtained from B. Bachmann (Hillman & Fraenkel, 1975). After SDS–PAGE had shown an inactive GAPDH present in this host (see Results), a new *E. coli* strain was constructed, in which the *gap* gene was inactivated by transposon insertion. The transposon Tn10 of phage λ1098 carrying tetracycline resistance, kindly provided by N. Kleckner (Harvard University), was introduced into the chromosome of *E. coli* wild-type strain W3110 (Bachmann, 1972) according to the "λ-hop" procedure (Way et al., 1984). Screening for a *gap*$^-$ mutant strain was carried out by replica plating on M63 minimal medium containing both malate and glycerol and M63 minimal medium containing only glucose as carbon source (Hillman & Fraenkel, 1975). One out of 10 000 colonies (denoted W3CG) growing on the glycerol/malate minimal medium did not grow on the glucose minimal medium. Activity assays (see below) and SDS–PAGE showed that this strain does not produce the *E. coli* GAPDH.

DNA Contructions and Mutagenesis. A plasmid which contains the cDNA encoding most of the GAPDH gene from chicken heart muscle, but missing the 60 NH_2-terminal amino acids, was a generous gift by H. Arnold (Universität Hamburg). The coding sequence was completed at the NH_2-terminus according to the known DNA sequence (Dugaiczyk et al., 1983; Domdey et al., 1983) with synthetic oligonucleotides. For expression in *E. coli*, the GAPDH gene was subcloned into an expression vector, which places GAPDH synthesis under the control of the *trp* promoter. This vector pCGAP1 also contains the β-lactamase gene as a selectable marker and the intergenic region of the bacteriophage f1 (Beck & Zink, 1981). For site-directed mutagenesis, single-stranded DNA of the plasmid pCGAP1 could thus be prepared and used directly as template.

GAPDH Purification. For production of chicken GAPDH, *E. coli* W3CG harboring the wild-type or a mutant plasmid was grown in 1 L of Luria broth (LB) (Lennox, 1955) supplemented with 50 μg/mL ampicillin, 15 μg/mL tetracycline, and 40 μg/mL indolyl-3-acrylic acid (Serva) for induction of the *trp* promoter.

The cells were harvested after overnight growth at 37 °C, suspended in 50 mM Tris-HCl (pH 7.0) containing 3 mM 2-mercaptoethanol and 1 mM EDTA, and disrupted in a French pressure cell. Next, a 60–90% $(NH_4)_2SO_4$ fractionation was carried out. The purification of the GAPDH was

achieved by NAD affinity chromatography (Harris et al., 1980) as follows. The material precipitating with 90% $(NH_4)_2SO_4$ was dissolved in 200 mM sodium phosphate (pH 8.0) containing 1 mM EDTA and 3 mM 2-mercaptoethanol and directly applied onto an NAD affinity column (AGNAD type 3, Pharmacia). After the column was washed with 100 mM sodium phosphate (pH 8.0) containing 1 mM EDTA, 3 mM 2-mercaptoethanol, and 700 mM NaCl, GAPDH was eluted with the same buffer containing 500 mM NaCl and 10 mM NAD. To remove NAD from the enzyme, GAPDH was dialyzed extensively against 50 mM sodium phosphate (pH 8.0) containing 1 mM EDTA and 3 mM 2-mercaptoethanol at 4 °C. The yield of purified GAPDH was about 10 mg per 1 L of *E. coli* culture.

Enzyme Assay. Enzyme activity was measured according to Velick (1955). The reaction was started by adding about 0.1 μg of GAPDH to the assay mix containing 45 mM sodium pyrophosphate (pH 8.5), 3 mM 2-mercaptoethanol, 10 mM sodium arsenate, 1 mM NAD, and 1 mM D-glyceraldehyde 3-phosphate at 25 °C. The change in absorbance at 340 nm was followed. Kinetic constants were calculated from initial rates. In the determination of kinetic parameters, the concentrations of the respective fixed substrates for the oxidative phosphorylation were 1 mM NAD or 0.2 mM GAP. At higher concentrations of the fixed substrate, substrate inhibition is seen as described previously (Furfine & Velick, 1965; Orsi & Cleland, 1972). K_m and V_{max} were determined from Lineweaver–Burk double-reciprocal plots.

For monitoring the enzyme activity in the urea denaturation experiments, 10 μL (0.1 μg) of each protein/urea solution was added to the assay mix. In these experiments, the assay mix contained, in addition to the above components, a small amount of urea to bring each assay mix to an identical final urea concentration of 50 mM. At this low urea concentration, the activity of native enzyme was not inhibited.

