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CATALYTIC PROPERTIES AND STABILITY OF A *PSEUDOMONAS SP.*101 FORMATE DEHYDROGENASE MUTANTS CONTAINING CYS-255-SER AND CYS-255-MET REPLACEMENTS

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SUMMARY: Two mutants of bacterial formate dehydrogenase from *Pseudomonas sp.*101 (EC 1.2.1.2, FDH) - C255S (FDH-S) and C255M (FDH-M), were obtained and its properties were studied. Both mutations provided the high resistance to inactivation by Hg^{2+}. Slow inactivation of mutants by DTNB reveals the presence in FDH molecule of another essential cysteine residue. Specific activities of FDH, FDH-S and FDH-M were 16, 16 and 9.5 U/mg of protein, respectively. K_m on formate was 7.5, 7.5 and 20 mM and K_m on NAD^+ - 0.1, 0.3 and 0.6 mM for FDH, FDH-S and FDH-M, respectively. Mutations of Cys255 on Ser or Met resulted in increasing of enzyme stability at 25 °C and decreasing of thermostability (above 45 °C). Data obtained show that Cys255 is unique residue for providing both enzyme thermostability and catalytically optimal binding of coenzyme.

NAD^+-dependent formate dehydrogenases (EC 1.2.1.2., FDH) are widely occurred in methanol-utilizing yeast and bacteria. All FDHs purified [1-8] have cysteine residue essential for enzyme activity. In the case of NAD^+-dependent formate dehydrogenase from bacterium *Pseudomonas sp.*101 (former names *Bacterium sp.*1 and *Achromobacter parvulus* T1) it was shown that essential cysteine residue is Cys255 [9]. According to X-ray analysis [10], this residue is placed in coenzyme-binding domain and takes part in binding of adenosine moiety of NAD^+. But exact information about role of Cys255 in FDH functioning is not available now. It can be obtained by the method of site-directed mutagenesis.

In this work we present our results on study of influence of mutations C255S and C255M on catalytic properties and stability of FDH. Ser is structural analog of Cys and change of Cys255 on Ser will give information about role of sulfur atom in Cys in FDH stability and coenzyme binding. Insert of Met with more long side chain in compare with Cys can provide information about structural requirements for binding of adenosine moiety of NAD(H).

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MATERIALS AND METHODS

To introduce mutation C255S, a 30-base oligonucleotide was synthesized (5'-CGTGGT-GACGCTGAATTCCCCGCTGCACCC-3') with two mismatches (bold letters). One mismatch provided change of Cys255 on Ser (TGC->TCC). The second mismatch was insignificant (AAC and AAT are codons of Asn). It was made to introduce EcoRI site (underlined in oligonucleotide sequence) to facilitate mutant clones selection. Mutation C255M was done with 30-mer oligonucleotide (5'-CGTGGTGACGCTGAAC**ATG**CCGCTGCACCC-3') and mutant C255S DNA as a template (remove of EcoRI site). Mutation reactions were carried out using DNA mutagenesis kit ver. 2.1 (Amersham International, plc.). To produce single strand DNA, 1.3 kb AsuII DNA fragment from plasmid pFDH4 [11] with the part of FDH gene was cloned in M13tg130 phage. Screening procedure was performed by restriction analysis and DNA sequencing on Applied Biosystems Automated DNA Sequencer model 370A. 658 b XhoI-Bsp119 fragments with mutations were cloned into pFDH2 or pFDH4 plasmids digested by XhoI and Bsp119 restrictases (plasmids pFDH2M1 and pFDH4M1, and pFDH2M3 and pFDH4M3 for mutations C255S and C255M, respectively). In plasmids pFDH2 and pFDH4 the FDH gene is under control of *lac*- and tandem of *lac*- and *taq*- promoters, respectively [12]. Plasmids with mutants were used for transformation *E.coli* AB2463 cell line. Cells were grown overnight at 37 °C in LB medium containing ampicillin, streptomycin and rymphopicin at concentration 50 μg/ml.

