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一、中文摘要：

為了探討二氫硫辛酸去氫 (E3)的反應機制，反以本研究利用定點突變的方式創造出兩種 E3 的突變蛋白質，分別是 V188A, V188S 及 V188G 經大量表達此三突變蛋白質以及純化後發現其專一性的活性分別是正常 E3 的 27.3%, 7.9%及 1.6%。經 FAD 含量分析後發現其 FAD 含量並沒有因為突變取代而大量降低，分別是正常 E3 的 101%, 95%及 97.5%。分子量分析的結果顯示，突變蛋白仍保持其具有生物活性的雙體構造。動力學的研究顯示 V188A 正反應的 Kcat 下降至 36.2 % 而逆反應則維持正常大約為 94%。V188S 正反應的 Kcat 下降至 9.1%而逆反應則下降為 6.4 %。V188G 正反應的 Kcat 下降至 2.1%而逆反應則下降為 9.6%。其中 V188A 正反應中 NAD^+之 Km 增加大約 1.5 fold，V188G 正反應中 NAD^+之 Km 增加大約 2.8 fold。FAD 之氧化還原電位中點則由 E3 之-314mV 上升至 -304(V188A), -300(V188S)及-270(V188G)。此結果說明 V188 參與酵素的正逆反應中電子由 FAD 轉移至 NAD^+之機制。

關鍵詞：二氫硫辛酸去氫 ，定點突變，酵素動力學

Abstract

To investigate the reaction mechanism of human dihydrolipoamide dehydrogenase (E3), three mutant human E3s, V188A, V188S and V188G, were over-expressed, purified and characterized. The specific activities of both mutant proteins are 27.3%, 7.9% and to that of the wild-type E3. The FAD content analysis indicated that these three mutant E3s have about 101%, 95% and 97.5% of FAD content compared to that of wild-type E3. The molecular weight analysis showed that these three mutant proteins form the dimer. Kinetics data demonstrated that the Kcat of forward reaction of mutant proteins were decreased to about 36.2 % (V188A), 9.1%(V188S) and 2.1% (V188S). The Kcat of reverse reaction of V188A protein was remain normal (about 94%) while V188S protein was decreased (about 6.4 %) and V188G was decreased (about 9.6 %) compared to that of wild-type. For V188A and V188G mutant E3s, the Km for NAD^+ in the forward reaction increased by about 1.5 and 2.8 fold. The mid-point oxidation-reduction potential of mutant proteins were increased from –314 mV(E3) to –304(V188A), -300(V188S) and –270(V188G). It suggests V188 involved in electron transfer between FAD and NAD^+ of the catalytic function of the enzyme.

Keywords: Dihydrolipoamide Dehydrogenase, site-directed mutagenesis, enzyme kinetics.

二、Introduction

Mammalian dihydrolipoamide dehydrogenase [EC1.8.1.4], a common component of pyruvate dehydrogenase (PDC), α-ketoglutarate dehydrogenase (αKGDC), and branched-chain α-keto acid dehydrogenase complexes (BCKADC) (1-3), contains both an active disulfide and FAD that catalyzes the following reaction:

Dihydrolipoamide + NAD^+ + H^+ ←→ Lipoamide + NADH

The entire route of the electron transfer of the E3 is describes below.

DHL ←→ Active disulfide ←→ FAD ←→ NAD^+

In the forward reaction, the direction of electron flow is from dihydrolipoamide, through active disulfide, FAD to NAD^+. While in the reverse reaction, the electron flow is from NADH, FAD, through active disulfide, to lipoamide.

X-ray crystallographic and structural studies from Azotobacrer vinelandii (4), Pseudomonas putida (5) and Pseudomonas fluorescens, (6) and homologous comparison of the structure of glutathione reductase to the amino acid sequence of the E3 (7) shows that E3 has 4 different domains: the FAD, the NAD^+, the center, and the interface domain. Each FAD domain contains one FAD molecule. These domains of the molecule form the homodimer. The FAD and NAD domain of one subunit and the interface domain of the other subunit formed the active center, where V188 is located in the N-terminal region of αV α helix of the NAD binding domain.

In this study, with the use of site-directed mutagenesis, threeo mutant proteins, V188A, V188S and V188G were created. Following the analyses of molecular weight, FAD contents and initial velosity kinetics of the mutant proteins, V188 shows their effects of catalytic function in the electron transfer between FAD and NAD^+.

