Disruption of a Calmodulin Central Helix-like Region of 10-Formyltetrahydrofolate Dehydrogenase Impairs Its Dehydrogenase Activity by Uncoupling the Functional Domains*

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10-Formyltetrahydrofolate dehydrogenase (FDH) is composed of three domains and possesses three catalytic activities but has only two catalytic centers. The amino-terminal domain (residue 1–310) bears 10-formyltetrahydrofolate hydrolase activity, the carboxyl-terminal domain (residue 420–902) bears an aldehyde dehydrogenase activity, and the full-length FDH produces 10-formyltetrahydrofolate dehydrogenase activity. The intermediate linker (residues 311–419) connecting the two catalytic domains does not contribute directly to the enzyme catalytic centers but is crucial for 10-formyltetrahydrofolate dehydrogenase activity. We have identified a region within the intermediate domain (residues 384–405) that shows sequence similarity to the central helix of calmodulin. Deletion of either the entire putative helix or the central part of the helix or replacement of the six residues within the central part with alanines resulted in total loss of the 10-formyltetrahydrofolate dehydrogenase activity, whereas the full hydrolase and aldehyde dehydrogenase activities were retained. Alanine-scanning mutagenesis revealed that neither of the six residues alone is required for FDH activity. Analysis of the predicted secondary structures and circular dichroic and fluorescence spectroscopy studies of the intermediate domain expressed as a separate protein showed that this region is likely to consist of two α-helices connected by a flexible loop. Our results suggest that flexibility within the putative helix is important for FDH function and could be a point for regulation of the enzyme.

One of the major enzymes of folate metabolism, 10-formyltetrahydrofolate dehydrogenase (FDH) is a natural fusion of two unrelated genes (1). The enzyme consists of two catalytic domains, the amino- and carboxyl-terminal, connected by a linker of ~100 amino acids (1–3). The amino-terminal domain of FDH (residues 1–310) can function as 10-formyltetrahydrofolate (10-formyl-THF) hydrolase and has sequence similarity to several other enzymes using 10-formyl-THF as a substrate (3). The carboxyl-terminal domain is an aldehyde dehydrogenase homologous protein (1, 2). It reveals up to 50% homology to proteins from the aldehyde dehydrogenase family and possesses its own aldehyde dehydrogenase activity (1, 2, 4). FDH also possesses another activity, 10-formyl-THF dehydrogenase (5–7), that is the result of concerted action of both functional domains (3, 8). This activity is the major metabolic function of the enzyme (9). The intermediate domain of FDH (Fig. 1) seems to have no function except simply to connect the two functional domains (1). However, the two domains produce the dehydrogenase activity only when they are combined in one polypeptide, whereas the mixture of the domains expressed separately is unable to catalyze the reaction (3). This suggests that the intermediate sequence is an essential part of the enzyme that brings the functional domains into correct orientation to produce a new activity.

The intermediate domain sequence as a whole has no identity to any known protein. A data base search revealed, however, that the fragment between residues 366 and 405 in the intermediate domain is 35% identical to a sequence from calmodulin-like protein identified in rice (10). The identity rises to 45.5% for residues 384–405 (Fig. 2A). It is interesting that in the calmodulin structure this sequence forms a long central α-helix that serves as a linker connecting two globular domains of calmodulin (11). The crystal structure of calmodulin (12, 13) showed that the helix is straight and is not surrounded by any other α-helices or β strands (Fig. 2B). NMR studies of calmodulin, however, have revealed a non-helical region at residues 78–81 (14). It has been further shown that the central helix is disrupted into two helices connected by a long flexible loop (residue 74–82, Fig. 2C) (15). Thus, rather than a rigid spacer, the linker serves more as a flexible tether between two domains, allowing them to swing around (16, 17). Movement in the central helix changes calmodulin conformation from an elongated dumbbell to a compact globular form (18). Such conformational changes occur during binding of target peptides (15), which is an important mechanism in calmodulin function (16, 19, 20).

