A Thermally Sensitive Loop in Clostridial Glutamate Dehydrogenase Detected by Limited Proteolysis*

Suren Aghajanian‡, Manushak Hovsepyan‡, Kieran F. Geoghegan§, Boris A. Chrunyk§, and Paul C. Engel‡§

From the ‡Department of Biochemistry and Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland, and §Pfizer Global Research and Development, Groton, Connecticut 06340

The structural flexibility and thermostability of glutamate dehydrogenase (GDH) from Clostridium symbiosum were examined by limited proteolysis using three proteinases with different specificities, trypsin, chymotrypsin, and endoproteinase Glu-C. Clostridial GDH resisted proteolysis by any of these enzymes at 25 °C. Above 30 °C, however, GDH became cleavable by chymotrypsin, apparently at a single site. SDS-PAGE indicated the formation of one large fragment with a molecular mass of ~44 kDa and one small one of <10 kDa. Proteolysis was accompanied by the loss of enzyme activity, which outran peptide cleavage, suggesting a cooperative conformational change. Proteolysis was prevented by either of the substrates 2-oxoglutarate or l-glutamate but not by the coenzymes NAD1 or NADH. Circular dichroism spectroscopy indicated that the protective effects of these ligands resulted from fixation of flexible regions of the native structure of the enzyme. Size-exclusion chromatography and SDS-PAGE studies of chymotrypsin-treated GDH showed that the enzyme retained its hexameric structure and all of its proteolytic fragments. However, circular dichroism spectroscopy and analytical ultracentrifugation showed global conformational changes affecting the overall compactness of the protein structure. Chymotrypsin-catalyzed cleavage also diminished the thermostability of GDH and the cooperativity of the transition between its native and denatured states. N-terminal amino acid sequencing and mass spectrometry showed that heat-induced sensitivity to chymotrypsin emerged in the loop formed by residues 390–393 that lies between helices α15 and α16 in the folded structure of the enzyme.

Studies of stability in proteins generally encompass their capacity to retain the native state across wide ranges of temperature, ionic strength, pH, or concentration of denaturant. In the case of enzymes, there must be a balance between stability and flexibility so that they can combine an ability to survive their environment with the ability to perform their catalytic function, which frequently requires rapid reversible conformational changes (1).

Limited proteolysis can be used to probe local structure and conformational transitions in enzymes. It can also generate fragments that retain certain functional properties of the original enzyme, allowing identification of individual functional domains. This method in combination with classical protein chemistry methods has been used widely to define the structural organization of some proteins and to locate exposed and flexible regions of their native structure (2–10).

This study examines effects of limited proteolysis at different temperatures on the structural and catalytic properties of glutamate dehydrogenase (GDH) from Clostridium symbiosum, a homo-hexameric enzyme for which high resolution crystal structures with and without bound substrate are available (Protein Data Bank accession numbers 1BGV and 1HRD) (11, 12). The underlying assumption is that the emergence of proteolytic cleavage sites in the early stages of unfolding can indicate the relative flexibility and stability of substructures or domains within the overall structure. The objective of this study is to identify flexible or accessible regions in the enzyme structure with a view to the design of modified forms of clostridial GDH with improved structural stability. Some of this work has been presented as a poster at a Biochemical Society Meeting (Galway, Ireland), and a brief summary was published previously (13).

EXPERIMENTAL PROCEDURES

Materials—Grade II NAD1 (free acid), NADH (dissodium salt), 2-oxoglutarate (disodium salt), and endoproteinase Glu-C were obtained from Roche Diagnostics. l-Glutamic acid, Tris[Tris(hydroxymethyl)-aminomethane], 1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin, and 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK)-treated chymotrypsin (the latter two from bovine pancreas) were purchased from Sigma. Sephadex G-25 (fine), Sepharose CL-6B, and the Superdex 200 HR 10/30 column were from Amersham Biosciences. All other chemicals were analytical reagent grade.

Enzyme Preparation—Escherichia coli TG1 pGS516 cells carrying the C. symbiosum GDH gene (14) were grown and harvested according to the method described elsewhere (15). Clostridial GDH was purified by a single step affinity-chromatographic procedure (16) modified as described elsewhere (17). The enzyme stored in 60% ammonium sulfate at 4 °C was desalted on a Sephadex G-25 column before use.

