Thermostable chimeric PQQ glucose dehydrogenase

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Abstract The thermal stability of PQQ glucose dehydrogenases (PQQGDHs) which were chimeras with more than 95% made up of the N-terminal region of *Escherichia coli* PQQGDH and the rest made up of the C-terminal region of *Acinetobacter calcoaceticus* PQQGDH was investigated. Among the chimeric PQQGDHs, E97A3 (*E. coli* 97% and *A. calcoaceticus* 3%) and E95A5 were found to possess higher thermal stability than parental *E. coli* PQQGDH. Further detailed characterization of the thermal stability was carried out, focusing on E97A3. E97A3 showed a more than 3-fold and 12-fold increase in half life time at 40°C, compared with the PQQGDHs of *E. coli* and *A. calcoaceticus*, respectively. Using transition state theory, the increase in the free energy of inactivation observed in E97A3 was compared with those of the *E. coli* and *A. calcoaceticus* parental enzymes. The region responsible for this stabilization was also discussed.

Key words: PQQ (pyrroloquinoline quinone); Glucose dehydrogenase; Chimeric enzyme; Thermal stability; Protein engineering

1. Introduction

Glucose dehydrogenases (GDHs) containing pyrroloquinoline quinone (PQQ) are widely found in various Gram negative bacteria [1]. Except for a water soluble PQQGDH (PQQGDH-B) in *Acinetobacter calcoaceticus* [2], they are all single peptide membrane-binding proteins with MWs of about 87 kDa. In spite of their high homologies in primary structure, their enzymatic characteristics, such as holo-enzyme form stability, bivalent metal requirement for holo-enzyme formation, and substrate specificity, are strain-specific. The authors have been carrying out protein engineering of PQQGDHs in order to improve the enzymatic characteristics, such as operational stability, sensitivity, selectivity and so on, considering their potential for diagnostic uses, such as biosensor components [3–6]. In order to elucidate the region responsible for PQQ binding, we have focused on one set of PQQGDH structural genes; *E. coli* PQQGDH, which easily loses its prosthetic group in the presence of EDTA, and *A. calocoaceticus* PQQGDH, which is very stable during EDTA treatment. Based on homology analysis of the putative PQQ binding site, we previously reported a mutant constructed by site directed mutagenesis with increased EDTA tolerance [6]. We have also succeeded in the construction of various chimeric PQQGDHs which differ in EDTA tolerance, by homologous recombination of *E. coli* and *A. calcoaceticus* PQQGDH structural genes ([8] and Sode et al., submitted for publication). We found that one such chimera, E97A3, of which 97% was the N-terminal region of *E. coli* PQQGDH and 3% was from *A. calcoaceticus* PQQGDH, showed exceptional stability during purification and storage.

Here, we report the increased thermal stability of a chimeric enzyme, E97A3, compared with other similar chimeric enzymes and the parental enzymes, *E. coli* and *A. calcoaceticus* PQQGDH. We characterize differences in thermodynamic properties between E97A3 and the parental enzymes, and discuss the implications with regards to increasing stability of enzymes by protein engineering.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli PP2418, the PQQGDH structural gene (gcd) of which was disrupted by insertion mutagenesis [9], was used as the host strain for the expression of *E. coli* PQQGDH, *Acinetobacter calcoaceticus* PQQGDH, and chimeric PQQGDHs, using an expression vector, pTrc99A (Pharmacia, Upsla, Sweden) with the corresponding structural genes inserted [8].

2.2. Enzyme purification

Solubilized membrane fractions containing each PQQGDH and purified enzyme samples were prepared following our previous report [7]. Samples found to be electophoretically homogeneous by silver staining on SDS-PAGE were utilized for kinetic studies.