Purification procedure for native recombinant and mutant FDHs included cell disruption by sonication on ice, ammonium sulfate fractionation, hydrophobic chromatography on Phenyl Sepharose Fast Flow (Pharmacia) and gel-filtration through Ultrogel AcA44 (LKB) [12]. Expression of full length FDH protein was controlled by Western-blot analysis as described earlier [13]. FDH concentration was determined by dot-blot analysis on nitrocellulose membrane (0.22 μm) (Bio-Rad) using rabbit polyclonal or mouse monoclonal anti-FDH antibodies and native FDH as a standard [13]. Concentration of active sites of native and mutant FDHs was determined by titration with potassium azide in the presence of 1.5 mM NAD^+ as described previously [14].

FDH activity was measured using automated Reaction Rate Analyzer, model 2086 Mark II (LKB), at 340 nm in 0.1 M phosphate buffer, pH 7.0, at 37 °C as described in [7]. Kinetic constants were obtained from duplicate or triplicate measurements of initial rates under conditions of varying of one substrate and saturating concentration of the second substrate after analysis using method of non-linear regression [15].

Chemical modification of native and mutant FDHs by DTNB was carried out in 0.1 M phosphate buffer, pH 7.9, at 25 °C. Enzyme and DTNB concentrations were 5 and 60 μM, respectively. At certain time intervals 25 μl aliquots were taken and enzyme activity was measured as described above. Incubation of FDH (5 μM) with Hg^{2+} was made in 0.1 M phosphate buffer, pH 7.0, at 25 °C with varying $Hg(CH_3COO)_2$ concentrations.

Thermal inactivation experiments were carried out by incubating of 1 ml solution of either native or mutant enzyme (0.5 mg/ml) in 0.1 M phosphate, pH 7.0, over temperature range 45 - 65 °C. At certain time intervals, 25 μl aliquots were removed and residual activity was determined.

RESULTS AND DISCUSSION

Expression and purification of mutant enzymes. In cells *Pseudomonas sp.*101 FDH is subjected to posttranslation modification and total preparation of wild-type FDH presents five isoforms with pI 4.6-5.2 [7]. As has been reported earlier [12], all isoforms have the same K_m on NAD^+, and K_m on formate increases with decreasing of pI. Recombinant FDH expressed in *E.coli* is the enzyme with 400 amino acid subunits and product of translation of full length gene [11,12]. Its properties coincide with properties of most basic isoform of wild-type FDH (pI 5.2). So, to make the correct comparison of properties of wild-type and mutant enzymes, native recombinant FDH was used as a standard.

After cloning of inserts with mutations into expressing vectors pFDH2 or pFDH4, the entire FDH coding region was determined by double-stranded dideoxy sequence analysis and correct substitutions of C255S (FDH-S) and C255M (FDH-M) were confirmed and no other

Table I. Kinetic properties of wild-type and mutant formate dehydrogenases

Enzyme	k_{cat} (s^{-1})	K_m on NAD^+ (mM)	K_m on formate (mM)
native FDH	10	0.11 ± 0.02	7.5 ± 1.0
C255S FDH	10	0.30 ± 0.04	7.5 ± 1.4
C255M FDH	6	0.65 ± 0.09	20 ± 3

alterations were detected. According to Western-blot analysis level of mutants expression in *E.coli* AB2463 was the same as for native FDH. Mutant enzymes were purified to apparent homogeneity as judged by SDS-polyacrylamide gel electrophoresis. There were no differences in behavior of native and mutant FDH during purification procedure. Cys255 is not a part of FDH antigenic determinants [16], and polyclonal and monoclonal anti-FDH antibodies can be used for quantitation of mutant proteins.