Determination of Protein Concentration—The enzyme concentration was determined spectrophotometrically at 280 nm by using an absorption coefficient of 1.05 liter/g cm (16).

Enzyme Assay—GDH activity was measured spectrophotometrically at 25 °C (UVikon 941 Pius, Kontron Instruments S.p.A., Milan, Italy) by recording the change in absorbance at 340 resulting from the production of NADH. Assay solution contained 1 mM NAD1 and 40 mM l-glutamate in 0.1 M potassium phosphate buffer, pH 7.0, containing also 1 mM EDTA.

Quaternary Structure Analysis—Size-exclusion chromatography of GDH samples was conducted on a Amersham Biosciences fast protein liquid chromatography system using a Superdex 200 HR 10/30 column equilibrated with 50 mM potassium phosphate buffer, pH 7.0. For calibration of the column, the following molecular mass marker proteins were used: ferritin (450 kDa), bovine serum albumin (68 kDa), hen egg

© 2003 by The American Society for Biochemistry and Molecular Biology, Inc.

Received for publication, June 19, 2002, and in revised form, October 29, 2002
Published, JBC Papers in Press, October 31, 2002, DOI 10.1074/jbc.M206099200

The abbreviations used are: GDH, glutamate dehydrogenase (EC 1.4.1.2); LC-MS, liquid chromatography-mass spectrometry.
The presence of chymotrypsin (residual GDH activity after 30 min incubation with trypsin and chymotrypsin were 100 and 95% activity or the structural integrity of the enzyme as measured in 100 mM potassium phosphate, pH 7.0, there was practically no effect on either GDH activity.

Circular Dichroism Measurements—CD spectra of native and chymotrypsin-treated samples of GDH (both 0.2 mg/ml in 50 mM potassium phosphate buffer, pH 7.0) were run simultaneously at 50,000 rpm in the Beckman XLI analytical ultracentrifuge at 4 °C, with a bandwidth of 0.1 nm and a response time of 4 s.

Amino Acid Sequence Analysis—The samples of GDH treated by proteases were subjected to SDS-polyacrylamide gel electrophoresis. Gels were electroblotted onto Sequi-Blot polyvinylidene difluoride membrane (Bio-Rad), and proteolytic fragments were detected by staining with Coomassie Brilliant Blue. Bands were subjected to sequence analysis by automated Edman degradation on an Applied Biosystems Procise 494 protein sequencer.

RESULTS

PROTEOLYTIC CLEAVAGE AND PROTECTION BY SUBSTRATES—When native clostridial GDH (1 mg/ml) was incubated with trypsin or chymotrypsin (0.1 mg/ml) at 25 °C in 100 mM potassium phosphate, pH 7.0, there was practically no effect on either GDH activity or the structural integrity of the enzyme as measured by SDS-PAGE (Fig. 1, lanes a and b).

When native clostridial GDH (1 mg/ml) was incubated with trypsin or chymotrypsin (0.1 mg/ml) at 25 °C in 100 mM potassium phosphate, pH 7.0, there was practically no effect on either GDH activity or the structural integrity of the enzyme as measured by SDS-PAGE (Fig. 1, lanes a and b). Activities after 30-min incubation with trypsin and chymotrypsin were 100 and 95%, respectively. An increase in the incubation temperature to 50 °C, however, resulted in rapid inactivation of GDH in the presence of chymotrypsin (residual GDH activity after 30 min was <4%), although virtually full enzyme activity (~98%) remained after similar treatment with trypsin. SDS-PAGE showed no changes in the structure of GDH after trypsin treatment (Fig. 1, lane a2), but during incubation with chymotrypsin, a large intermediate fragment of ~45 kDa was rapidly further degraded into fragments too small for detection on the gel (Fig. 1, lane b2).

Chymotryptic cleavage of native GDH could be largely prevented by the addition of the substrate 2-oxoglutarate (Fig. 2). This protective effect was enhanced by the coenzyme NAD", presumably by forming the catalytically non-productive complex (enzyme-NAD"-2-oxoglutarate), even though NAD" on its own had little effect (Fig. 2). Similar results were obtained with l-glutamate and NADH singly and in combination (data not shown). Corresponding to the structural protection, in these experiments there was also retention of catalytic activity.

