Vol. 211, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 6, 1995 Pages 268-273

ELUCIDATION OF THE REGION RESPONSIBLE FOR EDTA TOLERANCE IN PQQ GLUCOSE DEHYDROGENASES BY CONSTRUCTING *ESCHERICHIA COLI* AND *ACINETOBACTER CALCOACETICUS* CHIMERIC ENZYMES

Koji SODE*, Hiromi YOSHIDA, Kazunori MATSUMURA, Tomoko KIKUCHI, Mika WATANABE, Nozomu YASUTAKE, Shunsuke ITO and Hiroyuki SANO

Department of Biotechnology, Faculty of Technology,
Tokyo University of Agriculture and Technology,
2-24-16 Naka-cho, Koganei-shi, Tokyo 184 , JAPAN

Received April 27, 1995

SUMMARY We constructed various chimeric PQQ glucose dehydrogenases (PQQGDHs) from an EDTA-sensitive PQQGDH from *Escherichia coli* and an EDTA-tolerant PQQGDH from *Acinetobacter calcoaceticus* by homologous recombination of their structural genes. The EDTA tolerance of the resulting chimeric enzymes was investigated. Our results demonstrated that EDTA tolerance of PQQGDHs can be completely altered by substituting each corresponding region. The EDTA tolerance of *A.calcoaceticus* PQQGDH is mostly within a region composed of about 90 amino acid residues located between 45 and 56 % of the distance from the N-terminal region.

Membrane binding glucose dehydrogenases (GDHs), which possess pyrroloquinoline quinone (PQQ) as the prosthetic group, have been found in variety of Gram negative bacteria (1). They have highly homologous primary structures, but the enzymatic characeristics are strain specific. Although their tertiary structures have not yet been studied, elucidation of the region responsible for each enzymatic character will be carried out by homology analysis together with appropriate mutagenesis studies.

Two types of PQQGDH have been proposed (2), Type I PQQGDHs, which are easily inactivated by EDTA treatment, and Type II PQQGDHs, which are stable toward EDTA treatment. Based on homology analyses between *E.coli* PQQGDH (Type I) and *A.calcoaceticus* PQQGDH (Type II), we previously reported a site directed mutagenesis study (3) on the putative PQQ binding site (1), which resulted in the slight increase in the EDTA tolerance, but far lower than that of *A.calcoaceticus* PQQGDH. Therefore, the restriction of the much limited region responsible for EDTA tolerance, not only based on

*To whom all correspondence should be addressed. Fax.; 81-423-88-7027.

0006-291X/95 $5.00

homology analyses, but supported by experimental results will be essential, prior to systematic side directed mutagenesis studies. Recently, the construction and characterization of chimeric proteins within a gene family is recognized as a versatile and powerful method in order to elucidate the region responsible for each enzymatic character (4,5).

In this paper, we aimed to restrict the region responsible for EDTA tolerance in PQQGDHs. For this purpose, we constructed various chimeric PQQGDHs from *E.coli* and *A.calcoaceticus* enzymes using homologous recombination, and investigated their EDTA-tolerance to restrict the region.

MATERIALS AND METHODS

Bacterial strains *Escherichia coli* PP2418, the PQQGDH structural gene of which was inactivated by insertion mutagenesis, was kindly provided by Prof. N.Goosen (Leiden Univ., Netherlands) (6). *E.coli* JC8679 (ATCC47001) and *Acinetobacter calcoaceticus* IFO12552 were obtained from the American Type Culture Collection and the Institution of Fermentation, Osaka, respectively.

Construction of chimeric PQQGDHs In order to construct chimeric PQQGDHs from *E.coli* and *A.calcoaceticus* enzymes, we first carried out PCR amplification of each structural gene according to the sequence data (6,7).

The *E.coli* PQQGDH structural gene was amplified as in the previous study (3,6), but using the following oligonucleotide primers to create suitable restriction enzyme sites.

EcII; (forward)5'-GGCCATGGCAATTAACAATACAGGCTCGCGACGA-3'
*Nco*I

(reverse)5'-GGCCATGGCTACGCTAGCTGTGCAATCCATTGATT-3'
*Nco*I

EcIII; (forward)5'-CCGGATCCGCAATTAACAATACAGGCTCGCGACGA-3'
*Bam*HI

(reverse)5'-GGAAGCTTCTACGCTAGCTGTGCAATCCATTGATT-3'
*Hin*dIII

The *A.calcoaceticus* IFO12552 derived PQQGDH gene was amplified using oligonucletide primers designed from the sequence data of the *A.calcoaceticus* L.M.D.79-41 PQQGDH-A structural gene (7).

