Analysis of acidic surface of *Halofex mediterranei* glucose dehydrogenase by site-directed mutagenesis

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Abstract Generally, halophilic enzymes present a characteristic amino acid composition, showing an increase in the content of acidic residues and a decrease in the content of basic residues, particularly lysines. The latter decrease appears to be responsible for a reduction in the proportion of solvent-exposed hydrophobic surface. This role was investigated by site-directed mutagenesis of glucose dehydrogenase from *Halofex mediterranei*, in which surface aspartic residues were changed to lysine residues. From the biochemical analysis of the mutant proteins, it is concluded that the replacement of the aspartic residues by lysines results in slightly less halotolerant proteins, although they retain the same enzymatic activities and kinetic parameters compared to the wild type enzyme.

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1. Introduction

The basis of knowledge of biochemical and biophysical properties of halophilic enzymes is an interesting question. Since the first amino acid composition determinations, it has become clear that halophilic enzymes present a higher proportion of acidic over basic residues, an increase in small hydrophobic residues, a decrease in aliphatic residues and lower lysine content than their non-halophilic homologues [1–5]. Since then, structural analyses have revealed two significant differences in the characteristics of the surface of the halophilic enzymes that may contribute to their stability in high salt. The first of these is that the excess of acidic residues are predominantly located on the enzyme surface leading to the formation of a hydration shell that protects the enzyme from aggregation in its highly saline environment [6–10]. The second is that the surface also displays a significant reduction in exposed hydrophobic character, which arises not from a loss of surface-exposed hydrophobic residues but from a reduction in surface-exposed lysine residues [11]. Furthermore, in several studies, the authors have concluded that it is the precise structural organization of surface acidic residues that is important in halophilic adaptation and not only the increase in acidic residue content, since these residues form clusters that bind networks of hydrated ions [12–14]. Nevertheless, the number of high resolution structures that permit the details of the protein solvent interactions to be seen is limited and the role of the reduction in the surface lysines has been largely ignored.

As a model enzyme to study the effect of lysines on halophilic adaptation, we have chosen the glucose dehydrogenase (GlcDH) from the extremely halophilic Archaeon *Halofex mediterranei*. This protein catalyses the oxidation of β-D-glucose to gluconic acid, preferentially using NADP⁺ as a coenzyme. Biochemical studies have established that the enzyme is a dimer, and that it requires the presence of divalent ions to enhance its enzymatic activity [15]. The gene encoding GlcDH from *Hfx. mediterranei* has been cloned and sequenced in our laboratory, and the sequence analysis has revealed that GlcDH belongs to the zinc-dependent medium-chain alcohol dehydrogenase (MDR) superfamily. Comparisons of the amino acid sequence of the halophilic GlcDH against the sequences of other glucose dehydrogenases belonging to the MDR superfamily, have shown an increase of acidic over basic residues in the halophilic enzyme, which has also been observed in other halophilic proteins. In addition, we have developed an heterologous overexpression system for the cloned gene [16], which have allowed us to crystallize the protein under conditions that closely mimic those experienced by the enzyme in the cell of the halophile [17,18]. More recently, the structure of the *Hfx. mediterranei* GlcDH has been solved at 1.6 Å resolution and the results reveal features of potential significance about the characteristics of the surface of this protein. Its decoration with acidic residues is only partially neutralized by bound potassium counterions and shows the expected reduction in hydrophobic character, surprisingly not from changes associated with the loss of exposed hydrophobic residues but rather arising from a loss of lysines. The structure also presents a highly ordered, multilayered solvation shell that can be seen to be organized into one dominant network covering much of the exposed surface accessible area to an extent not seen in almost any other protein structure solved [19].

With the aim of determining the influence of the reduction in surface lysine residues on the properties of the halophilic enzyme, we have constructed mutants of the *Hfx. mediterranei* GlcDH. Three surface aspartic residues of the 27 per subunit were changed to lysine residues (D172K, D216K and D229K), in such a way that four mutant enzymes were obtained, the triple mutant and the three corresponding single...
mutants. In the present paper, we report the construction, overexpression, purification and biochemical analysis of the GlcDH mutant proteins.

