Stability-increasing mutants of glucose dehydrogenase

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Four stability-increasing mutants of glucose dehydrogenase from *Bacillus megaterium* were purified together with the wild-type enzyme; each has a single amino acid change of Glu-96 to Gly, Glu-96 to Ala, Gln-252 to Leu, or Tyr-253 to Cys. These mutant enzymes are more heat resistant at pH 6.5 than the wild-type enzyme; the replacement of Glu-96 by Ala increases the thermostability by about 20°C. The mutant enzymes are also resistant to inactivation in alkaline solutions. The replacement of Glu-96 by Gly or Ala protects the enzyme from alkaline inactivation almost completely. The kinetic constants for the activities of these mutant enzymes do not differ significantly from those of the wild-type enzyme.

Glucose dehydrogenase; Stability-increasing mutant; Subunit interaction

1. INTRODUCTION

Glucose dehydrogenase (EC 1.1.1.47) from *Bacillus megaterium* is a tetrameric enzyme with four identical subunits [1]. The enzyme is inactivated in alkaline solutions, and the inactivation is due to the reversible dissociation of the tetramer into inactive protomers [2-4]. In our previous work [5], we isolated a glucose dehydrogenase gene from *B. megaterium* IWG3, and obtained 8 kinds of mutant genes coding for stability-increasing mutant enzymes. The amino acid substitutions in these mutant enzymes must have much information about the roles of these amino acid residues in the stabilization or destabilization of the inter-subunit interaction and/or tertiary structure of the protomer.

Abbreviations: E96G, E96A, Q252L, and Y253C are glucose dehydrogenase mutants with the amino acid changes of Glu-96 to Gly, Glu-96 to Ala, Gln-252 to Leu, and Tyr-253 to Cys, respectively; kb, kilobase pair

In this work, we have purified from *Escherichia coli* cells the wild-type enzyme and the following four mutant enzymes: E96G, E96A, Q252L, and Y253C. These mutant enzymes have single amino acid substitution of different types, and they should provide much clearer information than other mutants with two or more substitutions.

2. EXPERIMENTAL

2.1. *Materials*

Restriction endonucleases (*Eco*RI and *Pst*I) and T4 DNA ligase were purchased from Toyobo Co. Ltd (Osaka); DNase was from Sigma (St. Louis, MO). NAD was a generous gift from Kohjin Co. Ltd (Tokyo). NADP was purchased from Kohjin Co. Ltd (Tokyo); DEAE-Sepharose CL-6B was from Pharmacia (Uppsala). Other chemicals were obtained from Nakarai Tesque (Kyoto). *E. coli* KP3998 (F⁻, *hsdS20*(r̄ᴮm̄ᴮ) *ara-14 proA2 lacⁱ galK2 rpsL20 xyl-5 mtl-1 supE44*, lambda⁻) [6] and pKP1500 [6] were generous gifts from Dr Taiji Imoto (Kyushu University). pGDA2, a hybrid plasmid containing a glucose dehydrogenase gene from *B. megaterium* IWG3, and the other related plasmids containing mutant dehydrogenase genes were prepared as described previously [5]. The *Eco*RI-*Pst*I 0.9-kb DNA fragments of pGDA2 and the related plasmids were reinserted into pKP1500 by the methods as described previously [5] for hyperproduction of the enzymes.