三、EXPERIMENTAL PROCEDURES

Construction of expression vectors

The construction of the expression vector and overexpression of human E3 in E. coli has been described previously (8). In order to create the V188A, V188S and V188G mutants, the mutagenic primer 5'-GGT GCA GGA GCA ATA GGT GTA -3' , 5'-GGT GCA GGA TCA ATA GGT GTA -3' and 5'-GGT GCA GGA GGA ATA GGT GTA -3' were used with the C-terminal primer LS (5'-GCG CGC GTC GAC TCA AAA GTT GAT TGA TTT -3') for an initial round of PCR with pQE-9:E3 as the template. The product of this PCR reaction was used as a primer with the N-terminal primer L1 (5'-GCG CGC GGA TCC GCA GAT CAG CCG ATT-3') for another round of PCR to generate the mutated version of E3. This PCR product was digested with the restriction endonuclease BamH1 an Sal1, and ligated to the expression vector pQE-9 previously digested with BamH1and Sal1, to generate pQE-9:E3 V188A, V188S and V188G. The sequence of the mutated E3s were confirmed by restriction endonuclease digestions and by DNA sequencing analysis (377 DNA Sequencer, Perkin-Elmer).

Overexpression and purification of E3s

A single colony containing pDM1.1 and pQE-9 with the appropriate insert (wild-type or mutant E3) was used to inoculate an 2ml of seed culture in Luria-Bertani (LB) broth containing 100 mg/ml ampicillin (9). This, after overnight culture at 37℃, was used to inoculate the main culture (200ml LB broth containing 100 mg/ml ampicillin). This culture was incubated at 37℃ with good aeration for 6 hours. At this point of time, isopropylthiogalactoside was added to a final concentration of 2 mM and the incubation was continued overnight at 30℃ with a vigorous aeration. The cells were harvested by centrifugation at 5,000 x g for 15 min at 4℃. The cell pellet was washed with 50 mM sodium phosphate buffer, pH 7.4 and the cells were pelleted once again, by centrifugation as before. The washed cell pellet was resuspended in the same buffer, and EDTA and lysozyme were added to a final concentration of 0.25 mM and 1 mg/ml respectively and the preparation was incubated on ice for 30 min with stirring occasionally. The cells were then broken by French press treatment at 20,000 pounds/square inch. The crude lysate was then subjected to further purification as described previously (8) with the following modifications. The elution of E3 from the Ni-nitrotriacetate agarose matrix was used imidazole stepwise from 0-100 mM with 10mM increase for each step instead of 0-100 mM gradient described previously (8). The eluted fractions were assayed for E3 activity and pooled for dialysis. After overnight dialysis with 50mM phosphate buffer, pH 7.4, 0.25mM EDTA and 50mM NaCl, proteins were applied to a DEAE-column, which was pre-equilibrium with water and eluted with the dialysis buffer. The eluted fractions were assayed for E3 activity and used for further analysis.

Determination of the FAD content

The procedure for the determination of the FAD content of E3 and its mutants has been described previously (8). Briefly, E3 protein (0.5 and 1 mg/ml) was subjected to UV-visible absorption spectroscopy (U3000, Hitachi). The absorption at 450 nm was used for the determination of FAD content. Standard curve was obtained by known concentrations of FAD.

Determination of molecular weight by molecular seiving

The experiment was performed by using the method of molecular sieving of High Performance Liquid Chromatography (HPLC). The column used for molecular sieving was Superdex 200(Pharmacia, Sweden). The mobile phase of HPLC was 50 mM potassium phosphate, pH 8, 200 mM NaCl. The pump was Hitachi L6200A. The outlet of the proteins was detected at UV 280nm by uv-vis spectrophotometric detector. The data was converted to ASCII file by Hitachi Model D-6500 DAD system manager and replotted with Origin program (MicroCal Inc.). The molecular standards are Bovine serum albumin (443Kd), Alcohol dehydrogenase(200Kd), β amylase(66Kd), Apoferritin(29Kd), and cytochrome c (12Kd).

