Electrostatic interactions across the dimer–dimer interface contribute to the pH-dependent stability of a tetrameric malate dehydrogenase

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Abstract **Malate dehydrogenase (MDH) from the moderately thermophilic bacterium *Chloroflexus aurantiacus* (CaMDH) is a tetrameric enzyme, while MDHs from mesophilic bacteria usually are dimers. Using site-directed mutagenesis, we show here that a network of electrostatic interactions across the extra dimer–dimer interface in CaMDH is important for thermal stability and oligomeric integrity. Stability effects of single point mutations (E25Q, E25K, D56N, D56K) varied from −1.2°C to −26.8°C, and depended strongly on pH. Gel-filtration experiments indicated that the 26.8°C loss in stability observed for the D56K mutant at low pH was accompanied by a shift towards a lower oligomerization state.**

Key words: Malate dehydrogenase; Oligomerization; Thermal stability; Electrostatic interactions

1. Introduction

Recent structural studies on malate dehydrogenases (MDHs) from the phototrophic green bacteria *Chlorobium vibrioforme* (CvMDH), *Chlorobium tepidum* (CtMDH), and *Chloroflexus aurantiacus* (CaMDH) have shown that these enzymes have tetrameric quaternary structures [1]. Structurally, these MDHs show a high degree of similarity to lactate dehydrogenases [2–4] and to tetrameric MDHs isolated from Archaea [5–7] (see also [8]). The tetramer consists of two dimers, where each dimer is highly similar to dimeric MDHs found in mesophilic organisms such as *Escherichia coli* [9] and pig [10,11] (Fig. 1). The dimer–dimer interfaces in the MDHs from *Chlorobium* and *Chloroflexus* contain conspicuous networks of salt bridges that may contribute to stabilization of their tetrameric quaternary structures. Studies on several proteins (e.g. from thermophilic microorganisms) suggest that the tetrameric quaternary structure and the reinforcement of this structure by networks of electrostatic interactions may be beneficial for protein stability [12–20].

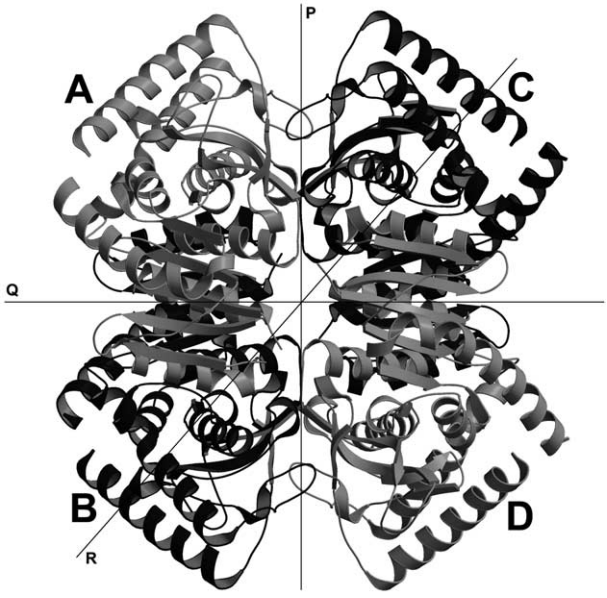

Fig. 1. Structure of tetrameric CaMDH with symmetry axes. The *Q* axis relates the two subunits in the dimers whereas the *P* and *R* axes relate the two dimers which comprise the full tetramer. The four monomers are marked A–D.

