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Circular permutation within the coenzyme binding domain of the tetrameric glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus*

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Abstract

A circularly permuted (cp) variant of the phosphorylating NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Bacillus stearothermophilus* has been constructed with N- and C-termini created within the coenzyme binding domain. The cp variant has a k_{cat} value equal to 40% of the wild-type value, whereas K_m and K_D values for NAD show a threefold decrease compared to wild type. These results indicate that the folding process and the conformational changes that accompany NAD binding during the catalytic event occur efficiently in the permuted variant and that NAD binding is tighter. Reversible denaturation experiments show that the stability of the variant is only reduced by 0.7 kcal/mol compared to the wild-type enzyme. These experiments confirm and extend results obtained recently on other permuted proteins. For multimeric proteins, such as GAPDH, which harbor subunits with two structural domains, the natural location of the N- and C-termini is not a prerequisite for optimal folding and biological activity.

Keywords: circular permutation; dehydrogenase; NAD binding domain; tetrameric enzyme

The three-dimensional structures of proteins are determined by their amino acid sequences. However, the general rules that govern the relationship between the primary and the three-dimensional structures of a protein remain largely to be defined. Various experimental approaches can be used to address this question. Introducing mutations by site-directed mutagenesis at defined locations in the primary structure can enable one to study the effect of specific mutations on the stability or folding efficiency of a protein. Another more global approach consists of producing protein variants by a circular permutation of the amino acid sequence. This concept was originally developed by Goldenberg and Creighton (1983) on bovine pancreatic trypsin inhibitor by coupling chemical and enzymatic approaches. More recently, Kirschner and colleagues generalized this approach, performing the permutation on the protein nucleotidic sequence by genetic engineering (Luger et al., 1989; Buchwalder et al., 1992). Eight circularly permuted proteins, out of which six were enzymes, have now been described (Luger et al., 1989; Buchwalder et al., 1992; Horlick et al., 1992; Yang & Schachman, 1993; Zhang et al., 1993; Hahn et al., 1994; Kreitman et al., 1994; Mullins et al., 1994). So far, circular permutations had been done on monomeric proteins, with the exception reported recently of a trimer (Yang & Schachman, 1993). The results obtained on the six permuted enzymes suggest that, independent of the structure of the protein (mono- or multidomain, mono- or multimeric), the location of the new N- and C-termini, provided they are harbored by surface-accessible loops, and the exact nature and length of the linking polypeptide effect little change in enzymatic activity, stability, or structure. In order to generalize these results, in particular to oligomeric enzymes, we have constructed a cp variant of the phosphorylating NAD-dependent GAPDH. GAPDH is a homotetramer with subunits harboring two structural domains. The nucleotide binding domain, which includes the Rossmann fold, spans from residues 0 to 148 and 313 to 333, residues 149–312 constituting the catalytic domain. The three-dimensional structure of the tetrameric GAPDH active form has been determined by high-resolution X-ray crystallography (Moras et al., 1975; Skarzynski et al., 1987; Lin et al., 1993; O. Dideberg, pers. comm.). This paper describes the biochemical and structural

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; C_m, midpoint concentration of chemical denaturation; cp, circularly permuted; T_m, midpoint temperature of thermal inactivation; 3-CAPAD, 3-(chloroacetyl) pyridine adenine dinucleotide; G3P, glyceraldehyde-3-phosphate; m, cooperativity of the transition.

characterizations of a cp GAPDH protein from *Bacillus stearothermophilus*, with N- and C-termini designed in the cofactor binding domain.

Results

Design and biochemical characterization of cp GAPDH

The design of the cp GAPDH was based on the knowledge of the refined high-resolution X-ray structure of GAPDH from *B. stearothermophilus* (Skarzynski et al., 1987). According to this structure, the N- and C-termini are exposed on the surface of the tetramer in close proximity, with a distance of 11.6 Å between the two α-carbons of Ala 0 and Leu 333, which should allow their connection with a 4–5-amino acid flexible link. As known from the X-ray structure, the GAPDH N- and C-termini are not involved in secondary structures. Indeed, the first β-strand of the Rossmann fold domain begins at position 2, whereas the C-terminal α-helix, which also belongs to the coenzyme binding domain, ends at position 330. Such is also the case for the lobster GAPDH (Moras et al., 1975) and the GAPDH from *Escherichia coli* (O. Dideberg, pers. comm.). The choice of the G_3A peptide linker in the cp GAPDH was based on the propensity of glycine residues to accommodate β-turns. The substitution of Ala 0 for Gly was also done and expected to bring more flexibility to the peptide linker.

