A monomeric mutant of *Clostridium symbiosum* glutamate dehydrogenase: Comparison with a structured monomeric intermediate obtained during refolding

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Abstract

The refolding of *Clostridium symbiosum* glutamate dehydrogenase (GDH) involves the formation of an inactive structured monomeric intermediate prior to its concentration-dependent association. The structured monomer obtained after removal of guanidinium chloride was stable and competent for reconstitution into active hexamers. Site-directed mutagenesis of *C. symbiosum* *gdh* gene was performed to replace the residues Arg-61 and Phe-187 which are involved in subunit-subunit interactions, as determined by three-dimensional structure analysis. Heterologous over-expression in *Escherichia coli* of the double mutant (R61E/F187D) led to the production of a soluble protein with a molecular mass consistent with the monomeric form of clostridial GDH. This protein is catalytically inactive but cross-reacts with an anti-wild-type GDH antibody preparation. The double mutant R61E/F187D does not assemble into hexamers. The physical properties and the stability toward guanidinium chloride and urea of R61E/F187D were studied and compared to those of the structured monomeric intermediate.

Keywords: double mutant; glutamate dehydrogenase; guanidinium chloride; monomeric intermediate; protein folding

Glutamate dehydrogenase (GDH) catalyzes the reversible deamination of L-glutamate to 2-oxoglutarate using NAD(P)⁺ as coenzyme. X-ray analysis at high resolution has been reported for GDH from *Clostridium symbiosum* (Baker et al., 1992) and from other microbial sources (Korber et al., 1993; Stillman et al., 1995; Yip et al., 1995). The enzyme is composed of six identical subunits and each subunit is formed by two domains separated by a deep cleft where the catalytic activity takes place (Baker et al., 1992). The two domains are linked by a hinge which allows the flexibility required for catalysis (Stillman et al., 1993). The quaternary struc-

ture assembly is characterized by a 32 symmetry and the enzyme is considered to be a dimer of trimers (Baker et al., 1992).

Glutamate dehydrogenases from various sources have been the object of detailed denaturation studies (e.g., Bell & Bell, 1984; West & Price, 1988; Aghajanian et al., 1995). The investigation on bovine and clostridial enzyme led to the conclusion that the process of dissociation into monomers and loss of secondary structure induced by guanidinium chloride (GdmCl) was irreversible (Bell & Bell, 1984; West & Price, 1988; Tashiro et al., 1982). These conclusions were based on the observations that catalytic activity, the most sensitive probe for the native structure assembly (Tsou, 1986), was not restored by the removal of denaturant. However, several proteins that are devoid of catalytic or biological function during reconstitution or under particular solvent conditions, nevertheless, show a native-like structure (Rehaber & Jaenicke, 1993; Lilie et al., 1995). In particular, the regain of a native-like structure does not necessarily imply full reconstitution of the native active state, as reported for the folding intermediates which are formed during the renaturation process of several monomeric and oligomeric proteins (Schmid, 1992; Price, 1994). The occurrence of inactive structured monomers and trimers in the refolding pathway

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Abbreviations: GDH, glutamate dehydrogenase; GdmCl, guanidinium chloride; SM, structured monomer.

of GDH from the hyperthermophilic archaeon *Pyrococcus furiosus* has been recently reported (Consalvi et al., 1996). The similarity between the structure of *P. furiosus* and *C. symbiosum* GDH (Yip et al., 1995) led us to investigate the structural properties of the *C. symbiosum* enzyme following GdmCl denaturation.

This paper reports the characterization of a monomeric intermediate formed in the refolding of *C. symbiosum* GDH after removal of GdmCl, preliminary to the formation of the fully reactivated hexamer. This structured monomeric intermediate (SM) of the reconstitution process has been trapped, purified, and studied by UV fluorescence, CD spectroscopy, size exclusion chromatography, and equilibrium sedimentation analysis.

To gain information about the contribution of subunit assembly in the complete tertiary folding of either or both domains and in the correct domain pairing necessary for catalysis, a double monomeric mutant (R61E/F187D) of clostridial GDH has been constructed on the basis of the knowledge of the intersubunit contact regions (Britton, 1992; Pasquo et al., 1996). The physical properties of the structured monomer (SM) and of the monomeric double mutant have been studied and compared with those of the native hexameric enzyme.