Kinetics properties. Enzyme reaction catalyzed by FDH from *Pseudomonas sp.*101 proceeds through equilibrium random Bi-Bi mechanism [15] and changes in values of k_{cat} and K_m on substrates will reflect the changes in the velocity of the elemental step of hydride transfer in ternary complex (FDH-NAD^+-formate) and in binding of formate and NAD^+ with corresponding binary complexes (FDH-NAD^+) and (FDH-formate). Table I shows the kinetic properties of purified mutant FDHs. Specific activity of mutants determined by dot-blot analysis and by titration of active sites by azide was 100 and 60% from activity of native FDH for FDH-S and FDH-M, respectively. FDH-S has the same affinity to formate as FDH but K_m on NAD^+ is 3-fold higher. FDH-M has more lower kinetic characteristics. In the case of FDH-M substitution C255M resulted in decreasing both specific activity and affinity to substrates. These data show that Cys255 does not influence on catalytic step and formate binding. Cys255 is placed rather far from catalytic and formate binding domain of active site [10], but this residue plays important role in coenzyme binding. 3-fold increasing of FDH-S K_m on NAD^+ can not be a result of structural changes because Ser and Cys are structural analogs. Structure of adenosine-binding part of coenzyme-binding domain of FDH is very important for providing catalytically optimal conformation of whole protein globular. Distortion of this part of NAD^+-binding domain by inserting of Met with more long side chain decreases both enzyme activity and affinity to NAD^+ and formate.

Resistance to inactivation by Hg^{2+} and DTNB. Change of Cys255 on Ser or Met resulted in very high resistance of both mutants, FDH-S and FDH-M, to inactivation by Hg^{2+} (fig. 1). At equimolar concentration of enzyme active sites and Hg^{2+} native FDH loses full activity after 20 min incubation. At the same conditions activity of FDH-S and FDH-M was not changed after 32 h incubation. Partial loss of enzyme activity (20% from initial value) was observed after 24 h incubation in the presence 50-fold excess of Hg^{2+} (data not shown).

At the presence of 12-fold of modifying agent wild-type enzyme was inactivated after 1 min. Speed of inactivation of mutant enzymes by DTNB was about 60-times slower in compare with native FDH (fig. 2). Dependence of residual enzyme activity on time is linearized in coordinates *ln*(v) - t, i.e. kinetics of the loss of enzyme activity corresponds to the kinetics of first order reaction. NAD^+ and formate at saturating concentrations only partially protect

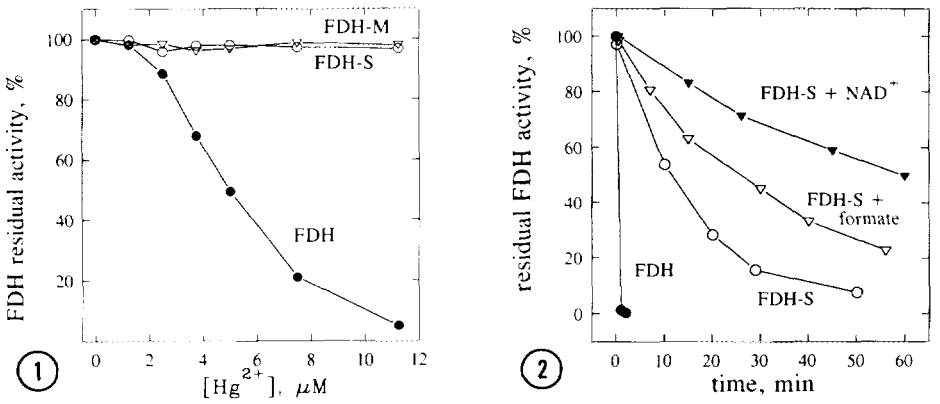

Fig. 1. Dependence of activities of FDH (filled circles) FDH-S (open circles) and FDH-M (triangles) on Hg^{2+} concentration. 0.1 M phosphate buffer, pH 7.0, 25 °C, enzyme concentration 10 μM. Enzyme activity was measured after 30 min incubation.

Fig. 2. Inactivation by DTNB of native FDH (filled circles), free FDH-S (open circles) and FDH-S in the presence of 0.3 M formate or 5 mM NAD^+ (open and closed triangles, respectively). 0.1 M phosphate buffer, pH 7.9, 25 °C. Enzyme and DTNB concentrations were 5 and 60 μM, respectively.