2. Materials and methods

2.1. 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) and E. coli XL1-Blue (Stratagene) were employed in site-directed mutagenesis experiments. And E. coli BL21(DE3) (Novagen) was used as the expression host. E. coli strains were grown in Luria-Bertani medium at 37 °C with shaking at 180 rpm. Antibiotic-resistant plasmids were maintained selected for in solid and liquid media by the addition of 100 μg ampicillin/ml.

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

2.2. Choice of the mutation

The choice of the three mutations was carried out by means of direct observation of the protein structure (2B5V, 2B5W). Moreover, multiple sequence alignments were performed by use of the CLUSTAL W program [20].

2.3. Site-directed mutagenesis

The gene encoding the halophilic GlcDH (AJ251111) was cloned into pGEM-11Zf(+). The GlcDH triple mutant was sequentially performed using the GeneEditor in vitro Site-Directed Mutagenesis System (Promega). 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 a 37 °C. The single mutants D216K GlcDH and D344K GlcDH were carried out by two enzymes, Klenow DNA polymerase (Stratagene) for amplification of mutational vectors and DpnI (Fermentas) for methylated parental vector digestion. After transforming into E. coli XL1-Blue, putative mutants were screened by dideoxynucleotide sequencing with parental vector digestion. After transforming into E. coli XL1-Blue, putative mutants were screened by dideoxynucleotide sequencing with parental vector digestion.

The mutagenic primers are summarized in Table 1.

2.4. Plasmid purification

Plasmids were prepared using CONCERT Rapid Plasmid Miniprep System Kit (Life Technologies) or the alkaline lysis method [21]. The purity of the DNA was determined spectrophotometrically by the ratio A260/A280.

2.5. Protein preparation

Expression, renaturation and purification of recombinant mutants were as previously described for wild type halophilic GlcDH [16]. The purity of the proteins was checked by running SDS-PAGE. No protein contamination was detectable after Coomassie blue staining of the gel. Protein concentration was determined by the method of Bradford [22].

2.6. Enzymatic assays

Glucose dehydrogenase was assayed as described previously [15]. All measurements were performed in triplicate.

2.6.1. Determination of Km for glucose and NADP+. The kinetic parameters, Km and Vmax were determined as described previously [15,16].

2.6.2. Effect of salt concentration on enzymatic activity. The dependence of enzymatic activity on the concentration of KCl and NaCl were conducted at 40 °C in 20 mM Tris–HCl buffer pH 8.8 in the concentration range of 0–4.0 M of salt. The protein samples were in 50 mM phosphate buffer pH 7.3 with 1 mM EDTA and 2 M KCl or NaCl according to the activity buffer. The results are expressed as the percentage of the activity relative to the highest activity obtained.

2.6.3. Effect of salt concentration on enzymatic stability. Salt concentration stability studies were carried out at room temperature (25 °C) and at 40 °C. Purified preparations of enzyme in 2.0 M KCl were quickly diluted with 50 mM potassium phosphate buffer pH 7.3 and 1 mM EDTA to obtain 0.25 and 0.5 M KCl concentrations. The final protein concentrations were then about 20 μg/ml. Samples were removed at known time intervals, cooled on ice, and the residual enzymatic activity was then measured in the standard buffer containing KCl instead of NaCl. The results are expressed as the percentage of the activity relative to that existing before incubation.

2.6.4. Differential scanning calorimetry (DSC). DSC experiments were performed using a VP-DSC microcalorimeter (MicroCal). Temperatures from 40 °C to 90 °C were scanned at a rate of 60 °C/h using 50 mM potassium phosphate buffer pH 7.3 containing 1 mM EDTA and 0.5 M or 2.0 M KCl, which also served for baseline measurements. Prior to scanning, all samples of protein and buffer were degassed under vacuum using a ThermoVac unit (MicroCal). The protein concentrations were in the range of 50–80 μM (approximately 4–6 mg/ml). The data were analyzed using ORIGIN software v 7.0.