To retard the secondary cleavage of the proteolytic fragments, the proteolysis by chymotrypsin was carried out at intermediate temperatures. The SDS-PAGE pattern of GDH fragments obtained by chymotrypsin treatment at 30, 35, and 40 °C shows (Fig. 3) that proteolysis could be achieved even at 30 °C, although the rate of digestion depended strongly on the incubation temperature. For incubation temperatures of 30, 35, and 40 °C, residual GDH activities after 20 and 60 min of incubation were as follows: at 30 °C, 85 and 40%; at 35 °C, 38 and 22%; and at 40 °C, 15 and 1%. Almost complete fragmentation of the native subunit was obtained at 40 °C over 60 min of incubation. Three major bands could be seen on the SDS gel: (a) a band in the native subunit position (50 kDa); (b) a large fragment ~10% smaller than the native subunit; and (c) a band near the bottom of the gel corresponding to a molecular mass of <10 kDa. In contrast to the situation at 50 °C, proteolytic fragments obtained at lower incubation temperatures appeared to be more stable. SDS-PAGE experiments on 12% SDS gels with the use of smaller amounts of protein to give sharp bands provided an estimate of 44 kDa for the size of the large GDH fragment.

INFLUENCE OF BUFFER COMPOSITION—Inactivation of bovine liver GDH by trypsin is much faster in Tris buffer than in phosphate buffer (19), confirming the view that these two buffers have strikingly different effects on the structure and functional properties of this enzyme. In search of possible similar effects, the proteolysis of clostridial GDH by chymotrypsin was carried out in both 50 and 100 mM Tris or potassium phosphate buffer, pH 7.0. At higher concentrations of buffers, clostridial GDH showed better resistance against chymotrypsin digestion (data not shown). Also, in the potassium phosphate buffer, clostridial enzyme and its proteolytic fragments were more stable than they were in the Tris buffer. However, the presence of 150 mM NaCl in the incubation mixture dramatically increased the stability of clostridial GDH against chymotryptic digestion in either one of these buffers (data not shown).
An attempt was made to correlate the protective effects of specific ligands and NaCl with the changes in enzyme conformation observed by CD. Thermal denaturation curves of clostridial enzyme were measured by CD (near-UV region, 280 nm) in the presence of 50 mM 2-oxoglutarate or L-glutamate or 150 mM NaCl (data not shown). In the absence of these ligands, a gradual increase of the CD signal was seen above 35–40 °C (as in Fig. 4). However, in the presence of NaCl, the CD signal stayed unchanged up to 50 °C. Moreover, in the presence of 2-oxoglutarate or l-glutamate, conformational changes were detected only above 55 °C. The effects of NAD+/NADH with or without 2-oxoglutarate and l-glutamate could not be measured by this method because of the high absorbance of these coenzymes. Nevertheless, these results suggest that the protection by specific ligands 2-oxoglutarate and l-glutamate as well as NaCl against chymotryptic digestion results from immobilization of flexible regions of the native structure.

**Effect of the Chymotrypsin Treatment on Enzyme Quaternary Structure**—Native clostridial GDH exists as a homo-hexamer composed of identical subunits (11, 20) with a molecular mass of 49,165 Da predicted from the gene sequence (14). To discover the effect of chymotryptic digestion on the hexameric structure, 1 mg/ml native clostridial GDH in 50 mM potassium phosphate, pH 7.0, was incubated at 40 °C with chymotrypsin (final concentration, 0.1 mg/ml). Aliquots withdrawn after 0-, 15-, 30-, and 45-min incubation were cooled on ice. Proteolysis was stopped immediately by the addition of phenylmethylsulfonyl fluoride (2500-fold molar excess relative to the proteinase). Samples were prepared for analysis by size-exclusion chromatography and SDS-PAGE, and their specific activities were also measured. The results summarized in Table I showed that after 15 min >50% GDH subunits were already digested and 16% of the subunits remained uncleaved after 45 min of incubation. However, the most striking conclusion from the results of Table I was that the loss of GDH activity proceeded faster than proteolytic cleavage. For example, the residual GDH activity after 15-min incubation was 23%, although only
half of the enzyme subunits were cleaved. This possibly implies that cleavage leads to cooperative conformational responses in neighboring uncleaved subunits.