Enzyme assays

Protein concentration was determined by Bradford method (10) using Bio-Rad Protein assay kit. The assay for detecting E3 activity and the determination of kinetic parameters were followed eviously (8) with the modifications to the ranges of the final concentrations of substrates and enzyme amounts used in the reactions. Kinetic parameters for the wild-type and mutant E3s were determined for both the forward and reverse reactions where possible, using appropriate range of varying substrate and cofactor concentrations. For the forward reaction (the assay buffer was: 50mM Potassium phosphate, 0.25mM EDTA pH 7.4), these were 0.1 to 3 mM range of NAD^+, 0.1 to 3 mM range of dihydrolipoamide with 100ng of proteins. In the reverse reaction (the assay buffer was: 50mM Potassium phosphate, 0.25mM EDTA pH 6.8), the kinetic parameters were determined with 0.01 to 0.3 mM range of NADH, 0.1 to 3 mM range of lipoamide with 100ng of proteins. All kinetics were done in triplicate, and double-reciprocal plots were drawn and the kinetics parameters were calculated with Grafit software (Erithacus Software, Ltd.).

四、Results and Discussion

Purification of overexpressed proteins

The purification of E3 was essentially as described earlier (8) with the modifications described in the previous section. The purification table of each protein is shown in Table 1. The purity of these proteins is examined by SDS-page(data not shown). All three proteins were expressed in significantly high amounts for structural and functional characterization. The specific activities of V188A, V188S and V188G were 27.3%, 7.9% 及 1.6% of that of wild-type E3 under standard E3 assay condition(50mM Potassium phosphate pH7.4, 0.25mM EDTA, 1.5 mM NAD^+, and 1 mM DHL). The change of E3 activity from mutant E3s may due to the FAD content, self-dimerization, enzyme-substrates binding and substrate turnover rate of the forward/reverse reaction. Therefore we performed the following studies to measure FAD contents, molecular weight and kinetic parameters of the mutant proteins.

The measurement of FAD contents

The FAD contents and protein monomer/FAD molar ratio were showed in the table 2. The FAD contents of V188A, V188S and V188G were 101%, 95% and 97.5% of that of wild-type E3. The protein monomer/FAD molar ratio of E3 is about 1:1. These data suggested that affinity of the enzyme for FAD is normal in V188A and only slightly reduced in V188S and V188G. This finding suggests that amino acid substitutions of V188 cause little alterations of the structure in the FAD binding region. Only slightly reduces the FAD-protein interaction and the dissociation of this cofactor from the active E3 dimer.

The measurement of Molecular weight

To examine whether the mutation affected the ability of self-dimerization of mutant proteins, the proteins was siezed using the method of molecular sieving of High Performance Liquid Chromatography (HPLC). The results are shown in the Figure 1. The retention time of E3 and all mutants are 28 min which represent the molecular weight of about 110KD. This result demonstrated that the amino acid substitutions had no effect in the self-dimerization and the mutant proteins formed in dimer.

Kinetic parameters

The kinetic parameters were determined for wild-type E3 and the three mutants described in this study for both the forward and reverse reactions catalyzed by this enzyme. These results are showed in Fig.2 and summarized in Table 3. In figure 2, both forward and reverse reaction of wild-type and mutant proteins have the "ping-pong" mechanism. From table 3, Kinetics data demonstrated that the Kcat of forward reaction of mutant proteins were decreased to about 36.2 % (V188A), 9.1%(V188S) and 2.1% (V188S). The Kcat of reverse reaction of V188A protein was remain normal (about 94%) while V188S protein was decreased (about 6.4 %) and V188G was decreased (about 9.6 %) compared to that of wild-type. For V188A and V188G mutant E3s, the Km for NAD^+ in the forward reaction increased by about 1.5 and 2.8 fold. In table 4, the mid-point oxidation-reduction potential of mutant proteins were increased from –314 mV(E3) to –304(V188A), -300(V188S) and –270(V188G).

In conclusion, the V188 does not involved in self-dimerization, FAD-binding and the reaction mechanism of both direction of the protein, but participates in the electron transfer between FAD and NAD^+ of the reaction mechanism.

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Figure 1. Molecular sieving on HPLC of E3 and E3 mutant proteins. Bovine serum albumin (443Kd), Alcohol dehydrogenase(200Kd), β amylase(66Kd), Apoferritin(29Kd), and cytochrome c (12Kd) were served as molecular standard. The mobile phase is 50 mM potassium phosphate, pH 8, 200 mM NaCl. The outlet of the protein was monotored at UV 280 nm.

Figure 2. Double-reciprocal plots of initial velocities for wild-type E3, V188A, V188S and V188G in the forward reaction (NAD^+ reduction) and those for wild-type E3, V188A, V188S and V188G in the reverse direction (NADH oxidation).

