Basis for Half-of-the-Site Reactivity and the Dominance of the K487 Oriental Subunit over the E487 Subunit in Heterotetrameric Human Liver Mitochondrial Aldehyde Dehydrogenase[†]

Jianzhong Zhou and Henry Weiner*

Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907-1153

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ABSTRACT: Human liver mitochondrial aldehyde dehydrogenase is a tetrameric enzyme composed of 4 identical 500 amino acid containing subunits arranged such that the protein is a dimer of dimers. No kinetic evidence for subunit interactions has been reported. However, the enzyme exhibits half-of-the-site reactivity in that there is a pre-steady-state burst of 2 mol of NADH per mole of enzyme. A variant of the enzyme, found in Asian people, contains a lysine rather than a glutamate at position 487. This enzyme has a high K_M for NAD^+ and a low specific activity. In heterotetramers composed of both subunit types, it appeared that the lysine-containing subunit was dominant over the glutamate-containing subunits. To allow for the separation of various heterotetrameric forms of the enzyme, surface residues were changed. Each of the five possible tetrameric forms of the modified enzyme was isolated and characterized with respect to steady-state kinetics and pre-steady-state burst magnitudes. The data best fit a model where in each dimer pair there is one functioning and one nonfunctioning subunit. Further, the lysine subunit affects the properties only of its dimer partner. Residue 487 is located at the dimer interface, and the glutamate forms salt bonds with two arginine residues. One is to Arg^{264} in the same subunit; the other is to Arg^{475} located in the other subunit. Most likely the presence of a lysine affects these salt bonds so the lysine subunit can cause the other subunit to become essentially nonfunctional.

Liver mitochondrial aldehyde dehydrogenase is a homotetrameric enzyme whose three-dimensional structure has been determined. The structures of the beef (*1*) and the human (*2*) enzyme in the presence of NAD^+ make it appear that the subunits are identical, yet the enzyme functions with half-of-the-site reactivity (*3*). However, in the tetramers that make up the unit cell, the nicotinamide ring of NAD^+ is found in two different locations (*1, 2*), as it was in the tetrameric sheep cytosolic enzyme (*4*). This is the only structural evidence to suggest that all four subunits may not be functionally identical. Thus far no kinetic evidence has been presented to show that there might be cooperativity between subunits during the course of the reaction. However, by converting an arginine at position 475 to a glutamine, an enzyme exhibiting positive cooperativity in NAD^+ binding was produced (*5*). Further, some Asian people possess an altered form of the enzyme where a lysine rather than a glutamate is found as residue 487 (*6, 7*). The recombinantly expressed Oriental variant was found to have a high K_M for NAD^+ and a low specific activity (7−10% compared to the active form) (*8*). Most Asian people who possessed the variant were found to be heterozygotic with respect to ALDH[1] in that they possessed genes coding for both the active glutamate enzyme and the essentially inactive lysine-containing enzyme (*9*) yet they had much less than 50% of the activity expected for a person expressing an active and an inactive isozyme. We showed that in recombinantly expressed heterotetramers of unknown composition the lysine subunit was dominant over the glutamate subunit (*10*). Thus, though there is no kinetic evidence with the homotetrameric enzyme to show that cooperativity or subunit interactions exist, evidence for the phenomenon does.

The structure of the enzyme reveals that the tetramer is composed of two pairs of dimers (*1, 2*). The oligomerization domain is comprised of three-stranded antiparallel β-sheets, which are involved in subunit interactions in both the dimer and the tetramer. Each dimer pair has contact between residues 487 and 475, the two residues that were found to affect the behavior of the enzyme and are located in the oligomerization domain. Though the enzyme is composed of a pair of dimers, it is not known if the half-of-the-site reactivity or the dominance of the K487 subunit is a result of an interaction within the dimer pair or is a result of interactions within the entire tetramer. We previously attempted to resolve this question by either coexpressing cDNAs coding for the E487 and the K487 forms on one plasmid (*10*) or transducing HeLa cells with both of these cDNAs (*11, 12*). Unfortunately, no discrete bands of differing

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* To whom correspondence should be addressed. Telephone: (765) 494-1650. Fax: (765) 494-7897. Email: hweiner@purdue.edu.