In size-exclusion chromatography for all three proteolysed samples, a small amount of material increasing with the time of proteolysis eluted from the Superdex 200 HR 10/30 column in a position corresponding to a molecular size much smaller than that of the clostridial GDH monomer. However, in all three cases, the great majority of protein material eluted in exactly the same position as native hexameric enzyme. For example, in sample 3 (Table I), almost 80% of the enzyme retained its hexameric structure, although >95% of the original activity was lost. SDS-PAGE of these hexameric fractions (Fig. 5) showed that they contained both major fragments of the digested enzyme. In this connection, it is important to note that retention of hexameric structure and of all fragments is not the result of internal disulfide linkage among these fragments or subunits. Clostridial GDH contains only two cysteine residues (14) in each monomer (Cys-144 and Cys-320) which are not involved in either intrasubunit or intersubunit disulfide linkage in the native hexamer (11). Moreover, it is clear from the sequence (Fig. 6) that any single peptide fragment cleaved from either the N or C terminus of the clostridial enzyme must have a molecular size of at least 15 kDa to be able to accommodate one of these cysteine residues.

N-terminal Amino Acid Sequence Analysis—The N-terminal amino acid sequence was determined for all three bands from lane 3 of the SDS gel (Fig. 5). The two upper bands had an identical N-terminal sequence (SKYVDVIAE . . . ), which is the N-terminal sequence of native GDH (Fig. 6) (14), indicating that the N-terminal region of the clostridial enzyme is quite resistant to attack by chymotrypsin. It appears that chymotrypsin cleaves a peptide bond ~50 residues in from the C terminus of the native protein. The N-terminal sequence of the lower band was identified as TAAEVSKLH . . . , indicating cleavage of the peptide bond between residues Trp-393 and Thr-394 in the GDH sequence (Fig. 6).

Mass Spectrometry—LC-MS was used to determine the molecular masses of the chymotryptic fragments of clostridial GDH. Hexamer fractions of samples 1 and 4 (samples of GDH after 0- and 45-min incubation with chymotrypsin, respectively, Table I) eluted from the Superdex 200 column were loaded separately onto a reverse phase column attached to a mass spectrometer. Undigested GDH (sample 1) eluted from the column as a single peak with a retention time of 77.6 min (data not shown). Mass analysis of this peak gave a value of 49,173 Da within 0.02% of the theoretical mass of the native subunit of clostridial GDH (theoretical mass 49,165 Da). In contrast, sample 4 gave two main peaks (Fig. 7A). The first small peak eluted with a retention time of 68.0 min and clearly showed a molecular mass of 5,907 Da (Fig. 7B). This corresponds closely to the mass of peptide fragment-(394–449) of clostridial GDH (theoretical mass 5,908 Da). The second larger peak eluted with a retention time of 76.8 min and had a back shoulder, suggesting the presence of multiple species. The estimated molecular mass of the major component (Fig. 7C, 43,278 Da), was within 0.01% of the theoretical mass of the peptide fragment-(1–393) of clostridial enzyme (theoretical mass 43,275 Da). Minor components with molecular masses of 43,007, 43,513, and 49,178 Da can also be seen in Fig. 7C. The mass of 49,178 Da corresponds to the undigested subunit. In fact, an analysis of the back shoulder of the second peak showed a higher proportion of material with a molecular mass of 49,178 Da (data not shown), indicating the presence of undigested subunits in this peak. The nature of the component.

