Autonomous folding of the excised coenzyme-binding domain of D-glyceraldehyde 3-phosphate dehydrogenase from *Thermotoga maritima**

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Abstract

An important question in protein folding is whether compact substructures or domains are autonomous units of folding and assembly. The protomer of the tetrameric D-glyceraldehyde-3-phosphate dehydrogenase from the hyperthermophilic bacterium *Thermotoga maritima* has a complex coenzyme-binding domain, in which residues 1–146 form a compact substructure with the last 31 residues (313–333). Here it is shown that the gene of a single-chain protein can be expressed in *Escherichia coli* after deleting the 163 codons corresponding to the interspersed catalytic domain (150–312). The purified gene product is a soluble, monomeric protein that binds both NAD⁺ and NADH strongly and possesses the same unfolding transition induced by guanidinium chloride as the native tetramer. The autonomous folding of the coenzyme-binding domain has interesting implications for the folding, assembly, function, and evolution of the native enzyme.

Keywords: CD spectroscopy; coenzyme-binding domain; glyceraldehyde 3-phosphate dehydrogenase; protein engineering; protein folding

Structural domains in proteins were originally defined as tertiary substructures of continuous segments of the polypeptide chain. They are assumed to play an important role as units both of folding and of evolution (Jaenicke & Buchner, 1993). Consequently, the study of the folding behavior of artificially excised domains should yield interesting insights into the mechanism of folding of multidomain proteins (Wetlaufer, 1981). Domains that exercise a measurable function, say, the specific binding of a ligand, are particularly useful in this regard, because they provide for an experimental measure of function.

Glyceraldehyde-3-phosphate dehydrogenase is a homotetrameric protein, where the amino-terminal domain (residues 1–147, cf. Fig. 1A) is mainly responsible for binding NAD⁺ or NADH (Mercer et al., 1976; Murthy et al., 1980; Griffith et al.,

1983; Skarzynski et al., 1987). It contains the dinucleotide-binding fold that is conserved in several dehydrogenases using NAD⁺, NADP⁺, or FAD as coenzymes (Ohlson et al., 1974; Rossmann et al., 1975; Brändén, 1980; Baker et al., 1992).

The coenzyme-binding domains appear to be structurally relatively independent already in the native enzyme, because they extend radially outward from the core of the tetramer and maintain only few side-chain interactions with it. Residues 148–311 constitute the catalytic domain that binds the substrate and also mediates the quaternary contacts between the 4 subunits. The carboxyl-terminal segment (312–333) forms the long helix α_3 that interacts intimately with the coenzyme-binding domain. This additional interaction provides for a rigid orientation between the 2 domains and converts the coenzyme-binding domain into a discontinuous domain.

The binding of NAD⁺ to GAPDH from *Bacillus stearothermophilus* occurs with negative cooperativity. It appears that NAD⁺ binds first to the apoenzyme conformation via its adenosine moiety. This interaction triggers a conformational change (Skarzynski & Wonacott, 1988), which leads to a closure of the active site cleft with a hinge passing approximately through residues 150 and 310.

We decided to study the coenzyme-binding domain of GAPDH to test the prediction, based on inspection of the known structure

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Abbreviations: GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; bGAPDH, GAPDH from *Bacillus stearothermophilus*; tGAPDH, GAPDH from *Thermotoga maritima*; GdmCl, guanidinium chloride; IPTG, isopropyl β -D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride.

Fig. 1. The coenzyme-binding domain of GAPDH. Numbering as for bGAPDH (Branlant et al., 1989; for alignment with tGAPDH see Schultes et al. [1990]). **A:** Model of the main-chain topology of tGAPDH residues 1 (N) to 146 and 312 to 333 (C). Cylinders, α -helices; arrows, β -strands; connections, loops. The top right-hand face of the domain depicts bound NAD⁺ in the configuration observed in bGAPDH (Biesecker et al., 1977). The carboxamide group of nicotinic acid is close to the original position of C 149. **B:** Amino acid and nucleotide sequence of the linker region. Below is the sequence of the primer used for deletion mutagenesis.

of bGAPDH, that it would (1) fold autonomously to a soluble, folded, monomeric protein, (2) exhibit native-like structure, and (3) bind both NAD⁺ and NADH. Because Biro et al. (1990) had shown that the carboxyl-terminal helix α_3 is important for the thermostability of GAPDH from *Methanothermobacter thermophilus*, we decided to fuse helix α_3 via an artificial, flexible linker peptide to the carboxyl terminus of the coenzyme-binding domain. Thus the naturally discontinuous domain would be converted to a continuous one.

