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Study of zinc protein ligands in a halophilic enzyme

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ABSTRACT

Glucose dehydrogenase (EC 1.1.1.47) from the halophilic Archaeon *Haloferax mediterranei* belongs to the medium-chain alcohol dehydrogenase superfamily and requires a zinc ion for catalysis. The zinc ion is coordinated by a histidine, a water molecule and two other ligands from the protein or the substrate, which vary during the catalytic cycle of the enzyme. In many enzymes of this superfamily one of the zinc ligands is commonly cysteine, which is replaced by an aspartate residue at position 38 in the halophilic enzyme. This change has been only observed in glucose dehydrogenases from extremely halophilic microorganisms belonging to the Archaea Domain. This paper describes biochemical studies and structural comparisons to analyze the role of sequence differences between thermophilic and halophilic glucose dehydrogenases which contain a zinc ion within the protein surrounded by three ligands. Whilst the catalytic activity of the D38C GlcDH mutant is reduced, its thermal stability is enhanced, consistent with the greater structural similarity between this mutant and the homologous thermophilic enzyme from *Thermoplasma acidophilum*.

KEYWORDS: Archaea, catalytic zinc, glucose dehydrogenase, structural analysis

INTRODUCTION

Zinc is an essential element for life and is involved in 10% of the chemical reactions catalyzed by enzymes. Most of these enzymes use zinc as a catalytic cofactor, and are zinc-dependent enzymes in the strictest sense. Zinc enzymes have been found in all six classes of enzymes defined in the EC system, hydrolases being the superior group followed by transferases and oxidoreductases. In these metaloenzymes, the zinc ion acts mainly as a catalytic ion and/or structural component. The role of the zinc ion in these enzymes has been the subject of extensive biochemical and structural studies. Protein zinc ligands in catalytic zinc sites present different frequencies depending on the different enzyme classes. Specifically, the occurrence of histidines ranges from 30% in transferases to 73% in lyases, cysteine represents 31% of protein ligands in oxidoreductases, although it is not present in isomerases, and the sum of aspartate and glutamate is 60% in isomerases while it constitutes only 22% in oxidoreductases [1, 2].

Glucose dehydrogenase (GlcDH) from the extremely halophilic Archaeon *Haloferax mediterranei* is an oxidoreductase enzyme that belongs to the zinc-dependent medium-chain alcohol dehydrogenase (MDR) superfamily [3]. This superfamily includes sorbitol dehydrogenases, xylitol dehydrogenases and alcohol dehydrogenases. Biochemical [4] and structural analysis of the halophilic enzyme [5, 6, 7] reveals the presence of one catalytic zinc ion per subunit. Of the three protein ligands to the zinc in enzymes of the MDR superfamily, one of the cysteine residues (Cys46 in horse liver alcohol dehydrogenase, HLADH) and the histidine (His67 in HLADH) are very strongly conserved in the sequence. In some family members, the second

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cysteine (Cys174 in HLADH) is replaced by a glutamate, as in *Thermoplasma acidophilum* GlcDH (Glu155) [8] and in rat sorbitol dehydrogenase (Glu174) [9]. Structural studies on different substrate complexes of *Hfx. mediterranei* GlcDH show that during the enzyme's catalytic cycle, a water molecule and a histidine residue (His63) remain coordinated to the zinc throughout. However, whilst the tetrahedral coordination of the zinc is preserved during catalysis, the zinc ion and the water molecule both move by up to 1.5 Å, and the remaining two ligands are drawn from a combination of Asp38, Glu64, Glu150 and the substrate, therefore changing the process of the catalysis [7]. This type of motion has not previously been observed for any enzyme in the MDR family. In addition, the replacement of a conserved cysteine by a carboxyl, as seen in all GlcDHs from haloarchaea available to date, for example, *Halobacterium* sp1 GlcDH [10], *Haloferax volcanii* GlcDH [11], *Haloquadratum walsbyi* GlcDH [12] or *Halogeometricum borinquense* GlcDH [13], is clearly uncommon. The change of the Cys to Asp at position 38 in the halophilic enzyme could be an adaptive response to the halophilic environment, as such enzymes have an increase of acidic residues.