2.2. Purification of glucose dehydrogenases

E. coli KP3998 cells harboring a plasmid containing the wild-type or each mutant gene of glucose dehydrogenase were grown on LB medium [7] (2 l) containing 0.15 mg/ml ampicillin. The cells were suspended in 40 ml of 50 mM sodium phosphate, pH 6.5, containing 2 M NaCl and DNase (final 5 μg/ml), and disrupted with a French pressure cell (SLM Instruments, Inc.). The supernatant obtained by centrifugation was heated at 60°C for 20 min, and the precipitate was removed by centrifugation. The supernatant was dialyzed against 50 mM sodium phosphate, pH 6.5, and applied to a DEAE-Sepharose CL-6B column (4.2 × 45 cm) equilibrated with the same buffer. The column was washed with 500 ml of 50 mM sodium phosphate, pH 6.5, and the enzyme was eluted with an NaCl gradient of 0–0.4 M (total 2 l). Active fractions were pooled, concentrated by ultrafiltration, dialyzed against 50 mM sodium phosphate, pH 6.5, containing 2 M NaCl, and used as the purified enzyme. The homogeneity of the purified enzymes (wild type, E96G, E96A, Q252L, and Y253C) was checked by SDS-polyacrylamide gel electrophoresis [8]. When other minor bands were observed, the enzyme solution was heated again at 60°C for 20 min, and the supernatant was used as the purified enzyme after checking the homogeneity as described above.

2.3. Enzyme assay

Initial rates of enzyme reactions were measured using a Hitachi 220A spectrophotometer with a magnetic stirrer and a thermostatted cell compartment. The reactions were recorded as the increase in absorbance at 340 nm (NADH or NADPH concentration); the NAD(P)H concentration was calculated using a molar absorption coefficient of 6300 $M^{-1} \cdot cm^{-1}$. Unless otherwise stated, the assay mixture contained 75 mM Tris-HCl, pH 8.0, 2.0 mM NAD, and 0.1 M D-glucose. The initial-rate measurements were made at least in triplicate with a reproducibility of less than 5% error. The kinetic constants were estimated using the computer program (SEQUEN) of Cleland [9]. The concentrations of NAD(P) and glucose dehydrogenase (tetramer) were measured spectrophotometrically using the molar absorption coefficients of 18000 $M^{-1} \cdot cm^{-1}$ at 260 nm and 132000 $M^{-1} \cdot cm^{-1}$ at 280 nm [10], respectively, assuming a relative molecular mass of 112400 [5] for the enzyme.

3. RESULTS

The four mutants of glucose dehydrogenase, E96G, E96A, Q252L, and Y253C, were selected by increasing heat resistance at 60°C [5]. Fig.1 shows that the heat resistance of the wild-type enzyme is clearly increased by any one of the amino acid substitutions observed in these mutant enzymes. The order of the thermostability at pH 6.5 is Y253C < Q252L < E96G < E96A, and the replacement of Glu-96 by Ala increases the thermostability by about 20°C. It is noteworthy that E96A is more stable than E96G; this difference in the stability is due to the presence of the methyl group at position 96.
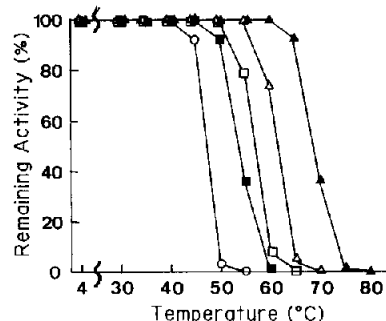

Fig.1. Thermostability of wild-type and mutant glucose dehydrogenases. The enzymes were incubated at the indicated temperature for 20 min in 50 mM phosphate, pH 6.5, and the remaining activity was measured as described in section 2. (○) Wild-type; (△) E96G; (▲) E96A; (□) Q252L; (■) Y253C.

These thermostable mutant enzymes are also resistant to inactivation in alkaline solutions (fig.2A). The amino acid replacement of Tyr-253 by Cys increases the stability at pH higher than 8, and the replacement of Glu-96 by Gly or Ala stabilizes the enzyme even more in this pH range. It is interesting that E96G and E96A show similar pH stability in contrast to the difference in thermostability. The type of the stabilization due to the replacement of Gln-252 by Leu seems to be different from that of the others; this replacement shifts the pH-stability curve to the alkaline side by about one pH unit. For the pH stability in the acidic region, on the other hand, E96G and E96A show similar stability to the wild-type enzyme, and Y253C is less stable in this region; but Q252L is more stable than the others.