Based on the presumption that flexible and variable loops, located on the surface of the GAPDH tetramer, would be less involved in the folding and structural stability of the protein as well as in the enzymatic activity, cleavage was introduced between Val 66 and Asn 67. This cleavage is located between the $\beta_{B''}$ (residues 63–66) and β_C (residues 71–74) strands of the coenzyme binding domain (Fig. 1) in a region where peptide insertions are known to occur for GAPDHs from *Caenorhabditis elegans* and *Caenorhabditis briggsae* (Yarbrough et al., 1987; Lee et al., 1992).

The gene for the permuted protein was sequenced to ensure the absence of unexpected mutations. The permuted GAPDH was expressed under the control of the *E. coli* gap A promoter (Charpentier & Branlant, 1994) and purified using usual procedures as for isolating the wild-type GAPDH (Corbier et al., 1994). The molecular weight of 36,305 determined by mass spectrometry for cp GAPDH was in agreement with the calculated 36,303 assuming the presence of a Met residue at the new N-terminus, as expected from the N-terminal processing rules recently defined for *E. coli* (Hirel et al., 1989). The N-terminal amino acid sequence—M N_{67} G K E I I V K A E—confirmed that the permuted protein begins with a Met residue.

Fig. 1. Ribbon and arrow representation of a *B. stearothermophilus* wild-type GAPDH monomer. The cleavage site is between residues 66 and 67. Dotted lines indicate the secondary structures surrounding the cleavage site. Nomenclature for β-strands and α-helices in the coenzyme binding domain is derived from the nomenclature used for the dogfish lactate dehydrogenase and lobster GAPDH (Rossmann et al., 1973) and also used by Biesecker et al. (1977) for *B. stearothermophilus* GAPDH. It includes the two strands added in the PDB entry 1gd1 (Skarzynski et al., 1987), named in this paper $\beta_{B'}$ and $\beta_{B''}$, but excludes one α-helix not present in the PDB entry. Numbering is according to the PDB entry: β_A 2–7; α_B 10–18; β_B 29–32; α_C 36–45; $\beta_{B'}$ 56–60; $\beta_{B''}$ 63–66; β_C 71–74; α_D 84–87; β_D 90–94; α_E 102–110; β_E 115–118; $\beta_{E'}$ 126–129; β_F 144–147. The figure was generated with RasMol (R. Sayle, RasMol: A program for the visualisation of protein and nucleic acid structures, ftp://ftp.dcs.ed.ac.uk/pub/rasmol) and MOLSCRIPT (Kraulis, 1991).

Table 1. *Kinetic parameters and rate of NADH dissociation for wild-type and cp GAPDHs from B. stearothermophilus*

	Wild-type GAPDH	cp GAPDH
k_{cat} (s^{-1})[a]	70 ± 5	20 ± 5
K_m G3P (mM)[a]	0.8 ± 0.1	0.54 ± 0.04
K_m NAD (mM)[a]	0.15 ± 0.02	0.045 ± 0.008
K_m P_i (mM)[a]	16 ± 3	14 ± 3
k_{off} NADH (s^{-1})[b]	70 ± 5	29 ± 4

[a] Kinetic parameters: experiments were performed in 50 mM triethanolamine buffer, 2 mM EDTA, 50 mM K_2HPO_4, pH 8.9.

[b] Rates of NADH dissociation (k_{off}) from the binary complex GAPDH–NADH were determined using lactate dehydrogenase and pyruvate as a trapping system. The reaction was performed in 50 mM Tris, 2 mM EDTA buffer, pH 8.5 with an ionic strength adjusted to 0.15 M. In the same buffer, in the presence of 50 mM K_2HPO_4, the k_{cat}'s of wild-type and cp GAPDHs are 70 s^{-1} and 24 s^{-1}, respectively.

Enzymatic properties

Kinetic parameters for the wild-type and cp GAPDHs are summarized in Table 1. The K_m value for NAD and the k_{cat} of the cp GAPDH are decreased 3.3- and 3.5-fold, respectively, compared to wild type. NADH dissociation rates of the GAPDH–NADH binary complexes were determined using lactate dehydrogenase as an NADH trapping system (Clermont et al., 1993). Values of 70 s^{-1} and 29 s^{-1} were obtained for the wild-type and cp GAPDHs, respectively (Table 1), and appear to correlate closely with the k_{cat} values.

To determine whether a decreased K_m could be considered indicative of a higher affinity of NAD for the permuted enzyme, the K_D macroscopic dissociation constants for NAD and NADH were determined using 3-CAPAD as an affinity-labeling probe

Table 2. *Dissociation constants of 3-CAPAD, NAD, and NADH for wild-type and cp GAPDHs from B. stearothermophilus*[a]

	Wild-type GAPDH	cp GAPDH
k (min $^{-1}$)	3.2 ± 0.5	2.8 ± 0.4
K_D 3-CAPAD (μM)	18.2 ± 4.0	7.2 ± 2.1
K_D NAD (μM)	0.94 ± 0.17	0.24 ± 0.06
K_D NADH (μM)	0.16 ± 0.03	0.06 ± 0.002

[a] See the Materials and methods for experimental conditions. Inactivation rate (k) and dissociation constants (K_D) of 3-CAPAD were determined from curves representing the double reciprocal plot of the apparent inactivation rate (k_{app}) versus 3-CAPAD concentration. NAD and NADH dissociation constants were determined from protection experiments against inactivation by 3-CAPAD, by plotting k_{app}^{-1} versus NAD and NADH concentrations at a fixed concentration of 3-CAPAD.