Results

Construction, purification, and molecular mass determination of the double mutant *gdh* R61E/F187D

A double mutant gene was achieved by taking advantage of the unique *Bst* EII restriction site located in the gene position 701, corresponding to D165 in the aminoacid sequence (Fig. 1). Ptae 85

plasmids carrying single mutant *gdh* genes F187D and R61E were digested with *Sal* I and *Bam* HI. The DNA bands, thus obtained, were extracted from 1% agarose gel, further digested with *Bst* EII and run again on 1% agarose gel. The 0.5 Kb band from the R61E gene was ligated with the 0.8 Kb band of F187D. The double mutant gene, thus obtained, was subcloned into ptae 85 and then transformed into PA340 *Escherichia coli* cells.

The entire R61E/F187D *gdh* gene has been sequenced in order to check for unwanted secondary mutations. Sonication of the *E. coli* cells harboring the double mutant *gdh* gene and SDS PAGE of the cell extract showed overexpression of a soluble protein of the right subunit molecular mass but without activity. A three-step purification of the cell extract led to an enzymatically inactive protein when assayed up to 100 μ g/mL under the standard conditions used for the wild-type enzyme (see Materials and methods). SDS PAGE analysis of the purified double mutant showed a single band with a molecular mass of 49 kDa consistent with the monomeric form of clostridial GDH. Because of the lack of catalytic activity, immunological detection on SDS PAGE was performed to monitor and confirm purification. The double mutant R61E/F187D cross-reacted with antibodies raised against the wild-type GDH.

Determination of molecular mass of the double mutant under native conditions was performed by equilibrium sedimentation experiments conducted at 4 or 20 °C and 18,000 rpm (Fig. 2A). At both temperatures the average molecular mass increases with the increase in initial concentration from 0.04 to 0.8 mg/mL with values falling between 68 and 82 kDa. A fit of the data to a monomer-dimer equilibrium yields an association constant $K_{1,2} = 8.0 \pm 0.8 \text{ mL mg}^{-1}$. This value can be utilized to establish that

Fig. 1. Strategy for protein engineering to produce double mutant R61E/F187D *gdh* gene of *C. symbiosum* from single mutants R61E and F187D. This strategy is explained in Results.

Fig. 2. Sedimentation equilibrium data of the double mutant R61E/F187D (A) and the structured monomer (B). The data were fitted to a single species, yielding the Z-average molecular weight. Experimental conditions: (A) 20 °C and 18,000 rpm, 40 $\mu\text{g/mL}$ initial concentration; (B) 4 °C and 17,000 rpm, 20 $\mu\text{g/mL}$ initial concentration.

under the conditions used for far-UV CD, fluorescence, and activity measurements the weight fraction of monomers is more than 85%. Determination of molecular mass of the double mutant under native conditions was also performed by size exclusion chromatography as described in Materials and methods. The elution profile from Superose 12 revealed a single species with an elution volume (V_e = 10.0 mL) close to that expected for a 49 kDa monomer. This V_e is similar to that of the structured monomer obtained after injection onto Superose 12 of the denaturation mixture in 5 M GdmCl (V_e = 10.1 mL). The Superose 12 elution pattern of the double mutant enzyme following concentration in a Centricon-30 device up to 6.0 mg/mL showed neither aggregates nor protein species eluting with a V_e corresponding to association states higher than the monomer. The double mutation thus achieved the construction of a monomeric form of *C. symbiosum* GDH protein which is catalytically inactive.

Isolation and characterization of *C. symbiosum* GDH structured monomer

Incubation of *C. symbiosum* GDH for 1.5 h at 0.5 mg/mL in 5 M GdmCl in 50 mM phosphate pH 7.0 containing 1 mM dithio-DL-threitol and 0.1 mM EDTA induced its unfolding (West & Price, 1988) and loss of structure, as indicated by the changes in ellipticity at 222 nm and in the intrinsic fluorescence properties. No further changes were observed after 24 h or at GdmCl concentrations above 5 M. Refolding of the enzyme was started by direct injection onto a Superose 12 column to remove GdmCl as described in Materials and methods. The elution profile showed the