[1] Abbreviations: ALDH, aldehyde dehydrogenase; E487, ALDH with a Glu residue at position 487; K487, Oriental variant, the form of ALDH found in many Asian people that has a lysine at position 487 rather than a glutamate; E, double mutation of E487 with R34E/K35E mutations; K, double mutation of K487 with Q362K/E363K mutations; IEF, isoelectric focusing; SDS−PAGE, polyacrylamide gel electrophoresis with sodium dodecyl sulfate.

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p*I* were observed between the bands of the E487 and K487 forms after isoelectric focusing (*10*−*12*). The difference in p*I* between the E487 and K487 enzymes is only 0.2 unit. Because of the small differences in net charge among subpopulations of the protein, they could not be resolved well by current isoelectric focusing systems. Therefore, it is not certain whether a K487 subunit could inactivate an E487 subunit in a dimer pair or all the E487 subunits in the heterotetramer. To try to understand the half-of-the-site reactivity and the dominant effect of the K487 subunit over the E487 subunit, heterotetramers were created in which the two subunits became quite different in net charges. This was done so that we could isolate the individual ALDH heterotetrameric forms. By determining the properties of the individual heterotetramers, we found that K487 subunit could affect the E487 subunit in the same dimer but not the E487 subunit in the other dimer pair of the tetrameric ALDH. Similarly, it will be shown that the basis for the half-of-the-site reactivity is that in the dimer pair, one subunit is functioning while the other is not.

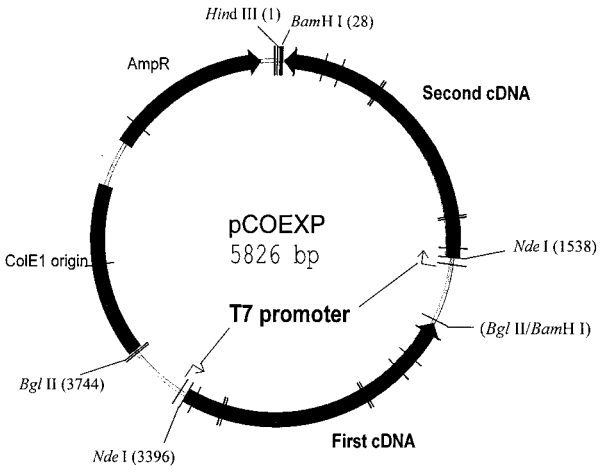


FIGURE 1: Construct of the coexpression plasmid. The pCOEXP contains cDNAs for both R34E/K35E E-subunit and Q362K/E363K K-subunit to coexpress heterotetrameric aldehyde dehydrogenases. The plasmid could be constructed so E487 is first followed by K487 cDNA or K487 first followed by E487 cDNA.

MATERIALS AND METHODS

Materials. Sequenase version 2.0 kit was obtained from U. S. Biochemical Corp.; Magic Minipreps DNA purification system and T4 DNA ligase were from Promega Corp.; restriction enzymes were from New England Biolabs; *Taq* DNA polymerase was from Boehringer Mannheim; NAD^+ and NADH were from Sigma; propionaldehyde was from Aldrich; IEF standards were from Bio-Rad; agarose IEF and Pharmalyte were from Pharmacia Biotech Inc.

Mutagenesis. Oligonucleotides 5′TGGCACGATGCCGTCAGCGAGGAAACATTCCCCACCGTCA3′ and 5′TACATCAACACGGGGAAGAAAAAGGGGGCGAAGCTGCTGT3′ were synthesized to obtain the double mutant enzymes R34E/K35E and Q362K/E363K, respectively. Site-directed mutagenesis was performed in vitro with a mutagenesis kit (Bio-Rad), following the manufacturer's instructions. E487 and K487 ALDH cDNA on a pT7-7 plasmid were used as a template for the R34E/K35E and Q362K/E363K mutants, respectively. The resulting mutant cDNAs were sequenced to verify the mutations. These plasmids were transferred into BL21(DE3) pLysS cells for expressing homotetrameric mutated E487 or K487 enzyme (*2*, *5*).