Here, we have studied MDH from *C. aurantiacus*, the most stable of the tetrameric MDHs previously studied in our laboratory. The dimer–dimer interface in CaMDH contains a prominent network of charged residues belonging to monomers related by the *R* symmetry axis shown in Fig. 1 (A–D and B–C interfaces). The core of this network consists of two acidic residues, Glu25 and Asp56, on the one monomer and two basic residues, Lys242 and Lys244, on the other monomer (Fig. 2). Structure-based alignments of CaMDH, CvMDH, and CtMDH [1] show that two of these charged residues (Glu25 and Lys242) only occur in CaMDH, a finding that could explain why this enzyme is the most stable one. We have probed the importance of electrostatic interactions across the dimer–dimer interface for integrity of the quaternary structure and for thermal stability by studying the effects of replacing Glu25 and Asp56 by residues that are either neutral or have an opposite charge. The results show that the mutated residues contribute to both thermal stability and stability of the quaternary structure, indicating that these two properties are closely related. The results also provide

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Abbreviations: MDH, malate dehydrogenase; CaMDH, *Chloroflexus aurantiacus* MDH; CtMDH, *Chlorobium tepidum* MDH; CvMDH, *Chlorobium vibrioforme* MDH

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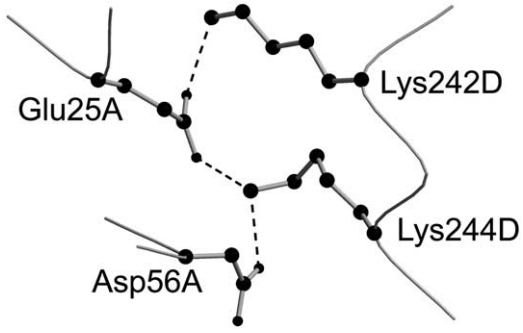

Fig. 2. Details of the electrostatic network between monomers A and D (Glu25 and Asp56 belong to A; Lys242 and Lys244 belong to D). The dotted lines connect atoms that are within 3 Å of each other. Note that this network occurs four times: twice between A and D and twice between B and C. Fig. 1 and this figure were drawn using the programs Molscript [27] and Raster3D [28].

clues as to how and why the stability of CaMDH depends on pH.

2. Materials and methods

2.1. Site-specific mutagenesis and protein purification

The gene for CaMDH was cloned into the plasmid pMDH4 as described previously [21]. Site-directed mutations were introduced into a pUC19 subclone of pMDH4, using the Quick Change™ site-directed mutagenesis kit from Stratagene. After verification of the DNA sequence of the mutated gene fragment, this fragment was used to construct a pMDH4 variant containing an intact mutated *mdh* gene. Wild-type and mutant genes were overexpressed in *E. coli* DH5α and the proteins were purified as described previously [21,22].

After purification, the enzyme was dialyzed against 20 mM potassium phosphate buffer pH 7.5 using Slide-A-Lyser® dialysis cassettes, cut-off 10 000 Da (Pierce). Protein concentrations were determined using the Bio-Rad protein assay based on the Bradford dye-binding procedure [23], and bovine serum albumin as standard. Enzyme purity was verified using SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis).

2.2. Enzyme characterization

MDH activity was assayed at 340 nm and 45°C using a Shimadzu UV-265 recording spectrophotometer, as described previously [22]. The standard assay mixture contained 20 mM phosphate buffer pH 7.5, 0.2 mM oxaloacetate, 0.15 mM NADH and enzyme in a total volume of 1 ml.

Circular dichroism (CD) spectra were recorded by using a Jasco J-810 spectropolarimeter (Jasco) calibrated with ammonium-D-camphor-10-sulfonate (Icatayama Chemicals). The conditions used were: *T*, 23°C; path length, 0.1 cm; protein concentration, 0.10 mg/ml; buffer, 10 mM potassium phosphate buffer pH 7.5; scanning, five times, at 20 nm/min. The data were averaged and the spectrum of a protein-free control sample was subtracted.