presence of a single protein peak with an elution volume (V_e = 10.1 mL) close to that of a 49 kDa monomer. Equilibrium sedimentation experiments were conducted at 4 °C and 17,000 rpm and were limited to an initial concentration of 20 $\mu\text{g/mL}$ (Fig. 2B) in view of the tendency of the structured monomer to polymerize into hexamers (see below). Under the conditions used the Z-average molecular mass is 83 ± 12 kDa. Fitting the data to a monomer-trimer equilibrium gives an association constant $K_{1,3} = 330 \pm 30 \text{ mL}^2 \text{ mg}^{-2}$. From this value, it can be established that under the conditions used for far-UV CD, fluorescence, and activity measurements the weight fraction of monomers is more than 85%. The protein showed the same intrinsic fluorescence emission and far-UV CD spectra as the native enzyme (Fig. 3), but was catalytically inactive. This species henceforward is described as structured monomer (SM). Concentration of SM above 6.1×10^{-7} M (30 $\mu\text{g/mL}$) in Centricon-30 resulted in the appearance of activity. Size exclusion chromatography analysis suggests this is due to assembly to a hexamer. Upon storage in buffer B at 20 °C the ability of SM to produce the hexamer following concentration is retained for approximately 24 h. The hexamers obtained after this concentration step and the removal of 10% aggregates by ultracentrifugation at $50,000 \times g$ showed the same spectral and catalytic properties and the specific activity (32 U/mg, forward reaction) as the native GDH.

Physical properties of R61E/F187D and of SM

The structures of R61E/F187D and SM were compared according to several criteria. They showed similar elution profiles from a

Fig. 3. Far-UV CD spectra of native hexamer, structured monomer (SM) and monomeric double mutant R61E/F187D of *C. symbiosum* GDH. Spectra were recorded at 20°C in a 0.2 cm quartz cuvette at 1.0×10^{-6} M protein concentration for the native hexamer (—) and R61E/F187D (····) and in a 0.5 cm quartz cuvette at 5.5×10^{-7} M protein concentration for SM (---).

calibrated Superdex 75 column (Fig. 4). The elution volumes of the double mutant ($V_e = 7.2$ mL) and of SM ($V_e = 7.0$ mL) are between those of ovalbumin ($V_e = 7.5$ mL, 45 kDa) and bovine serum albumin ($V_e = 6.7$ mL, 66 kDa), indicating a hydrodynamic volume slightly more expanded than that expected from the molecular mass of 49 kDa determined by SDS PAGE. The secondary structure of the two proteins was very similar, as monitored by far-UV CD, and comparable with that of the native hexamer (Fig. 3). The intrinsic fluorescence emission spectra of the native enzyme, SM and R61E/F187D were superimposable, indicating a similar shielding of aromatic residues from the solvent.

Figure 5 represents a comparison of the fluorescence titration curves of the two monomers and of the native hexameric GDH with anilinonaphthalene-8-sulfonic acid (ANS). Both monomers bind the fluorescent probe to a higher extent compared to the hexamer. Saturation behavior is observed for all the forms but for both monomers the final value of ANS emission intensity at 465 nm is higher with respect to the native enzyme with a saturation midpoint at a higher ANS concentration. The quantum yield and the fluorescence emission maximum of ANS are directly related to the polarity of the medium. In our results the fluorescence emission maximum of ANS in the presence of the native enzyme is centered at 510 nm and in the presence of either monomer at 470 nm (data not shown). This suggests that the observed fluorescence changes of the probe in the presence of the monomers are caused by the differences in polarity of the environment of bound ANS molecules and probably by an increased binding stoichiometry. These data are consistent with the increased accessibility of hydrophobic sites when the enzyme is in the monomeric state.

The effective acrylamide quenching constants for R61E/F187D and SM and the native hexamer are 9.3, 9.4, and 5.7 M^{-1} , respec-

Fig. 4. Elution profile of the double mutant (R61E/F187D) and structured (SM) monomer of *C. symbiosum* GDH from Superdex 75. Elution was performed with buffer B containing 0.1 M NaCl. Flow rate was 0.4 mL/min, ovalbumin (OA) 45 kDa, $V_e = 7.5$ mL; bovine serum albumin (BSA) 66 kDa, $V_e = 6.7$ mL; double mutant (R61E/F187D), $V_e = 7.2$ mL; structured monomer, $V_e = 7.0$ mL. The column was calibrated as described in Materials and methods.

tively. A quantitative interpretation of the reported quenching constants is not possible since *C. symbiosum* GDH is a highly heterogeneous emitting system. However, qualitatively the data suggest that the overall protein dynamics and the accessibility of tryptophans are very similar in the two monomers which both differ from the native hexamer.