Construction of Coexpression Plasmid. The coexpression plasmids were constructed to carry two ALDH cDNAs randomly (Figure 1) similarly as previously done (*10*). Each construct was first made on a pT7-7 plasmid containing only one cDNA with the double mutation described above. One gene was cut out from the pT7-7 plasmid with *Bgl*II and *Hin*dIII restriction enzymes. This DNA fragment contained the full-length ALDH cDNA as well as its T7 promoter and ribosome binding site and was cloned into the other pT7-7 plasmid that was digested with *Bam*HI and *Hin*dIII by taking advantage of the compatible cohesive ends of *Bgl*II and *Bam*HI. Thus, each cDNA had its own T7 promoter and ribosome binding site. *E. coli* DH5α (Bio-Rad) was transformed with this coexpression plasmid, and restriction analysis using different enzymes was carried out to verify the construction.

Purification of Heterotetramer. *E. coli* BL21(DE3) pLysS cells harboring the ALDH expression vector were grown at 37 °C in 2 × YT medium (1.6% tryptone, 1.0% yeast extract, and 0.5% NaCl) containing both ampicillin (50 μg/mL) and chloramphenicol (50 μg/mL). When the optical density at 600 nm reached about 0.5, isopropyl-1-thio-β-D-galactopyranoside (final concentration, 0.5 mM) was added to the culture medium. The cultures were then grown at 16 °C overnight, and the cells were harvested by centrifugation (*2*).

After disruption of the cells by French press, cell debris and genomic DNA were removed by centrifugation at 40 000 rpm for 1 h in a Beckman 60Ti rotor. The enzymes were separated by DEAE-Sephacel chromatography on a Pharmacia Biotech FPLC with a linear gradient of 0−0.3 M NaCl in an elution buffer containing 10 mM sodium phosphate, pH 7.4, 1 mM EDTA, and 0.025% β-mercaptoethanol. The flow rate was 1.0 mL/min. The fractions containing individual heterotetrameric ALDHs were pooled and then purified through a 4′-hydroxyacetophenone affinity column (*13*). Finally, the individual heterotetramers were further purified by DEAE-Sephacel chromatography as described above to remove any trace of other heterotetramers. The separated heterotetramers were stable, and subunit exchange did not occur.

Slab-Gel IEF. Agarose (1%) IEF was performed on a Pharmacia flat-bed electrophoresis apparatus using Pharmalyte (pH 3−10) at 10 °C. The gel was stained either for activity or for protein with Coomassie Blue (*14*). The concentrations of NAD^+ and propionaldehyde were 5 mM and 140 μM, respectively, in the activity staining solution prepared in 100 mM sodium phosphate (pH 7.4).

Fluorescence Assay for the Dehydrogenase Activity. The dehydrogenase activity assays were performed by measuring the rate of increase in the fluorescence of NADH in 100 mM sodium phosphate (pH 7.4) at 25 °C (*8*).

Pre-Steady-State Burst of NADH Formation. The pre-steady-state burst magnitude of NADH formation was determined with an Aminco filter fluorometer as we previously have done (*3*, *15*). Enzyme and NAD^+ were incubated in 100 mM sodium phosphate (pH 7.4) to establish a fluorescence baseline. Concentrations of NAD^+ were 1 mM for the homotetrameric E487 enzyme and 5 mM for heterotetrameric and homotetrameric K487 enzymes. At a

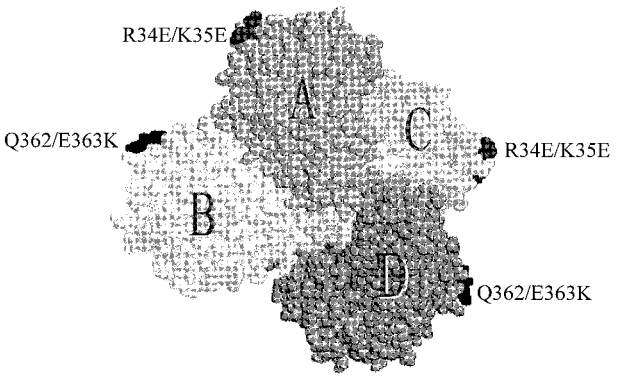


FIGURE 2: Positions of mutant R34E/K35E and Q362K/E363K on a heterotetramer. A, B, C, and D indicate individual subunits.