In this work we chose GAPDH from the hyperthermophilic bacterium *Thermotoga maritima* for study. The native tetramer is extremely stable toward thermal unfolding ($T_m > 105^\circ\text{C}$; Wrba et al., 1990; Rehder & Jaenicke, 1992, 1993). Its sequence is readily aligned to that of bGAPDH, because 63% of the residue positions are identical (Schultes et al., 1990). Thus, it appeared safe to use the known structure of the former protein to model the structure of the latter (Böhm, 1992), and the genetic construction was based on the known coding sequence of tGAPDH. The coenzyme-binding domain was expressed in *Escherichia coli* cells as a soluble, monomeric protein that binds both NAD⁺ and NADH.

Results

Strategy

The model of the structure of the homotetrameric tGAPDH (Böhm, 1992), which is based on the known structure of holo-bGAPDH (Skarzynski et al., 1987), was used to design the discontinuous coenzyme-binding domain of tGAPDH. As shown in Figure 1A, the domain comprises residues 1–149 (numbering for bGAPDH as in Biesecker et al. [1977] and Branlant et al. [1989]), fused with a linker of 3 residues to the carboxyl-terminal helix α_3 (residues 313–333). With the exception of a hydrogen bond between N180 and the pyrophosphate moiety of NAD⁺, all other direct interactions with the coenzyme involve residues of the coenzyme-binding domain. The distance between C α atoms of C149 and N313 is 5.9 Å. Figure 1B presents the sequence of the short linker chosen to replace the deleted catalytic domain (residues 150–312).

This linker sequence was selected by computer modeling to provide for minimal length yet optimum flexibility. The coenzyme-binding domain was then subjected to energy minimization, which confirmed that the docking of the carboxyl-terminal α -helix is unimpaired. This deletion also eliminates the catalytic residue C149 to avoid disulfide bond formation with C146, as well as 1 of the 2 tryptophan residues of tGAPDH (W310, Schultes et al., 1990). However, residues N313 and E314, which participate in an extensive network of hydrogen bonds to the carboxamide moiety of NAD⁺ (Skarzynski et al., 1987), are preserved in the construct.

The region coding for the catalytic domain (residues 150–312) was deleted by hybridizing single-stranded DNA of pAT1 with the mutagenic oligonucleotide shown in Figure 1B as a primer for synthesis of the second strand. The DNA was ligated and used to transform *Escherichia coli* JM 83 to ampicillin resistance. Restriction analysis of quick-screen DNA from single colonies led to the desired deletion mutant.

Viewing bGAPDH along its P-axis (Skarzynski et al., 1987), it is evident that the subunit contacts within the tetrameric quaternary structure are provided mainly by the catalytic domains and their S-loops, whereas the coenzyme-binding domains extend radially into the solution. Thus one would predict that deleting the catalytic domain of the subunit should result in a stable, monomeric protein. Significant contributions to the stability of the protomer appear to come from ion pairs, all of which turn out to be conserved in both bGAPDH and tGAPDH (R10–D47; R102–D125; R320–D323; R10–E314; Skarzynski et al., 1987). As judged from the distribution of surface charges, the solubility of the coenzyme-binding domain should be similar to that of other small globular proteins (Böhm, 1992).

Expression and purification of the coenzyme-binding domain

Cell growth

The transformed cells were grown at 37°C in 10 L of LB-medium (Lennox, 1955) containing ampicillin (100 µg/mL). Af-

ter reaching an optical density $OD_{550} = 1.0$, IPTG was added to a final concentration of 1 mM, and growth was continued for a further 16 h. Cells were harvested by centrifugation.