Thermal unfolding/denaturation curves were determined by recording the change in CD signal at 222 nm during heating. Temperature was controlled using a Peltier-type temperature control system (TPC-423S/L, Jasco) and a heating rate of 1°C/min. The protein concentration was 0.10 mg/ml and the path length 0.1 cm. The following buffers were used: 10 mM sodium acetate buffer pH 4.4, and 10 mM potassium phosphate buffer pH 6.0 and pH 7.5. After baseline correction [24], each unfolding curve was smoothed (means-movement method, convolution width 25) and normalized, using the computer program Origin 7.0 (OriginLab Corporation). Apparent melting temperatures (T_m) were determined from the transition midpoint visible in the first derivative of the unfolding curve.

The quaternary structures of CaMDH variants were analyzed by size-exclusion chromatography using a Superdex 200HR 10/30 PC column (Pharmacia Biotech) and the SMART micropurification chromatography system (Pharmacia Biotech). The column was equilibrated with an appropriate buffer (10 mM sodium acetate pH 4.4 or 10 mM potassium phosphate pH 7.5) and calibrated with aldolase (158 kDa), bovine serum albumin (67 kDa) and ovalbumin (43 kDa). The CaMDH variants were analyzed by applying 10 µl samples with a protein concentration of 1 mg/ml. The flow rate was 40 µl/min and the elution profile was monitored at 280 nm.

3. Results and discussion

3.1. Mutant production and general characterization

Structural analysis of the four mutations using the CaMDH crystal structure and the molecular modelling program WHAT IF [25] indicated that all new side chains could be accommodated in the structure in favorable rotamers without introduction of steric overlap. All four mutant enzymes, E25Q, E25K, D56N, and D56K, were produced in normal amounts by the producer strains and could be purified with the standard procedure developed for the wild-type enzyme. Except for the D56K mutant, which displayed a four-fold decrease in specific activity, the specific activities of the mutants were close to that of wild-type CaMDH. The CD spectra of the mutants (not shown) were similar to that of the wild-type enzyme, indicating that no major structural changes had occurred.

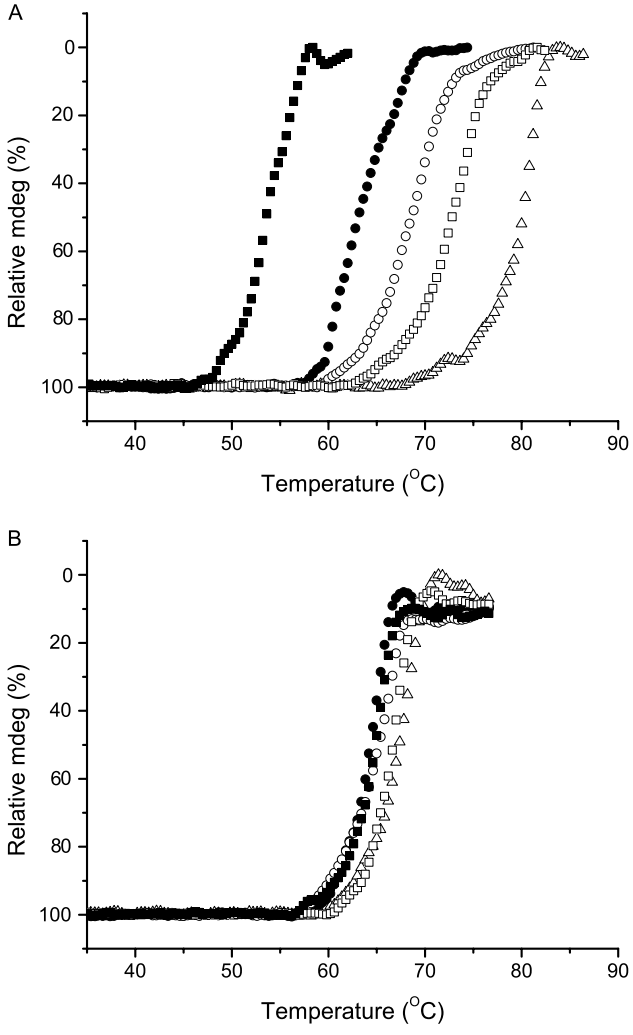

Fig. 3. Thermal unfolding of CaMDH (△) and the E25Q (○), E25K (●), D56N (□) and D56K (■) variants at pH 4.4 (A) and pH 7.5 (B).