AcI; (forward)5'-GGCCATGGATCAACCTACTTCAAGATCAGGTTTAACG-3'
*Nco*I

(reverse)5'-CCGTCGACTTATTTGTTATCTGGTAAGGCATAAGCCAC-3'
*Sal*I

These oligonucleotides were synthesized using a DNA synthesizer (ABI Model 391). PCR amplification was carried out using Taq polymerase (Promega Co.) under the following conditions; denaturation; 95 °C, 1 min, annealing; 55 °C, 2 min, polymerization; 72 °C, 3 min, 30 cycles, using genomic DNA obtained from *E.coli* DH5α and *A.calcoaceticus* IFO12552 as templates. The PCR product was cloned into an expression vector, pTrc99A (Pharmacia, Sweden), after digestion with the corresponding re-

striction enzyme(s) (*Nco*I for EcII, *Bam*HI and *Hin*dIII for EcIII, *Nco*I and *Sal*I for AcI) and was transformed into *E.coli* PP2418. Transformants were identified by their ability to complement the PQQGDH activity of *E.coli* PP2418. Plasmids containing genes encoding active PQQGDH were then taken up. The plasmids encoding the structural genes of *E.coli* DH5 α PQQGDH were designated as pGEcII, pGEcIII, and the plasmid encoding the structural gene of *A.calcoaceticus* IFO12552 was designated pGAcI (Figure 1), and they were sequenced using an automated DNA sequencer (DSQ-1 Shimadzu, Japan). The Cloned fragment from pGAcI was digested with *Nco*I and *Sal*I, made blunt-ended, and inserted into the multi cloning site of pUC19 after *Sma*I digestion (the *Nco*I site was reformed by the ligation). The plasmid obtained was designated pUCAc (Figure 1).

Chimeric PQQGDHs were constructed according to Ogawa et al.(8). PQQGDH genes from *E.coli* or *A.calcoaceticus* obtained as described above, were cloned in tandem (pGEAc and pGAcE; Figure 1). After digestion with appropriate restriction enzymes (*Kpn*I and *Sma*I for pGEAc, *Nru*I and *Sal*I or *Msc*I and *Sal*I for pGAcE), linearized plasmids were transformed into JC8679 for the homologous recombination. Plas-

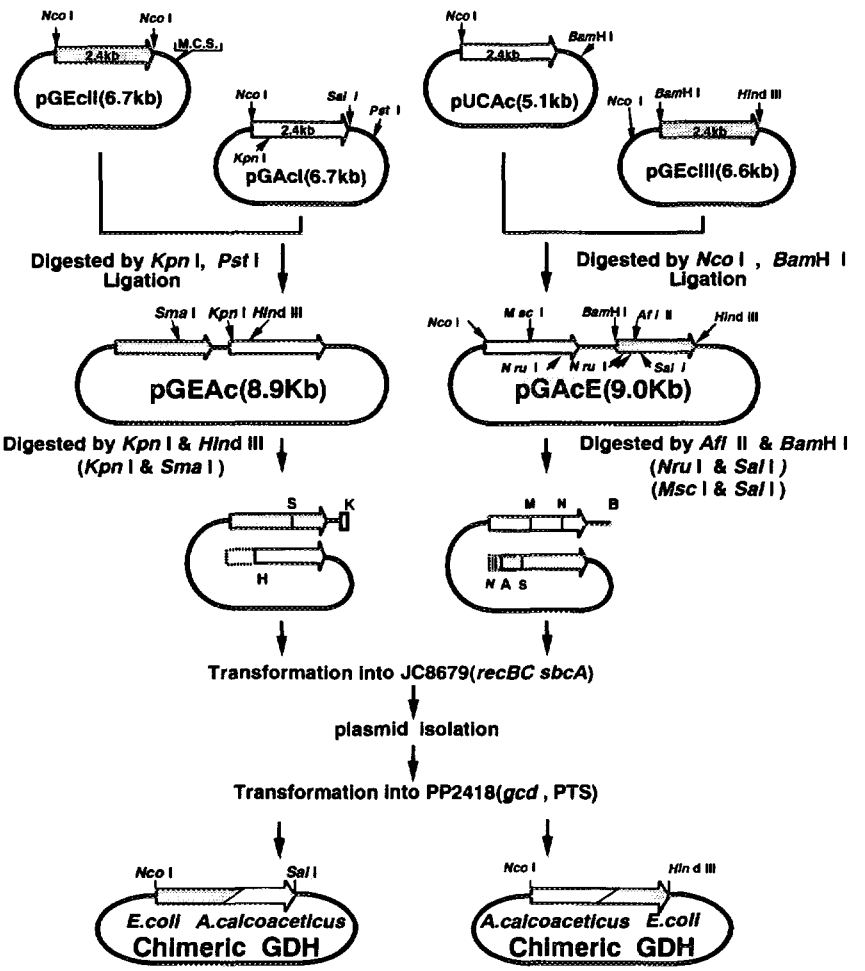

Figure 1. Construction of chimeric PQQGDH genes.