Although the similarity of the intermediate domain with calmodulin (Fig. 1A) is lower than with the calmodulin-like protein (36.4% and 45.5%, correspondingly), it is 63.6% in both cases if conservative changes are taken into account. We suggest that the “calmodulin-like” region of the intermediate domain with its flexible central part plays an important role in coupling the two catalytic domains of FDH to produce dehydrogenase activity. In the present work we apply a site-directed mutagenesis approach to explore the role of this region in FDH function.
Materials—10-Formyl-5,8-dideazafolate (10-formyl-DDF) and 5,8-
dideazafolate (5,8-DFF) were obtained from B. Hymowitz, Depart-
ment of Pharmaceutical Chemistry, Medical University of South Caro-
olina. All media were obtained from Difco or Invitrogen. SDS-PAGE
standards and Sephacyrl S-300 were purchased from Amersham Bio-
sciences. Other chemicals were obtained from Sigma.

Generation of the Construct for Intermediate Domain Expression—The
construct for the intermediate domain expression was generated in
two steps from pRSET vector with cloned FDH cDNA (21). This con-
struct has a unique NdeI restriction site upstream of the 5‘-translated
region of the FDH, which overlaps the ATG start codon, and a unique
ScaI restriction site on 3‘-translated region, which overlaps the TGA
stop codon. In the first step, the second NdeI restriction site was intro-
duced to vector immediately downstream of the intermediate domain
coding sequence using site-directed mutagenesis. The sequence be-
tween two NdeI sites was excised, and vector was religated through
internal NdeI sites. This approach has created an in-frame ATG start
codon next to which was a triplet coding for the first amino acid in the
intermediate domain sequence. In the second step, the second ScaI
restriction site was introduced into the vector immediately downstream
of the intermediate domain coding sequence using site-directed mu-
tagenesis. The sequence between two ScaI sites was excised, and vector
was religated through internal ScaI sites. This approach has created an
in-frame TGA stop codon immediately downstream of a triplet coding
for the last amino acid in the intermediate domain sequence.

Site-Directed Mutagenesis—The QuikChange site-directed mutagen-
esis kit (Stratagene) was used to generate a construct for the interme-
diate domain expression and to introduce desirable mutations within
the intermediate domain site. Site-directed mutagenesis was carried out
directly on expression constructs: pRSET with cloned intermediate
domain cDNA or pVL 1393 with cloned FDH cDNA (22). Experiments
were performed according to the manufacturer’s protocol. Plasmids
were isolated by using a Quantum Prep kit (Bio-Rad) and sequenced to
identify clones carrying the mutation. The entire coding region of the
clones carrying the mutations was sequenced to ensure the absence of
random mutations.

Expression of the Intermediate Domain and Its Mutants—Esche-
richia coli BL21 (DE3) cells (Novagen) were transformed with the cor-
responding expression vector according to the manufacturer’s protocol,
and the cells were grown in 4 mL of NZCYM medium containing ampic-
illin (50 μg/mL) overnight at 37 °C with shaking. The 100 mL of NZCYM
medium containing ampicillin were inoculated with the overnight cul-
ture and incubated at 37 °C with shaking until A600 = 0.6 (about 6 h)
followed by induction with isopropyl β-D-thiogalactopyranoside (1 mM
concentration). Three hours after induction, the cells were har-
vested by centrifugation (18,000 × g, 15 min), and the insoluble fraction
was removed by centrifugation, and the soluble fraction of the
material was precipitated by centrifugation (18,000 × g, 15 min), and
the soluble sample was subjected to the

size-exclusion chromatography procedure on a Sephadex G-100 column
(1.5 × 100 cm). The column was equilibrated with 20 mM Tris-HCl
buffer, pH 7.5, containing 10 mM 2-mercaptoethanol and 1 mM NaCl.
About 5 mg of protein was loaded onto the column in a volume of about
3 mL. Chromatography was carried out at 20 mL/h flow rate, and fractions
of 4 mL were collected.