Thermal Denaturation Experiments. Thermally induced irreversible denaturation was monitored by activity measurements. Wild-type and mutant enzymes were incubated at a protein concentration of 500 μg/mL (3.8 μM) in 50 mM sodium phosphate (pH 8.0) containing 1 mM EDTA and 1 mM 2-mercaptoethanol at each temperature for 10 min. After being chilled on ice and a 1:40 dilution with incubation buffer preincubated at 25 °C, the enzyme activity was immediately measured at 25 °C.

Urea-Induced Denaturation Followed by Fluorescence. For urea denaturation experiments, GAPDH was equilibrated at a final protein concentration of 25 μg/mL (0.2 μM) for 2 h at 25 °C in 50 mM sodium phosphate, 1 mM EDTA, and 1 mM 2-mercaptoethanol (pH 8.0) containing the appropriate urea concentration. The urea solution was freshly prepared for each experiment. The extent of unfolding was found to be identical after 2–20 h in incubations with the same urea concentration. The refolding experiments were carried out by diluting samples of unfolded protein (obtained by a 2-h incubation in a 5 M urea solution) into a buffer containing a low urea concentration to give the appropriate final concentration of urea. The final protein concentration was 0.2 μM.

Fluorescence measurements were carried out with an RF5000 fluorometer (Shimadzu) at 25 °C. The effect of urea on the intrinsic protein fluorescence was monitored in two different ways. First, the change of fluorescence intensity was measured at an emission wavelength of 360 nm. Second, the wavelength of the emission maximum was determined by recording the emission spectrum from 300 to 400 nm. In all experiments, the excitation wavelength was 280 nm.

CD Spectroscopy. The protein denaturation by urea was monitored by circular dichroism measurements on a Dichrograph Mark IV (Jobin Yvon) instrument at 25 °C with cuvettes of 0.1-cm path length (0.3 mL). The protein solution (1 μM) was equilibrated for 12 h in 50 mM sodium phosphate, 1 mM EDTA, and 1 mM 2-mercaptoethanol (pH 8.0) at the appropriate urea concentration at 25 °C. A spectrum of only buffer and urea at the same concentration was subtracted from each protein spectrum.

Dilution. The dilution experiment was carried out by incubating the protein in a concentration range from 10^{-6} to 10^{-9} M for 2 h at 25 °C in 10 mM sodium phosphate, 1 mM EDTA, and 1 mM 2-mercaptoethanol (pH 8.0). The change in fluorescence emission at 360 nm was monitored. The excitation wavelength was 280 nm.

Numbering. To keep the numbering consistent with that of the lobster enzyme (Moras et al., 1975; entry 1GPD in the Protein Data Bank), we do not count the NH_2-terminal methionine present in the recombinant enzyme. In this numbering, residue 24 is a deletion in the lobster enzyme and a lysine residue in the chicken enzyme, but counted in both.

Results

Expression and Purification of Recombinant Chicken GAPDH. The gene of chicken GAPDH which had earlier been obtained by cDNA cloning (Arnold et al., 1982) extended from amino acid 60 to the C-terminus. It was completed with synthetic oligonucleotides according to an independently derived cDNA sequence (Dugaiczyk et al., 1983). DNA sequencing showed that at position 196, a glycine codon was present instead of a codon for aspartic acid in the published sequence. All further constructions therefore contain a glycine residue at position 196. A vector was constructed in which the chicken gene is expressed under the control of the *trp* promoter.

To be able to characterize the recombinant protein, the *E. coli* GAPDH must be completely absent. Expression was initially investigated in a *gap*$^-$ strain (Hillman & Fraenkel, 1975) that apparently carried one or several point mutations in the *E. coli* genome inactivating the enzyme. An NAD affinity purification was developed, but it was discovered that the inactive *E. coli* GAPDH copurified with the recombinant enzyme. The identity of the host-derived (enzymatically inactive) protein as GAPDH was confirmed by determining the NH_2-terminal sequence according to Eckerskorn et al. (1988). The first three amino acids of the recombinant chicken protein were also sequenced and found to be identical with the expected sequence, but containing the NH_2-terminal methionine.