2.3. Thermoinactivation of PQQGDHs

The time course of irreversible thermoinactivation was measured by incubating 56 ml of apo-enzyme solution (60 $mU \cdot ml^{-1}$) in 10 mM phosphate buffer, pH 7.0, containing 0.2% Triton X-100, at each temperature. Samples taken every 2 or 5 min were stored at 4°C for 1 min. The remaining PQQGDH activity was assayed as described previously [7], after holo-enzyme formation for 1 h in a 10 mM phosphate buffer, pH 7.0, containing 10 mM $MgCl_2$ and 5 μM PQQ. The first order rate constant, k, and the half life time of thermoinactivation ($t_{1/2}$) of irreversible thermoinactivation was obtained by linear regression in semilogarithmic coordinates at each temperature.

According to transition state theory, enthalpy (ΔH^*), entropy (ΔS^*), and free energy of thermoinactivation ($\Delta G^*$of thermoinactivation were obtained.

Results

3.1. Thermal stability of C-terminal chimeric PQQGDHs

Fig. 1 shows the time course of thermal inactivation at 40°C of chimeric PQQGDHs (E99A1, E97A3 and E95A5) and parental *E. coli* PQQGDH. Thermoinactivation was adequately describable by first-order kinetics, from linear regression of $\ln(A/A_0)$ against time. The chimeric PQQGDHs, E97A3 and E95A5, showed higher thermal stability than either the parental enzyme, or the other chimera, E99A1. In the further study, we selected E97A3 as the representative thermostable chimeric PQQGDH, and used a purified sample for analysis of thermodynamic parameters.

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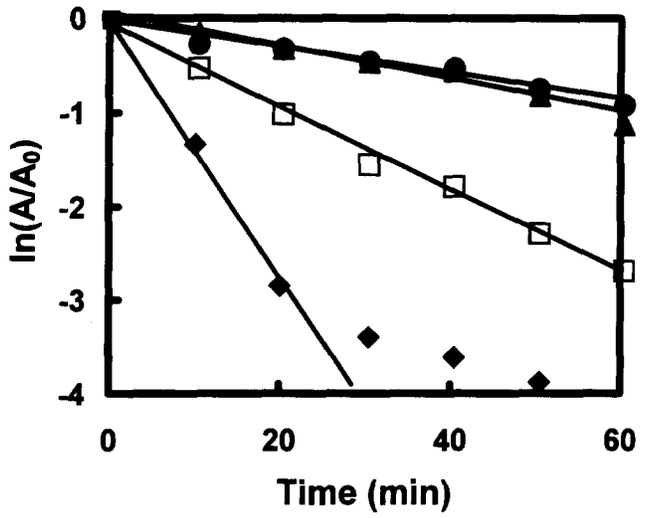

Fig. 1. Time course of thermal inactivation (40°C) of PQQGDHs. A, Enzymatic activity; A_0, enzymatic activity of unheated sample. (▲) E97A3, (●) E95A5, (□) *E. coli* PQQGDH, (◆) E99A1.

3.2. Thermal stability of the chimeric PQQGDH, E97A3

The thermal stability of E97A3 was investigated. Fig. 2 shows the residual activity of PQQGDHs after 10 min. of incubation at each temperature. Over the range we tested, the chimeric PQQGDH, E97A3, showed the highest thermal stability. Among the samples we tested, *A. calocoaceticus* PQQGDH was most sensitive to thermoinactivation.

The inactivation time courses of purified PQQGDHs were also described by first-order kinetics. This was confirmed at every temperature we studied. The first order rate constant (k) and half life time ($t_{1/2}$) of E97A3 at 40°C were 2.6×10^{-4} s^{-1} and 2.7×10^3 s, whereas those of *E. coli* and *A. calcoaceticus* were 8.4×10^{-4} s^{-1} (8.2×10^2 s), and 3.1×10^{-3} s^{-1} (2.1×10^2s), respectively. This indicated that E97A3 was more than 3 times as stable as *E. coli* PQQGDH and 12 times more stable than *A. calcoaceticus* PQQGDH.