FDH-S against inactivation (fig. 2). The same results were obtained for FDH-M too (data not shown).

Results on chemical modification of mutant enzymes suppose the presence of the second cysteine residue which is essential for enzyme activity. This residue does not take part in binding of NAD^+ or formate because substrates do not provide protection of enzyme against inactivation. Changes in the speed of inactivation in the presence of substrates may be the result of big conformational changes occurred on production of binary (enzyme-substrate) complexes [10]. Analysis of positions of cysteine residues in FDH globular shows that the most probable residue is Cys5. This residue is placed in ß-sheet which provides shielding of active site from solution [10].

Stability of mutant enzymes. Mutation C255S produces enzyme with higher stability at room temperature in compare with native FDH. At the absence of stabilizing agents wild-type enzyme inactivated after 72 h due to oxidation of essential cysteine residue [7]. In the case of mutant enzymes this mechanism of inactivation does not work. Loss of FDH-S activity was 50% after 30 days (720 h). FDH-M was 5-times less stable as FDH-S.

Mutations of Cys255 influence on enzyme stability in urea (fig. 3). Native FDH preserved more than 80% of initial activity after 2 h incubation in 7 M urea. FDH-M and FDH-S were completely inactive under this conditions.

Both mutant enzymes are less thermostable as native FDH. Constants of thermoinactivation (k_{in}) for FDH-S and FDH-M were 3- and 7.5-fold higher, respectively, in compare with wild-type FDH. NAD^+ and formate do not stabilize native and mutant enzymes at high temperature. According to the theory of activating complex the dependence of k_{in} on T is described by

$$k_{in} = kT/h \cdot \exp[-(\Delta H^{\#} - R\Delta S^{\#})/(RT)],$$

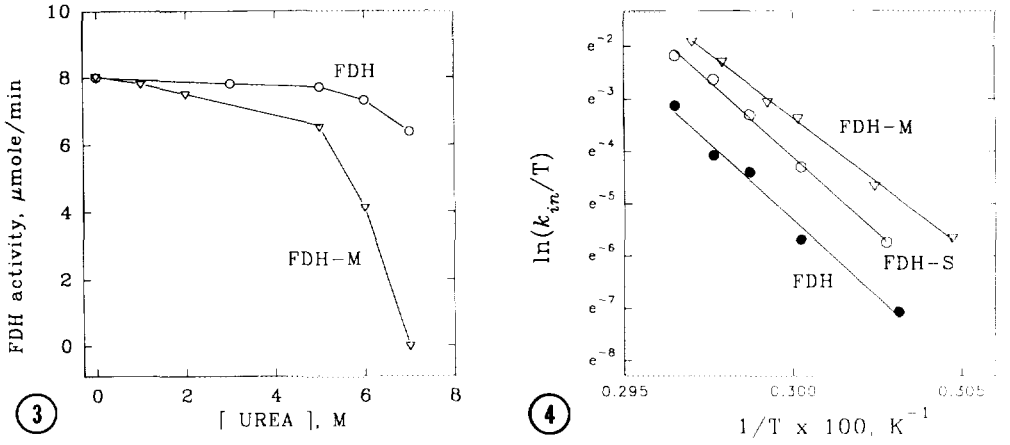

Fig. 3. Influence of urea concentration on activity of native FDH and FDH-M. Enzymes were incubated during 2 h at various urea concentrations in 0.1 M phosphate buffer, pH 7.0, at 25 °C. After 4 and 8 h of incubation practically the same values of activity were observed.