(Table 2) as already described (Clermont et al., 1993). Dissociation constants of 0.94 and 0.24 μM for NAD, 0.16 and 0.06 μM for NADH, and 18.2 and 7.2 μM for 3-CAPAD were determined for the wild-type and cp GAPDHs, respectively.

Structure and stability of the cp GAPDH

The secondary structures of the wild-type and cp GAPDHs were compared by CD spectroscopy. As shown in Figure 2, the far-UV CD spectrum of cp GAPDH is almost superimposable with that of the wild type (Suzuki & Imahori, 1973) with a minimum at 220 nm. GuHCl denaturation of wild-type and cp GAPDHs was followed at 25 °C, from both CD measurements at 220 nm and activity measurements (Fig. 3). The transition curves obtained from the measure of either molar ellipticity or residual activity are highly coincident, suggesting that no significant concentrations of intermediates are present at equilibrium and, hence, that a two-state mechanism could be assumed for analyzing the data. The unfolding of both wild-type and cp GAPDHs was shown to be reversible in the presence of 1 mM NAD. More than 85% of the initial activity was recovered by a 100-fold dilution of the proteins. The C_m values were shifted from 1.21 M GuHCl for the wild-type to 0.97 M for the cp GAPDH. The m values for the wild-type and cp GAPDHs were 2.53 and 2.38 kcal mol^{-1} M^{-1}, respectively. Compared to wild-type levels, the thermodynamic stability of the cp GAPDH is reduced by 0.7 kcal/mol (Table 3).

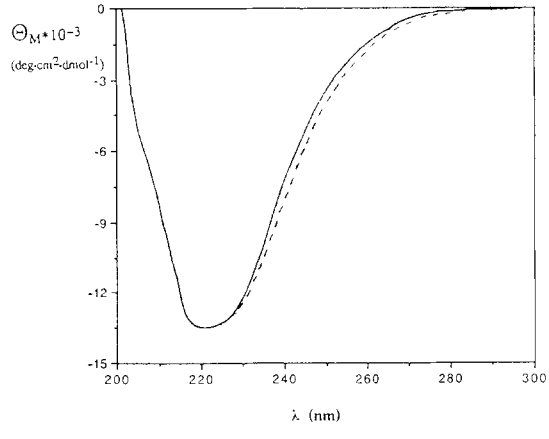

Fig. 2. Far-UV CD spectra of wild-type (———) and cp (— — —) GAPDHs. Measurements were made in 50 mM Tris buffer, 2 mM EDTA, 1 mM DTT, pH 8.0. Enzyme concentrations were 8.3 μN.

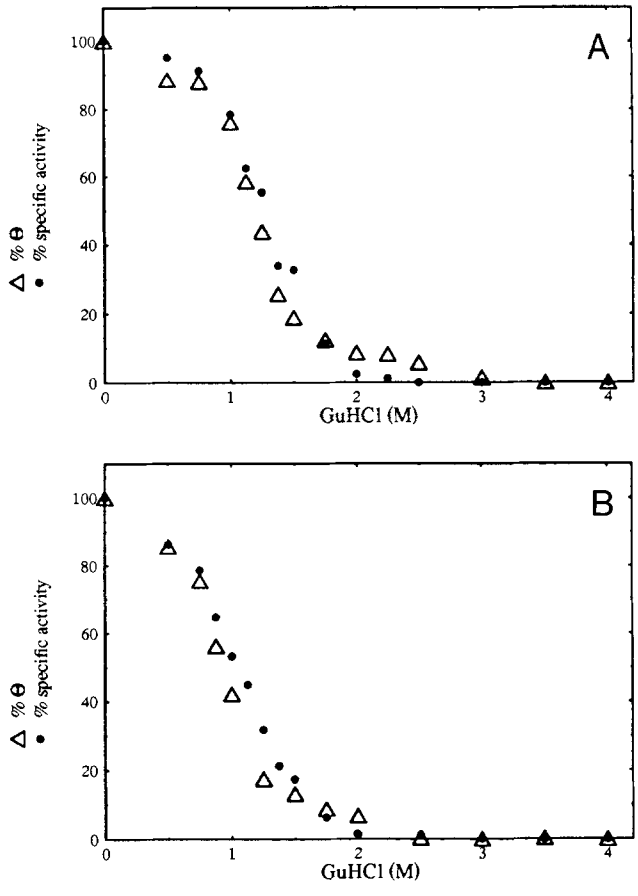

Fig. 3. Evolution of the specific activity and of the molar ellipticity at 220 nm as a function of denaturant concentration. Measurements were performed after 24 h incubation. Results are expressed as percentage of residual activity (●) or molar ellipticity (△) as compared to a control carried out in the absence of denaturant. **A:** Wild-type enzyme. **B:** cp enzyme.