Protein purification

Forty grams of wet cell paste was suspended at 0 °C in 200 mL of 50 mM sodium phosphate buffer pH 7.0, containing 5 mM EDTA, 5 mM cysteamine hydrochloride, and 1 mM PMSF. Two cycles in a French press at 18,000 psi were applied to disrupt the cell suspension. Intact cells and cell debris were removed by centrifugation at ≤ 4 °C (30 min at 15,000 rev/min in Sorvall SS34 rotor). During the whole procedure, cells, homogenates, and solutions were kept on ice. The whole purification procedure was monitored by SDS-polyacrylamide gel electrophoresis and immunoblotting after each purification step.

Ammonium sulfate precipitation

Powdered ammonium sulfate was added to the crude extract in small portions during 2 h, under continuous stirring at 4 °C. PMSF was added repeatedly to a concentration of about 1 mM. After 40% saturation was attained, stirring was continued for a further 12–16 h. The precipitated protein was then removed by centrifugation at 4 °C (30 min at 15,000 rev/min in Sorvall SS34 rotor).

Hydrophobic chromatography

The ammonium sulfate supernatant was applied to a 60-mL column (4 × 4.7 cm) of phenyl-Sepharose (FF High Sub), equilibrated with 50 mM sodium phosphate buffer, pH 7.0, containing 5 mM EDTA, 5 mM cysteamine hydrochloride, and 30% saturated ammonium sulfate. The coenzyme-binding domain was quantitatively retained on the column. After extensive washing with the equilibration buffer, elution was accomplished using a linear gradient decreasing from 30 to 0% ammonium sulfate saturation in the same buffer. The protein was eluted at about 15% saturation.

Chromatography on blue-Sepharose

The pooled fractions of the phenyl-Sepharose column were dialyzed against 50 mM Tris-HCl buffer, pH 8.0, containing 5 mM EDTA and 5 mM cysteamine hydrochloride. For further purification, the dialysate was applied to a 5-mL HiTrapBlue column, equilibrated with the same buffer, and the protein was fully retained on the column. Subsequent elution with a linear gradient from 0 to 1 M NaCl recovered the protein at about 0.5 M NaCl, resulting not only in efficient purification but also in a drastic increase in the concentration of the protein.

Gel permeation chromatography

The final purification and approximate size determination made use of gel filtration. To this purpose, the pooled fractions were dialyzed against 50 mM Tris-HCl buffer, pH 8.0, containing 5 mM EDTA and 5 mM cysteamine hydrochloride, and subsequently concentrated to a total volume of 600 μ L, using Centricon 10 centrifugation. The protein solution was applied to a Superdex 75 prep-grade column (1.6 × 60 cm), equilibrated with the above Tris buffer containing 100 mM NaCl. Fractions containing the pure protein were pooled. The total amount of protein was 6.5 mg with a purity of 98%, as judged by SDS-polyacrylamide gel electrophoresis (Fig. 2). The protein was stored at -70 °C after shock-freezing in liquid nitrogen.

Fig. 2. Purification of the coenzyme-binding domain. Polyacrylamide gel electrophoresis in the presence of SDS. Proteins were stained with Coomassie blue. Lanes 1 and 7, marker proteins with indicated $10^{-3} M_r$ values; lane 2, crude extract; lane 3, ammonium sulfate precipitate; lane 4, pooled fractions from phenyl-Sepharose; lane 5, pooled fractions from HiTrap Blue; lane 6, pooled fractions from gel filtration step.