3.2. Thermal stability and oligomerization state

Thermal denaturation of the three MDH variants was monitored by CD spectroscopy at pH 4.4, 6.0 and 7.5 (Figs. 3 and 4). Unfolding was in all cases irreversible, meaning that only apparent T_m values could be determined. In all cases, stability was positively correlated with protein concentration (not shown), which is indicative of a situation where dissociation/association reactions are important for stability (e.g. [26]).

The thermal stability of the wild-type was considerably higher at pH 4.4 and 6.0 than at pH 7.5 (Fig. 4). All mutations reduced the thermal stability, but the magnitude of the effects was strongly dependent on pH (Fig. 4). At pH 7.5, mutational effects were small, whereas they ranged from −7.2°C (D56N) to as much as −26.8°C (D56K) at pH 4.4. The fact that mutational effects were large and strongly pH dependent show that electrostatic interactions involving the titratable side chains of Glu25 and Asp56 are indeed important for the stability of CaMDH.

The increasing stability of wild-type CaMDH with lower pH suggests that there is an unfavorable surplus of negative charge on the enzyme at higher pH (e.g. pH 7.5). A reduction of this surplus could lead to the higher stability that is observed for the wild-type enzyme at pH 6.0 and 4.4. Accordingly, removal of negative charge (E25Q, D56N) and even charge reversal (E25K, D56K) had only modest effects on stability at pH 7.5, whereas much more drastic effects were obtained at pH 6.0 and 4.4 (Fig. 4).

Taking into account the considerable beneficial effect of low pH on stability in the wild-type enzyme, one might have assumed that a mutation like E25Q would actually stabilize the enzyme at pH 7.5. The fact that this is not the case indicates that the assumed positive effect of charge removal is outweighed by negative side effects in this case.

Gel-filtration experiments performed at pH 4.4 (Fig. 5A) and pH 7.5 (Fig. 5B) showed almost identical retention times for wild-type and all mutants under both conditions, except for D56K. The observed retention times were compatible with the notion, derived from X-ray crystallography, that the enzymes are tetrameric (Fig. 5). Apparently, the equilibrium between the tetramer and lower oligomerization states lies almost exclusively on the tetramer side under the conditions of the experiments. The only exception was the D56K mutant, which, at pH 4.4, showed a second peak with increased retention time. This second peak corresponds to a lower oligomerization state of CaMDH, most probably the dimer (Fig. 5). In this connection, it is interesting to note that at pH 4.4 the apparent T_m of the D56K mutant is as much as 10°C lower than the T_m of any other mutant at any pH value tested. The gel-filtration results show that this exceptionally low thermal stability is correlated with reduced stability of the tetrameric state of the enzyme.

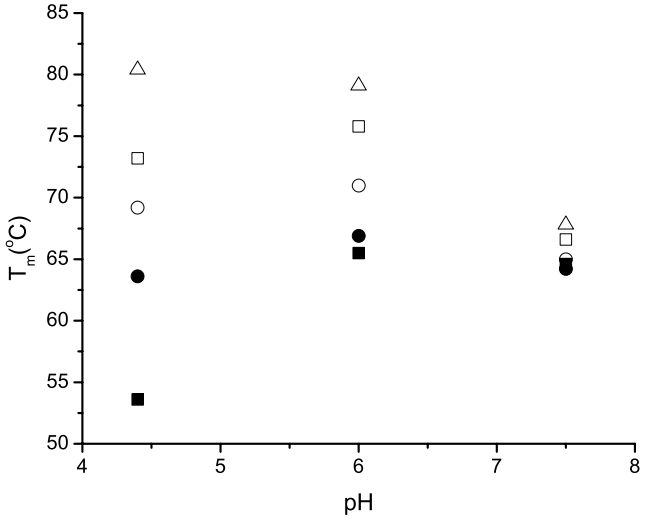

Fig. 4. pH dependence of the apparent T_m for CaMDH (△) and the E25Q (○), E25K (●), D56N (□) and D56K (■) mutants. Values presented are the averages of two independent measurements; in all cases the results of these two measurements differed by less than 0.5°C.