Analysis of the Mutant Proteins—SDS-PAGE was carried out accord-
ing to the method of Laemmli (23) in 8.0% or 15.0% gel. Protein con-
centration was determined using Bradford protocol. Plasmids
were isolated by using a Quantum Prep kit (Bio-Rad) and sequenced to
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illin (50 μg/mL) overnight at 37 °C with shaking. The 100 mL of NZCYM
medium containing ampicillin were inoculated with the overnight cul-
ture and incubated at 37 °C with shaking until A600 = 0.6 (about 6 h)
followed by induction with isopropyl β-D-thiogalactopyranoside (1 mM
concentration). Three hours after induction, the cells were har-
vested by centrifugation (18,000 × g, 10 min) and resuspended in 2 mL
buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 0.1% Triton X-100).
The suspension was chilled on ice and sonicated (three times for 45 s).
The insoluble material was precipitated by centrifugation (18,000 × g, 15
min). The supernatant was removed by centrifugation (18,000 × g, 15
min) and the dissolved pellet was

in continuos stirring for 1 h at 4 °C. The supernatant and the dissolved pellet were
examined for the presence of the protein of interest by SDS-PAGE.

Purification of the Intermediate Domain and Its Mutant—

Expressed wild type intermediate do-
mam was carried out in the Protein Core facility at the Medical
University of South Carolina using automated Edman degradation with
gas phase analysis on an LF-3000 (Beckman) or an Applied Biosys-
stem 470A (PerkinElmer Life Sciences) protein sequencer.

Assay of Enzyme Activity—All assays were performed at 30 °C in a
Shimadzu 2401PC double-beam spectrophotometer. For measurement of
hydroxylase activity the reaction mixture contained 0.05 mM Tris-HCl,
pH 7.8, 100 mM 2-mercaptoethanol and 5 mM of substrate, 10-formyl-
DDF. 10-Formyl-DDF is an alternative, stable substrate for the enzyme
(25). The reaction was started by the addition of enzyme (1–2 μg) in a
final volume of 1 mL and incubated for 1 minute at 37 °C. The reactions
were terminated by adding 50 mM CHES buffer, pH 9.4, 1 mM NADP
and enzyme in a total volume of 1 mL. Activity was estimated from the
increase in absorbance at 340 nm.

MALDI Mass Spectrometry—Expressed wild type intermediate do-
mam and its mutants were analyzed at the Medical University of South
Carolina mass spectrometry facility using matrix-assisted laser de-
sorption/ionization mass spectrometry (MALDI-MS). A time-of-flight
MALDI (Voyager-DE STR from Applied Biosystems) was used in linear
mode to detect average ion masses. Samples were prepared by mixing 1
part protein solution (0.5–2.5 μg/μL) with 3 parts matrix (50 mM o-
cyano-4-hydroxycinnamic acid in 70% acetonitrile and 0.1% trifluoro-
aetic acid); 0.5 μL of each sample mixture, containing 5–25 μμL of protein, was then spotted on to air dried MALDI sample plates.

Fluorescence Studies—Fluorescence experiments were done on a Hi-
tachi F-2500 fluorescence spectrophotometer. Emission fluorescence
spectra of wild type intermediate domain and its mutants were recorded by scanning from 300 to 460 nm with fluorescence excita-
tion at either 280 or 295 nm. Protein samples (0.1 mg/mL) were in
20 mM Tris-HCl buffer, pH 7.5. All experiments were done at 20 °C.