Physical properties

Molecular mass

The calculated molecular mass of the construct amounts to 19,195 Da. Gel permeation chromatography in the last step of purification allows the approximate molecular mass of the protein to be estimated. Using cytochrome *c*, chymotrypsinogen, ovalbumin, bovine serum albumin, and aldolase as standards, the apparent M_r is approximately 19,000. Sedimentation velocity experiments in the analytical ultracentrifuge yielded 1 single band with a sedimentation coefficient $s_{20,w} = 2.40 \pm 0.02$ S. The α subunit of tryptophan synthase, which is of similar size and known structure, possesses a similar $s_{20,w}$ value (2.5; Adachi et al., 1974). Application of the meniscus depletion sedimentation equilibrium method led to $M_r = 19,690 \pm 1,200$. There was no indication for nonlinearity, even at the bottom of the cell. Evidently, the protein is homogeneous and does not associate or aggregate. This holds also at acid pH, where the $s_{20,w}$ value decreased but the M_r value remained unaltered. The decrease of $s_{20,w}$ at pH 1.0 (1.86 ± 0.04 S) may be attributed to the expansion of the molecule, which accompanies acid denaturation (see below).

Spectral characteristics

Figure 3 summarizes the spectral properties of the coenzyme-binding domain. To ascertain that the purified protein was devoid of bound coenzyme it was denatured in 6 M GdmCl in 50 mM sodium phosphate buffer, pH 7.0, plus 0.1 mM EDTA (standard buffer). Removal of both the denaturant and material of low molecular mass was accomplished by repeated dilution with standard buffer, using Centricon ultrafiltration and gel filtration (cf. Rehder & Jaenicke, 1992). The UV absorption spectrum of the renatured material was indistinguishable from the original spectrum. The absorption maximum at 276 nm and the shoulder at 280 nm (Fig. 3A) may be attributed to the excess of tyrosine (total of 6) over tryptophan (total of 1) residues (Schultes et al., 1990). The extinction coefficient calculated according to Wetlaufer (1962) amounts to $15,279 M^{-1} cm^{-1}$. The A_{280}/A_{260} ratio of 1.9 exceeds the value (1.78) determined for native tetrameric apo-GAPDH (Rehder & Jaenicke, 1992).

Fig. 3. Spectra of the coenzyme-binding domain of tGAPDH: 50 mM sodium phosphate buffer, pH 7.0, containing 0.1 mM EDTA, 20 °C. **A:** Absorption spectrum, 1.4 mg protein/mL, $d = 1$ cm. **B:** Fluorescence emission spectrum in arbitrary units, 5 μ g protein/mL, $\lambda_{exc} = 280$ nm. —, Native protein; ---, protein in the presence of 6 M GdmCl. **C:** Far-UV CD spectrum, 5 μ g protein/mL, $d = 0.1$ cm. **D:** Near-UV CD spectrum, 5 μ g protein/mL, $d = 0.1$ cm.

Maximum fluorescence emission is observed at 334 nm, which must be due to the single tryptophan residue (W84, Fig. 3B), compared to 327 nm for the native tetramer of GAPDH. Upon denaturation in 6 M GdmCl, both the native enzyme and its coenzyme-binding domain show a shift of their fluorescence maxima to 350 nm, which corresponds to the emission of fully exposed Trp residues.

The far-UV and near-UV CD spectra (Fig. 3C,D) closely resemble those observed for native and recombinant tGAPDH (Wrba et al., 1990; Tomschy et al., 1993), except for a slight decrease in the amplitudes of the maxima and minima, which may be attributed to differences in the number of aromatic residues as well as to uncertainties in protein concentration. Based on the far-UV CD, the secondary structure of the protein was estimated at 35% α -helix, 18% β -structure, and 47% β -turns and random coil (cf. Böhm et al., 1992). These values are in good agreement with the model in Fig. 1A). In summarizing the spectral data, we conclude that the overall structure of the isolated coenzyme-binding domain must be closely similar to that of the native enzyme. This conclusion is buttressed by experiments probing the conformational stability of the 2 proteins.