Fig.2B shows the pH-stability profiles in the presence of 2 M NaCl. The addition of NaCl stabilizes all the enzymes in the whole pH range examined. In the alkaline region, the effects of NaCl are great for the wild-type enzyme and Q252L, but is rather small for E96G, E96A, and Y253C; in the presence of 2 M NaCl, Y253C is the most unstable enzyme.

The effects of these amino acid substitutions on the catalytic activity of glucose dehydrogenase were then studied. We measured the kinetic constants of the wild-type and mutant enzymes in the general initial-rate equation:

$$k_{cat}[E]/v = 1 + K_a/[A] + K_b/[B] + K_{ia}K_b/([A][B])$$

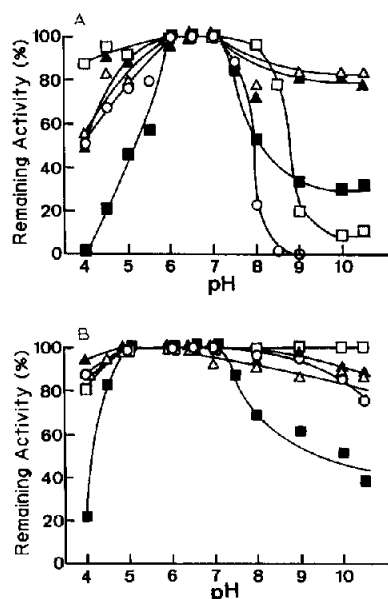where E is the enzyme, A is NAD or NADP, B is

Fig.2. Effects of pH on the stability of wild-type and mutant glucose dehydrogenases. The enzymes were incubated at 30°C for 20 min in the following buffer solutions without (A) or with (B) 2 M NaCl: 75 mM acetate, pH 4–5.5; 75 mM phosphate, pH 6–7.5; 75 mM Tris-HCl, pH 8 and 8.5; 75 mM glycine-NaOH, pH 9–10.5. (○) Wild-type; (△) E96G; (▲) E96A; (□) Q252L; (■) Y253C. After the incubation at each pH, A_{280} and the activity were measured as described in section 2.

D-glucose, k_{cat} is the turnover number, K_a and K_b are the limiting Michaelis constants for NAD(P) and D-glucose, respectively, and K_{ia} is the dissociation constant of the enzyme-NAD(P) complex. Table 1 shows the results for the assay system in which NAD or NADP was used as a coenzyme. The k_{cat} values of E96G and E96A are similar and about 30% of that of the wild-type enzyme, the value of Y253C is about 60%, and the value of Q252L is similar to that of the wild-type enzyme. The values of K_a, K_b, and K_{ia} of the mutant enzymes are not much different from those of the wild-type enzyme, except that the K_b values of Y253C are about 5–8 times larger than that of the wild-type enzyme. These results indicate that these amino acid substitutions do not change the catalytic efficiency and the affinities for NAD, NADP, and D-glucose of this dehydrogenase very much.

4. DISCUSSION

It has been reported that the inactivation of glucose dehydrogenase in alkaline solutions is due to the reversible dissociation of the tetramer into inactive protomers [2–4]. As all the mutant en-

Table 1

Kinetic constants of wild-type and mutant glucose dehydrogenases

Enzyme	Coenzyme	k_{cat} (s^{-1})	K_a (mM)	K_b (mM)	K_{ia} (mM)
Wild-type	NAD	390 ± 11	0.37 ± 0.03	9.5 ± 0.5	0.65 ± 0.04
E96G	NAD	114 ± 4	0.17 ± 0.05	16 ± 2	0.42 ± 0.14
E96A	NAD	97 ± 2	0.31 ± 0.04	8.7 ± 1.0	0.82 ± 0.20
Q252L	NAD	310 ± 8	0.19 ± 0.02	9.0 ± 0.5	0.24 ± 0.03
Y253C	NAD	200 ± 5	0.25 ± 0.03	55 ± 3	0.41 ± 0.03
Wild-type	NADP	260 ± 3	0.027 ± 0.003	11 ± 0.5	0.075 ± 0.014
E96G	NADP	92 ± 0.7	0.0076 ± 0.0018	18 ± 0.4	0.073 ± 0.006
E96A	NADP	98 ± 0.9	0.0093 ± 0.0020	13 ± 0.5	0.096 ± 0.009
Q252L	NADP	300 ± 2	0.026 ± 0.008	10 ± 0.2	0.028 ± 0.002
Y253C	NADP	186 ± 5	0.037 ± 0.004	85 ± 3.6	0.042 ± 0.003