In order to investigate the mode of zinc binding to halophilic GlcDH, the combination of the site-directed mutagenesis, overexpression technologies, biochemical analysis and structural studies were used. This report describes the construction of two site-directed mutants of *Hfx. mediterranei* GlcDH in order to study both the zinc binding and structural analysis.

MATERIALS AND METHODS

Strains, culture conditions and vectors

Escherichia coli NovaBlue (Novagen) was used as host for plasmids pGEM-11Zf(+) and pET3a. *E. coli* BMH71-18 [*mutS*] (Promega) was employed in site-directed mutagenesis experiments. *E. coli* BL21(DE3) (Novagen) was used as the expression host. *E. coli* strains were grown in Luria-Bertani medium at 37 ºC with shaking at 180 rpm. Plasmids were selected in solid and liquid media by the addition of 100 µg ampicillin/ml.

Vector pGEM-11Zf(+) (Promega) was used for cloning genes and carrying out site-directed mutagenesis experiments. The expression vector pET3a was purchased from Novagen.

Site-directed mutagenesis

Site-directed mutations were introduced into genes cloned in pGEM-11Zf(+). The synthetic oligonucleotide primers (Applied Biosystems and Bonsai Technology) were designed to replace Asp38 with Cys or Ala (Table 1). Site-directed mutagenesis was performed using the GeneEditorTM *in vitro* Site-Directed Mutagenesis System (Promega).

This method works by simultaneous annealing of two oligonucleotide primers to one strand of a denatured plasmid; one primer introduces the desired mutation in the gene, and the other primer mutates the beta-lactamase gene, increasing the resistance to alternate antibiotics.

The protocols supplied with the kit were followed but with one modification; the length of the DNA denaturation stage was increased from 5 min at room temperature to 20 min at 37 ºC as previously described [14].

Putative mutants were screened by dideoxy-nucleotide sequencing with ABI3100 DNA sequencer (Applied Biosystems).

Protein preparation

Expression, renaturation and purification of recombinant mutants were carried out as previously described for wild-type halophilic GlcDH [3].

Table 1. Synthetic primer used for site-directed mutagenesis.

Name	Sequence
GlcDH D38A	5' CGG CGT C**GC A**GG GAC C 3'
GlcDH D38C	5' CGG CGT C**TG C**GG GAC C 3'

The mutant codons are shown in bold-type.

Enzyme and protein assays

The mutant GlcDH activity was assayed spectrophotometrically as previously described [15]. The kinetic constants were obtained from at least triplicate measurements of the initial rates at varying concentrations of D-glucose and $NADP^+$. Kinetic data were fitted to the sequential ordered BiBi equation with the program SigmaPlot 9.0. Protein concentration was determined by Bradford method [16] using bovine serum albumin as standard. SDS-polyacrylamide gel electrophoresis was performed according to the method described by Laemmli [17] using a 12% gel.

Effect of EDTA concentration

The samples at different NaCl concentration were incubated with increasing EDTA concentration for 5 min at room temperature. After the incubation, the residual activities of the enzymes were measured in the activity buffer defined previously [15].

Effect of the temperature on enzymatic stability and activity

The samples at different NaCl concentrations were incubated at various temperatures: 55, 60, 65, 70 and 80 ºC. Aliquots were analysed at set times to measure the residual activity. Furthermore, enzymatic activity was assayed between 25 and 75 ºC at the same conditions described previously.

Structure comparisons

The structures of wild-type and mutant *Hfx. mediterranei* GlcDHs were extracted from the protein data bank as considered appropriate. The structures used were D38C/NADP/Zn, 2B5W; WT/NADP/Zn, 2VWQ; WT/NADP/Zn/Glucose, 2VWH; WT/NADP/Zn/lactone, 2VWG; and WT/NADPH/Zn, 2VWP [5, 6, 7, 8, 18].