Discussion

We have engineered a cp variant of GAPDH from *B. stearothermophilus* that can be produced in high yields in *E. coli* as an active tetrameric protein. The permuted variant shows a 3–4-fold decrease in K_m and K_D values for NAD as well as a 3.5-fold decrease of the k_{cat} value. As discussed previously for the wild-type enzyme (Clermont et al., 1993), the similar values found for the NADH dissociation rate of the binary GAPDH–NADH complex and the k_{cat} value strongly suggest a similar NADH dissociation rate for both the binary and ternary complexes, assuming the NADH release from the acylenzyme intermediate is the rate-limiting step. The identical values found for the k_{cat} and the NADH dissociation rate from the binary GAPDH–

Table 3. *Thermodynamic parameters of wild-type and cp GADPHs from B. stearothermophilus for GuHCl-induced denaturation*[a]

	Wild-type GAPDH	cp GAPDH
C_m (M)	1.21 ± 0.05	0.97 ± 0.05
m (kcal/(M-mol))	2.53 ± 0.11	2.38 ± 0.13
$\Delta\Delta G$ (kcal/mol)		0.74 ± 0.20

[a] Denaturation studies were carried out as described in the Materials and methods. The fraction of unfolded protein was plotted as a function of the GuHCl concentration. Thermodynamic parameters were calculated using the linear extrapolation method.

NADH complex of the cp enzyme also support a behavior of the cp GAPDH similar to that of the wild-type GAPDH. The lower k_{cat} value observed for the variant protein could therefore be due to a lower release rate of the bound NADH, which could explain the better binding of NADH and NAD to the cp GAPDH.

The similar rates of 3-CAPAD inactivation for wild-type and cp GAPDHs suggest that, prior to inactivation, the orientation of the Cys 149 side chain and of the chloromethyl group of 3-CAPAD and thus of its pyridinium ring are the same in the wild-type and cp GAPDH binary complexes. The fact that the intensity of the Racker band (ϵ_{360} = 1,060 M^{-1} cm^{-1}, curves not shown) was found to be the same for the wild-type and the cp GAPDHs is indicative of a similar location and orientation of the nicotinamide ring of NAD in the active site of the wild-type and the cp GAPDHs. Indeed, this absorption band was interpreted as a charge transfer between the sulfur atom of Cys 149 and the nicotinamidium (Mougin et al., 1988) and taken as a probe for efficient positioning of the nicotinamidium within the catalytic domain. Therefore, the lower K_D values of 3-CAPAD, NAD, and NADH for the cp GAPDH compared to wild type would be rather the consequence of a slight difference in the positioning of the adenosine moiety and could reflect a change in the relative conformational flexibility of the coenzyme-binding domains and catalytic domains.

However, the small differences observed for the K_m, K_D, and k_{cat} values between wild-type and cp GAPDHs show that the structures of the active sites, and in particular of the Rossmann fold and the catalytic domain, are almost identical in these two proteins. Furthermore, the CD data, showing that the far-UV CD spectra of wild-type and cp GAPDHs are superimposable, indicate that the permutation has no effect on the secondary structure of the GAPDH variant. The fact that near-UV CD, absorbance, and fluorescence emission spectra (data not shown) are identical for both structures supports the idea that the environment of aromatic side chains of tyrosines and tryptophans is similar in both structures, in particular for the two tryptophans located at positions 84 and 310 in the coenzyme binding domain.

Thermal inactivation followed by loss of activity indicated a T_m value of 82 °C and 77 °C for the wild-type and cp GAPDHs, respectively. Under similar conditions, the GAPDH from *E. coli* had a T_m value of 66 °C (curves not shown). These results show that the stability of the cp variant is reduced relative to the native thermophilic GAPDH but is still higher than that of the mesophilic *E. coli* counterpart. This finding was confirmed by GuHCl unfolding studies, which showed a reduction in stability of the cp protein by about 0.7 kcal/mol. So far, there have been only two other examples of permuted proteins where the thermodynamic stability was quantified (Zhang et al., 1993; Mullins et al., 1994). In both cases, a small reduction of about 1–2 kcal/mol was observed when compared to the wild type, as we found here for cp GAPDH. The destabilizing effect seen could either be attributed to strains due to the linker peptide connecting the original termini or to the newly created N- and C-termini. Answering this question will clearly need a biochemical characterization of additional cp proteins. A systematic analysis of the linkage peptide, for instance, would be very informative in this regard. In the case of GAPDH, Val 66 and Asn 67, which were chosen as the new C- and N-termini, respectively, are implicated in a hydrogen bonding network involving both the peptide backbone and the amino acid lateral chains (Fig. 4). The permuted construction creates a break in a β-turn and introduces a Met residue at the new N-terminus and a negative charge at the new C-terminus of Val 66. These changes are expected to induce local structural rearrangements that could be responsible for the reduction in thermodynamic stability of the cp GAPDH. These rearrangements may be similar to those recently observed when comparing crystal structures of a cp β-glucanase and the corresponding wild-type protein (Hahn et al., 1994). Differences between the two structures were small and located mainly near the old and new polypeptide chain termini. Unfortunately, no data on the thermodynamic stability of the cp β-glucanase are available from this study.