### Table I

| Sample | Incubation time | Residual activity | Amount of intact subunits | Amount of enzyme retaining hexameric structure |
|--------|-----------------|-------------------|---------------------------|-----------------------------|
| 1      | 0               | 100               | 100%                      | 100%                        |
| 2      | 15              | 23                | 49%                       | 88%                         |
| 3      | 30              | 4                 | 23%                       | 78%                         |
| 4      | 45              | 1                 | 16%                       | 66%                         |

a Calculated from 12% SDS-PAGE of samples.  
b Calculated from elution profiles of samples on a Superdex 200 HR 10/30 column.
with the mass of 43,513 Da is not obvious, but the mass of 43,007 Da could be correlated with the predicted mass (43,002 Da) of the fragment-(1–391) of the clostridial enzyme. Attempts to find a fragment with a molecular mass of 6182 Da (corresponding to the residual peptide fragment-(392–449)) failed. This was anticipated because of the good accessibility of the peptide bond between Trp-393 and Thr-394, resulting in the initial formation of the peptide-(394–449) with a mass of 5908 Da. However, as shown later, after chymotrypsin treatment of clostridial GDH, the C-terminal fragment of the peptide-(1–393) becomes very flexible and it could be accessible for further attack by chymotrypsin as well as by other proteases. In this particular case, chymotrypsin probably attacked the peptide bond between Leu-391 and Ser-392 of the peptide fragment-(1–393) with the release of a dipeptide and formation of the fragment-(1–391).

Physical Properties of GDH after Chymotryptic Cleavage—As seen above, chymotrypsin cleaved clostridial GDH mainly between Trp-393 and Thr-394 with the formation of two polypeptide fragments-(1–393 and 394–449). However, the digested enzyme retained its hexameric structure and contained both of these fragments. Properties of this nicked enzyme were investigated by CD and analytical ultracentrifugation. As a control, the native enzyme was analyzed under the same conditions. Fig. 8 shows CD spectra measured at 20 °C for native and chymotrypsin-treated samples of clostridial GDH in the near-UV (A) and far-UV (B) regions. It is clear that the chymotrypsin treatment affected the secondary structure of clostridial GDH. In the near-UV (Fig. 8A), the CD spectrum of the treated enzyme showed an elevated ellipticity reflecting changes in the environment of aromatic side chains. Such changes in the near-UV CD spectra were anticipated from the experimental results discussed above. However, chymotrypsin-treated enzyme showed a decreased CD signal in the far-UV region (Fig. 8B), indicating more widespread changes in the secondary structure. Analytical ultracentrifugation studies yielded sedimentation coefficients of 13.2 and 11.7 S, respectively, for the native and chymotrypsin-treated enzymes (data not shown). These results point to an increase in relative hydrodynamic radius of the proteolysed samples and thus also to substantial changes in the compactness of the digested enzyme.

To find out the effect of chymotrypsin digestion and consequent conformational changes on the thermal stability of clostridial GDH, the CD scans at 220 nm were monitored continuously with increasing temperature. This experiment showed (Fig. 9) a melting point of chymotrypsin-digested GDH of 56.5 °C in comparison with 62.2 °C for the native enzyme, indicating a significant decrease in structural stability of the protein after cleavage. Also, denaturation of the chymotrypsin-treated enzyme occurred over a wider temperature range, suggesting a less cooperative transition from the folded to the unfolded state than in the native enzyme.

Further Digestion of Chymotrypsin-treated GDH with Other Proteinases—As outlined above, the treatment of clostridial GDH with chymotrypsin produces major conformational changes affecting both stability and cooperativity. The most flexible region of the enzyme appears to be the loop lying between helices α15 and α16 of the enzyme (Figs. 6 and 10), as this was the first point where proteolysis occurred as the temperature was raised. As discussed above, apart from the residue Trp-393, chymotrypsin attacks also the peptide bond between Leu-391 and Ser-392, although much more slowly. However, it was not clear whether this minor cleavage at the position Leu-391 required prior cleavage at Trp-393. Even though the fragment-(392–449) was not seen, this could merely reflect rapid secondary cleavage at Trp-393. However, taking into consideration that one of the potential tryptic digestion sites (Arg-390) lies just beside Leu-391 and the fact that native clostridial GDH is quite resistant to trypsin digestion (even at
50 °C, it seems probable that the second chymotryptic site became available only after digestion at Trp-393. To test this view, the hexameric fraction of the chymotrypsin-treated enzyme was incubated over 45 min with trypsin at 25 °C. SDS-PAGE of the incubation mixture showed virtually no difference between samples before and after incubation with trypsin (data not shown). However, LC-MS analysis of the same incubation mixture clearly revealed the following two GDH fragments: 1) 5910 Da, which is the same 394–449 chymotryptic fragment of the enzyme, and 2) 42,895 Da, which corresponds within 0.01% with the mass of the tryptic fragment-(1–390) of the clostridial enzyme (theoretical mass 42,889 Da). These results first of all indicate that both chymotryptic fragments of clostridial enzyme (1–393 and 394–449) retain their compact secondary structure and remain resistant to trypsin with the exception of a small segment (tripeptide) at the C terminus of 1–394, which becomes exposed and accessible to digestion. Secondly, they support the above assumption that the second chymotryptic digestion site in clostridial enzyme (Leu-391) is a result of conformational changes in the loop area between helices a15 and a16 of the GDH structure caused by the first cleavage at Trp-394.