3. Results and discussion

3.1. Choice of the halophilic GlcDH mutations

At the start of the project, an initial GlcDH structure had been solved at medium resolution. Based on direct observation of this structure, we selected three aspartic residues (D172, D216 and D344) of the GlcDH/27 per subunit found in the protein surface, which present at least the carboxyl oxygens exposed to the solvent. The three selected residues are considered as surface acidic residues and they are located in different regions of the protein surface. Later, the 1.6 Å resolution GlcDH structure revealed that the side-chain carboxyl of D172 is involved in interactions with a cluster of surface water molecules near a bound potassium counter-ion. In contrast, the side-chain carboxyl of D216 forms interactions with surface waters in a region in which no counter-ion can be seen. And, finally, the side-chain carboxyl of D344 lies on the surface where it interacts with the solvent but also makes hydrogen bonds to the nearby side-chains of T346 and T347 (Fig. 1).

Moreover, multiple alignments (data not shown) with other GlcDH sequences belonging to the MDR superfamily have shown that the acidic residue D216 from Hfx. mediterranei GlcDH is conserved in any other halophilic microorganisms.

Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcDH D172K</td>
<td>5’ GTT CGA CTA GAA ACC GTC GTC GGC 3’</td>
</tr>
<tr>
<td>GlcDH D216K</td>
<td>5’ CGG ACC CGA CAA TCA AAA TCA TCG AG 3’</td>
</tr>
<tr>
<td>GlcDH D344K</td>
<td>5’ GAT GAC AAG GAC ACC ACT ATA AAA ACC C 3’</td>
</tr>
<tr>
<td>216 Forw</td>
<td>5’ GCC CGG ACC CGA CAA TCA AAA TCA TCG AGG AAC TCG 3’</td>
</tr>
<tr>
<td>216 Rev</td>
<td>5’ GCA GTT CCT CGA TGA TTT TTA TTA TCG GAT CGG GGC 3’</td>
</tr>
<tr>
<td>344 Forw</td>
<td>5’ GAA GCA GCA TCC GAT GAC AAG GAC ACC ACT ATA AAA ACC GGC 3’</td>
</tr>
<tr>
<td>344 Rev</td>
<td>5’ GCC GAT TTT TAT AGT GAT GTC GTC ATC GAA TCG TCC TCC 3’</td>
</tr>
</tbody>
</table>

The mutant codons are shown in bold-type.
as Haloarcula marismortui, Halobacterium halobium and Haloferax volcanii. However, the residue D344 is only conserved in the Hfx. volcanii GlcDH, and the residue D172 is not present in any halophilic GlcDH. At the locations corresponding to D172, D216 and D344 in wild type Hfx. mediterranei GlcDH, there are non-acidic residues in 100% of the non-halophilic GlcDH sequences analysed. Therefore, the presence of these acidic residues in the GlcDH from Hfx. mediterranei could be an adaptive response to the halophilic environment.

3.2. Site-directed mutagenesis and expression of the mutant proteins

The triple mutant GlcDH was created with GeneEditor in vitro Site-Directed Mutagenesis System (Promega). The GlcDH gene was excised from pCR2.1-HmGDH [16] with the endonuclease BamHI, and cloned into the pGEM-11Zf(+) mutagenesis vector. The mutations were introduced one by one using the primers GlcDH D172K, GlcDH D216K and GlcDH D344K (Table 1). The single mutant D172K GlcDH was achieved as the first step in the construction of the triple mutant GlcDH. And the mutants D216K and D344K GlcDHs were constructed by PCR using the Pfu Turbo DNA polymerase and the pair of primers 216Forw and 216Rev, and 344Forw and 344Rev, respectively. Subsequently, the treatment with the endonuclease DpnI was carried out at 37°C for 1 h with the aim of digesting the parental DNA. Every mutation introduced in the Hfx. mediterranei GlcDH gene was confirmed by DNA sequencing.