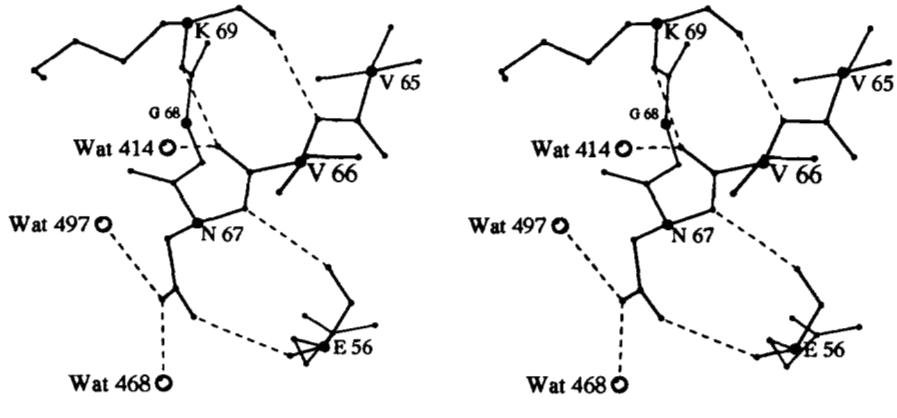

Fig. 4. Stereo view of hydrogen bonds of residues Val 66 and Asn 67 in the O monomer of *B. stearothermophilus* GAPDH (Skarzynski et al., 1987). Dashed lines indicate the following hydrogen bonds: Val 66 N–Lys 69 O; Val 66 O–Lys 69 N; Val 66 O–Wat 414; Asn 67 N–Glu 56 O; Asn 67 Oδ1–Glu 56 N; Asn 67 N–Wat 468; and Asn 67 N–Wat 497. The figure was generated with RasMol (R. Sayle, RasMol: A program for the visualisation of protein and nucleic acid structures, ftp://ftp.dcs.ed.ac.uk/pub/rasmol) and MOLSCRIPT (Kraulis, 1991).

The circular permutations described so far have been performed on various proteins harboring one or two domains. They were made in a different class of proteins, $\alpha\beta$ proteins ($\beta\alpha$ barrel, $\beta\alpha\beta\alpha\beta$ nucleotide binding motif), or in proteins containing mainly or only β structures. GAPDH is the second example of an interconversion done within the NAD(P) binding domain and on a protein postulated to be only active as a multimer. From the nine examples of permuted proteins described so far, it is tempting to conclude that the interconversion approach could be applied to all proteins whatever their structures and multimeric states, provided that chain termini are close to each other and that loops, where new N- and C-termini would be created, are accessible on the surface of the protein. In this regard, it would also be interesting to introduce new N- and C-termini on loops located at the subunit interfaces within multimeric proteins. Indeed, it is not known yet to what extent the location of newly created N- and C-termini at subunit interfaces could influence the assembly efficiency of a monomeric chain into an active quaternary structure. GAPDH is presently a good model for approaching this question.

Materials and methods

Construction of a cp gene of GAPDH

The cp GAPDH protein was obtained by circular permutation of the GAPDH gene. For that purpose, the GAPDH gene was subjected to site-directed mutagenesis using the following oligonucleotides: a, 3′ TTC CTC CTT TTA TTC CCG CGG CCG CTC TTT CAC CCT TAG 5′; b, 3′ TTG TTG AAC CAG CAG ATT ATT GTA TAC TTG CCG TTT CTT TAG 5′; and c, 3′ CGG AGC TTT CCC GAG CCG CCG CCG CGG TTG GAC GAA CCA CGA 5′. Oligonucleotides a and b removed the ATG and stop codons, respectively, while creating *Nar* I restriction sites. Oligonucleotide c was designed to create new C- and N-termini in Val 66 and Asn 67, respectively, as it inserts two successive stop codons and an *Nde* I site that provides the ATG translation initiation codon (Fig. 5B). A fourth oligonucleotide was used to remove a *Nar* I restriction site present within the GAPDH coding sequence (data not shown). The mutated GAPDH gene was digested to completion by the *Nar* I and