To eliminate the genomically encoded GAPDH completely, a transposon mutagenesis with a derivative of Tn10 (Way et al., 1984) was carried out. The selection for transposition was resistance to tetracycline. The screening for GAPDH inactivation was the inability to grow on minimal medium with glucose as sole carbon source, but the ability to grow on minimal medium with malate and glycerol (Hillman & Fraenkel, 1975). The mutant strain so obtained (denoted W3CG) does not show any detectable GAPDH activity or any GAPDH protein after affinity purification. A recently discovered putative second *gap* gene in *E. coli* (Alefounder & Perham, 1989), whose role and regulation are unclear, does not seem to markedly rescue this defect. Interestingly, the cells of strain W3CG do not grow on rich medium. This phenotypic behavior is consistent with previous data on *E. coli* strains missing glycolytic enzymes (Irani & Maitra, 1977). The GAPDH expression plasmid confers the ability to grow on rich

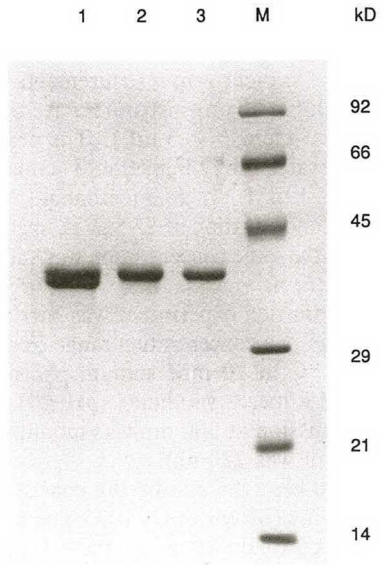

FIGURE 1: SDS–polyacrylamide gel (12.5%) stained with Coomassie brilliant blue. (Lane 1) Chicken GAPDH purified from *E. coli* DF221 (pCGAP1); (lane 2) chicken GAPDH purified from *E. coli* W3CG (pCGAP1); (lane 3) chicken GAPDH purified from chicken muscle (Sigma); (lane M) protein molecular weight marker.

medium or on M63/glucose medium.

With the *E. coli* host W3CG harboring the plasmid pCGAP1, the recombinant chicken GAPDH could be purified to homogeneity by NAD affinity chromatography. The recombinant enzyme is kinetically indistinguishable from the protein purified from chicken muscle (data not shown) and shows identical mobility on SDS–PAGE (Figure 1). Since the chicken enzyme restores all growth defects of *E. coli* W3CG, it must be functional in vivo. Thus, we have developed a rapid purification and screening system for mutants of chicken GAPDH.

Construction of Mutants. The GAPDH molecule from chicken is a tetramer of 4 identical subunits of 332 residues each, not counting the NH_2-terminal methionine, which is present only in the recombinant enzyme. Each subunit contains seven helices (Figure 2), and there are five glycine residues within these helical regions: at positions 12, 106, 111, 215, and 316. It was therefore of interest to investigate the consequences of substituting these helical glycine residues. We have not considered the glycine residues at the ends of the helices (i.e., positions 9, 23, 112, 166, 209, and 265). As can be seen from Figure 2, residues 12, 106, 111, and 215 are in contact with the solvent, whereas residue 316 is buried in the