The four mutant genes were excised from pGEM-11Zf(+) using the restriction enzymes BamHI and NdeI and ligated into similarly cut pET3a expression vector. The resulting constructs were transformed into the expression host E. coli BL21(DE3), and the expression assays were performed as described previously [16]. The four mutant proteins were obtained as inclusion bodies which were solubilized using 20 mM Tris–HCl buffer pH 8.0, 8 M Urea, 50 mM DTT and 2 mM EDTA, like wild type GlcDH. The refolding of each mutant protein was achieved by rapid dilution in 20 mM Tris–HCl buffer pH 7.4, 1 mM EDTA and KCl or NaCl in the concentration range of 1–3 M. The wild type and triple mutant GlcDHs behave identically in the refolding process under the conditions assayed. The profiles for the triple mutant protein are like the wild type GlcDH, independently of concentration and type of salt. The three single mutants also presented the same profiles. In the presence of NaCl the recovery of activity was always higher than with KCl and the highest enzymatic activity was obtained at 3 M NaCl. Furthermore, at low salt concentration the recovery of activity is lower than at high salt concentration. No activity was recovered at 1 M KCl or NaCl. Thus, the mutations introduced on the protein surface do not appear to affect refolding in either the triple mutant or the single mutant proteins.

The purification of the GlcDH mutants were carried out as described previously [16]. However, after 3–4 days, protein precipitation was observed in the fractions of triple mutant GlcDH whose protein concentration was greater than 1 mg/ml. This problem was solved by decreasing protein concentration or reducing the salt concentration through a dialysis against the buffer containing 1 M NaCl or KCl. This fact indicates that the halophilic properties of the triple mutant protein have been altered, since the wild type and single mutant proteins are stable for months under these conditions.

3.3. Properties of the mutant enzymes

The kinetic parameters of the mutant proteins were determined and compared to those that had previously been obtained of wild type GlcDH (Table 2). Their $K_m$ values for NADP$^+$ and glucose are essentially similar and no significant differences in the values for $V_{max}$ were detected. These results indicate that the kinetic parameters were not affected by the mutations. It is unlikely, therefore, that the mutations in
position 172, 216 and 344 influenced the active site or the integrity of the enzyme. Similar results were obtained with malate dehydrogenase from *Har. marismortui* [12] and dihydrolipoamide dehydrogenase from *Hfx. volcanii* [13] when residues on the surface were mutated.

The dependence of enzymatic activity on the concentration of NaCl is shown in Fig. 2. The triple mutant GlcDH shows its maximum activity in a buffer with 0.50–0.75 M NaCl while the wild type protein has its maximum activity with 1.5 M NaCl. Furthermore, at low salt concentration the activity of the triple mutant enzyme is higher than the activity of the wild type GlcDH, whereas at higher salt concentrations it is lower than the wild type protein. With the purpose of determining if the observed behaviour in the triple mutant protein is due to the presence of just one mutation or of the three modifications, these experiments were also performed with each single mutant protein. The mutants D172K GlcDH and D216K GlcDH show the same profiles as the triple mutant enzyme. In striking contrast, the behaviour of the D344K mutant protein is very similar to the profile obtained with the wild type GlcDH. These results suggest that the D344K modification does not disturb the halophilic characteristics of the GlcDH and, therefore, the behaviour of the triple mutant GlcDH in the salt concentrations assayed could be due to the introduction of the mutation D172K and D216K. The profiles obtained using buffers with KCl are very similar.

At optimal salt concentration, the activities of the wild type and mutants GlcDH are very close and the kinetic parameters are very similar too. Therefore, it appears that the different mutations introduced in the GlcDH only have influence on the dependence of enzymatic activity on the salt concentration. Although, in similar studies with the dihydrolipoamide dehydrogenase from *Hfx. volcanii* the introduction of only one mutation (E243Q, E423S or E423A) resulted in enzymes less active than the wild type enzyme and with different kinetics parameters. Although when the E423 was replaced by another acidic residue (aspartic residue) the mutant was only slightly less active than the wild type enzyme [13]. Based on these results, Jolley and co-workers also support the view that it is the precise structural organization of acidic residues that is important in halophilic adaptation and not only the increase in acidic residue content [12,13,24].

