Characterization of Two Naturally Occurring Mutations Close to Cofactors in Human Dihydrolipoamide Dehydrogenase

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Dihydrolipoamide dehydrogenase (E3) (dihydrolipoamide: NAD^+ oxidoreductase; EC 1.8.1.4) exists as a common component in three α-keto acid dehydrogenase complexes (pyruvate, α-ketoglutarate and branched-chain α-keto acid dehydrogenase complexes)[1] and the glycine cleavage system.[2] It catalyzes the reoxidation of the dihydrolipoyl group of the acyltransferase components of the α-keto acid dehydrogenase complexes and the reoxidation of that of the hydrogen-carrier protein of the glycine cleavage system. It is a homodimeric enzyme containing one FAD as a prosthetic group at each subunit.[3] Two naturally occurring mutations near cofactors in human E3 were reported. One mutation was from E3 deficient patients of Ashkenazi Jewish.[4] A common mutation of Gly-194 to Cys was found in their E3 sequences. Their clinical symptoms were the recurrent attacks of vomiting, abdominal pain, neonatal suffering, muscle weakness and exertional fatigue. Another mutation in a boy having reduced E3 activity was also reported.[5] At the age of ten weeks, his clinical symptoms were vomiting, progressive hypotonia and a severe lactic acidosis. He possessed a mutation of Met-326 to Val in his E3 sequence.

Site-directed mutagenesis method has been a useful tool for the structure-function study of human E3 and other proteins.[6-11] By the site-specific mutations of Gly-194 to Cys and Met-326 to Val, the effects of these naturally occurring mutations close to cofactors in human E3 structure and function were examined. Site-directed mutagenesis was performed using a mutagenesis kit according to the provided protocol. PCRs were carried out using the human E3 expression vector pPROEX-1:E3 as a template in a programmable PCR machine with the appropriate primers. Whole DNA sequence of human E3 coding region was sequenced to verify the integrity of DNA sequences and the mutations.

Purification of the Val-326 mutant was unsuccessful. There was not enough of the Val-326 mutant present to be purified when its expression was induced in *E. coli*. The SDS-PAGE gel showed that very small amounts of the mutant were present in the soluble fraction. This indicated that the mutant could have problems in its structural stability and/or proper folding processes while it was expressed in *E. coli*. The Met-326 is located very close to both FAD and NAD^+ in the E3 structure as shown in Figure 1A and involved in several direct interactions with them. The substitution of the Met-326 with smaller Val probably causes structural changes that affect more directly the bindings of near FAD and NAD^+ to the enzyme (Figure 1B). These structural changes could be detrimental enough to destroy the stable expression of the enzyme in *E. coli*.

The Cys-194 mutant was expressed in *E. coli* enough to be purified and characterized even though the amounts of purified enzyme were about 12-fold lesser than those of normal enzyme. The purification of the Cys-194 mutant was performed using nickel affinity column according to the provided protocol. SDS-PAGE gel showed that the mutant was highly purified. E3 assay was performed at 37 °C in a 50 mM potassium phosphate buffer (pH 8.0) containing 1.5

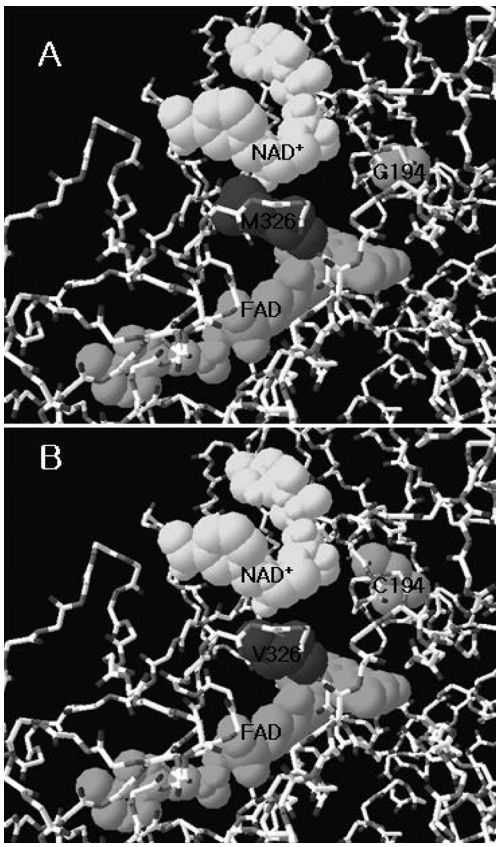

Figure 1. The location of Gly-194 and Met-326 in human E3 structure. The FAD, NAD^+ and mutated residues are shown in space-filled structures and other residues are shown in backbone structures. A; Before the mutations. B; After the mutations.