RESULTS AND DISCUSSION

Site-directed mutagenesis and expression of mutant proteins

Site-directed mutagenesis was carried out to replace the Asp38 residue by cysteine and alanine in the recombinant glucose dehydrogenase using GeneEditorTM invitro Site-Directed Mutagenesis System. The GlcDH gene was excised from pET3a expression vector [3] using *BamHI* and *NdeI*, and cloned into the pGEM-11Zf(+) mutagenesis vector. The mutagenic oligonucleotides described in the 'Materials and Methods' were used, confirming all mutations by DNA sequencing.

The mutant gene were excised from pGEM-11Zf(+) using *BamHI* and *NdeI* and ligated into similarly cut pET3a expression vector. The resulting constructs were transformed into *E. coli* JM109 to provide enough DNA for transformation into *E. coli* BL21(DE3). After expression, both mutant proteins were obtained as inclusion bodies, like wild-type GlcDH.

The mutant enzymes were refolded and purified as described previously (Figure 1). In both mutants, the activity was lower than in the wild-type protein, with the D38A mutant being inactive. This data suggests that Asp38 is an important residue and that the mutation to Ala leaves the enzyme seriously compromised. With respect to the D38C mutant, the maximum activity observed following the refolding process was approximately 30% of the activity with the wild-type enzyme.

Both proteins were purified and concentrated using the method described previously [3].

Characterization of GlcDH D38C

The kinetic parameter values for mutant D38C GlcDH were determined and compared with those obtained for wild-type GlcDH (Table 2). K_{mNADP} differences are not significant, however, the mutation led to a significant increase of the K_m for glucose. Moreover, as the K_{cat} and $K_{cat}/K_{mglucose}$ parameters show, the catalytic efficiency of mutant protein is less than the catalytic efficiency of wild-type GlcDH. These results indicate that the replacement of Asp38 with Cys in the GlcDH probably affect not only catalytic zinc binding site but also the active site of the protein, decreasing its affinity for glucose and its V_{max}, and consequently its catalytic efficiency.

The zinc ion in the wild-type enzyme can be removed by EDTA treatment yielding an inactive enzyme [4]. In order to compare the strength of zinc binding in wild-type and in the D38C mutant, a similar treatment was carried out. Figure 2 shows that zinc is more weakly bound in the D38C mutant since the EDTA concentration needed to inactivate the enzyme is lower than in the

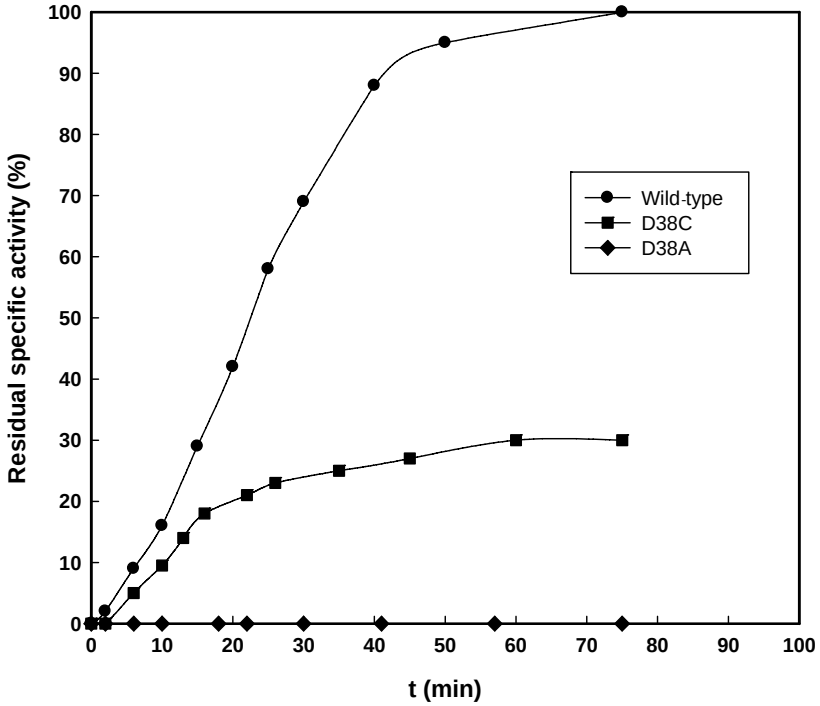

Figure 1. Refolding the D38C and D38A GlcDH mutants. The refolding buffer was 20 mM Tris-HCl buffer pH 7.4, with 1 mM EDTA and 2 M NaCl. (●) Wild-type, (■) D38C, (◆) D38A.