Table 1

Purification of wild-type and mutant E3 enzymes

Protein was calculated for 0.4-litter preparation

Fraction	E3			V188A			V188S			V188G		
	Protein	Specific activity	Recover of activity	Protein	Specific activity	Recover of activity	Protein	Specific activity	Recover of activity	Protein	Specific activity	Recover of activity
	mg	units/mg	%	mg	units/mg	%	mg	units/mg	%	mg	units/mg	%
Supernatant	108.8	74.5	100	67.2	60.07	100	175	7.8	100	181.6	2.3	100
Ni-column	20.8	300	77	25.6	115.5	73	18.24	52	70	12	5.65	16.2
DE52-column	15.4	421	80	20	115	56	16	33.4	40	7	6.6	11

Table 2

FAD content of wild-type and mutant E3 enzymes

Protein was calculated for 500 ug/ml(9.82 nmole/ml)

	FAD content	Molar Ratio (FAD/E3)	Percentage
	nmole/ml		%
E3	9.22	0.938	100
V188A	9.8	0.99	101
V188S	8.75	0.89	95
V188G	8.8	0.896	97.5

Table 3

Steady-state kinetic parameters of wild-type E_3 and mutant E_3 enzymes

Forward Reaction

	E_3	V188A	V188S	V188G
Reaction Mecnism	ping-pong	ping-pong	ping-pong	ping-pong
Kcat (1/sec)	505	183	46	10.8
Km (DHL)(mM)	0.38	0.19	0.09	0.14
Km (NAD)(mM)	0.22	0.32	0.04	0.61

Steady-state kinetic parameters of wild-type E_3 and mutant E_3 enzymes

Reverse Reaction

	E_3	V188A	V188S	V188G
Reaction Mecnism	ping-pong	ping-pong	ping-pong	ping-pong
Kcat (1/sec)	519	488	33	50
Km (LA)(mM)	0.56	1.13	1.78	0.83
Km (NADH)(mM)	0.04	0.19	0.28	0.068

Table 4

Oxidation-Reduction Reaction Table

	E3(mV)	V188A(mV)	V188S(mV)	V188G(mV)
Active Disulfide (290nm)	-264	-280	-275	-250
FAD (454nm)	-314	-304	-300	-270

Figure 1.