Transition state theory yielded values of enthalpy (ΔH^*) and entropy (ΔS^*) of enzyme inactivation. From these, the free energy of thermoinactivation (ΔG^*) was determined for each PQQGDH (Fig. 3). DH* of E97A3 increased markedly in comparison with that of *E. coli* GDH, whereas the increase in ΔS^* was small, resulting in a higher ΔG^* than for *E. coli* PQQGDH. These thermodynamic parameters are summarized in Table 1.

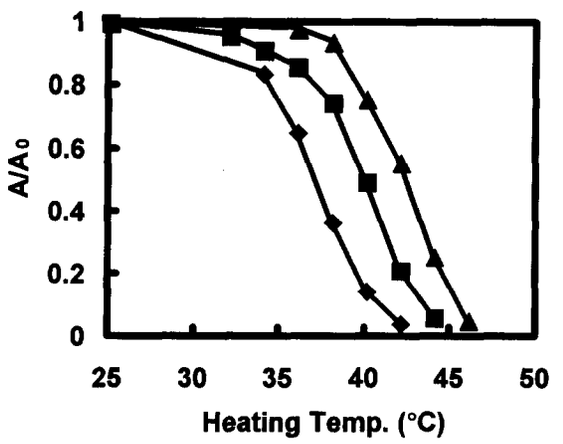

Fig. 2. The thermal stability of PQQGDHs. After 10 min of incubation at each temperature, the residual activity was determined at 25°C. (▲) E97A3, (■) *E. coli* PQQGDH, (◆) *A. calcoaceticus* PQQGDH.

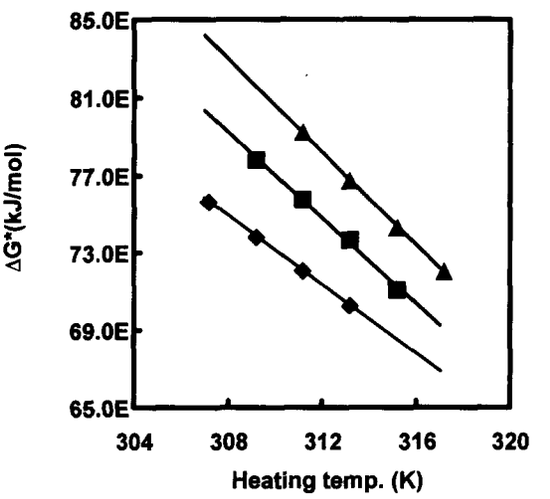

Fig. 3. The free energy of inactivation of PQQGDHs. (▲) E97A3, (■) *E. coli* PQQGDH, (◆) *A. calcoaceticus* PQQGDH.

4. Discussion

In this paper, we reported the improved thermal stability of *E. coli*/*A. calcoaceticus* chimeric PQQGDHs, in comparison with both parental enzymes. Thermal stabilities of E97A3 and

Table 1
Thermodynamic parameters for wild-type and chimeric-type

Strain	ΔH^* (kJ/mol)[a]	ΔS^* (kJ/deg·mol)[b]	ΔG^* (kJ/mol) at 40°C
E97A3	452	1.20	77.0 ± 0.1
A. calcoaceticus	350	0.89	70.5 ± 0.1
E. coli	422	1.11	73.9 ± 0.1

Strain	$\Delta\Delta H^*$ (kJ/mol)	$\Delta\Delta S^*$ (kJ/deg·mol)	$\Delta\Delta G^*$ (kJ/mol) at 40°C
E97A3 to *A. calcoaceticus*	102	0.31	6.5 ± 0.1
E97A3 to *E. coli*	30	0.09	3.1 ± 0.1

[a,b] All r^2-values > 0.99 (r = correlation coefficient).
$\Delta\Delta H^*$, $\Delta\Delta S^*$ and $\Delta\Delta G^*$ are the difference in enthalpy, entropy, and free energy of thermoinactivation between E97A3 and parental enzymes, *E. coli* and *A. calcoaceticus* PQQGDHs, respectively.