Table 2. Kinetic parameters of recombinant wild-type GlcDH and the D38C mutant.

	K_{mNADP^+} (mM)	$K_{mglucose}$ (mM)	V_{max} (U/mg)	K_{cat} (min^{-1})	K_{cat}/K_m ($mM^{-1}min^{-1}$)
Wild-type	0.035 ± 0.004	2.8 ± 0.3	397 ± 15	31 ± 1	11.10 ± 1.60
D38C	0.044 ± 0.010	12.4 ± 2.3	83 ± 9	7 ± 1	0.52 ± 0.14

wild-type enzyme, and this inactivation was independent of salt concentration. For wild-type enzyme the capacity of EDTA to sequester the zinc is lower than for the D38C mutant and it is salt concentration dependent. In the three NaCl concentrations tested, the enzyme lost approximately 80% of its activity in the presence of 0.25 mM EDTA, and it was completely inactive at concentrations higher than 2 mM. However, in the case of the wild-type GlcDH, the EDTA necessary to sequester zinc atom at 3 M NaCl is higher than at 1 mM, and hence the behavior of this protein is dependent on the salt concentration. At concentrations above 4 mM of the chelating agent, the enzyme is completely inactive, regardless of the NaCl concentration. Therefore, the substitution of Asp38 by Cys38 in the protein has weakened the binding of the zinc ion. The Asp residue at position 38 in the halophilic glucose dehydrogenase instead of Cys, which is commonly found at the analogous position in other members of the medium chain dehydrogenase family, could represent a halophilic adaptation.

The replacement of Asp38 by Cys38 makes the binding of catalytic zinc ion of the halophilic GlcDH very similar to that presented by the thermophilic GlcDHs and other MDR family proteins. In order to clarify if Cys38 instead of Asp38 modifies the thermal characteristics of the enzyme at different salt concentrations, the effect of the temperature on enzymatic stability and activity were determined. Generally at low salt

concentration, halophilic proteins are less stable. High temperatures can contribute to their destabilization under these conditions. At high salt concentrations, halophilic proteins are stable; but stability can be perturbed by several factors, such as high temperatures. The thermal inactivation results illustrate that both the wild-type and the D38C mutant proteins show higher thermostability when the concentration of NaCl is raised. However, the D38C GlcDH appears to be slightly more thermostable than wild-type GlcDH at the NaCl concentration assayed. The half-lives calculated for each protein under the different conditions are shown at Table 3. In general, at temperatures of 60-70 ºC the D38C mutant shows a half-life higher than that of wild-type GlcDH. No reliable comparisons can be made at 80 ºC, as at that temperature total inactivation of the enzyme is