Fig. 4. Dependence of k_{in} of thermodenaturation process versus reciprocal absolute temperature for wild-type and mutant FDHs.

where T is absolute temperature in K, k and h - constants of Bolzmann and Planck, respectively, R - universal thermodynamic constant, $\Delta H^{\#}$ and $\Delta S^{\#}$ - activating parameters of changes of enthalpy and entropy for the process of enzyme thermoinactivation. This dependence can be linearized in coordinates [$\ln(k_{in}/T)$] - 1/T (fig. 4). As follows from fig. 4, increase of speed of

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PSEU_FDH  - AKVLCVLYDDPVDGYPKTYARDDLPKIDHYPGGQTLPTPKAIDFTPGQLLGSVSG -55
HANS_FDH  -  KVVLVLYDAGK------HAQDEE----------------------RLYGCTEN -25

PSEU_FDH  - ELGLRKYLESNGHTLVVTSDKDGPDSVFERELVDADVVISQPFWPAYLTPERIAK -110
HANS_FDH  - ALGIRDWLEKQGHDVVVTSDKEGQNSVLEKNISDADVIISTPFHPAYITKERIDK -80

PSEU_FDH  - AKNLKLALTAGIGSDHVDLQ--SAIDRNVTVAEVTYCNSISVAEHVVMMILSLVR -163
HANS_FDH  - AKKLKLLVVAGVGSDHIDLDYINQSGREISVLEVTGSNVVSVAEHVVMTMLVLVR -135

PSEU_FDH  - NYLPSHEWARKGGWNIADCVSHAYDLEAMHVGTVAAGRIGLAVLRRLAPFDVH-L -217
HANS_FDH  - NFVPAHEQIISGGWNVAEIAKDSFDIEGKVIATIGAGRIGYRVLERLVAFNPKEL -190

PSEU_FDH  - HYTDRHRLP-ESVEKELNLTWHATREDMYPVCDVVTLNCPLHPETEHMINDETLK -271
HANS_FDH  - LYYDYQSLSKEAEEKVGARRVHDIKEL-VAQADIVTINCPLHAGSKGLVNAELLK -244

PSEU_FDH  - LFKRGAYIVNTARGKLCDRDAVARALESGRLAGYAGDVWFPQPAPKDHPWRTMPY -326
HANS_FDH  - HFKKGAWLVNTARGAICVAEDVAAAVKSGQLRGYGGDVWFPQPAPKDHPWRSMAN -299

PSEU_FDH  - -----NGMTPHISGTTLTAQARYAAGTREILECFFEGRP-IRDEYLIVQGGALAG -375
HANS_FDH  - KYGAGNAMTPHYSGSVIDAQVRYAQGTKNILESFFTQKFDYRPQDIILLNGKYKT -354

PSEU_FDH  - TGAHSYSKGNATGGSEEAAKFKKAV -400
HANS_FDH  - -------KSYGADK            -361
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Fig. 5. Alignment of amino acid sequences of formate dehydrogenases from bacterium *Pseudomonas sp.*101 (PSEU_FDH) [11] and yeast *Hansenula polymorpha* (HANS_FDH) [17]. The character to show that two aligned residues are identical is '|'. The character to show that two aligned residues are similar is '.' Amino acids said to be 'similar' are: A,S,T; D,E; N,Q; R,K; I,L,M,V; F,Y,W

FDH-S thermoinactivation is due to only increase of $\Delta S^{\#}$ component. In the case of FDH-M, both parameters, $\Delta H^{\#}$ and $\Delta S^{\#}$, are changed.

CONCLUSION

Data obtained show that the position of Cys255 in FDH globular is unique for providing of effective catalysis of enzyme reaction and high thermostability. This unique position is confirmed by homology comparison with another FDH from methanol-utilizing yeast *Hansenula polymorpha* (fig. 5). Bacterial and yeast enzymes have seven and three cysteine residues per subunit, respectively. These enzymes present rather high (48.9%) level of homology. As follows from fig. 5 only in two cases we can see coincidence in positions of Cys. One pair, Cys255 - Cys228 is placed inside the region with very high homology (80% identical plus 20% of similar residues). This region is a part of coenzyme-binding domain. In the case of the second pair, Cys288 - Cys261, only left part of this region presents high level of homology. According to data of X-ray analysis Cys288 in bacterial FDH is far from the active site of enzyme and can not participate in enzyme catalysis or binding of substrates [10].

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