Analysis of Conformation by CD Spectroscopy—Far-UV CD spectra
(190–250 nm) were obtained using Jasco J710 spectropolarimeter (East-
on, MD) at a resolution of 0.2 nm with a bandwidth of 2 nm. For each
sample, ten spectra were collected and averaged. The concentration of
wild type and mutant proteins was about 0.05 mg/mL in 50 mM potassium
phosphate, pH 7.0, containing 10 mM NaCl. Protein concentration was
determined using a calculated molar extinction coefficient (28). Spectra
were recorded at 25 °C using cylindrical quartz cuvette with a path
length of 0.02 cm. The protein spectra were corrected by subtracting a

FDH Intermediate Domain

Recombinant viral stock was amplified in Sf9 cells to produce high titer
virus stock. High Five cells were seeded as a monolayer in 25-cm2 flasks
(final volume). Three hours after induction, the cells were har-
vested by centrifugation (18,000 × g, 15 min), and the soluble sample was subjected to the

size-exclusion chromatography procedure on a Sephadex G-100 column
(1.5 × 100 cm). The column was equilibrated with 20 mM Tris-HCl
buffer, pH 7.5, containing 10 mM 2-mercaptoethanol and 1 mM NaCl.
About 5 mg of protein was loaded onto the column in a volume of about
3 mL. Chromatography was carried out at 20 mL/h flow rate, and fractions
of 4 mL were collected.

Analysis of the Mutant Proteins—SDS-PAGE was carried out accord-
ing to the method of Laemmli (23) in 8.0% or 15.0% gel. Protein con-
centration was determined using Bradford protocol. Plasmids
were isolated by using a Quantum Prep kit (Bio-Rad) and sequenced to
identify clones carrying the mutation. The entire coding region of the
clones carrying the mutations was sequenced to ensure the absence of
random mutations.
null
solution. All constructs for the intermediate domain expression had an extra nucleotide triplet encoding for methionine in an amino-terminal end that was necessary to initiate translation. Analysis of the amino-terminal sequences of purified proteins revealed that they do not have a terminal methionine and start from serine (Ser-311 in the FDH sequence). The first methionine, however, is often not retained at the amino terminus of the proteins expressed in prokaryotes (30). Thus, the intermediate domain was expressed as an original sequence presented in the full-length FDH.

Comparison of Wild Type and Mutant Intermediate Domains—We compared CD spectra of the intermediate domain and its mutants. These experiments were carried out to detect changes in the secondary structure elements within the domain induced by the mutations. CD spectra in the far-UV range reflect content of secondary structure components in the proteins with the strongest and most characteristic spectrum for the α-helix (31). These experiments revealed significant shifts in CD plots indicating changes in secondary structure element composition (Fig. 3). The spectra were analyzed using a method that allows quantitative calculation of the content of the secondary structure elements (29). Data derived from this analysis (Table II) revealed that introduction of alanine residues in the putative flexible region resulted in an increase of α-helical content with a decrease in random coil content compared with the wild type intermediate domain. These results indicate that the entire putative helix (about 22 amino acid residues) is likely to be straight in the mutant. The other mutant, 22del, in which the putative long α-helix was deleted, showed a notable decrease in content of α-helical region compared with the wild type protein. This decrease was proportional to the relative contribution of this fragment in the α-helical portion of the intermediate domain. Results on the last mutant, 6del, suggest that the deletion of the putative flexible region within the helix stabilized the entire helix, which probably becomes straight similar to 6Ala mutant.

To further explore whether or not conformational changes occur due to the introduced mutations, we compared the emission fluorescence spectra of the intermediate domain and its 6Ala mutant. The intermediate domain has one tryptophan and two tyrosine residues that allow one to measure its intrinsic fluorescence. We performed these experiments at excitation wavelengths of 278 and 295 nm. We observed significant differences in the fluorescence spectra of the mutant compared with the spectrum of the wild type intermediate domain (Fig. 4). There was stronger shift in the fluorescence maximum with an excitation at 278 nm (from 322 to 344 nm) than with excitation at 295 nm (from 332 to 341 nm). These results suggest that the tryptophan and the tyrosines become more susceptible to solvent in 6Ala mutant than in the wild type intermediate domain.