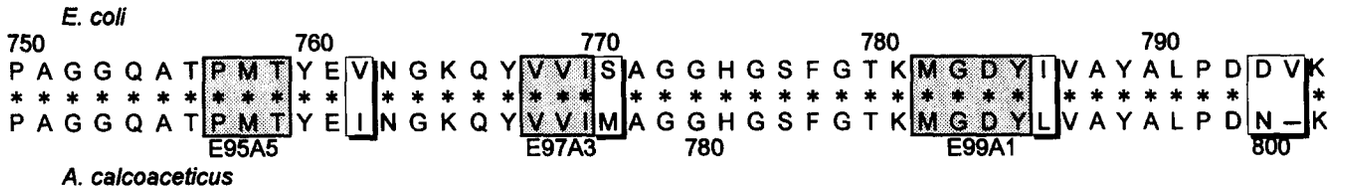

Fig. 4. C-Terminal regions of *E. coli* and *A. calcoaceticus* PQQGDHs and the site of recombination in each chimera. Shaded boxed residues are the site of recombination in each chimera. Open boxed residues are distinct in the two sequences. *Shows the identical residues.

E95A5 were found to be higher than those of both parental enzymes. As shown in Fig. 2, and Tables 1, *A. calcoaceticus* PQQGDH was less stable than *E. coli* PQQGDH, and the thermal stability of chimeric PQQGDHs obtained was therefore not due to the characteristic property of *A. calcoaceticus* PQQGDH.

Fig. 4 shows the C-terminal regions of *E. coli* and *A. calcoaceticus* PQQGDHs, and the site of recombination. Based on the *E. coli* PQQGDH sequence, E97A3 had 4 substitutions in the C-terminal sequence; S771M, I786L, D794N, and V795 deletion. As a result, it caused the increase in ΔG^* of thermal inactivation ($\Delta\Delta G^*$; 3.1 kJ·mol^{-1}). An additional substitution, V762I, occured in E95A5. It is quite interesting that the difference between E97A3 and E99A1 was only one amino acid residue (S771M), although E97A3 was more stable than the parental enzyme whereas E99A1 was less stable.

There is considerable interest in improving the stability of proteins by protein engineering. The common approaches so far reported can be divided into 4 strategies: reducing the ΔS^* of inactivation [10]; increasing in hydrophobic interaction in the interior core region [11]; reducing the water-accessible hydrophobic surface area [12]; and stabilizing the dipoles of the helical structure [13]. The $\Delta\Delta S^*$ of E97A3 between *E. coli* PQQGDH was positive, and thermal stabilization, therefore, did not come from decrease in ΔS^*. In contrast, the increase in ΔG^* in this case was the result of an increase in ΔH^* ($\Delta\Delta H^*$; 30 kJ/mol). The increase in ΔG^* ($\Delta\Delta G^*$; 3.1 kJ/mol) was relatively small in comparison with various recently reported various site directed mutagenesis studies in which thermal stability was enhanced by increasing hydrophobic interaction [11]. The amino acid residue at V795 observed in *E. coli* PQQGDH was deleted in the chimeras. S771M substitution caused increase in hydrophobicity, although I786L substitution resulted in decreased hydrophobicity. D794N substitution caused elimination of negative charge. Therefore, it should be noted that the cause of increased thermal stability of E97A3 was the result of a complex of negative and positive effects on thermal stability. Considering that E99A1 was less stable than parental PQQGDH, V795 deletion, and D794N and I786L substitutions may contribute to the decrease in the thermal stability, whereas S771M in E97A3 may enhance thermal stability due to the increase in hydrophobic interaction. Further studies based on site directed mutagenesis of the previously mentioned residues will elucidate the role of each substitution in the changing thermalstability.

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