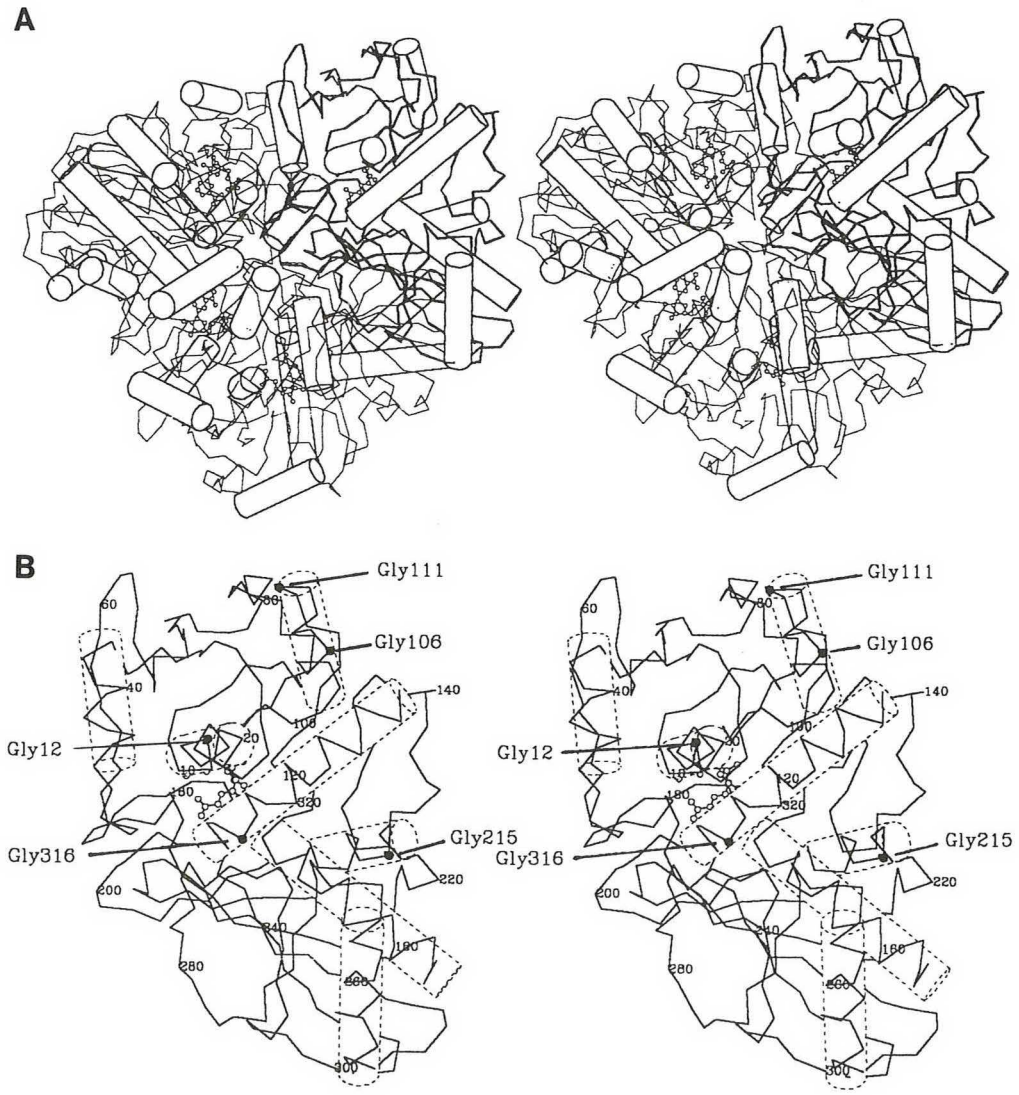

FIGURE 2: (A) α-Carbon tracing of the GAPDH tetramer from lobster illustrating the interactions between the subunits. One subunit is shown in thicker lines. The bound NAD molecules are shown by open circles. (B) α-Carbon tracing of one subunit in the same orientation as the emphasized subunit in (A). The locations of the mutations described in the text are shown. The α-helices are indicated in (B) with a dashed line. The diagrams were obtained with the plot program of Lesk and Hardman (1982).

Table I: Kinetic[a] and Denaturation[b] Properties of Wild-Type and Mutant GAPDH

enzyme	k_{cat} (s^{-1})	$K_{M,NAD}$ (μM)	$K_{M,GAP}$ (μM)	T_{50} (°C)	$[urea]_{50}$ (M)
wild type	231	30	260	50	1.4
G106A	nd[c]	nd	nd	50	nd
G106A/G111A	316	27	230	51	nd
G215A	231	27	260	47	1.2
G316A	233	33	220	57	1.8

[a] K_M and k_{cat} values were determined as described under Materials and Methods. [b] T_{50} and $[urea]_{50}$ are the values at which 50% of the enzyme activity is retained after thermal and urea denaturation, respectively (taken from Figures 3 and 4, respectively). [c] Not determined.

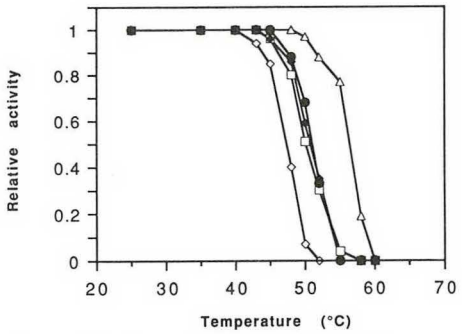

FIGURE 3: Irreversible thermal denaturation of wild-type and mutant GAPDH. The denaturation was monitored by measurement of enzymatic activity as described under Materials and Methods. The protein concentration during the incubation at different temperatures was 500 μg/mL for all proteins. Wild type (□), G215A (◊), G316A (△), G106A (♦), G106A/G111A (●).

core of each subunit in the center of the long C-terminal helix. This helix is part of the catalytic domain but has contacts with the NAD binding domain. Gly-215 is part of a helix that is close to a contact site between the two domains of each subunit. None of the glycine residues considered here is involved directly in intersubunit contacts. Due to its small steric requirements, alanine was chosen to replace the glycine residues. Alanine is also a residue that has a high helix propensity (Schulz, 1988). The helices of lobster GAPDH containing Gly show no large deviation from standard α-helices (except the NH_2-terminal helix). There is no prominent kink at Gly positions 106, 111, 215, or 316.

