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Effect of surface electrostatic interactions on the stability and folding of formate dehydrogenase from *Candida methylica*

Emel B. Ordu[a,b,c], Richard B. Sessions[d], Anthony R. Clarke[d], Nevin Gül Karagüler[a,b,*]

[a] Istanbul Technical University, Faculty of Science and Letters, Department of Molecular Biology and Genetics, Istanbul, Turkey
[b] Istanbul Technical University, Molecular Biology-Biotechnology & Genetics Research Center, 34469 Istanbul, Turkey
[c] Yıldız Technical University, Faculty of Science and Letters, Department of Molecular Biology and Genetics, Istanbul, Turkey
[d] University of Bristol, Department of Biochemistry, Bristol BS8 1TD, Avon, United Kingdom

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ABSTRACT

NAD$^+$-dependent formate dehydrogenase (FDH-EC 1.2.1.2) is an important enzyme to regenerate valuable NADH required by NAD$^+$-dependent oxidoreductases in enzyme catalysis. The limitation in the thermostability of FDH enzyme is a crucial problem for development of biotechnological and industrial processes, despite of its advantages. In this study, to investigate the contribution of surface electrostatic interaction to the thermostability of FDH from *Candida methylica* (*cm*FDH) N187E, H13E, Q105R, N300E, N147R N300E/N147R, N187E/Q105R, N187E/N147R,Y160R, Y302R, Y160E and Y302E mutants were designed using a homology model of *cm*FDH based on *Candida boidinii (cb)* by considering electrostatic interactions on the protein surface. The effects of site-specific engineering on the stability of this molecule was analyzed according to minimal model of folding and assembly reaction and deduced equilibrium properties of the native system with respect to its thermal and denaturant sensitivities. It was observed that mutations did not change the unfolding pattern of native *cm*FDH and increased numbers of electrostatic interactions can cause either stabilizing or destabilizing effect on the thermostability of this protein. The thermodynamic and kinetic results suggested that except relatively improved mutants, three out of the nine single mutations increased the melting temperature of *cm*FDH enzyme.

1. Introduction

Elucidation of the temperature resistance of proteins is essential for stability and protein folding studies. Thus, the novel enzymes can be redesigned to work at high temperatures for industrial and biotechnological applications [1]. The lack of thermostability is one of the limiting factor of proteins for development of biotechnological and industrial processes. Although there are several successful examples for stabilization of proteins, there is no general method to improve the protein stability, as the mechanism of thermostabilization is not yet clearly understood [2]. When the amino acid sequences of homologous proteins from thermophilic and mesophilic organisms are compared it can be shown that thermostable proteins have more salt bridges and surface charge than their mesophilic counterparts. The results found in the literature emphasize that studies to increase protein thermostability are mainly concentrated on optimizing the electrostatic interactions on the protein surface among all other strategies [3–6]. Compared with positions buried in the core, stabilizing surface mutations are less likely to disrupt the tertiary structure, which may be considered as the evidence of evolutionary selection [7,8]. Electrostatic interactions also affect the protein flexibility that is significant for movement of residues with respect to each other and their environment. The changes in electrostatic interaction of charged side chains of residues are critical for protein folding and stability thus biological activity of the protein. Protein folding is a hierarchical system in which protein reach firstly to molten globule state by hydrophobic collapsing and then specific electrostatic interactions are optimized to achieve the native folding. As a result of folding, interactions between charged and polar groups and their environment are formed or broken during the assembly processes [9,10]. Since folding is related to many other biological processes, the failure of protein folding and formation of aggregates in the cell is possibly the reason of many neurodegenerative diseases [11,12].

The native formate dehydrogenase (FDH) which has relatively high activity is known as the best candidate for the NADH regeneration system. Although there are many advantages of using FDH for NADH regeneration, thermostability problem is a fact and needs to be addressed. Several strategies based on using bacteria and yeast [13,14] have been applied to increase the thermostability of FDH.

* Corresponding author at: Istanbul Technical University, Faculty of Science and Letters, Department of Molecular Biology and Genetics, Istanbul, Turkey.
Tel.: +90 212 2857264; fax: +90 212 2856386.
E-mail address: karaguler@itu.edu.tr (N.G. Karagüler).

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However a common strategy to enhance thermostability is to have more favorable surface electrostatic interactions, effect of surface electrostatic interactions on the FDH from a yeast source has not been investigated yet.

Therefore, we attempt to investigate the effect of surface electrostatic interactions on the thermostability and folding characteristics of *Candida methylica* FDH by using site directed mutagenesis. Investigation of FDH folding and stability would also be considered as an illuminative model in terms of diseases mentioned above beside industrial benefits of this enzyme [15].

2. Material and methods

2.1. Computer modelling

A new homology model of *cm*FDH was made using a holo dimer of *cb*FDH [16] taken from the crystal structure 2J6I (subunits A and D). The sequences of *cm*FDH and *cb*FDH are 96% identical and align with no gaps; hence the model was constructed by making the 13 residue changes in each subunit of the template. Hydrogen atoms were added to the model consistent with pH 7 followed by a 10 Å layer of water molecules. This system was refined by energy minimization while tethering the backbone atoms to their initial positions. The tethering force was reduced in subsequent rounds of minimization and removed for the final 2000 conjugate gradient steps. Molecules were manipulated with Insight II (2005) and energy calculations performed with Discover 2.98 (Accelrys) using the CVFF.