2.4kb ; *E.coli* PQQGDH gene.

2.4kb ; *A.calcoaceticus* PQQGDH gene.

mids from transformants were then extracted, and transformed into PP2418. Transformants containing active chimeric PQQGDHs were then selected using EMB agar plates. Plasmids were isolated from each transformant and their PQQGDH sequence were analyzed.

Multi-chimeric enzymes, such as E10A49E41, were constructed with basically the same principle as above, but using the plasmid containing E10A90 chimera, constructed from pGEAc. This plasmid was digested with *Pst*I and a *Nco*I fragment of pGEcII containing the EcII region was inserted, after it was made blunt ended. After *Sma*I digestion (for the construction of E10A87E3 and E10A74E16) or *Sal*I and *Msc*I double digestion (for the construction of E10A35E55 and E10A49E41), the plasmid was transformed into *E.coli* JC8679. Multi-chimeric enzymes were recovered similarly as described above.

Analysis of EDTA-tolerance of PQQGDHs EDTA-tolerance of each enzyme was analyzed using a solubilized membrane fraction prepared from *E.coli* PP2418 containing either pGEcI, pGEcII. pGEcIII, pGAcI, or from pTrc99A containing constructed chimeric PQQGDHs. The membrane fractions were obtained using a French pressure cell followed by ultracentrifugation. By adding 1 % Triton X-100, in a 10 mM potassium phosphate buffer (P.B) pH 7.0, PQQGDHs were solubilized on ice for 30 min. After ultracentrifugation, the supernatant was dialyzed against 10 mM P.B., pH 7.0, containing 0.2 % Triton X-100. The enzyme solution thus obtained was incubated in 10 mM P.B. pH 7.0, in the presence of 5 μM PQQ, 1 mM $MgCl_2$ and 0.2 % Triton X-100 at 25 °C for 1 hour to form the holo-enzyme. Holo-enzyme samples were then incubated in the presence of 10 mM EDTA. After 30 min of incubation at 25 °C, the residual PQQGDH activity was determined. PQQGDH activity measurement were carried out as follows. Enzyme samples were incubated in 10 mM P.B. pH 7.0, 0.6 mM phenazine methosulfate (PMS), 0.06 mM 2,6-dichrolophenolindophenol (DCIP) and 0.2 % Triton X-100. 198 μl of prepared sample was added to 2 μl of 2 M glucose. PQQGDH activity was determined using the decrease in absorbance of DCIP at 600nm.

RESULTS AND DISCUSSION

The residual activity of PQQGDHs was examined after 30 min of incubation in a buffer containing 10 mM EDTA. *E.coli* PQQGDH (EcI, EcII and EcIII) showed no activity after the incubation, therefore they were sensitive to EDTA treatment. In contrast, *A.calcoaceticus* PQQGDH was EDTA tolerance showing more than 80 % of initial activity after the EDTA treatment.

Therefore, we further utilized these structural genes as starting materials for the construction of chimeric PQQGDHs, in order to investigate the regions responsible for EDTA tolerance. Using homologous recombination , 12 types of chimeric PQQGDHs were constructed, including 8 types of EA chimeras possessing the N-terminal region of *E.coli* and the C-terminal region of the *A.calcoaceticus* PQQGDH structural gene, and also 4 types of AE chimeras possessing the N-terminal region of *A.calcoaceticus* PQQGDH and the C-terminal region of the *E.coli* PQQGDH structural gene. The amino acid sequences and the sites of recombination are shown in Figure 2. The EDTA tolerance of the constructed chimeras are listed in Figure 3. The EDTA-tolerance of these chimeras demonstrated that 4 of them, E10A90 (composed of 10 % of the *E.coli*

Figure 2. Alignment of sequences of *E.coli* PQQGDH and *A.calcoaceticus* PQQGDH.

———— show the sites of recombination in each chimera.

– – – – – – shows the region responsible for EDTA tolerance.