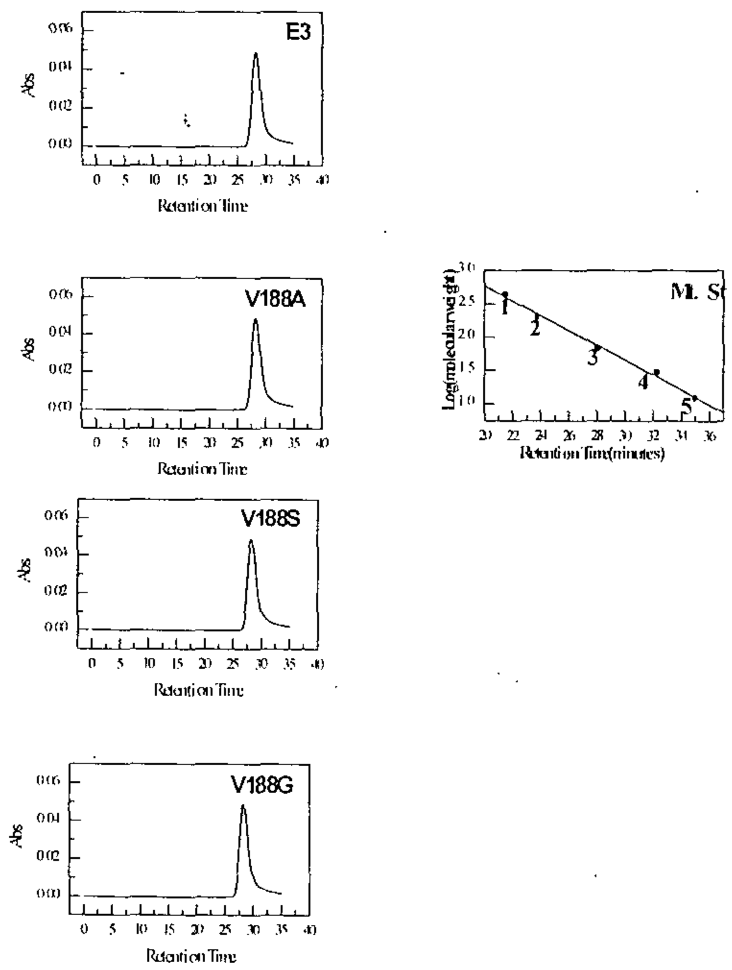

Figure 2. Forward reaction

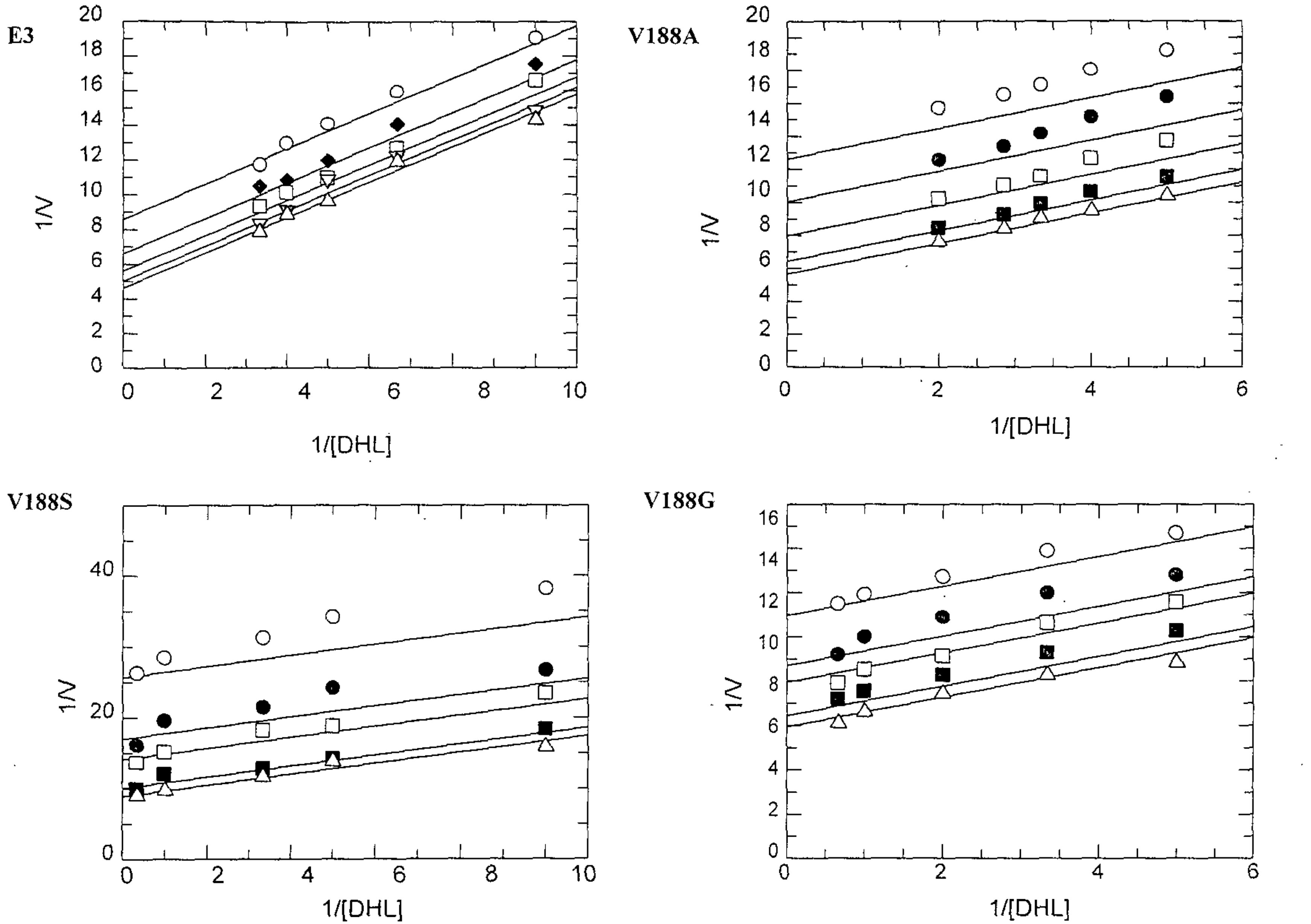

Figure 2. Reverse reaction