A further attempt was made to estimate the extent of this most flexible section of the clostridial GDH molecule. Fig. 6 shows that several glutamic acid residues are located around the chymotryptic and tryptic digestion sites of clostridial GDH. Specifically, Glu-389 is located at the end of helix a15 and just beside the tryptic cleavage site (Arg-390), and two others, Glu-396 and Glu-397, at the beginning of helix a16 are just two residues away from the main chymotryptic digestion site Trp-393. However, incubation of chymotrypsin-treated enzyme with endoproteinase Glu-C had no effect on the molecular masses of either of the polypeptide fragments, 1–393 or 394–449. This finding indicates that the proteolysis by chymotrypsin does not expose any new sites for cleavage by Glu-C and that over the temperature range studied only the tripeptide loop-(391–393) (Figs. 6 and 10) becomes susceptible to proteolysis.

DISCUSSION

The results described here show parallels in some respects with earlier studies on limited proteolysis of glutamate dehydrogenase from bovine liver (19, 21–25). Chymotrypsin treatment produced a cleavage product with a molecular size ~5000 Da smaller than the native enzyme (according to SDS-PAGE) but resulted in severalfold activation (22, 24). This intermediate had reportedly lost the ability to respond to allosteric acti-
vation by ADP (22), although the extent and direction of regulatory changes depended on the pH and composition of the assay mixture (23, 25). As in the present case, the chymotryptic cleavage produced no change in the quaternary structure or molecular weight of the enzyme. Also, the bovine liver enzyme was very resistant to trypsin, but the single chymotryptic cleavage opened up the enzyme to further attack and inactivation by trypsin.

In the case of the bovine enzyme, it is not yet clear at which end of the polypeptide chain the cleavage occurs, but in clostridial GDH, we are now able to locate the site precisely not only in the sequence but also in the three-dimensional structure. The loop in question comes between two long helices, $\alpha_{15}$ and $\alpha_{16}$, highlighted in Fig. 10.

Native clostridial enzyme exists as a homo-hexamer with a 32-structural symmetry (11, 20) and, as shown above, retains its hexameric structure even after treatment with chymotrypsin. Fig. 11 represents the crystal structure of the hexamer of the clostridial enzyme and shows locations of these thermally sensitive loops, which are in a large cavity round the 3-fold axis of the hexamer. Thus, there are two such cavities, one for each trimer (each containing three sensitive loops), on either side of the GDH molecule. It is obvious from the structure of GDH (Fig. 11) that the molecule of chymotrypsin would not readily be able to approach the cleavage site because it is too large to penetrate deep into the cavity. This must imply that as the temperature is raised, the sensitive loop becomes in some way more accessible. One possibility would be that there is a transient dissociation to monomers, which would then make the loop fully accessible. If so, however, it would be difficult to explain the failure to observe cleavage by chymotrypsin at a number of other potential sites such as those in the loop joining $\alpha_{16}$ and $\alpha_{17}$ (e.g., Tyr-420, Tyr-424). Also, trypsin was found only to cleave at Arg-390 after the chymotryptic cleavage at Trp-393, whereas in a monomer, not only this site but also a number of others might be readily accessible even without prior proteolysis by chymotrypsin.