The study of the near-UV CD spectrum was feasible only for R61E/F187D because it did not associate upon concentration while the SM promptly associated to active hexamers above $30 \mu\text{g/mL}$. A comparison between the near-UV CD spectra of the native hexameric enzyme and R61E/F187D (Fig. 6) indicates that the dichroic activity of the double mutant is reduced but still present over the whole spectral range. The notable decrease of negative ellipticity in the 275–280 nm bands of tyrosines and in the 290 nm band of tryptophans makes the phenylalanine 262 nm band and the tryptophan 292 nm band more evident, despite their decreased amplitude with respect to the native form.

Unfolding transitions for the monomeric mutant R61E/F187D and the structured monomer of *C. symbiosum* GDH

The unfolding of the monomeric mutant R61E/F187D and of the structured monomer (SM) at increasing GdmCl concentrations was investigated by monitoring the changes in far-UV CD spectra at

Fig. 5. Interaction of the monomeric mutant R61E/F187D (●), structured monomer (▲), and native hexamer (■) of *C. symbiosum* GDH with the fluorescent probe ANS. The changes in fluorescence intensity at 465 nm (390 nm excitation wavelength) were monitored at 20 °C 10 min after each addition of the probe. Protein concentration was 5.0×10^{-7} M.

222 nm (Fig. 7A) and the changes in intrinsic fluorescence emission (Fig. 7B). The progressive decrease of molar ellipticity at 222 nm (Fig. 7A) occurred in a multi-step transition. The main amplitude of the ellipticity changes was observed between 0.23

Fig. 6. Near-UV CD spectra of monomeric mutant R61E/F187D (A) and native hexamer (B) of *C. symbiosum* GDH. Spectra were recorded at 20 °C in a 1.0 cm quartz cuvette at 5.6×10^{-5} M protein concentration.

and 1.22 M GdmCl with transition midpoints centered at 0.70 and 0.67 M GdmCl for R61E/F187D and SM, respectively. The amplitude of the ellipticity transition between 0 and 0.23 M GdmCl was larger for SM compared to that observed for the double mutant. No further changes in ellipticity were observed above 1.75 and 1.10 M GdmCl for the monomeric double mutant and SM, respectively. The decrease in the intrinsic fluorescence emission at 344 nm at increasing GdmCl concentrations showed comparable profiles (Fig. 7B), over the same denaturant concentration range as for the ellipticity changes. The midpoints for the second transitions were similar and corresponded to 0.63 M GdmCl for SM and 0.64 M GdmCl for the monomeric double mutant. On the other hand, the shift in the maximum emission wavelength from 343 to 358 nm (Fig. 7B) appeared to be a two-state process with transition midpoints at 1.20 M GdmCl for R61E/F187D and 1.10 M GdmCl for SM. No aggregation to particles detectable by right-angle light scattering at 480 nm occurred at any denaturant concentration since no changes in 90° scattering signals were observed.

The urea-induced unfolding transitions of the two monomers showed a broad multi-step profile. Between 0 and 0.50 M denaturant an abrupt change in the 222 nm ellipticity occurred for both monomers (Fig. 7C), with a larger amplitude for the structured monomer compared to the monomeric mutant. In the case of SM, above 0.50 M urea a second larger transition was observed with a midpoint at 1.35 M denaturant concentration. The monomeric mutant R61E/F187D showed two unfolding transitions of similar amplitude over the ranges 0.50–2.52 M and 2.52–5.14 M urea, with midpoints at 1.65 M and 3.25 M, respectively. The decrease in the intrinsic fluorescence emission intensity (Fig. 7D) was similar to that observed by monitoring ellipticity changes. Interestingly, the shift in the maximum fluorescence emission wavelength (Fig. 7D) showed a two-state transition with midpoints at 1.94 M and 2.90 M urea for SM and R61E/F187D, respectively.

Discussion

The refolding of *C. symbiosum* GDH after removal of GdmCl occurs through the formation of an inactive structured monomeric intermediate which, upon increasing protein concentration, promptly associates into active hexamers. The active site of *C. symbiosum* GDH is located in a cleft between the two domains and does not contain structural elements directly involved in subunit-subunit interaction (Baker et al., 1992). The lack of catalytic activity of SM may be then attributed either to an incomplete tertiary fold of either or both domains, or to an incorrect domain pairing.