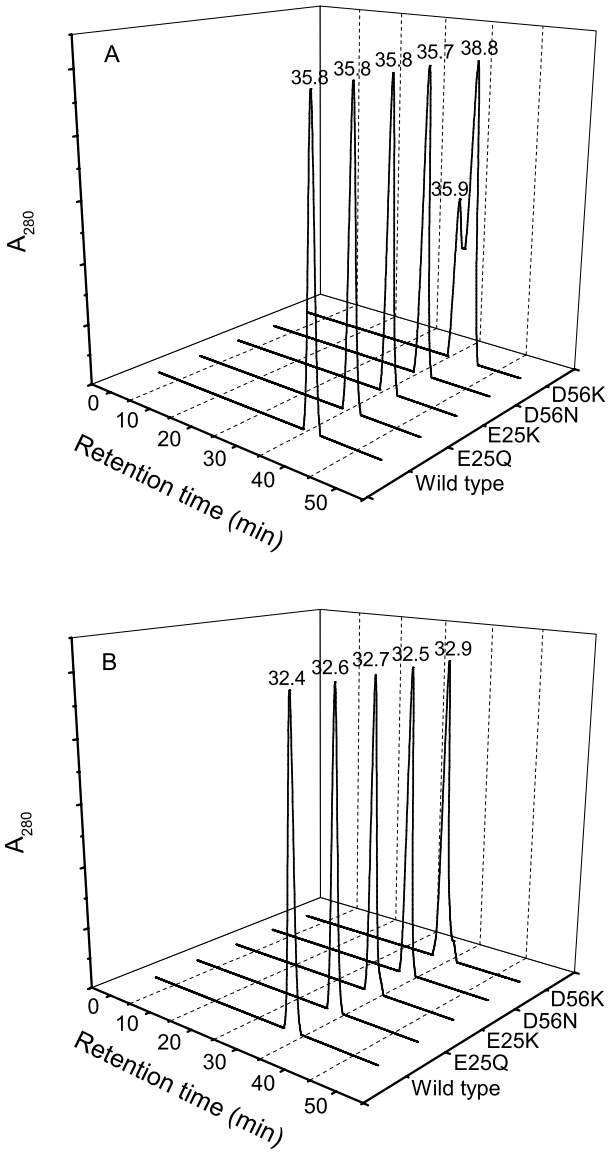

Fig. 5. Gel-filtration of CaMDH variants at pH 4.4 (A) and pH 7.5 (B). Standard proteins had the following retention times (in minutes): aldolase, 158 kDa, 34.7 at pH 4.4, 32.8 at pH 7.5; bovine serum albumin, 67 kDa, 38.0 at pH 4.4, 35.0 at pH 7.5; ovalbumin, 43 kDa, 40.9 at pH 4.4, 37.1 at pH 7.5. The molecular masses of the CaMDH tetramer and dimer are 130.8 and 65.4 kDa, respectively.

4. Concluding remark

The present study shows that the quaternary structure of CaMDH is reinforced by a network of electrostatic interactions and that this reinforcement contributes to protein stability. The results add to existing knowledge derived from similar studies on other oligomeric proteins [13–19] by showing that the effects of single point mutations in the subunit interface may be quite large and by revealing that these effects may be strongly dependent on pH. Furthermore, the pH-dependent thermal destabilization was found to be linked to a destabilization of the tetrameric state. Thus, the present results provide a clear example of the potentially large contribution of oligomerization to protein stability and of the role that electrostatic interactions may play to achieve this.

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