Therefore, it is difficult to escape the conclusion that the increased temperature leads to a specific conformational arrangement that makes the target loop available to chymotrypsin. Inspection of the structure shows that the two helices, $\alpha_{15}$ and $\alpha_{16}$, which are linked by the sensitive loop, are not directly involved in the formation of the intersubunit contacts, and in principle, such a conformational rearrangement might happen by a large movement of these two helices, $\alpha_{14}$ and $\alpha_{15}$, outward from the 3-fold axis, projecting the loop into a much more exposed position.

In connection with this loop, it is interesting to note that the major structural difference between bacterial and mammalian GDHs is that the large 48-amino acid insertion between $\alpha_{15}$ and $\alpha_{16}$, and starting right after residue Trp-393 (14). The recently determined crystal structure of the bovine GDH in a complex with NADH, glutamate and GTP have revealed that this 48-residue insertion forms an “antenna” structure lying immediately adjacent to the 3-fold axis of the hexamer, and it has been suggested that this domain of the mammalian enzyme is involved in the regulation by allosteric effectors GTP and ADP as well as by coenzymes NAD$^+$ and NADH (26, 27). It was also suggested that allosteric effects of these regulators on enzyme activity (activation by ADP and inhibition by GTP) are realized via conformational changes in this regulatory domain affecting the energy required for the enzyme to open and close the active center cleft during the catalytic cycle. On the other hand, these allosteric regulators GTP and ADP have opposite effects not only on the catalytic activity but also on the ability of this enzyme to associate into high molecular weight aggregates by polymerization of hexamers along the 3-fold axis of the enzyme (28, 29). It is reasonable to surmise that the realization of such effects by GTP and ADP requires substantial conformational changes in the antenna domain, which is involved in the contact area among hexamers. Therefore, the two helices of the bovine enzyme flanking this antenna domain (corresponding to the clostridial helices $\alpha_{15}$ and $\alpha_{16}$) must have enough flexibility to allow these conformational changes. Clearly, one cannot reliably extrapolate from the complex bovine GDH structure to the simpler clostridial structure. Nevertheless, the helices in question are a conserved feature, and since the clostridial GDH also displays allosteric behavior (15, 30, 31), it is inconceivable that these flexible helices also play a key role in subunit interaction in the bacterial enzyme.

An interesting question in relation to the catalytic mechanism of clostridial GDH is the role of glutamate. Crystallography has suggested that the amino acid substrate triggers a closure of the cleft separating the two domains of the monomer to enclose the active site of the enzyme (12). A comparison of the structures for the free clostridial enzyme (open) and for the enzyme-glutamate complex (closed) reveals that this hinge closure causes remarkably little change in the location of the sensitive loop. Hinge closure alone cannot therefore explain the pronounced protection by glutamate against proteolysis by chymotrypsin. However, the structure of the enzyme-glutamate complex shows that the carboxylate groups of the bound substrate interact directly with Ser-380 and indirectly via Lys-113 with Asn-373 (12). Both of these residues are located in the helix $\alpha_{15}$ and it seems reasonable to suggest that the complexation with glutamate may impede any tendency for $\alpha_{15}$ and $\alpha_{16}$ to swing outward as suggested above, thus protecting the enzyme from chymotrypsin digestion.

Acknowledgments—We thank Dr. J. G. Stroh (Pfizer Global Research and Development, Groton, CT) for access to the mass spectrometer and useful discussions, Michele H. Rosner for analytical ultraacentrifugation studies, and Anthony J. Lanzetti for protein sequence analysis. We are grateful to the members of the Sheffield University crystallography group (Sheffield, United Kingdom) including Prof. D. W. Rice and Drs. P. J. Baker, T. J. Stillman, and K. L. Britton for providing the coordinates of the C. symbiosum GDH hexamer.