Stability estimated from unfolding transitions

Making use of the change in fluorescence accompanying denaturation (cf. Fig. 3B), the relative fluorescence intensity at 330 nm was determined, varying (1) the GdmCl concentration, (2) the temperature, and (3) the pH value. As illustrated in Figure 4A, GdmCl causes a highly cooperative equilibrium transition of the coenzyme-binding domain, with the midpoint of the transition at $(C_{GdmCl})_{1/2} = 2.2$ M. It coincides precisely with the one observed for intact tGAPDH (Rehaber & Jaenicke, 1992; Tomschy et al., 1993). This observation indicates that there is no significant contribution of domain interactions to the overall stability of the native tetramer, at least with regard to GdmCl. The denaturation and renaturation transitions of the coenzyme-binding domain coincide, so that the Gibbs free energy of sta-

bilization for the coenzyme-binding domain can be estimated by extrapolating the reversible equilibrium transition data to zero GdmCl concentration. As a result, we obtain $\Delta G_{GdmCl \rightarrow 0} = 28$ kJ/mol. Similar values have been reported for other proteins of similar size, e.g., the α -subunit of tryptophan synthase (Yutani et al., 1984), or fragments of thermolysin (Vita et al., 1989).

In the case of thermal denaturation, the comparison is complicated by the different methods used for monitoring denaturation: deactivation was measured for the intact enzyme; fluorescence emission for the coenzyme-binding domain (Fig. 3B). Figure 4B shows that the intact apoenzyme is stable up to 80 °C, with 50% deactivation at 90 °C. In contrast, the fluorescence transition of the coenzyme-binding domain attains 50% at 44 °C, and cooling does not yield the initial amplitude of fluorescence. It is likely that aggregation leads to partial loss of the protein. Normalizing the data with respect to the amount of renatured material allows the van't Hoff heat of denaturation to be estimated. The value of ΔH in the range of 312–323 K is 154 kJ/mol, again in accordance with data reported on proteins of similar size and topology (Yutani et al., 1984; Pfeil, 1986).

The pH-dependent stability profile was monitored by fluorescence changes at 330 nm (Fig. 4C). It is evident that both cooperativity and stability of the coenzyme-binding domain in the acidic pH range are reduced by comparison to those of intact tGAPDH. However, comparison of the normalized pH profiles is difficult because the intact native monomer contains 2 Trp residues, whereas the nucleotide-binding domain has only one. Moreover, the fluorescence emission maximum of the native GAPDH is not affected at alkaline pH, whereas that of the coenzyme-binding domain is shifted from 330 to 350 nm (data not shown).

Ligand binding

The binding of specific ligands to a protein indicates convincingly that it carries a native-like binding site (Herold et al., 1991). We therefore determined the equilibrium dissociation constant

Fig. 4. Unfolding transitions of the coenzyme-binding domain. Buffer and temperature as in Figure 3. Protein concentration 0.05 mg/mL. Fluorescence was excited at 280 nm and recorded at 330 nm. **A:** Reversible unfolding by GdmCl. Nucleotide-binding domain in the presence of 0.1 mM EDTA at 25 °C. ●, Unfolding by incubating the native protein at the indicated concentrations of GdmCl; ○, refolding by diluting the denatured protein in the presence of 6 M GdmCl to the indicated final concentrations of GdmCl. ---, tGAPDH in the presence of 5 mM EDTA and 5 mM cysteamine hydrochloride (Rehabe & Jaenicke, 1992). **B:** Thermal unfolding in the presence of 0.1 mM EDTA. Coenzyme-binding domain unfolding (●) and refolding (○); ---, tGAPDH unfolding measured by enzyme assay. **C:** pH-dependent unfolding. pH ranges and buffers: ●, 1–3 and 9–13, 50 mM glycine/H₃PO₄/NaOH; □, 2.5–4.5, 50 mM sodium citrate/HCl; ■, 4.0–5.5, 50 mM sodium acetate/acetic acid; △, 5.5–8.0, 50 mM sodium phosphate; ▲, 7–9, 50 mM Tris/HCl; ---, tGAPDH (cf. Rehabe & Jaenicke, 1992).