Enzymatic Properties. The effects of any of the Gly to Ala substitutions on the enzymatic properties were minimal (Table I). The K_M value for neither NAD nor GAP was significantly altered, and the turnover number, k_{cat}, was very similar to wild type. In the double mutant combining G106A *and* G111A, a slight increase in turnover number was observed. Only the substitution G12A was found to inactivate the enzyme. This mutant plasmid does not confer the ability to grow on rich medium to the strain W3CG (in contrast to the wild-type plasmid and all other mutants described here), and no protein bound to the NAD affinity column. This mutant protein was not further investigated.

Thermal Inactivation. To compare the effects of these substitutions on thermal stability, the purified proteins were incubated for 10 min at various temperatures under otherwise identical conditions, and the remaining enzymatic activity was measured (Figure 3). The mutants clearly fall into three groups. G106A and the double-mutant G106A/G111A behave like the wild-type enzyme with a midpoint of temperature stability of about 50 °C, whereas the substitution G215A destabilizes the protein (midpoint at 47 °C). The substitution G316A, on the other hand, strongly stabilizes the protein (midpoint at 57 °C). To elucidate the causes of these differences, three of the proteins were investigated further: the wild type, G215A as an example of a destabilizing mutant, and G316A as an example of a stabilizing mutant.

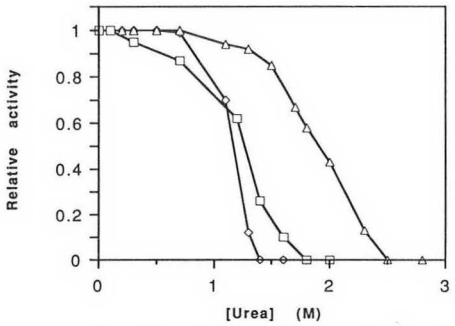

FIGURE 4: Urea-induced inactivation monitored by activity measurements as described under Materials and Methods. The proteins are designated as follows: wild type (□), G215A (◊), G316A (△).

Urea-Induced Unfolding Followed by Enzymatic Activity. After the enzyme had been exposed for 2 h to buffers containing increasing amounts of urea, the remaining enzymatic activity was measured. The effects observed during irreversible thermal denaturation are mirrored in the urea-induced unfolding experiment (Figure 4). The protein G316A is inactivated at higher urea concentration than the others, and G215A shows a somewhat steeper transition than the others (see below).

Urea Denaturation: Fluorescence Measurements. The effect of urea denaturation on the fluorescence properties of the enzyme is seen in Figures 5 and 6. First, between about 1 and 2 M urea, the intensity of the spectrum changes, but the emission maximum remains constant at 336 nm. At higher urea concentrations (around 4 M), the emission maximum changes from 336 to 349 nm. This clearly indicates the presence of (at least) two transitions in urea-induced unfolding, consistent with previous studies (Elödi & Jécsai, 1960).

The first transition was followed by measuring the fluorescence intensity at 360 nm (Figure 5), since the intensity difference between the native and denatured state was found to be largest there. The midpoint of the wild type is at about 1.5 M urea, and there is some hysteresis seen. The G215A mutant shows not only a transition at lower urea concentration (at 1.2 M) but also an almost complete absence of hysteresis and a shape of the transition curve resembling a classical two-state transition. On the other hand, in the more stable G316A mutant, the transition is less steep, the midpoint is higher (at about 1.8 M urea), and the hysteresis effect drastically increases.

In contrast to the first transition, the second transition is almost identical for the wild-type and the mutant proteins (Figure 6). This transition was followed by recording the maximum of the emission spectrum as a function of urea concentration in both directions. No hysteresis is observed, and a very steep transition can be seen. This transition is apparently not sensitive to the Gly to Ala mutations.