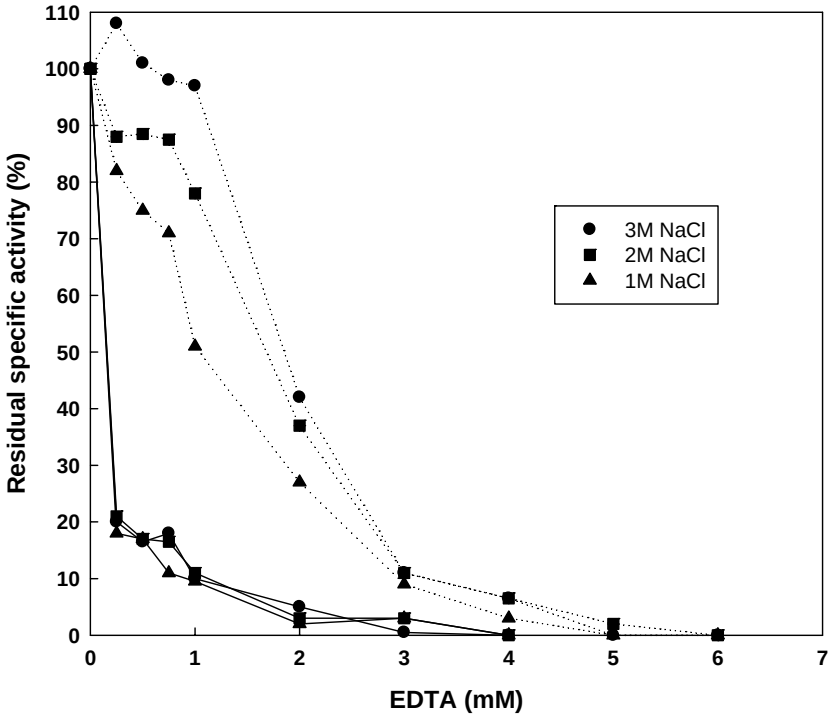

Figure 2. Deactivation of the wild-type (dashed line) and D38C mutant (solid line) GlcDH under various EDTA concentrations at different buffer salt concentrations.

Table 3. Half-life time at different temperatures and salt concentrations of wild-type GlcDH and D38C GlcDH.

	$t_{1/2}$ 1 M NaCl (h)		$t_{1/2}$ 2 M NaCl (h)		$t_{1/2}$ 3 M NaCl (h)	
	D38C	**Wild-type**	**D38C**	**Wild-type**	**D38C**	**Wild-type**
55 ºC	33.9	37.2	(a)	(a)	(a)	(a)
60 ºC	7.4	4.6	123.7	51.33	210.6	96.27
65 ºC	0.3	0.3	9.6	8.3	(a)	(a)
70 ºC	(b)	(b)	0.3	0.2	17.2	8.25
80 ºC	(b)	(b)	0.04	0.02	0.2	0.2

(a) The enzyme is stable under these conditions.
(b) The enzyme is stable for only a few minutes under these conditions.

achieved in a few seconds. Below 60 ºC the differences between the half-lives are not significant. Regarding studies of the effect of temperature on enzymatic activity we have observed that D38C GlcDH has shown maximum activity to higher temperatures. In particular wild-type GlcDH maximum activity is obtained at 56 ºC while D38C GlcDH maximum is found at roughly 70 ºC.

Comparison of the binary complexes of D38C GlcDH with NADP and zinc and wild-type GlcDH with NADPH and zinc

The high resolution structure of the binary complex of *Hfx. mediterranei* D38C GlcDH with NADP and zinc was compared to that of the NADPH/zinc wild-type complex. It was immediately apparent that there was a major difference between the ligands to the zinc between the wild-type and mutant structures. In the wild-type enzyme the four ligands to the zinc are two carboxyl oxygens of Glu64 and Glu150 and a nitrogen atom of His63 with the fourth position occupied by a water molecule. In the structure of D38C, Glu64 and His63 remain as zinc ligands but Glu150 is no longer bound by the zinc ion. Instead Glu150 makes a water mediated contact to the zinc ion and the sulphur of Cys38 completes the tetrahedral zinc coordination. A further difference is that the helix (α1) which has Cys38 at its N-terminal end is shifted by approximately 1.0 Å compared with its position in the wild-type enzyme. Comparing these structures the positions of His63, Glu64 and Glu150 are largely the same and the major differences (apart of the conformational changes associated with α1) is a shift in the position of the zinc ion (Figure 3). Interestingly, although both structures have one water molecule bound by the zinc ion, the water occupies different position in the coordination sphere of the zinc.