The effects of different salt concentration on the residual activity of wild type halophilic GlcDH and the four mutant proteins were measured after incubation at 25 °C and 40 °C. In the presence of 2 M KCl, neither wild type enzyme nor mutant proteins were inactivated at the temperatures assayed, in particular, at salt concentrations above 1 M the proteins are stable for weeks. Furthermore, as salt concentration increases, the proteins are more stable independently of the temperature. However, at low salt concentrations, small differences were observed in the stability of the proteins. The triple mutant GlcDH and each one of the single mutant proteins appear to

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>$K_{\text{NADP}}$ (mM)</th>
<th>$K_{\text{glucose}}$ (mM)</th>
<th>$V_{\text{max}}$ (U/mg)</th>
<th>$K_{\text{cat}} \times 10^3$ (min$^{-1}$)</th>
<th>$K_{\text{cat}}/K_{\text{glucose}} \times 10^3$ (mM$^{-1}$ min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type GlcDH</td>
<td>0.03 ± 0.004</td>
<td>2.8 ± 0.3</td>
<td>397 ± 15</td>
<td>31 ± 1</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>D172K GlcDH</td>
<td>0.036 ± 0.005</td>
<td>2.4 ± 0.4</td>
<td>347 ± 13</td>
<td>27 ± 1</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>D216K GlcDH</td>
<td>0.035 ± 0.005</td>
<td>2.2 ± 0.4</td>
<td>320 ± 13</td>
<td>25 ± 1</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>D344K GlcDH</td>
<td>0.056 ± 0.012</td>
<td>1.8 ± 0.5</td>
<td>322 ± 26</td>
<td>25 ± 2</td>
<td>14 ± 2</td>
</tr>
</tbody>
</table>

Fig. 2. Effect of NaCl on the activity of wild type GlcDH (●), triple mutant GlcDH (○) and single mutants: (A) D172K GlcDH (□), (B) D216K GlcDH (△) and (C) D344 K GlcDH (◇). The activity buffer was 20 mM Tris–HCl pH 8.8 with varying concentrations of NaCl.
be slightly more stable than the wild type protein at 0.25 and 0.50 M KCl. The behaviour of the proteins at 25 °C is similar, although a decrease in the temperature implies an increase of the period over which the enzymes are stable. The half-life time ($t_{1/2}$) for each protein (Table 3) was calculated from the logarithm of residual activity versus time plots. The results show that the mutant protein half-life times, either as a single alteration or altogether, are longer than wild type, both at 25 °C and 40 °C. However, there are not significant differences between the triple mutant GlcDH and the single mutant proteins, all of them present a similar half-life time under the conditions assayed.

With the purpose of studying the thermodynamic properties of the proteins, biocalorimetry experiments were carried out under two different KCl concentrations using a DSC. In the presence of 2 M KCl, wild type and single mutants GlcDH denaturing temperatures are in a range from 74.6 °C to 75.9 °C. However, the triple mutant enzyme shows a lower denaturing temperature, between 73.6 °C and 73.7 °C. In other words, the triple mutant enzyme is denatured at slightly lower temperatures than the wild type and single mutant GlcDHs in the presence of high salt concentration. At 0.50 M KCl, the results obtained do not reveal significant data. On the one hand, the protein denaturing temperatures are lower than those obtained in the presence of higher salt concentration, independently of protein type (Fig. 3), but this decrease was expected because the halophilic proteins are destabilized in the presence of low salt concentration. On the other hand, the denaturing temperature of the wild type and mutant enzymes are in a range from 59.8 °C to 60.7 °C, not showing any significant difference between the temperatures obtained for each protein.