Circular Dichroism. To further elucidate the nature of the transition, CD measurements were carried out. Figure 7 shows the molar ellipticity at 222 nm. The changes within the CD spectrum are also indicative of at least two transitions. Interestingly, the denaturation curves for the wild-type protein and the G316A mutant are almost superimposable. The G215A mutant, on the other hand, shows an earlier first transition than the wild type with a more pronounced plateau around 2–3 M urea. The midpoint of the first CD transition (at about 2 M urea) is higher than the fluorescence transition for the wild type. Furthermore, the CD transition is much

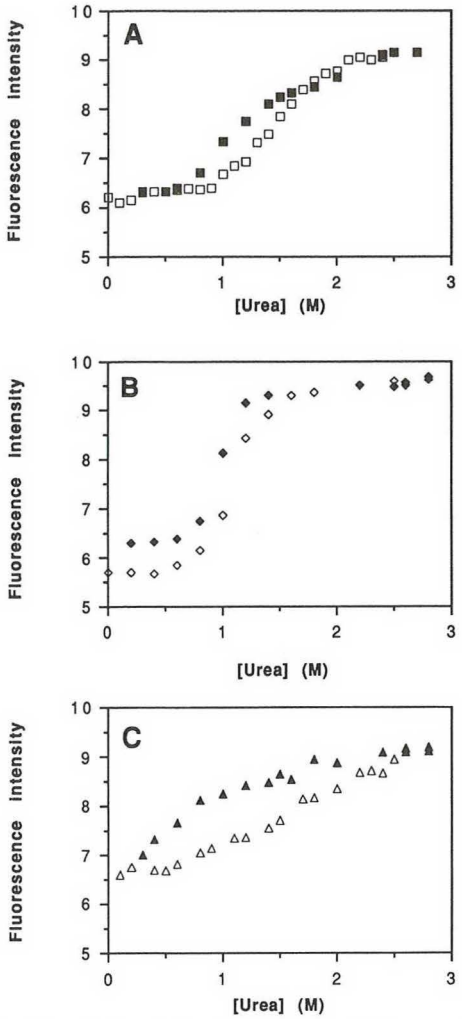

FIGURE 5: Urea-induced denaturation of wild-type and mutant GAPDH. This first transition was monitored by measuring the intensity of the intrinsic fluorescence at 360 nm. The fluorescence excitation was at 280 nm. The denaturation is denoted by open symbols, the renaturation by closed symbols. (A) Wild type; (B) G215A; (C) G316A.

steeper than the fluorescence transition for the wild type and G316A. The identity of the wild-type and the G316A curves in the CD measurements suggests that the Gly to Ala substitution does not influence the transition in which extensive secondary structure is lost but rather exerts an effect upon an earlier transition in the unfolding reaction as detected by fluorescence measurements. This would be unexpected if the Gly to Ala substitution stabilized the protein by having an effect on the entropy of the unfolded state.

Dilution. To distinguish the effects of the substitutions on the stability of a monomeric subunit from an effect on the interaction between subunits, dilution experiments were carried out with the native wild type, the G215A mutant, and the G316A mutant protein. The fluorescence intensity shows a transition around 10^{-8} M, but the transition is essentially identical for all three proteins (Figure 8). While the enzymatic assays were carried out at final concentrations below this transition, it has been observed that substrate and cofactor shift the equilibrium toward the tetramer (Hoagland & Teller, 1969). To verify that the dilution experiments actually do detect changes in subunit interaction energy, a mutant in which Tyr46 was changed to a lysine was also examined. This substitution lies at the subunit interface and shows a transition at about a 10-fold higher protein concentration (Figure 8). We conclude that the dilution experiment indeed measures the