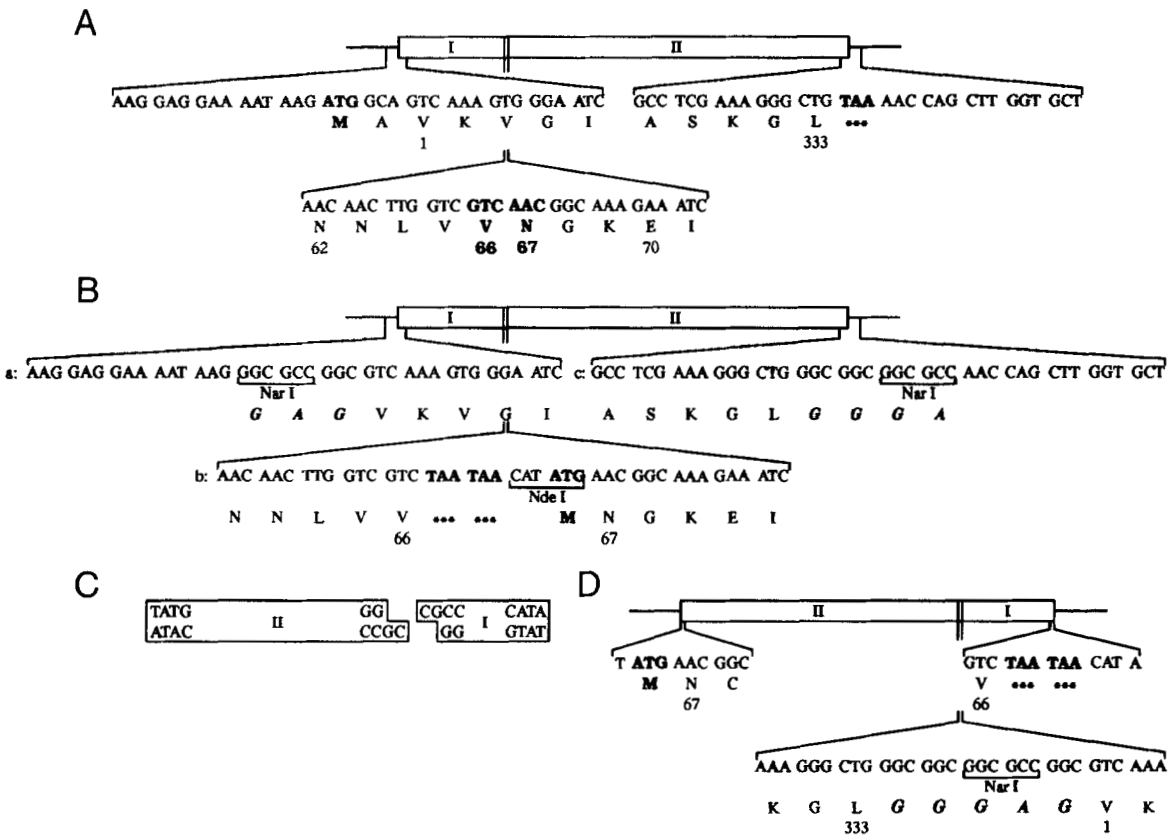

Fig. 5. Genetic construction of the cp gene of *B. stearothermophilus* GAPDH. **A:** Coding sequence of the GAPDH, schematically divided into domain I, N-terminal of Val 66, and domain II, C-terminal of Asn 67. Nucleotide sequences around the ATG and stop codons, as well as at the chosen cleavage site between Val 66 and Asn 67, are indicated. The stop codon is shown with stars. **B:** Generation of *Nar* I and *Nde* I restriction sites by site-directed mutagenesis. Oligonucleotides used for the site-directed mutagenesis are the following: a, 3′ TTC CTC CTT TTA TTC CCG CGG CCG CTC TTT CAC CCT TAG 5′; b, 3′ TTG TTG AAC CAG CAG ATT ATT GTA TAC TTG CCG TTT CTT TAG 5′; c, 3′ CGG AGC TTT CCC GAG CCG CCG CCG CGG TTG GAC GAA CCA CGA 5′. **C:** Schematic representation of the ligation reaction between the two *Nar* I cohesive half sites, present 3′ of domain II, and 5′ of domain I, while the *Nde* I half sites had been made blunt by filling with Klenow enzyme. **D:** Schematic representation of the cp variant of GAPDH obtained. DNA and amino acid sequences are shown at the newly created N- and C-termini as well as at the junction between the original N- and C-termini. The ATG and stop codons, as well as the linking peptide, are indicated in bold characters.