The structure of D38C GlcDH was then compared to that of the GlcDH from *T. acidophilum* in which the zinc ligands are similar, except that in the thermophilic GlcDH structure at 2.9 Å no water molecules can be identified. This comparison shows that in these two structures the position occupied by the zinc ions is more similar (Figure 4). Therefore, from a structural point of view the catalytic zinc binding site of D38C GlcDH is more closely related to the zinc binding site of the thermophilic GlcDH than to halophilic GlcDH. Whether this shift in position of the zinc ion in the active site of the halophilic enzyme and the differences of the nature of the zinc ligands represent a halophilic adaptation is unclear. Comparing the K_{mNADP} of the two enzymes they can be seen to be slightly different (0.044 ± 0.010 for D38C GlcDH and 0.113 ± 0.011 for *T. acidophilum* GlcDH). However the $K_{mglucose}$ are

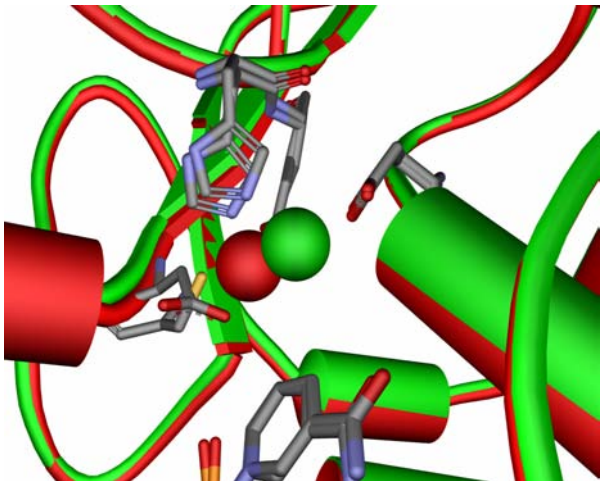

Figure 3. Superposition of catalytic zinc binding site between wild-type GlcDH in the presence of NADPH and zinc (red) and D38C GlcDH in the presence of NADP and zinc (green) using the LSQKAB software.

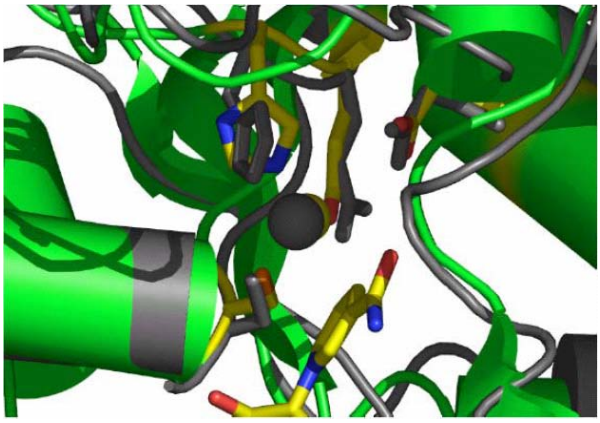

Figure 4. Superposition of catalytic zinc binding site between D38C GlcDH from *Hfx. mediterranei* (green and yellow) and GlcDH from *T. acidophilum* (grey) using the LSQKAB software.

closely related (12.4 ± 2.3 for D38C GlcDH and 10.0 ± 0.9 for *T. acidophilum* GlcDH). Moreover, at present it is not possible to say why the activity of D38C is lower than that of the wild-type enzyme. Clearly there are significant structural rearrangements which affect the active site region which may result in a modification to the functional properties of the enzyme. However, since mutations in the active site region of enzymes often lead to a significant drop in activity it is perhaps surprising that the activity of D38C is only around 60% lower than that of the wild-type enzyme.

CONCLUSIONS

The mutation of Asp38, a protein residue that lies close to the catalytic zinc ion, by Cys or Ala led to a significant reduction or abolition of activity. This confirms that Asp38 is important in catalysis forming a key aspect of the zinc binding site. The replacement of Asp38 by Cys, an amino acid commonly present at the same position in the proteins belonging to the MDR superfamily, results in the production of less efficient enzyme with lower enzymatic activity and catalytic efficiency. Furthermore this change appears to have a stabilizing effect on the ability of the protein to withstand high temperatures, producing an enzyme that is marginally more stable at high temperature as a consequence of imitating the zinc protein ligands present in thermophilic microorganisms. This data is in agreement with the structural analysis, which shows that the zinc binding site in thermophilic GlcDH is more like the D38C GlcDH than the wild-type *Hfx. mediterranei* GlcDH.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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