Table 1: Kinetic Properties of the Recombinantly Expressed Homotetrameric and Heterotetrameric Aldehyde Dehydrogenases

enzyme[a]	E487	K487	E_4	E_3K	E_2K_2	EK_3	K_4
$K_{M(NAD+)}$, μM	37	5600	32	34	48	4300	5200
$K_{M(prop)}$, μM	0.33	0.8	0.42	0.32	0.4	0.34	0.38
$k_{cat(app)}$, min^{-1}	180	9.5	200	95	24	10	7.8
p*I*	4.9	5.1	4.7	5.1	5.6	6.2	6.8
burst[b]	2	0	2	0.8	0.2	0	0

[a] E487 and K487 refer to the native and Oriental variant ALDHs, respectively. [b] Pre-steady-state burst magnitude, mol of NADH/mol of enzyme ± 0.2. Finding a burst of essentially 2 for E487 and 0 for K487 is consistent with what we previously reported (*3, 8, 15*).

time called zero, propionaldehyde (140 μM) was added to initiate the reaction. The extrapolated line intersecting at time zero gave the magnitude of the burst of NADH formation. By calibrating the fluorometer with various concentrations of NADH, it was possible to calculate the moles of NADH produced prior to reaching steady-state velocity (*3, 15*).

RESULTS

Separation of Individual Heterotetramers. Previous studies showed that it was very difficult to separate individual heterotetrameric forms of ALDH containing the E487 and K487 subunits (*10, 11*). The heterotetramic forms of ALDH could not be separated from each other by IEF or ion exchange chromatography. The reason was that there were just subtle differences in net charge among the subpopulations of the proteins. To separate the heterotetramers, we had to make the E487 subunit more negative and the K487 subunit more positive. Based on structural information (*1, 2*), several charged patches on the surface of human ALDH were found, and the charged amino acid residues were replaced by amino acids with opposite charges. These modified ALDHs, R34E/K35E and Q362K/E363K, were used to make heterotetramers (Figure 2). The p*I* of the E487 mutant with R34E/K35E, to be referred to as the E-subunit, was about 4.7 while the p*I* of the K487 mutant with Q362K/E363K, to be referred to as the K-subunit, was about 6.8. The mutated enzymes were found to have similar kinetic properties as their parent (Table 1). Thus, these two mutants could be used in the coexpression system to produce heterotetramers to allow for the separation of the individual heterotetrameric forms of ALDHs.

There was a second major electrophoretic difference between the mutated E- and K-subunits. The E-subunits migrated slower in SDS−PAGE than did the K-subunits

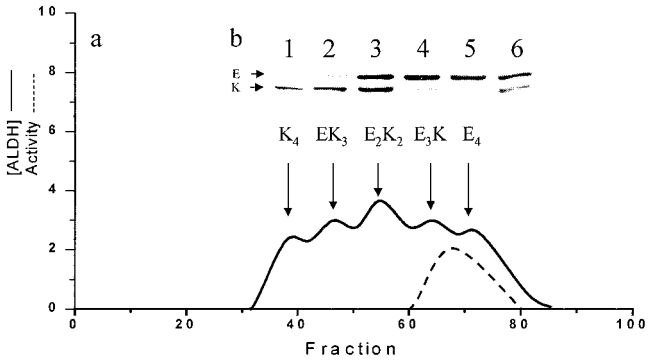


FIGURE 3: Separation of the coexpressed heterotetrameric aldehyde dehydrogenases. (a) First separation of the coexpressed E/K heterotetrameric ALDH on a DEAE-Sephacel column by FPLC. The five ALDH peaks, detected by Western blotting with anti-ALDH antibody, were labeled to indicate the corresponding subunit components as shown in (b). K_4 was eluted in 40−60 mM NaCl, EK_3 in 60−80 mM NaCl, E_2K_2 in 80−100 mM NaCl, E_3K in 100−120 mM NaCl, and E_4 in 120−140 mM NaCl. One peak of dehydrogenase activity was found located near the last two ALDH peaks. (b) Western blotting analysis of the ALDH peaks was performed after SDS−PAGE. Lanes 1−5 correspond to the five peaks from (a). Lane 6 contains a portion of the sample before separation. E-subunits migrated slower than did the K-subunits on SDS−PAGE for unknown reasons.

(Figure 3b). Mass spectroscopic analysis showed that the E-subunits had the expected molecular weight, identical to that of the K-subunit (data not shown). The reason for slower migration on SDS−PAGE is not known, but it has been shown that an alteration of even a single amino acid may change the mobility of some proteins in SDS−PAGE (*16, 17*). This altered migration, however, made it easy for us to identify the E-subunits and the K-subunits in heterotetramers.