of both NADH and NAD⁺ for the coenzyme-binding domain by monitoring the quenching of protein (i.e., tryptophan) fluorescence at 330 nm by NADH (Holbrook, 1972). Because the coenzyme-binding domain is monomeric and contains only a single Trp residue, fluorescence quenching must depend linearly on NADH binding. The numerical evaluation of the titration curve (Fig. 5A) was based on the equations given in Holbrook (1972). The determination of the dissociation constant for NADH yields $K_d^{\text{NADH}} = 2.8 \mu\text{M}$, as compared to the value of $\sim 0.05 \mu\text{M}$ observed for the saturation of the high-affinity sites of the complete native *Thermotoga* enzyme (Tomschy, 1993). In the case of the oxidized coenzyme, the competition between NADH and NAD⁺ was used to determine $K_d^{\text{NAD}^+}$ (Stinson & Holbrook,

Fig. 5. Binding of coenzymes to the coenzyme-binding domain of tGAPDH. Buffer and temperature as in Figure 3. Protein fluorescence emission at 330 nm in arbitrary units ($\lambda_{\text{exc}} = 280 \text{ nm}$); 0.26 μM protein concentration. **A:** ○, Quenching of protein fluorescence by NADH in the absence of NAD⁺; —, best fit with $n = 1$ and $K_d^{\text{NADH}} = 2.8 \mu\text{M}$. **B:** ○, Quenching of protein fluorescence in the presence of 385 μM NAD⁺; —, best fit with $n = 1$, $K_d^{\text{NADH}} = 2.8 \mu\text{M}$, and $K_d^{\text{NAD}^+} = 51 \mu\text{M}$.

1973). The apparent values of K_d^{NADH} were 4.9 μM in the presence of 38.5 μM NAD⁺ (data not shown) and 24 μM in the presence of 385 μM NAD⁺ (Fig. 5B). These changes correspond to $K_d^{\text{NAD}^+} = 51 \mu\text{M}$. Thus, our construct does indeed bind the coenzyme in its reduced and oxidized form with high affinity.

Discussion

Deletion of the region coding for the catalytic domain of the protomer of tGAPDH led to the expression in vivo of a soluble, monomeric protein. It binds both NAD⁺ and NADH with high affinity. As deduced from the sedimentation analysis, the axial ratio of the protein is 1.5, which is close to what is predicted from the model in Figure 1A. Taken together with the absorption, fluorescence, and CD spectra, the evidence confirms our expectations that the artificially continuous coenzyme-binding domain is folded as in the wild-type tetramer. Moreover, the contact areas responsible for assembling the tetramer of GAPDH seem to be located exclusively to the deleted catalytic domain.

Further work is required to compare the coenzyme-binding affinities of the domain to those of the native GAPDH tetramer. Preliminary studies indicate that NADH binds to the apoenzyme of tGAPDH with negative cooperativity ($K_1 \approx K_2 = 0.05 \mu\text{M} \ll K_3, K_4$; Tomschy, 1993). It is interesting that, in the case of bGAPDH, the K_M values for NAD⁺ (42 μM) and NADH ($\leq 3 \mu\text{M}$; Corbier et al., 1990) show a similar preference

for NADH. However, the average K_d value for all 4 NAD⁺ binding sites of bGAPDH (0.22 μ M) is much smaller than its K_M value.

The reversibility of denaturation proves that the domain folds autonomously, but its unfolding transitions at equilibrium vary according to the conditions used. The GdmCl-induced transition at 2.2 M GdmCl is indistinguishable from that of the wild-type GAPDH tetramer. This observation demonstrates that the domains of thermostable proteins can share the characteristically high stability with the parent protein, even if it is an oligomer. The superposition of the 2 transition curves indicates that the dissociation and unfolding of the tetramer is a highly cooperative process; that is, neither partially folded monomers nor dimers accumulate as significant intermediates. This relationship is in contrast to previous results for mesophilic oligomers, where, stepping down the structural hierarchy from the oligomer via the dimer and monomer to structural domains, similar truncations led to a drastic decrease in stability (Jaenicke et al., 1981; Opitz et al., 1987; Jaenicke, 1991). Whether specific salt effects may be involved remains to be shown. The thermal stabilities of the coenzyme-binding domain and intact tGAPDH differ widely with respect to both transition temperature and cooperativity. In the case of the pH dependence, the titrations of the tyrosine residues in the alkaline limb coincide, whereas the acid limbs of the profiles differ widely, possibly due to differences in the number and the environment of the fluorophores.