Nde I restriction enzymes. *Nde* I cohesive ends were filled using Klenow enzyme, and DNA fragments were gel purified. Selective ligation between fragments II and I (Fig. 5C) was achieved by taking advantage of the higher ligation efficiency of *Nar* I cohesive ends compared to the blunt *Nde* I ends.

The permuted GAPDH gene (Fig. 5D) was subcloned at the *Nde* I site of the pBS-gap A::Eco RI plasmid, which allows its expression under the control of the *E. coli* gap A promoter (Charpentier & Branlant, 1994).

Determination of the NADH dissociation rate

The rate of NADH dissociation was determined for the binary complex GAPDH-NADH, by using lactate dehydrogenase and pyruvate as a trapping system. A solution of 50 μN lactate dehydrogenase and 20 mM pyruvate was rapidly mixed with an equal volume of NADH (40 μM) and GAPDH (20 μN). As the rate of NADH oxidation by lactate dehydrogenase is higher (410 s^{-1} in our control experiments) than the NADH release from GAPDH, the resulting curves decompose into two phases: the first one represents the titration of NADH in excess, which is rapidly oxidized by the coupled system, and the second one corresponds to the titration of NADH that dissociates from the binary complex GAPDH-NADH (Clermont et al., 1993).

Dissociation constants for 3-CAPAD, NAD, and NADH

Inactivation of GAPDH by 3-CAPAD was carried out as described previously (Corbier et al., 1990) except that experiments were performed in 100 mM TES, 0.2 mM EDTA buffer, pH 8.4. The concentration of 3-CAPAD varied from 2.5 to 20 μM. In the protection experiments, NAD and NADH concentrations varied from 0.2 to 3 μM and from 0.01 to 0.2 μM, respectively, and 3-CAPAD concentration was fixed at 10 μM.

Thermodynamic measurements

Wild-type and cp GAPDHs (8.5 μN) were incubated at 25 °C for 24 h in 50 mM Tris buffer, 2 mM EDTA, 1 mM DTT, pH 8.2, containing varying concentrations of GuHCl. GuHCl concentrations were determined by refractive index measurements (Nozaki, 1970). Unfolding was monitored by CD at 220 nm. The fraction of unfolded protein was plotted against GuHCl concentration, assuming a two-step model. The difference in stability ($\Delta\Delta G$ value) was calculated by multiplying the difference between the C_m values by the average of the m values (Pace, 1986).

Other methods

Site-directed mutagenesis, production, and purification of wild-type and cp GAPDHs were carried out as described previously (Corbier et al., 1994). Protein concentrations are expressed per subunit (N). They were determined spectrophotometrically using a molar extinction coefficient of 1.31×10^5 M^{-1} cm^{-1} and 1.17×10^5 M^{-1} cm^{-1} for the holo- and apoenzymes, respectively. Enzyme assays were carried out at 25 °C on a Cary 2200 spectrophotometer. Initial rate measurements were performed according to Ferdinand (1964).

Molecular mass analyses were performed on a Bio-Q quadrupole mass spectrometer (Fisons, Manchester). Samples were solubilized in aqueous 50% (by volume) CH_3CN containing 1% HCOOH at a final concentration of 20 pmol/μL. Data acquisition was performed in the multichannel acquisition mode. The N-terminal amino acid sequence of cp GAPDH was determined using an Applied Biosystems 473A sequencer. CD spectra were determined using a Jobin Yvon Mark IV dichrograph at a protein concentration of 8.3 μN in 50 mM Tris, 2 mM EDTA, pH 8.0. The cell was maintained at 25 °C.

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References

Biesecker G, Harris IJ, Thierry JC, Walker JE, Wonacott AJ. 1977. Sequence and structure of D-glyceraldehyde 3-phosphate dehydrogenase from *Bacillus stearothermophilus*. *Nature 226*:328–333.

Buchwalder A, Szadkowski H, Kirschner K. 1992. A fully active variant of dihydrofolate reductase with a circularly permuted sequence. *Biochemistry 31*:1621–1630.

Charpentier B, Branlant C. 1994. The *E. coli gapA* gene is transcribed by the vegetative RNA polymerase holoenzyme $E\sigma^{70}$ and by the heat shock RNA polymerase $E\sigma^{32}$. *J Bacteriol 176*:830–839.

Clermont S, Corbier C, Mely Y, Gerard D, Wonacott A, Branlant G. 1993. Determinants of coenzyme specificity in glyceraldehyde-3-phosphate dehydrogenase: Role of the acidic residue in the fingerprint region of the nucleotide binding fold. *Biochemistry 32*:10178–10184.

Corbier C, Clermont S, Billard P, Skarzynski T, Branlant C, Wonacott A, Branlant G. 1990. Probing the coenzyme specificity of glyceraldehyde-3-phosphate dehydrogenases by site-directed mutagenesis. *Biochemistry 29*:7101–7106.