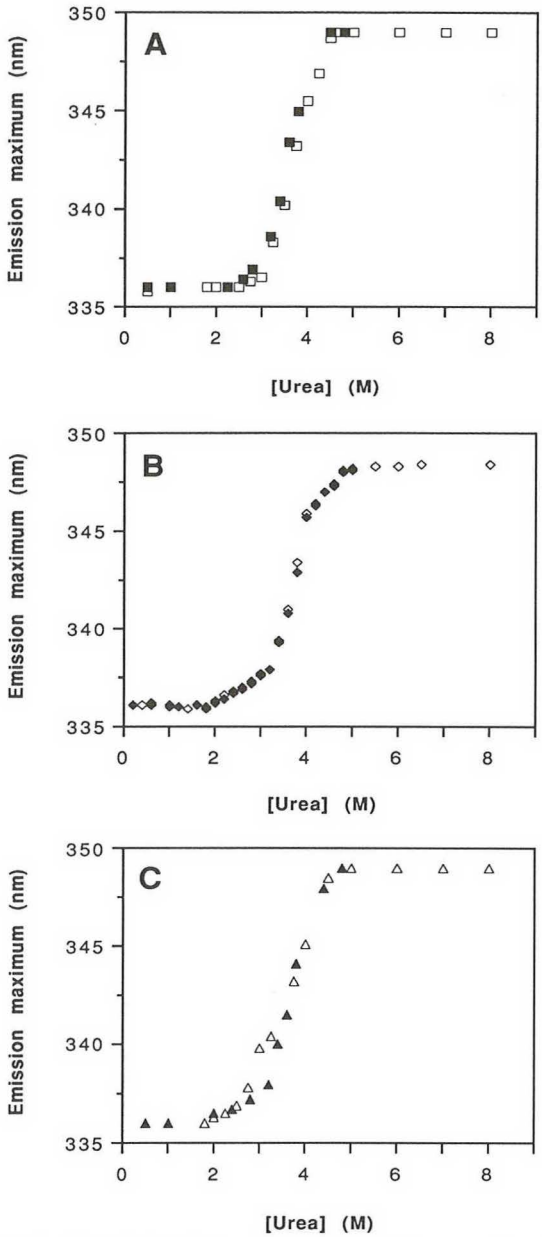

FIGURE 6: Urea-induced denaturation of wild-type and mutant GAPDH. This second transition was monitored by measuring the maximum of the fluorescence emission spectrum. The fluorescence excitation was at 280 nm. The same symbols were used as in Figure 5. (A) Wild type; (B) G215A; (C) G316A.

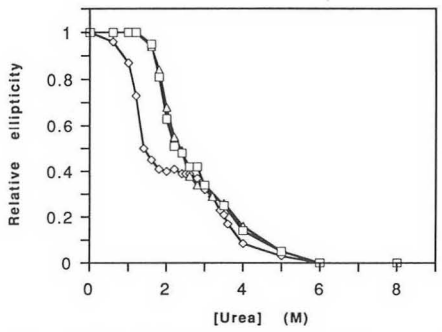

FIGURE 7: Urea-induced denaturation of wild-type and mutant GAPDH measured by CD spectroscopy as described under Materials and Methods. The transition was monitored at 222 nm. Wild type (□), G215A (◇), G316A (△).

interaction between the subunits and that the Gly to Ala substitutions do not have an effect on the association of the native subunits.

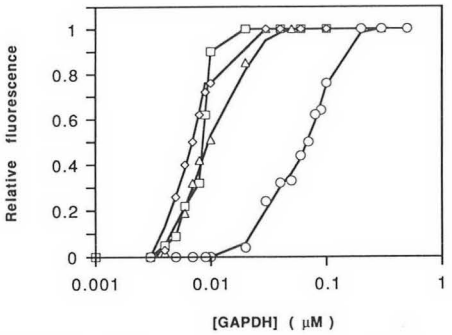

FIGURE 8: Stability of tetrameric GAPDH upon dilution monitored by the fluorescence intensity at 360 nm. Excitation was at 280 nm. The proteins are designated as follows: wild type (□); G215A (◇); G316A (△); Y46K (○).

DISCUSSION

Chicken GAPDH can be stabilized, left unchanged, or destabilized by single mutations of Gly to Ala in helical regions. Our analysis shows that the increase (or decrease) in stability against irreversible denaturation corresponds to changes seen in urea-induced denaturation. The urea-induced unfolding shows, as must be expected for a complex protein of this size, several transitions. The enzymatic activity disappears (also as expected) with the first transition. Neither the stabilizing mutation G316A nor the destabilizing one G215A influences the interaction between the subunits since the dilution-induced transitions are essentially superimposable. We propose therefore that the mutations affect an early step in the unfolding within a subunit.