2.2. The bacterial strains

Two different bacterial strains were used in this work namely, *JM*105 {F' traD36 proA$^+$ proB$^+$ laclq lacZΔM15/Δ (lac-pro) X111 thi rpsL (Strr) endA sbcB supE hsdR9} as a host cell and DH5α^{TM}-T1^R competent cells ([F$^-$80*lac*ZM15 (*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A]) supplied with the Invitrogen GeneTailor^{TM} Site-Directed Mutagenesis System.

2.3. Site directed mutagenesis

The Invitrogen Gene Tailor^{TM} Site-Directed Mutagenesis System was used to obtain mutant enzymes. The PCR reaction (at 94 °C for 2 min initial denaturation, 94 °C for 30 s, 55 °C for 30 s, 68 °C for 5 min 30 s, 30 cycles, and at 68 °C for 10 min final extension) was performed in the presence of 0.3 mM of each dNTP, 0.3 pmol of each primer and 0.02 unit of Platinum® *Taq* High Fidelity polymerase to give the altered *cm*FDH gene. The mutagenesis mixture was transformed into DH5α-T1 cells (supplied with the kit). The host cells circularizes the linear mutated DNA, and *Mcr*BC endonuclease in the host cell digests the methylated template DNA, leaving only unmethylated, mutated product. The double-stranded DNA extracted from mutated *cm*FDH gene was sequenced in the region of the mutation to check for the correct base change. Purified DNA samples were sequenced using an ABI Prism 3100-Avant automated sequencer at the Molecular Biology and Genetics Dept, ITU. The pQE-2 expression vector containing His-tagged mutant FDH gene was transformed into *JM*105 cells for over-expression of mutant FDH proteins.

2.4. Protein purification

Native and mutant *cm*FDH proteins from *Escherichia coli* over-expressing clones were made and purified as described previously [17] giving recombinant enzymes at >95% purity, as judged by Coomassie Brilliant Blue staining following SDS-PAGE. Protein concentrations were estimated at 280 nm from the extinction coefficient, 49,170 $cm^{-1}\,M^{-1}$, and expressed in terms of the monomeric protein concentration using monomer mass of 42,000 Da.

2.5. Steady state kinetics

The steady-state kinetic experiments of recombinant native and mutant *cm*FDHs were carried out by using Shimadzu 1700 double beam (10 mm path length) UV–vis spectrophotometer at 25 °C. The reaction mixture contained 20 mM Tris buffer at pH 8, 1 mM NAD^+, 0–40 mM formate and 0.4 μM enzyme (Mr 42,000). The initial rates at each formate concentration were determined at 340 nm by measuring the NADH production. Data were fitted to the Michaelis–Menten equation using Grafit 5.

2.6. Thermal denaturation

The enzymes were incubated at different temperatures between 30 °C and 70 °C by 2 °C intervals for 20 min as triplicate. Remaining activity measurements were performed at 25 °C in a reaction mixture containing 20 mM Tris Buffer at pH 8, 1 mM NAD^+, 2 mM formate and 0.4 μM enzyme.

2.7. Thermodynamics of the folding-unfolding transition

The susceptibility of the native and catalytically active mutant proteins to denaturation by guanidinium chloride (GdnHCl) and urea was monitored by using fluorescence spectroscopy. All experiments were performed in unfolding buffer including 20 mM Tris and 5 mM DTT, pH 8. Protein samples (0.1 μM) were incubated in unfolding buffer at a series of GdnHCl or urea concentrations at different temperatures (25, 30, 35, 40, and 45 °C) for 4 h. Urea stock solutions were freshly prepared before each experiment. The protein fluorescence intensity of each sample was measured between 300–400 nm using an excitation wavelength of 285 nm in a Spex Fluoromax spectrofluorometer. Maximum emission was observed at 350 nm. Fluorescence values obtained from each denaturant at each temperature were analyzed as previously described [18].

3. Results and discussion

3.1. Homology modelling

We used a homology model of *cm*FDH based on the crystal structure of *ps*FDH (2NAD) to design residue changes for our previous mutagenesis studies of *cm*FDH [19,20]. In this paper we describe a homology model based on the recent crystal structure of *cb*FDH (2J6I) [16]. The sequence identities between *cm*FDH and the template structures are 44% (2NAD) and 96% (2J6I) respectively. At these levels of sequence identity we would expect the model based on *ps*FDH to be good quality while that based on *cb*FDH should be essentially the same as an experimentally determined structure of *cm*FDH. Gratifyingly, we find that the two models are closely similar with the following Root Mean Squared Deviations of the Cα postions: complete dimer, 2.7 Å; NAD-binding domain, 1.7 Å; catalytic domain, 1.8 Å. Likewise, this result validates the designs and interpretations presented in our previous mutagenesis work.

3.2. Mutant design

Many studies have been undertaken to investigate the structural basis of thermostability in proteins [21]. While structural factors like increased compactness, extra disulphide bonds, extra salt bridges, shorter surface loops and increased helix content have all been correlated with increased stability in specific cases, it is only the slight increase of surface charged residues and slight changes in amino acid composition (increased Arg Tyr, decreased Cys Ser) that show a general correlation with increased thermostability [22]. However, it has already been observed that the effect of