PQQGDH N-terminal region and 90 % of the *A.calcoaceticus* PQQGDH C-terminal region), E32A68, A21E79 and A45E55, showed remarkable properties with regard to EDTA-tolerance. Although, other chimeras containing the N-terminal region of *E.coli* PQQGDH did not show EDTA-tolerance, E10A90 and E32A68 showed EDTA-tolerance. In contrast, A21E79 and A45E55 showed EDTA sensitivity, whereas the rest of the chimeras containing the *A.calcoaceticus* PQQGDH N-terminal region were EDTA-tolerant. E10A90 and E32A68 retained more than 70 % the initial activity, at a similar level to PQQGDH from *A.calcoaceticus*, AcI. In contrast, A21E79 and A45E55 completely lost activity, as did PQQGDHs from *E.coli*, EcII and EcIII.

These results indicated that by substitution of each corresponding region of the PQQGDH, EDTA-tolerance was completely altered. Focusing that E32A68 gained EDTA tolerance but as both A45E55 and E56A44 were EDTA-sensitive, the region responsible for EDTA-tolerance may be located between 45 and 56 % of the distance from the N-terminal region of *A.calcoaceticus* PQQGDH, corresponding to about 90 amino acid residues (Figure 2).

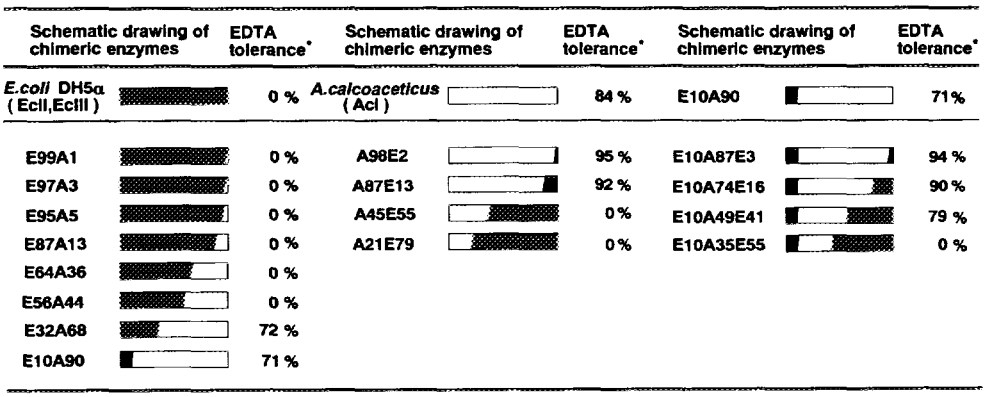

Figure 3. EDTA tolerance of chimeric PQQGDHs consisting of the *E.coli* PQQGDH and *A.calcoaceticus* PQQGDH genes.
* Residual activity after 30 min of incubation in the presence of 10 mM EDTA.

In order to confirm the importance of this region, we further constructed following 4 different multi-chimeric PQQGDHs based on E10A90; E10A87E3, E10A74E16, E10A49E41 and E10A35E55. Among these 4 multi-chimeric PQQGDHs, only E10A35E55 was EDTA-sensitive, whereas 3 other chimeras were tolerant. These results suggested that the region responsible for EDTA tolerance may be located between 45 and 59 % of the distance from the N-terminal region. This region, composed of about 112 amino acid residues, included the previously mentioned 90 amino acid residues. Therefore, we propose that the region composed of about 90 amino acid residues is responsible for EDTA tolerance of *A.calcoaceticus* PQQGDH.

In conclusion, we have succeeded in restricting the region responsible for EDTA tolerance in PQQGDHs. Further systematic site directed mutagenesis studies are expected based on the elucidated region.

ACKNOWLEDGMENTS

The authors thank Dr.J.G.Burgess and Dr.R.Thornhill in compiling this manuscript.

REFERENCES

1. Anthony, C. (1992) *Int. J. Biochem.* **24**, 29-39
2. Dokter, P., Frank, J.Jzn., and Duine, J.A. (1986) *Biochem. J.***239**, 163-167
3. Sode, K. and Sano, H. (1994) *Biotechnol. Lett.* **16**, 455-460
4. Ogawa, T., Shinohara, A., Ogawa, H. and Tomizawa, J. (1992) *J. Mol. Biol.* **226**, 651-660
5. Miyazawa, K., Kawaguchi, S., Okamoto, A., Kato, R., Ogawa, T. and Kuramitsu, S. (1994) *J. Biochem.* **115**, 568-577
6. Cleton-Jansen, A. M., Goosen, N., Fayet, O. and van de Putte, P. (1990) *J. Bacteriol.* **172**, 6308-6315
7. Cleton-Jansen, A. M., Goosen, N., Odle, G. and van de Putte, P. (1988) *Nucleic Acids Res.* **16**, 6228