Thornton and Sibanda (1983) have pointed out that most multidomain proteins possess "crossover linkages," in which the carboxyl terminus of the carboxyl-terminal domain interacts with the amino-terminal domain, thus bringing the terminal segments into spatial proximity. It is likely that such "straps" stabilize the relative orientation between the domains also in the case of GAPDH. Such assistance may be essential for catalysis, because each domain is responsible for binding its particular substrate, and their mutual orientation must be well defined to stabilize the transition state in an optimal way. Our results show that the interaction between the carboxyl-terminal helix α_3 and the coenzyme-binding domain does not require the presence of the intervening catalytic domain. Apparently a linker of sufficient length and flexibility allows the 2 substructures to interact as in the native protein. Similar studies, such as linking the V_H and V_L domains of antibodies (Bird et al., 1988; Huston et al., 1988) and "grafting" experiments involving GAPDH from *M. fervidus* (Biro et al., 1990) and γ_{II} crystallin (Mayr et al., 1994), demonstrate that interactions between native subdomains can override the effect of the non-native linkers.

The ability of the coenzyme-binding domain of tGAPDH to fold autonomously is reminiscent of the autonomous folding of the coenzyme-binding domain of the pyridoxal phosphate-dependent aspartate aminotransferase (Herold et al., 1991) and to the ATP-binding domain of yeast phosphoglycerate kinase (Minard et al., 1989). Although these data do not yield direct insights into the mechanism of folding and assembly of the wild-type GAPDH, they focus the interest on the coenzyme-binding domain (residues 1–147) that lacks the carboxyl-terminal helix α_3 . It contains the "Rossmann" fold that is common to GAPDH, lactate dehydrogenase, malate dehydrogenase, and L-alcohol dehydrogenase (Rossmann et al., 1974), implying relationship by evolution. It is the first to be synthesized on the ribosome, and its noncovalent interaction with the helix α_3 (cf. Fig. 1A) must wait until the entire protein chain has been syn-

thesized. Because folding of the monomer and its assembly to the tetramer are coupled processes, it is conceivable that the low-temperature intermediate that accumulates during the refolding and reassembly of unfolded tGAPDH (Rehaber & Jaenicke, 1992, 1993) lacks this important interaction.

Materials and methods

Chemicals and reagents

NAD⁺, NADH, and restriction enzymes were purchased from Boehringer Mannheim, ultrapure GdmCl from USB, Cleveland. Column material was obtained from Pharmacia. All common chemicals were products of A-grade purity purchased from Merck, Darmstadt. Quartz-bidistilled water was used throughout.

Bacterial strains and plasmids

Expression of the coenzyme-binding domain was performed in *E. coli* JM 83 (Yanisch-Perron et al., 1985), using the expression plasmid pAT1 (Tomschy et al., 1993).

DNA constructions and mutagenesis

Recombinant DNA techniques were based on Sambrook et al. (1989). *E. coli* JM 83 was used as host strain. Site-directed mutagenesis was carried out according to Kunkel et al. (1987) and Geisselsoder et al. (1987), using the helper phage M13K07 (Vieira & Messing, 1987) for the preparation of single-stranded DNA. For the site-directed mutagenesis of *Thermotoga gap* gene, the oligonucleotide 5' GGA GTA ACC GTA TTC ATT ACC ACC TCC AGA GGA CGC ACA GGA AAT 3' was synthesized using a 380A DNA synthesizer (Applied Biosystems).

Immunoblotting

Samples were separated on SDS polyacrylamide gels (Fling & Gregerson, 1986) and transferred onto nitrocellulose membranes. After incubation with rabbit anti-tGAPDH antibodies, the recombinant protein was detected making use of alkaline phosphatase conjugated with swine anti-rabbit antibodies (Blake et al., 1984).