The monomeric double mutant of *C. symbiosum* GDH (R61E/F187D) is inactive and does not assemble into states of association higher than dimer. These results suggest that the mutations introduced at position 61 and 187 are not consistent with hexamer formation. The production of the monomeric form of *C. symbiosum* GDH has been possible by combining two mutations at the subunit contact areas, F187D and R61E. The replacement of the hydrophobic residue F187 with the charged Asp (F187D) presumably leads to the destabilization of the trimer interface because of the requirement to bury an aspartate in a hydrophobic region of this interface in the mutant. The product of the mutation F187D is, therefore, a dimeric form of *C. symbiosum* GDH (Pasquo et al., 1996) which is also inactive. Inspection of the structure of *C. symbiosum* GDH (Fig. 8) has shown that Arg61 plays a key role in assembly of the two trimers with its side chain binding the -COOH

Fig. 7. GdmCl-induced (A,B) and urea-induced (C,D) equilibrium unfolding of the monomeric mutant R61E/F187D (●,○), and of the structured monomer (SM) (▲,△). A,C: The decrease in molar ellipticity at 222 nm was measured in 0.5 cm quartz cuvettes. B,D: Intrinsic fluorescence emission intensity at 343 nm (●,▲) and wavelength of maximum fluorescence emission ($\lambda_{\max}$, ○,△) were monitored with an excitation wavelength set at 290 nm. All experiments were performed at 20 °C and 5.5×10^{-7} M protein concentration.

of the C-terminus (W449) of the 2-fold related subunit through a salt bridge (Britton, 1992; A. Pasquo, pers. comm.). Thus, the introduction of the new acidic group at position 61 is again a driving force destabilizing the dimer interface. The two mutations combined lead to a monomeric form of GDH since the repulsive forces among residues at the subunit contact areas overcome the attractive forces tending to drive the association into hexamers.

A comparative structural analysis of the structured monomeric intermediate formed during the refolding of the hexameric *C. symbiosum* GDH and of the monomeric double mutant revealed sim-

ilarities between the two monomers and some remarkable differences with respect to the hexameric enzyme. The analysis of the far-UV CD and fluorescence spectra of the two monomers (SM and R61E/F187D) reveals that the secondary structure elements as well as the shielding of aromatic residues from the solvent are identical, and they are both comparable to those of the native hexamer. These results indicate that the two mutations only prevent interactions with other subunits but do not markedly affect the secondary structure which is indistinguishable from that of the SM and that of the native enzyme.

Fig. 8. A MOLSCRIPT diagram (Kraulis, 1991) showing a portion of the wild-type *C. symbiosum* GDH structure between two of the subunits at the dimer interface. The fold of the polypeptide chain, in each of the two monomers, is depicted as a ribbon but in contrasting styles: one as a shaded ribbon, the other in black. In each case the residues highlighted in ball and stick representation are involved directly in the intersubunit contacts at this interface. The side chain portrayed in solid black at the centre of the diagram indicates the position of one of the two mutations made to create the monomeric form of this GDH, namely R61. The residues numbered are those which are found in closest proximity to this mutated residue, are suffixed either A or B to distinguish between the two subunits shown. The salt bridge between R61 and the COOH of the C-terminus in the wild-type enzyme is also shown by the dashed line.

ANS binding results for the two monomers are very similar and differ from those for the native hexamer. Hydrophobic areas are more accessible in the two monomers as shown by the increased ANS fluorescence and shift of its maximal emission wavelength. The explanation of the increased fluorescence of the probe in the presence of either R61E/F187D or SM may be due to an increased quantum yield and/or to the increased stoichiometry of binding but cannot be attributed to an increased affinity for the probe itself. An increased accessibility of the hydrophobic core of SM and of the double mutant is shown by the acrylamide quenching constant values, identical for the two monomers but again different from that of the hexameric enzyme. The results obtained with ANS and with the uncharged quencher acrylamide suggest a less compact state of the monomeric forms.