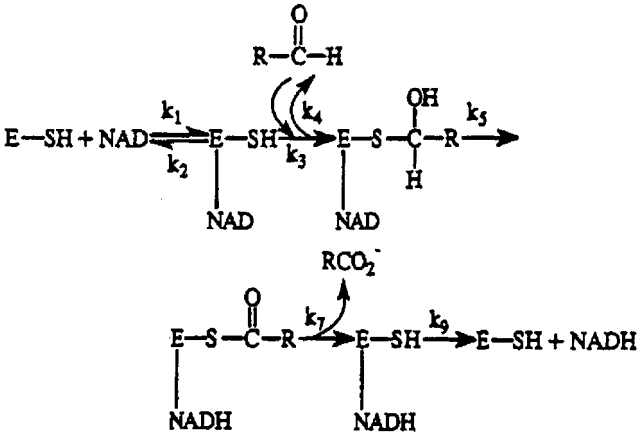
The cDNA cloned in the first position on the coexpression vector employed (Figure 1) was found to be expressed at a higher level than the one in the second position, even though each of them had their own T7 promoter and ribosome binding site. Cells possessing the plasmid with a different order of the two ALDH cDNAs were combined equally and grown together in the same flask. The enzymes were purified from the homogenate of this system. The coexpressed ALDHs were first separated by DEAE-Sephacel chromatography. There were five peaks of ALDH, determined by Western blotting, though they were not totally separated from each other (Figure 3a). Only one peak of catalytic activity was found; it was located where E_3K and E_4 eluted from the column (Figure 3a). Samples from each peak of ALDH proteins were separately pooled and purified by 4′-hydroxyacetophenone affinity chromatography (*13*). In each sample, one of the heterotetrameric forms of ALDH was the major isozyme, but a trace of other forms was still present. To remove those, each sample was run through the DEAE-Sephacel column again to further separate the individual heterotetramers from each other. A new sodium chloride gradient was used for each sample. A gradient corresponding to the range in which the different heterotetrameric ALDHs were eluted from the DEAE column was used. The purity and ratio of E-subunit to K-subunit could be easily determined on SDS−PAGE since the E-subunit migrated slower than the K-subunit. Native IEF also showed that the individual heterotetramers were separated (Figure 4) and had

a different p*I* (Table 1). Staining the IEF gel for catalytic activity revealed that E_4, E_3K, and E_2K_2 had activity although E_2K_2 had much lower activity than did the others (Figure 4).

Kinetic Studies of the Individual Heterotetrameric ALDHs. Like with the K_4 homotetramer, the K_M for propionaldehyde for E_3K, E_2K_2, and EK_3 heterotetramers was essentially identical to that of the E_4 enzyme. Only one K_M for NAD^+ was found with E_3K (34 μM) and E_2K_2 (48 μM), respectively; its value was similar to that of the E_4 form. The K_M for NAD^+ with EK_3 was about 4300 μM, which was similar to that of the K_4 homotetramer (Table 1).

The specific activity of the five tetrameric forms was determined. E_4 was assayed with 1 mM NAD^+ and 140 μM propionaldehyde. When the activity of K_4 and the heterotetramers was measured, 5 mM NAD^+ and 140 μM propionaldehyde were used, because of the high K_M for NAD^+ of K487 enzyme. The $k_{cat(app)}$ for E_3K, E_2K_2, and EK_3 decreased to 48%, 12%, and 5% of E_4, respectively (Table 1). Under these conditions, the $k_{cat(app)}$ of K_4 ALDH was about 3.9% compared with that of E_4 enzyme. It appeared that E_3K had half of the activity as did E_4 while EK_3 had essentially the same low activity as did K_4. The activity of E_2K_2 was higher than that of EK_3 and K_4, but was just 12% of E_4.

Pre-Steady-State Burst Magnitude of NADH Formation. It was shown that there was a pre-steady-state burst of NADH formation with liver mitochondrial ALDHs (*3*, *8*). This has been interpreted to imply that the rate-limiting step occurred after NADH was formed, which is deacylation (k_7 step) of a thioester intermediate.