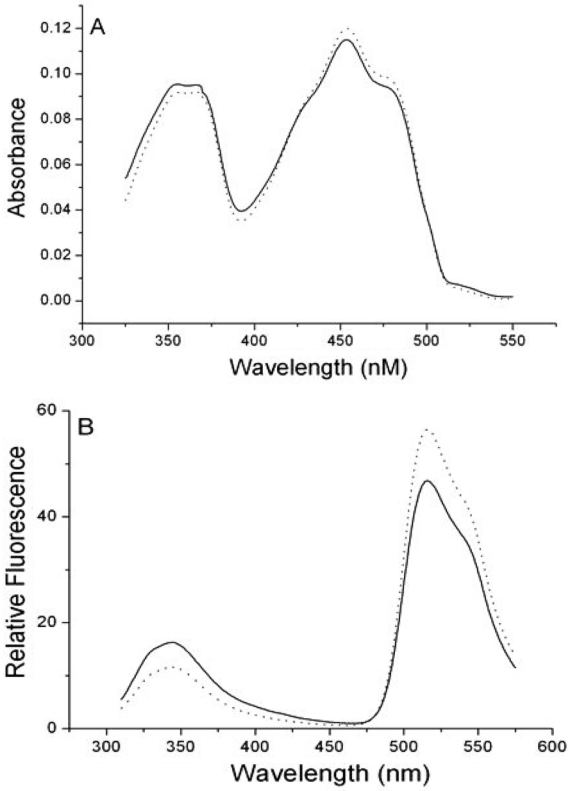

Figure 2. Spectroscopic properties of the Cys-194 mutant. A; UV-visible spectra of the mutant (solid line) and wild-type (dotted line) recombinant human E3s. B; Fluorescence spectra of the mutant (solid line) and wild-type (dotted line) recombinant human E3s.

mM EDTA with variable concentrations of the substrates. The k_{cat} value was determined as 674 s^{-1} which was about 25% lower than that (899 s^{-1}) of normal human E3. The K_m value for dihydrolipoamide was determined as 0.30 mM which was about 2.1-fold smaller than that (0.64 mM) of normal human E3, indicating that the mutation makes the enzyme more efficient to dihydrolipoamide. However, the K_m value for NAD^+ was determined as 1.23 mM which was about 6.5-fold larger than that (0.19 mM) of normal human E3, indicating that the mutation makes the enzyme less efficient to NAD^+. The Gly-194 is located in a hydrophobic environment close to NAD^+ as shown in Figure 1A. It also belongs to an α-helix which has residues that interact with both FAD and NAD^+. The introduction of larger and more polar Cys in the residue-194 likely causes a disturbance in the local structure of this region (Figure 1B). These structural changes could be responsible for these alterations in kinetic parameters of the mutant.

To examine any structural changes occurring in the Cys-194 mutant, UV-visible absorption and fluorescence spectroscopies were performed. Due to the prosthetic group FAD, human E3 has a characteristic UV-visible absorption spectrum of flavoproteins as shown in Figure 2A. The overall shape of the mutant spectrum (solid line) was similar to that of the wild-type human E3 spectrum (dotted line). However, slight differences were observed in overall ranges of spectra. Further clear evidence of the structural changes in the mutant came from the fluorescence study. When enzymes were excited at 296 nm, two fluorescence emissions were observed for both mutant and wild-type E3s, as shown in Figure 2B. The first emission from 305 nm to 400 nm was mainly due to Trp. The second emission from 480 nm to over 550 nm was due to FAD. In human E3, the Trp fluorescence was quenched due to energy transfer from Trp to FAD. When the fluorescence spectra were compared, a noticeable difference was found in the ratio between relative intensities of the first and second fluorescence emissions. The ratio (about 2.9) between relative intensities of the first and second fluorescence emissions of the mutant (solid line) was lower than that (about 4.9) of the wild-type enzyme (dotted line). This indicated that the energy transfer from Trp to FAD was disturbed in the Cys-194 mutant. The structural changes due to the mutation of Gly-194 to Cys could have affected the structure of human E3, interfering with the energy transfer from Trp residues to FAD.

In this study, the effects of two naturally occurring mutations close to cofactors in human E3 structure and function were examined using site-directed mutagenesis, E3 activity measurement and spectroscopic methods. The substitution of Met-326 with Val in human E3 was detrimental enough to destroy the stable expression of the enzyme in *E. coli*. The mutation of Gly-194 to Cys caused the structural changes which interfered with the efficient energy transfer from Trp residues to FAD. These structural changes could result in the altered kinetic parameters of the Cys-194 mutant. These findings indicated that both Met-326 and Gly-194 residues were important to the human E3 structure and function.

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