Alanine-scanning Mutagenesis—Using alanine-scanning mutagenesis we studied whether substitution of a single residue within the putative flexible regions will influence FDH activity or generate secondary structure changes within the intermediate domain. We have generated six mutants of the full-length FDH and six corresponding mutants of the intermediate domain protein. Thus, following mutants were expressed and purified: K394A, L395A, R396A, G397A, E398A, and D399A. The six mutants of the full-length FDH were expressed and purified as described for the wild type enzyme (22). All mutants showed a similar level of expression and the same elution profile when purified on 5-formyltetrahydrofolate affinity column (data not shown). They come off from the affinity column during elution with 20 mM folic acid that are the same conditions as for elution of the wild type enzyme (22). Assay of the enzyme activity of the purified mutants revealed that they all possessed three types of activities, hydrolase, dehydrogenase, and aldehyde dehydrogenase (Table I). Levels of all three activities were comparable to the activities of the wild type enzyme (Table I).

Expression of the six corresponding mutants of the intermediate domain revealed that all were expressed as insoluble proteins similar to the wild type intermediate domain. The mutants were solubilized from inclusion bodies, refolded, and purified by size-exclusion chromatography as described above. Each of the mutants came out of the column as a single peak with an apparent molecular mass of about 15 kDa (data not shown) that is similar to the elution profile of the wild type intermediate protein. This suggests that they were not aggre-
gated in solution. To ensure that the expressed intermediate domain was not truncated or otherwise degraded and to check for relative purity, we analyzed the wild type intermediate domain and its mutants using matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS). Results of MALDI-MS analysis showed that all of the expressed polypeptides behaved as a single molecular mass species and matched their respective calculated molecular masses (Table III). Amino-terminal sequence analysis revealed that each of the purified intermediate domain preparations contained only the intermediate domain protein. Thus, these experiments showed that expressed intermediate proteins were not degraded from the termini. CD spectra of all six mutants of the intermediate protein were close to each other and similar to that of the wild type protein in contrast to 6Ala mutant spectra (data not shown). The calculated content of the secondary structures confirmed close structural similarity between these mutants and the wild type intermediate domain (Table IV). These results suggest that neither of these single-point mutations induce substantial changes in the secondary structure.

DISCUSSION

In this study we have identified a region within the linker domain of FDH that might be responsible for the regulation of the concerted action of the two functional domains of the enzyme. FDH has an unusual structure. The folate binding site of the enzyme is located in its amino-terminal domain, whereas the dehydrogenase catalytic center is located in the carboxy-terminal domain (2, 3). Our previous studies have shown that the two functional domains of FDH, the amino- and carboxy-terminal, do not interact in the absence of the intermediate domain (3). This implies that the intermediate domain of FDH is necessary to hold the functional domains together to produce dehydrogenase activity. Thus, the intermediate domain, although unlikely to be directly involved in catalysis, is an important component of the enzyme machinery. Because connectivity between the two functional domains is critical for FDH activity, it is likely that unfavorable changes within the intermediate domain will uncouple the functional domains resulting in an inactive enzyme. The crystal structure of FDH is not available at present; therefore, it is difficult to draw conclusions about the intermediate domain structure. However, the sequence identity between part of the intermediate domain and calmodulin-like protein, suggests that they might have some common elements. It is interesting that the intermediate domain similar to calmodulin has an elevated content of the acidic residues, aspartate and glutamate: 7.3% and 14.5% for the intermediate domain and about 11 and 15% for calmodulin, respectively. The same values for the amino- and carboxy-terminal domains, however, are 4.5%/6.8% and 5.0%/6.4%, respectively. The identity of a part of the intermediate domain sequence with the calmodulin long central helix has attracted our attention, because this helix can undergo conformational changes that are important for calmodulin function (16–19).