An analysis of the tertiary structure of SM by near-UV CD was not feasible since it rapidly associates into hexamers when protein concentration is raised above 30 $\mu\text{g/mL}$ but was possible for the double mutant which does not associate. The decrease of negative ellipticity at the tyrosine and tryptophan bands in the near-UV CD spectrum of R61E/F187D may be possibly due to fluctuation of tertiary contacts and suggests a different tertiary structure arrange-

ment compared to the hexamer. This result, together with the ANS binding studies and the acrylamide quenching constants, is further evidence of a decreased compactness of the double mutant compared to the hexamer. The subunit-subunit interactions, which allow polymer assembly, seem to play an important role in the formation of the native tertiary contacts and may well be responsible for the increase in compactness of the protein. It is impossible to establish from these results if polymerization precedes or is consequent upon conformational change(s) into a proper tertiary structure.

The analysis of the GdmCl-induced unfolding of the two monomers reveals that the loss of helicity and the decrease in intrinsic fluorescence intensity occur in multi-state processes. These results suggest that different parts of the molecule, e.g., the two domains, show a different stability toward the denaturant and that they do not contribute to a single folding unit. In the GdmCl concentration range where helicity and intrinsic fluorescence are dramatically decreased, the maximum fluorescence emission wavelength is not shifted. This indicates that despite the absence of a "compact" structure the aromatic residues are shielded from the solvent and the observed quenching is mainly dynamic for the increased mobility of the aromatic side chains. It is interesting to compare the effect of urea on the helicity and on the intrinsic fluorescence for the two monomers (SM and R61E/F187D) with that exerted by GdmCl. Similarly to what was observed with GdmCl, the loss of helicity and fluorescence intensity at increasing urea concentrations both show a multi-state transition, whereas the shift of the maximum emission wavelength is a two-state process. This result indicates that, also when urea is used as denaturant, the loss of fixed structural contacts does not occur simultaneously with the exposure of the aromatic residues to the solvent. Upon increasing urea concentration the differences between the unfolding profiles of the two monomers are evident. The structural transitions of the double mutant are shifted toward higher urea concentrations with respect to those of the SM, when both CD and intrinsic fluorescence emission was monitored. The increased stability of R61E/F187D, with respect to SM, can also be observed in the GdmCl-induced unfolding but the differences in transition midpoints are larger when urea is used as denaturant. Despite the increased stability of the double mutant, both monomers are less stable than the hexamer, and more importantly, they are not a single folding unit. These observations indicate that the assembly step has a fundamental role in conferring the proper tertiary contacts and the correct pairing of the two domains required for catalysis. However, the lack of specific tertiary contacts does not prevent SM association into active hexamers indicating that all the specific areas involved in the subunit recognition and interaction are correctly formed. This type of highly structured but inactive state might be common to the folding of polymeric proteins and it may be involved in the chaperonin-assisted folding of oligomers (Ellis & Hartl, 1996; Lorimer, 1996).

The availability of a monomeric mutant of *C. symbiosum* GDH now offers the possibility of studying the interaction of this species with chaperones and the role of each domain in the folding of subunits.

Materials and methods

Chemicals

Restriction enzymes, T4 ligase, T4 polymerase, and T4 polynucleotide kinase were purchased from Northumbria Biologicals Lim-

ited (UK). Electrophoresis reagents were from Bio-Rad. Guanidinium chloride (Biochemika microselect) and all the other chemicals were from Fluka. All the chemicals for molecular biology and enzymology were of the highest purity available.

Bacterial strains and plasmids

The *E. coli* strains used for mutagenesis were: - *TG 1* [Δlac -*proAB*, *supE*, *thi1*, *hsd* D5/F'*traD*36, *pro* A⁻B⁺, *lacI*^q, *lac* Z Δ -M15], CJ236 [*dut1*, *ung1*, *thi1*, *relA*/pCJ105 (*cmr*)]. Bacteriophage M13mp18 and mp19 were from Boehringer (London). *E. coli* K12 [*thr* -1 *fhuA2* *leuB6* *lacY1* *supE44* *gal*-6 *gdh*-1 *hisG1* *rfbD1* *gal* P63D (*gltB*-F) 500 *rps* L19 *mal* T1 *xyl* -7 *mtl* -2 *arg* H1 *thi* -1] strain PA340 as well as the expression vector *ptac* 44 (Marsh, 1986) harboring the gene of glutamate dehydrogenase were kindly provided by Dr. J.K. Teller.