Initial rates were measured as described in section 2 except for the concentrations of NAD, NADP, and D-glucose. When NAD was used as a coenzyme, the ranges of NAD concentration are 0.21–1.79 mM for the wild-type enzyme, 0.67–3.71 mM for E96G and E96A, 0.32–1.89 mM for Q252L, and 0.31–1.92 mM for Y253C; the ranges of D-glucose concentration are 2.2–20 mM for the wild-type enzyme and Q252L, and 20–200 mM for E96G, E96A, and Y253C. When NADP was used as a coenzyme, the ranges of NADP concentration are 0.087–1.92 mM for the wild-type enzyme, 0.12–1.95 mM for E96G and E96A, and 0.045–1.92 mM for Q252L and Y253C; the ranges of D-glucose concentration are 2.5–50 mM for the wild-type enzyme, 20–100 mM for E96G, E96A, and Y253C, and 5–100 mM for Q252L. Results are expressed ± SE

zymes examined are more resistant against the alkaline dissociation, each amino acid substitution observed for the mutant enzymes must stabilize the tetrameric structure in alkaline solutions by strengthening intersubunit association. One of the possible mechanisms for the dissociation of the wild-type tetramer in alkaline solutions is the destabilization of the tetrameric structure by inter-subunit anion-anion repulsion that is not present in neutral solutions. Glu-96 is a strong candidate for one of the anions, because the replacement of Glu-96 by Gly or Ala prevents the alkaline inactivation of the enzyme almost completely, and the alkaline stability is as high as that of the wild-type enzyme in 2 M NaCl; the presence of 2 M NaCl must reduce electrostatic interactions between charged groups.

Tyr-253 may be the counterpart of Glu-96, because the tyrosyl residue ionizes in the alkaline region and can generate intersubunit anion-anion repulsion. The lower stability of Y253C than E96G seems to be due to complex effects of the replacement of Tyr-253 by Cys, a residue with a similar pK_a value but with a smaller size: this replacement may stabilize the tetrameric structure in alkaline solutions by shifting the position of the anionic group, but at the same time, may destabilize the structure by reducing some intersubunit hydrophobic interactions. This destabilization effect of this replacement seems to be expressed as the difference between the pH-stability curves for E96G and Y253C in fig.2A or the difference between the curves for the wild-type enzyme and Y253C in fig.2B. It should also be pointed out that the effects of NaCl on the alkaline stability of Y253C are much smaller than that of the wild-type enzyme; this suggests that the anion-anion repulsion in the alkaline region is much relieved by this substitution.

The mechanism of the stabilization by the substitution of Leu for Gln-252 is different from that of the other substitutions, because this substitution does not change the electric charge of the enzyme. Fig.2A shows that Q252L is more stable than the wild-type enzyme in the whole pH range, but is still inactivated in alkaline solutions.

In addition, Q252L is markedly stabilized by NaCl (fig.2B). Therefore, the mechanism of the stabilization by this substitution seems to be the strengthening of intersubunit hydrophobic interactions, and the fundamental cause of the alkaline instability of the wild-type is not eliminated by this substitution.

The glucose dehydrogenase mutants described in this paper clearly show that the stabilization of the oligomeric structures of enzymes is an effective method for preparing mutant enzymes with enhanced stability. It is surprising that the one point mutation of Glu-96 to Ala not only increases the thermostability by about 20°C, but also increases the alkaline stability and completely changes the pH-stability profile. These mutant enzymes should provide a good system for investigating intersubunit interaction. At present, we are investigating the effects of various amino acid substitutions on the dissociation constant of the tetramer.

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