REFERENCES

1. Creighton, T. E. (1984) Proteins-Structure and Molecular Properties, W. H. Freeman, NY
2. Fontana, A., Fassina, G., Vita, C., Dalzoppo, D., Zanami, M., and Zambonin, M. (1986) Biochemistry 25, 1847–1851
3. Loomes, K., and Jorrvall, H. (1991) Biochemistry 30, 8685–8670
4. Arnone, M. I., Birolo, L., Giamberini, M., Cubellis, M. V., Nitti, G., Marino, G., and Sanina, G. (1992) Eur. J. Biochem. 204, 1183–1189
5. Brockerhoff, S. E., Edmonds, C. G., and Davis, T. N. (1992) Protein Sci., 504–516
6. Cohen, S. L., Ferre-D’Amare, A. R., Burley, K. S., and Chait, B. T. (1995) Protein Sci. 4, 1088–1099
7. Pardaro, J., Consejero-Lara, F., Smith, R. A. G., Marshall, J. M., Punxing, C. P., and Dobson, C. M. (1996) Protein Sci. 5, 693–704
8. Arnold, U., Rucknagel, K. P., Schierhorn, A., and Ulrich-Hofmann, R. (1996) Eur. J. Biochem. 237, 862–869
9. Zappacosta, F., Pecci, A., Bianchi, E., Venturini, S., Sollazzo, M., Tramontano, A., Marino, G., and Pucci, P. (1996) Protein Sci. 5, 802–813
10. Nakagawa, N., Masui, R., Kato, R., and Karumitsu, S. (1997) J. Biol. Chem. 272, 22763–22773
11. Cohen, S. L., Britton, K. L., Engel, P. C., Farrants, G. W., Lilley, K. S., Rice, D. W., and Stillman, T. J. (1992) Proteins Struct. Funct. Genet. 12, 75–86
12. Stillman, T. J., Baker, P. J., Britton, K. L., and Rice, D. W. (1995) J. Mol. Biol. 254, 1131–1140
13. Aghajanian, S., Hovsepian, M., Geoghegan, K. F., Chrunyk, B. A., and Engel, P. C. (1995) Biochem. Soc. Trans. 26, 526
14. Teller, J. K., Smith, R. J., McPherson, M. J., Engel, P. C., and Guest, J. R. (1992) Eur. J. Biochem. 206, 151–159
15. Aghajanian, S., and Engel, P. C. (1998) Protein Eng. 11, 569–575
16. Syed, S. E-H., Engel, P. C., and Parker, D. M. (1991) Biochim. Biophys. Acta 1125, 125–130
17. Aghajanian, S. A., Martin, S. R., and Engel, P. C. (1995) Biochem. J. 311, 905–910
A Thermally Sensitive Loop in GDH

18. Laemmli, U. K. (1970) Nature 227, 680–685
19. Hucho, F., Rasched, I., and Sund, H. (1975) Eur. J. Biochem. 52, 221–230
20. Rice, D. W., Baker, P. J., Farrants, G. W., and Hornby D. P. (1987) Biochem. J. 242, 789–795
21. Malcolm, A. D. B., and Sommerville, J. (1974) Biochem. Soc. Trans. 2, 140–141
22. Beynon, R. J., and Kay, J. (1976) Int. J. Biochem. 7, 449–453
23. Aitchison, M. J., and Engel, P. C. (1980) Biochem. Soc. Trans. 8, 649
24. Place, G. A., and Beynon, R. J. (1982) Biochem. J. 205, 75–80
25. Aitchison, M. J., and Engel, P. C. (1983) Int. J. Biochem. 15, 79–85
26. Peterson, P. E., and Smith, T. J. (1999) Structure 7, 769–782
27. Smith, T. J., Peterson, P. E., Schmidt, T., Fang, J., and Stanley, C. A. (2001) J. Mol. Biol. 307, 707–720
28. Sund, H., Pilz, I., and Herbst, M. (1969) Eur. J. Biochem. 7, 517–525
29. Eisenberg, H., and Reisler, E. (1970) Biopolymers 9, 115–115
30. Syed, S. E.-H., Engel, P. C., and Martin, S. R. (1990) FEBS Lett. 262, 176–178
31. Wang, X.-G., and Engel, P. C. (1995) Biochemistry 34, 11417–11422
A Thermally Sensitive Loop in Clostridial Glutamate Dehydrogenase Detected by Limited Proteolysis
Suren Aghajanian, Manushak Hovsepyan, Kieran F. Geoghegan, Boris A. Chrunyk and Paul C. Engel

J. Biol. Chem. 2003, 278:1067-1074.
doi: 10.1074/jbc.M206099200 originally published online October 31, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M206099200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 7 of which can be accessed free at http://www.jbc.org/content/278/2/1067.full.html#ref-list-1