Sub-cloning strategies

Standard protocols (Sambrook et al., 1989) were used to subclone the wild-type and the mutated genes in the bacteriophage for mutagenesis and into the expression vector using the *Sal* I and *Bam* HI restriction sites unless differently stated. Both *gdh* genes, each carrying a single mutation R61E or F187D, were digested with the endonuclease BstE II and then run on an agarose gel. The 0.5 Kb band derived from the R61E gene and the 0.8 Kb band obtained from the F187D gene were extracted from the agarose gel with GeneClean II Kit (Bio 101, USA). The two fragments were ligated, subcloned into the expression vector and then transformed into the *E. coli* PA340 strain.

Enzymes

Overnight cultures (1 lt) of *E. coli* transformants harboring either the wild-type or the double mutant enzymes were harvested, sonicated in ice-cold conditions and then centrifuged in a Kontron T324 centrifuge for 30 min at $30,000 \times g$. The double mutant (R61E/F187D) was purified to homogeneity with a three-step purification protocol at 4°C. Two milliliters (20 mg) of the clarified supernatant of the sonicated cells was loaded onto an Amicon Blue A affinity column (1.2 $\times$ 5 cm) equilibrated with 20 mM phosphate pH 6.5 containing 0.1 mM phenylmethylsulfonyl fluoride, 0.2 mM EDTA and 10 mM 2-mercaptoethanol (buffer A). The enzyme was eluted with buffer A and with 0.1 M NaCl in buffer A. The fractions containing the enzyme were concentrated and dialyzed against buffer A on a YM 30 membrane (Amicon). The protein (5.0 mL, 5.0 mg) was then loaded onto a DEAE Sepharose Fast Flow (Pharmacia) (1.0 $\times$ 4 cm) ionic exchange column equilibrated with buffer A. After extensive washing with the same buffer the column was eluted stepwise with 0.05, 0.1, 0.18, and 0.25 M NaCl in buffer A. The 0.18 M NaCl fraction was concentrated and dialyzed against 40 mM phosphate pH 7.0 containing 0.2 mM dithio-DL-threitol and 0.1 mM EDTA (buffer B) and 1.5 mL (2.3 mg) were loaded onto a Superdex 200 HiLoad 16/60 (Pharmacia) column equilibrated with the same buffer. Typical yield of pure protein was 2.0 mg (10% yield). Recombinant *C. symbiosum* wild-type GDH was purified as described elsewhere (Teller et al., 1992). The protein concentration was determined as described by Teller et al. (1992) and refers to 49 kDa monomer. The structured monomer (SM) was obtained at 20°C by direct injection of the 5 M GdmCl-unfolding mixture containing 1 mM dithio-DL-threitol, 0.1 mM EDTA and 3.5 μ M recombinant wild-type GDH onto a Superose

12 column equilibrated and eluted with buffer B. The enzyme purity was checked on 12% SDS PAGE.

GDH activity was monitored on a Perkin Elmer Lambda 16 computerized spectrophotometer at 25°C by following the change of absorption at 340 nm due either to the formation or the consumption of the coenzyme. The activity assay was performed at 25°C in 0.1 M sodium phosphate pH 7.0, containing 40 mM L-glutamate and 1 mM NAD⁺ (forward reaction) or 0.1 mM NADH, 10 mM 2-oxoglutarate and 50 mM NH₄Cl (reverse reaction).

Western blotting

After 12% SDS and 7.5% native PAGE the double mutant (R61E/F187D) and the wild-type proteins were detected immunologically (Harlow & Lane, 1988) using rabbit serum antibodies raised against the wild-type clostridial GDH. The protein was transferred to a Hybond-C nitrocellulose membrane 0.45 mm (Amersham) in a MiniProtein II trans-blot electrophoretic cell (Bio-Rad) for 1 h. For immunodetection the filter was treated following the instructions of the amplified alkaline phosphatase Immuno-Blot kit (Bio-Rad).

Spectroscopic techniques

Circular dichroism (CD) spectra were recorded on a JASCO J-720 spectropolarimeter at 20°C. Measurements were performed in the far-UV (200–250 nm) with 0.2 and 0.5 cm pathlength quartz cuvettes and in the near-UV (250–310 nm) range with 1 cm pathlength quartz cuvettes. The results are the average of 2–3 spectra and are reported as the mean residue ellipticity $[\theta]$ assuming a mean residue mass of 110 Da per aminoacid residue.