Our experiments with site-directed mutagenesis of the intermediate domain suggest that the calmodulin long helix-like region of FDH is likely to be bent in a manner similar to that of the calmodulin structure (15, 18). All mutants of full-length

### Table III

<table>
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<tr>
<th>Intermediate domain</th>
<th>Calculated average mass</th>
<th>Observed average mass</th>
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<tr>
<td>Wild type</td>
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<td>K394A</td>
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<td>11980.1 Da</td>
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<td>L395A</td>
<td>12202.7 Da</td>
<td>12204.0 Da</td>
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</tr>
<tr>
<td>G397A</td>
<td>12174.7 Da</td>
<td>12168.4 Da</td>
</tr>
<tr>
<td>E398A</td>
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<td>12192.0 Da</td>
</tr>
<tr>
<td>D399A</td>
<td>12215.8 Da</td>
<td>12210.8 Da</td>
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</tbody>
</table>

### Table IV

<table>
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<th>β-Sheet</th>
<th>Turn</th>
<th>Coil</th>
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</thead>
<tbody>
<tr>
<td>Wild type</td>
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<td>22.2 ± 1.6</td>
<td>18.0 ± 0.9</td>
<td>29.2 ± 1.4</td>
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<tr>
<td>K394A</td>
<td>31.9 ± 2.2</td>
<td>20.4 ± 1.7</td>
<td>17.8 ± 1.0</td>
<td>28.1 ± 1.5</td>
</tr>
<tr>
<td>L395A</td>
<td>29.1 ± 1.8</td>
<td>23.1 ± 1.9</td>
<td>18.2 ± 0.9</td>
<td>20.2 ± 1.8</td>
</tr>
<tr>
<td>R396A</td>
<td>32.2 ± 1.5</td>
<td>20.1 ± 1.7</td>
<td>17.7 ± 0.9</td>
<td>28.1 ± 1.7</td>
</tr>
<tr>
<td>G397A</td>
<td>29.4 ± 2.1</td>
<td>23.2 ± 1.4</td>
<td>18.3 ± 0.9</td>
<td>29.2 ± 1.5</td>
</tr>
<tr>
<td>E398A</td>
<td>29.0 ± 1.9</td>
<td>23.8 ± 1.8</td>
<td>18.5 ± 1.1</td>
<td>29.5 ± 2.0</td>
</tr>
<tr>
<td>D399A</td>
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<td>20.7 ± 1.6</td>
<td>17.8 ± 1.2</td>
<td>28.4 ± 1.4</td>
</tr>
</tbody>
</table>

Fig. 4. Fluorescence spectra of wild type and 6Ala mutant intermediate domain. Spectra at excitation wavelengths of 295 nm (A) and 278 nm (B) are shown. Curves 1, wild type intermediate domain; curves 2, 6Ala mutant.
FDH retained hydrolase and aldehyde dehydrogenase activities similar to those for the wild type enzyme suggesting that both functional domains themselves were not affected by the mutations. This is consistent with our previous studies that have shown that the catalytic domains are capable of folding into functional proteins independently of each other or of the intermediate domain (2, 3). However, deletion of either the entire long helix-like sequence or just the putative flexible region of the sequence, or the change of all residues in the flexible region to alanines, resulted in an enzyme that lacks dehydrogenase activity. An explanation of these results is depicted on the schematic (Fig. 5). Because alanine has the highest propensity for helix formation (32), replacement of the residues within the flexible region with alanines has apparently straightened the helix by pulling apart the amino- and carboxyl-terminal domains. This should prevent concerted action of the two domains resulting in the loss of the dehydrogenase activity. It is less obvious what happened to the reciprocal orientation of the amino- and carboxyl-terminal domains after deletions are made within this region. We suggest that deletion of the entire “calmodulin-like” region in FDH resulted in more tight but much less flexible interactions between the two domains. Such an orientation apparently misoriented the folate binding site in the amino-terminal domain with respect to the dehydrogenase catalytic center located in the carboxyl-terminal domain (Fig. 5). We further suggest that deletion of the putative flexible region affected the reciprocal domain orientation in a manner similar to that when the region was replaced with alanine residues thus distancing the domains from each other.