Spectral analysis

All spectroscopic measurements were performed in standard 50 mM sodium phosphate buffer, pH 7.0, plus 0.1 mM EDTA. Ultraviolet absorption measurements at 1.4 mg protein/mL were performed using a Cary 118C spectrophotometer. CD spectra were monitored in a Jasco J500A CD spectropolarimeter: protein concentration 1.4 mg/mL; 1-mm cuvettes were used in the near- and far-UV. Fluorescence emission was determined in a Perkin-Elmer Fluoromax spectrofluorometer at a protein concentration of 5 μ g/mL. GdmCl-, temperature-, and pH-dependent denaturation transitions were measured in a Hitachi-Perkin Elmer MPF-3L spectrofluorometer detecting the change in fluorescence emission at 330 nm, using a constant excitation wavelength at 280 nm.

Stability

The reversible GdmCl-induced transition was measured at 25 °C in standard phosphate buffer (50 mM sodium phosphate, pH 7.0; 0.1 mM EDTA), varying the denaturant concentration. Denaturation: aliquots were incubated at different GdmCl concentrations at protein concentrations of 5 µg/mL for at least 48 h, then protein fluorescence was measured. Renaturation: the protein was denatured in 6 M GdmCl at a final concentration of 0.3 mg/mL for about 24 h. After dilution in standard phosphate buffer containing various denaturant concentrations, the final protein solutions (5 µg/mL) were kept for 48 h at 25 °C. Renaturation was recorded by measuring the protein fluorescence. The reversible temperature-dependent transition was conducted in standard phosphate buffer between 20 and 60 °C by first heating in 2 °C-steps up to 60 °C and then cooling down in the same way. The protein solution (50 µg/mL) was in a closed and stirrable cuvette. The protein fluorescence was measured after an incubation time of 5 min at the respective temperature. Parallel light scattering was measured at $\lambda_{\text{exc}} = \lambda_{\text{em}} = 360$ nm to detect the possible formation of aggregates. The pH-dependent unfolding was performed as described by Rehder and Jaenicke (1992) at a protein concentration of 5 µg/mL.

Binding of NADH and NAD⁺

The binding of NADH to the coenzyme-binding domain was monitored by the NADH-induced quench of protein fluorescence. Because saturation reduces the emission at 330 nm ($\lambda_{\text{ex}} = 280$ nm) by about 50%, NADH binding at a protein concentration of 0.26 µM was determinable with high accuracy. The titration experiment was carried out at 20 °C in a Fluoromax luminescence spectrometer (Spex Industries), using a stirrable cuvette (d = 0.5 cm) containing 1 mL of protein solution.

The measured fluorescence data were corrected for the decrease in intensity of the exciting and emitting radiation by the added NADH. Therefore we used the molar coefficients $\epsilon_{\text{NADH}}^{280} = 6,000 \text{ cm}^{-1} \text{ M}^{-1}$ and $\epsilon_{\text{NADH}}^{330} = 3,400 \text{ cm}^{-1} \text{ M}^{-1}$ respectively. The measurements were repeated in the presence of 38.5 µM and 385 µM NAD⁺ to determine the value of $K_d^{\text{NAD}^+}$ using the equation given by Stinson and Holbrook (1973).

Sedimentation analysis

Sedimentation experiments were performed in a Beckman Model E analytical ultracentrifuge using UV scanning. Double sector cells with sapphire windows were used in an AnG rotor. $s_{20,w}$ values were calculated from $\ln r$ versus time plots, correcting for temperature and water viscosity; sedimentation equilibrium according to Yphantis (1964) was evaluated from $\ln c$ versus r^2 plots making use of a computer program developed by G. Böhm, Regensburg. The partial specific volume of the protein was calculated from the amino acid composition ($v_p = 0.739 \text{ cm}^3 \text{ g}^{-1}$; Schultes et al., 1990).

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