Fluorescence and light scattering measurements were carried out at 20°C on a LS 50B Perkin Elmer spectrofluorimeter using 1-cm pathlength quartz cuvettes. Intrinsic fluorescence emission spectra were recorded between 300 and 400 nm with an excitation wavelength set at 295 nm. For light scattering measurements excitation and emission wavelength were both set at 480 nm.

Experiments with ANS were carried out at 20°C by adding aliquots of 0.5 mM ANS to 1 mL solution containing 0.5 μ M protein in 20 mM phosphate, pH 7.0. Ten minutes after the addition of ANS emission intensity was recorded at 465 nm (390 nm excitation wavelength).

Fluorescence quenching with acrylamide (Eftink & Ghiron, 1981) was performed at 20°C by adding increasing amounts of acrylamide (0–150 mM) to a solution containing 30 μ g/mL protein in 20 mM phosphate, pH 7.0. Emission spectra were recorded at 300–400 nm 10 min after the addition of the quencher (excitation was set at 290 nm). Effective quenching constants were obtained from modified Stern-Volmer plots analyzing $F_0/\Delta F$ versus $1/[\text{acrylamide}]$ (15 data pairs) (Lehrer, 1971).

All spectroscopic measurements were performed in buffer B.

Unfolding transition studies

The effect of GdmCl or urea on the helicity and on the intrinsic fluorescence emission of the monomers was studied at 20°C by adding aliquots of 8 M denaturant in buffer B to a solution containing 30 μ g/mL in buffer B of SM or R61E/F187D and adjusting for volume changes after each addition. Spectra were recorded 15 min after addition of denaturant; no further changes occurred after this time up to 16 h.

Molecular mass analysis

The association state of the enzyme was analyzed by size exclusion chromatography on a Superose 12 column (Pharmacia) at 20 °C equilibrated and eluted with buffer B with 0.1 M added NaCl at a flow rate of 0.4 mL/min using a Dionex gradient pump. The protein was applied to the column at a concentration not higher than 30 µg/mL. Elution was monitored at 280 and 226 nm. The Superose 12 column was calibrated with horse spleen apoferritin ($V_e = 8.1$ mL, 440 kDa), rabbit muscle aldolase ($V_e = 8.9$ mL, 161 kDa), horse liver alcohol dehydrogenase ($V_e = 9.7$ mL, 80 kDa), bovine serum albumin ($V_e = 9.9$ mL, 66 kDa), ovalbumin ($V_e = 10.3$ mL, 45 kDa), and horse heart cytochrome *c* ($V_e = 11.7$ mL, 12 kDa). The apparent molecular masses of the structured monomer and of the double mutant R61E/F187D were analyzed by size exclusion chromatography on a Superdex 75 (Pharmacia) at 20 °C eluted with buffer B containing 0.1 M NaCl at 0.4 mL/min and calibrated with bovine serum albumin ($V_e = 6.7$ mL, 66 kDa), ovalbumin ($V_e = 7.5$ mL, 45 kDa), carbonic anhydrase from bovine erythrocyte ($V_e = 8.6$ mL, 29 kDa), soybean trypsin inhibitor ($V_e = 9.0$ mL, 20 kDa), and horse heart cytochrome *c* ($V_e = 10.4$ mL, 12 kDa).

Sedimentation equilibrium experiments were conducted on a Beckman Optima XL-A analytical ultracentrifuge equipped with absorbance optics at 17,000 or 18,000 rpm and 4 or 20 °C. Data were collected at an appropriate wavelength (222, 230, or 280 nm depending on the initial protein concentration) and at a spacing of 0.001 cm with 10 averages in a step scan mode. Equilibrium was checked by comparing scans up to 24 h. Data sets were edited with REEDIT (J. Lary, National Analytical Ultracentrifuge Center, Storrs, CT) and fit individually or jointly with NONLIN (Johnson et al., 1981; PC version provided by E. Braswell, National Analytical Ultracentrifuge Center, Storrs, CT). Data from different speeds and from different wavelengths have been combined for global fitting. Fits to a single species give a Z-average molecular weight. For fits to an association scheme, the monomer molecular weight was held at the value determined from the sequence. For data collected at different wavelengths appropriate extinction coefficients were determined from absorbance values relative to 280 nm. All experiments were done in buffer B. The partial specific volumes of the proteins were calculated from their aminoacids composition and the density of the buffer was obtained by summing the density increments calculated for each component (Laue et al., 1992).

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