Predicted secondary structure of the intermediate domain showed indeed that regions between residues 379 and 393 and between 403 and 416 have a high tendency to form α-helices but a low tendency to form either β-sheets or random coils. In contrast, the region between residues 394 and 402 has a high tendency to form a random coil. This suggests that the region is likely to be represented by two α-helices connected by a loop. For the 6Ala mutant it has been predicted that the region between residues 380 and 415 that coincides with our hypothesis. We also modeled simulations of the original calmodulin-like 22-residue-long fragment and the fragment with six residue converted to alanines. This has been done using SYBYL 6.7 (TRIPOS Associates Inc., St. Louis, MO). In modeling simulations with the original fragment, adding turns to the putative six-residue flexible region results in a conformation with a lower minimum energy than with an α-helix. When the six residues were converted to alanine, the converse was true: modeled as a helix, the region reached a lower minimum energy. It should be also noted that the putative flexible region and adjacent upstream and downstream sequences are enriched with charged residues that create an opportunity for multiple salt bridge formations and hydrogen bonding. Indeed, the modeling revealed possible salt bridge formation when the putative helix is bent in the middle that apparently stabilizes it in the required conformation.

The results of CD spectroscopy studies of the intermediate domain, and its mutants expressed as separate proteins, correspond well to the predicted secondary structures. Better correspondence was observed, however, for the wild type intermediate domain and for the 6Ala mutant. Both theoretical predictions and experimental approaches showed an increase in α-helical content with a decrease in random coil content in this mutant compared with the wild type protein. A significant increase in α-helix content was also observed for another mutant, 6del. This suggests that this mutation resulted in the formation of a single long helix rather than two closely located helices connected by a shorter loop. Studies on calmodulin also revealed that deletion within the flexible region results in straightening of the central helix (33). A notable decrease in the α-helix content in the 22del mutant further suggests that the region is indeed represented mainly by an α-helix. The results of fluorescence spectroscopy studies are in accordance with the results of CD-spectroscopy studies. Comparison of the fluorescence spectra of the wild type intermediate domain and 6Ala mutant revealed that the tryptophan and tyrosines of the 6Ala mutant are more exposed to the solvent than in the wild type protein suggesting that the mutant has a more open conformation. The single tryptophan (Trp-332) of the intermediate domain is distant from the sequence mutated, whereas the two tyrosines (Tyr-380 and Tyr-409) are located in close proximity to this region flanking it from each side. This explains the stronger shift in the fluorescence maximum at 278 nm versus the one at 295 nm. With excitation at 295 nm, the spectrum reflects fluorescence of tryptophans, whereas with excitation at 278 nm there is a significant contribution of tyrosine residues to the fluorescence (34). Apparently, straightening the putative helix makes the nearby tyrosines more susceptible to solvent, whereas the more remote tryptophan is less affected. Although the truncated mutants also revealed significant changes in their intrinsic fluorescence, we did not analyze them in detail. Interpretation of such results would be more difficult, because conformational changes of these mutants might be the result of a difference in size and not just the result.
of changes in secondary structure content. Overall, these results support our explanation of the role of the region in FDH function.

We propose that flexibility within the calmodulin long helix-like region of FDH is associated with the enzyme mechanism. Our results imply that simple connectivity between the two functional domains is insufficient to produce the dehydrogenase activity. Due to its structural organization, FDH undergoes significant conformational changes during its catalytic activity. After substrate binding, it is likely that the enzyme changes conformation and couples the two functional domains to initiate the dehydrogenase reaction. In turn, opposite changes uncouple the domains after completion of the reaction to allow release of the product. For such a mechanism, the calmodulin long helix-like region of FDH is a suitable candidate to work as a hinge, allowing reciprocal movement of the functional domains. This region might also be a site for regulation of the enzyme. Thus, stabilization of the sequence in elongated conformation will distance the functional domains “turning off” the enzyme or “switching” from the dehydrogenase to hydrolase activity.

Acknowledgments—We thank Dr. E. Starr Hazard for help with the intermediate domain modeling, Dr. Erika Bullesbach for kind permission to use a CD spectrophotometer, and Dr. Zigmund Luka for the helpful discussion of CD spectra.

REFERENCES
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doi: 10.1074/jbc.M302948200 originally published online April 8, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M302948200

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