Corbier C, Michels S, Wonacott AJ, Branlant G. 1994. Characterization of the two anion-recognition sites of glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus* by site-directed mutagenesis and chemical modification. *Biochemistry 33*:3260–3265.

Ferdinand W. 1964. The isolation and specific activity of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. *Biochem J 92*:578–585.

Goldenberg DP, Creighton TE. 1983. Circular and circularly permuted forms of bovine pancreatic trypsin inhibitor. *J Mol Biol 165*:407–413.

Hahn DP, Piotukh K, Borriss R, Heinemann U. 1994. Native-like in vivo folding of a circularly permuted jellyroll protein shown by crystal structure analysis. *Proc Natl Acad Sci USA 91*:10417–10421.

Hirel PH, Schmitter JM, Dessen P, Fayat G, Blanquet S. 1989. Extent of N-terminal methionine excision from *Escherichia coli* proteins is governed by the side-chain length of the penultimate amino-acid. *Proc Natl Acad Sci USA 86*:8247–8251.

Horlick RA, George HJ, Cooke GM, Tritch RJ, Newton RC, Dwivedi A, Lischwe M, Salemme FR, Weber PC, Horuk R. 1992. Permuteins of interleukin 1β—A simplified approach for the construction of permuted protein having new termini. *Protein Eng 5*:427–431.

Kraulis PJ. 1991. MOLSCRIPT: A program to produce both detailed and schematic plots of protein structure. *J Appl Crystallogr 24*:946–950.

Kreitman R, Puri RK, Pastan I. 1994. A circularly permuted recombinant interleukin 4 toxin with increased activity. *Proc Natl Acad Sci USA 91*:6889–6893.

Lee YH, Huang XY, Hirsh D, Fox GE, Hecht RM. 1992. Conservation of gene organization and *trans*-splicing in the GAPDH encoding genes of *Caenorhabditis briggsae*. *Gene 121*:227–235.

Lin ZJ, Li J, Zhang FM, Song SY, Yang J, Liang SJ, Tsou CL. 1993. Structure of D-glyceraldehyde-3-phosphate dehydrogenase from *Palinurus versicolor* carrying the fluorescent NAD derivatives at 2.7 Å resolution. *Arch Biochem Biophys 302*:161–166.

Luger K, Hommel U, Herold M, Hofsteenge J, Kirschner K. 1989. Correct folding of circularly permuted variants of a $\beta\alpha$ barrel enzyme in vivo. *Science 243*:206–209.

Moras D, Olsen KW, Sabesan MN, Buehner M, Ford GC, Rossmann MG. 1975. Studies of asymmetry in the three-dimensional structure of lobster glyceraldehyde-3-phosphate dehydrogenase. *J Biol Chem 250*:9137–9162.

Mougin A, Corbier C, Soukri A, Wonacott A, Branlant C, Branlant G. 1988. Use of site-directed mutagenesis to probe the role of Cys 149 in the charge transfer transition in glyceraldehyde-3-phosphate dehydrogenase. *Protein Eng 2*:45–48.

Mullins LS, Wesseling K, Kuo JM, Garrett JB, Raushel FM. 1994. Transposition of protein sequences: Circular permutation of ribonuclease T1. *J Am Chem Soc 116*:5529–5533.

Nozaki Y. 1970. The preparation of guanidine hydrochloride. *Methods Enzymol 26*:43–54.

Pace CN. 1986. Determination and analysis of urea and guanidine hydrochloride denaturation curves. *Methods Enzymol 131*:266–280.

Rossmann MG, Adams MJ, Buehner M, Ford GC, Hackert ML, Liljas A, Rao ST, Banaszak LJ, Hill E, Tsernoglou D, Webb L. 1973. Molecular symmetry axes and subunit interfaces in certain dehydrogenases. *J Mol Biol 76*:533–537.

Skarzynski T, Moody PCE, Wonacott AJ. 1987. Structure of hologlyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus* at 1.8 Å resolution. *J Mol Biol 193*:171–187.

Suzuki K, Imahori K. 1973. Glyceraldehyde-3-phosphate dehydrogenase of *Bacillus stearothermophilus*. Kinetics and physicochemical studies. *J Biochem 74*:955–970.

Yang YR, Schachman HK. 1993. Aspartate transcarbamoylase containing circularly permuted catalytic polypeptide chains. *Proc Natl Acad Sci USA 90*:11980–11984.

Yarbrough PO, Hayden MA, Dunn LA, Vermersch PS, Klass MR, Hecht RM. 1987. The GAPDH gene family in the nematode *Caenorhabditis elegans*: Isolation and characterization of one of the genes. *Biochim Biophys Acta 908*:21–33.

Zhang T, Bertelsen E, Benvegnu D, Alber T. 1993. Circular permutation of T4 lysozyme. *Biochemistry 32*:12311–12318.