The data that we have presented indicate that the halophilic properties of the mutant proteins have been modified. Their enzymatic activity and kinetic parameters have been not affected by the mutations, however, the triple mutant protein and the single mutants, D172K GlcDH and D216K GlcDH, have reached their maximum activity at lower salt concentrations than the wild type GlcDH and D344K mutant enzyme. It appears that the D344K substitution has no effect on the salt activity profile. Strikingly, in all the cases the mutant proteins have been slightly more stable than the wild type GlcDH at low salt concentrations, although they require high salt concentration for maximum stability like malate dehydrogenase mutant from Har. marismortui [12]. The biocalorimetry analyses have revealed another difference. The single mutants and the wild type GlcDHs have showed similar denaturing temperature in the presence of 2 M KCl while the triple mutant enzyme has presented a lower denaturing temperature. Thus all three substitutions in the protein are apparently needed to significantly modify its denaturing temperature at high salt concentration. Probably, these data are the result of an alteration of the hydration shell, which is required for halophilic proteins to be stable at high salt concentrations. Analysis of the high resolution GlcDH structure has shown that the size and order of the hydration shell in the halophilic enzyme is significantly greater than in other non-halophilic proteins, and that the differences in the characteristics of the molecular

| Table 3 Half-life times of wild type and mutant GlcDHs in the presence of different KCl concentrations at 40 °C (A) and 25 °C (B) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | Wild type GlcDH | Triple mutant GlcDH | D172K GlcDH | D216K GlcDH | D344K GlcDH |
| (A) $t_{1/2}$ 40 °C (h) |                |                |                |                |                |
| 0.25 M KCl      | 14 ± 2          | 18 ± 3          | 23 ± 5         | 26 ± 8         | 25 ± 4         |
| 0.5 M KCl       | 86 ± 4          | 114 ± 9         | 95 ± 9         | 117 ± 8        | 114 ± 7        |
| >1 M KCl        | >1 week         | >1 week         | >1 week        | >1 week        | >1 week        |
| (B) $t_{1/2}$ 25 °C (h) |                |                |                |                |                |
| 0.25 M KCl      | 142 ± 22        | 246 ± 23        | 293 ± 30       | 219 ± 32       | 232 ± 30       |
| 0.5 M KCl       | 506 ± 50        | 613 ± 74        | 630 ± 113      | 660 ± 113      | 537 ± 75       |
| >1 M KCl        | >1 week         | >1 week         | >1 week        | >1 week        | >1 week        |

Fig. 3. Calorimetric traces of the thermal transition for wild type GlcDH (A) and triple mutant GlcDH (B). Thermal transitions were determined in 50 mM potassium phosphate buffer pH 7.3 with 0.5 M (continuous line) or 2 M KCl (dotted line).
surface arise not only from an increase in negative surface charge but also from the reduction in the percentage of hydrophobic surface area due to lysine side chains. Furthermore, these lysine residues retained in the halophilic enzyme tend to be more buried than the average seen in non-halophilic proteins [19]. This theory had previously been predicted from a comparative modelling study that compared a mesophilic glutamate dehydrogenase to a model of its homologous halophilic enzyme from *Hbt. salinarum* [11]. And recently, the nucleoside diphosphate kinase, containing hexa-His-tag at the N-terminal, from the halophilic archaeon *Hbt. salinarum* has been overexpressed and crystallized. Based on structural and biochemical studies, the authors have proposed that the introduction of additional arginine and/or histidine in the N-terminal of this protein could be the reason for solubility and activity of the enzyme in the low salt conditions of the *E. coli* cytosol [25,26], since the overexpressed halophilic enzymes are generally obtained as inclusion bodies or soluble and inactive. This fact also supports our results, because the introduction of positive residues leads to a protein which shows different properties at low salt concentrations.

In conclusion, the replacement of aspartic residues by lysine residues on the GlcDH surface have led to a modification of the halophilic properties of the mutant enzymes, D172K and D216K being the most significant mutations. Having established the GlcDH structure at the highest resolution to date for any water soluble halophilic enzyme, it would be fascinating to extend the structural analysis to the mutant proteins in order to examine the solvent interactions. Such analyses will enable any potential disruption of the solvent shell by the lysine sidechains to be seen.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.01.054.

**References**