To analyze whether the packing within the interior might have been changed, we calculated surfaces accessible to a probe sphere of 1.7-Å radius (Connolly, 1983) and found a rather large internal cavity adjacent to glycine-316. Clearly, the methyl group of alanine would point into this cavity and decrease its size. The hypothesis that an improved packing is the cause of the increased stability for this mutant protein is strengthened by the observation that a valine residue at position 316 is also tolerated. However, this substitution increases the thermal stability against irreversible denaturation only by 2 °C (data not shown), while alanine increases it by 7 °C. A quantitative analysis of the packing of GAPDH is complicated by the fact that the chicken enzyme structure had to be modeled from the known lobster structure (74% identity, one insertion of one amino acid at position 24 in the chicken enzyme) and that the lobster structure itself was solved to only 3.0-Å resolution. Nevertheless, the fact that the glycine to valine substitution cannot only be tolerated in the interior of this protein, but actually slightly stabilizes it, supports the hypothesis of an internal cavity at this position. Furthermore, all residues lining the cavity are identical in the enzymes from chicken and lobster and thus do not depend on any arbitrary choices in modeling the chicken enzyme from the lobster structure.

In the particular case of GAPDH, Gly to Ala substitutions apparently do not stabilize the protein by affecting the entropy of the unfolded state. This is most clearly demonstrated by the fact that only one of the substitutions has a stabilizing effect. We cannot rule out compensating effects of stabilization and destabilization, and we would not detect very small changes in free energy. However, as two mutants clearly had a dramatic effect on stability (one stabilizing, the other destabilizing), the various methods do detect changes in stability in this protein.

The unfolding transition affected by the G316A mutation is the one observable by fluorescence but not the one observable by CD. Therefore, the loss of secondary structure as measurable by CD does not seem to be different between wild type and the G316A mutant. The first fluorescence transition is less steep, and its midpoint occurs at a slightly lower urea concentration than the CD transition. Thus, this protein has a critical, stability limiting transition affected by the mutation, in which most of the native secondary structure is conserved. Since none of the mutations has any effect on the interactions of the native subunits in dilution experiments, this critical transition is likely to be a conformational transition within a subunit and still close to the native structure. This does not rule out that dissociation occurs very early in the unfolding pathway in the presence of small amounts of urea, after the native subunit has been slightly destabilized. Thus, the first observable fluorescence change may also contain a dissociation step.

The analysis of this first detectable folding intermediate in urea-induced denaturation is complicated by the distinct hysteresis effects seen especially with the more stable mutant protein. This observation suggests that aggregation phenomena or extremely slow transitions might be involved following the first transition. We have no information at present as to whether there is a distinct conformational transition within an intact tetramer or whether this conformational transition immediately leads to a dissociation of the tetramer in a cooperative process.

The exact cause of the destabilizing effect of G215A is not clear. Each subunit consists of two domains (residues 1–148 and 149–332), and glycine-215 is close to the interface between the domains. It is possible that this region is very critical for the stability of the subunits.

This particular protein and possibly other proteins of similar size and subunit structure can apparently be stabilized by improving the interior packing. It is possible that this was one of the first steps in the evolution of GAPDH toward thermophilicity. A direct test of this hypothesis is complicated by the fact that the structure of the *B. stearothermophilus* enzyme is resolved to higher resolution than that of the lobster and that many sequence changes have accumulated complicating the analysis of the interactions.

The mutation G316A does not affect enzyme catalysis, and the question thus arises why it has not occurred naturally. Interestingly, position 316 is a conserved glycine in most known GAPDH molecules. In the enzyme from *B. stearothermophilus*, the cavity is smaller due to a slightly different relative orientation of the backbone. Mutations elsewhere in the protein may thus have improved the packing in this region without changing the side chain corresponding to position 316 in this bacterial enzyme. Either the absence of stringent selection or other, still hidden beneficial effects outweighing stability might account for the persistence of glycine at that position in mesophilic GAPDH. Nature has probably introduced additional changes that stabilize the thermophilic GAPDH against chemical inactivation. These will only play a role, however, once the weakest point in the protein stability has been improved. In present day GAPDH from higher organisms, this seems to be the packing within the interior of the subunits.

We conclude from this work that Gly to Ala changes may stabilize different proteins for different reasons and that the entropy effect may only be seen in small single-subunit proteins. The observation of increase in stability by Gly to Ala substitutions has also been made in the λ repressor (Hecht et al., 1986) and in a neutral protease from *B. stearothermophilus* (Imanaka et al., 1986), but the mechanistic causes have not

yet been elucidated. An extreme view of this packing hypothesis would be that an improved packing has been a factor in all reported Gly to Ala mutants except those where the side chain is only in contact with solvent. This point must be clarified by further experimentation.

A general strategy to improve thermal stability cannot be formulated since it depends on the individuality of the protein. It is likely, however, that in larger multisubunit proteins the large interior surface will provide ample opportunity for improvement in packing, and that in many cases imperfect packing may be the main factor limiting stability.

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