Previously, it was shown that the magnitude for the burst with the recombinantly expressed E487 enzyme was essentially 2 mol of NADH per mole of tetrameric enzyme, and no burst was found with the recombinant K487 mutant (*8*). It was found in this study that the burst magnitude of the E_4 was again about 2 mol of NADH/mol of tetrameric enzyme and the K_4 showed no burst, as expected. The heterotetrameric E_3K enzyme had a burst of 0.8 mol of NADH/mol of the enzyme. Essentially no burst was found with EK_3. E_2K_2 had a very low burst magnitude, approximately 0.2 mol of NADH/mol of enzyme. From the burst data, it appeared that half of the subunits in E_3K heterotetramer functioned like an E487 enzyme while the other half functioned like a K487 enzyme. Each E−K pair in the heterotetramer exhibited no burst while the E−E pair had a burst of just 1, not 2, mol/mol of dimer.

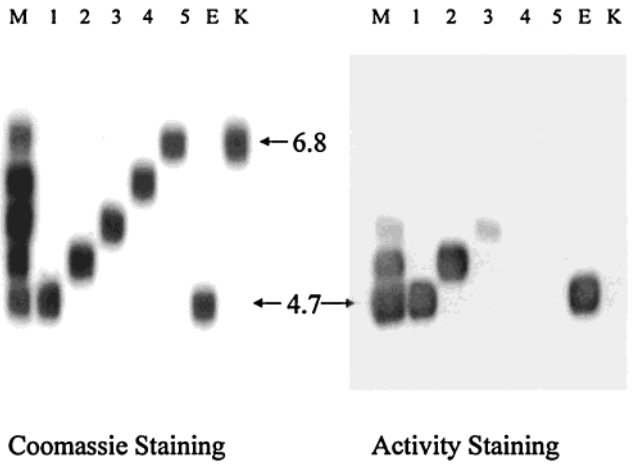


FIGURE 4: IEF of human homotetrameric and heterotetrameric aldehyde dehydrogenases. The homotetrameric and heterotetrameric ALDHs were subjected to IEF gel electrophoresis on a horizontal IEF chamber and stained with Coomassie blue and for activity. The p*I*s for E_4 (4.7) and K_4 (6.8) are indicated. Lane M, crude sample of coexpressed enzyme. Lanes 1−5, purified enzyme from peaks 1−5 (see Figure 3). Lane E, purified recombinantly expressed E_4 enzyme. Lane K, purified recombinantly expressed K_4 enzyme. An equal amount of protein was added to the IEF gel for lanes 1−5 and lanes E and K.

DISCUSSION

To understand the dominant effect of K487 subunit over E487 subunit, it was necessary to isolate the individual ALDH2 heterotetramers. To accomplish this, residues on the surface of the enzyme were changed so that the two subunit types would have greater charge differences between them. The homotetrameric forms of the modified ALDHs proved to have properties virtually indistinguishable from their parent form, even though the p*I*s were different from the parent enzymes. Using these mutants, it was possible to purify all five forms of the enzyme. The accidental finding that the K-subunits ran differently than did the E-subunit in SDS−PAGE allowed us to determine the subunit composition of each tetrameric form.

When the specific activity was measured in the presence of 5 mM NAD^+, K_4 possessed about 4% the activity of E_4. This NAD^+ concentration would saturate the E-enzyme but not K-enzyme. This concentration of NAD^+ was chosen since the velocity of E_4 started to decrease when the NAD^+ concentration was above 5 mM (*10*). It was not prudent to use higher concentrations of NAD^+ in the assay of the heterotetrameric enzymes as it then would have caused inhibition of the active E-subunit in the protein.

It was known that K-subunits had a dominant effect over E-subunits (*10*−*12*). However, it was not clear whether a K-subunit only affected the E-subunit in the same dimer pair or affected all the E-subunits in the tetramer. In the heterotetramer of EK_3, there are two types of dimer pairs, E−K and K−K. If the K-subunit did not affect the E-subunit, the enzyme could have 28% of the activity of E_4 enzyme (Table 2). Since the activity of EK_3 was the same as that of K_4, it implied that an E−K dimer behaved like a K−K dimer pair. The E_3K heterotetramer consisted of E−E and E−K dimer pairs. If the K-subunit did not affect the E-subunit in the E−K dimer, E_3K would have about 76% of the activity of E_4 enzyme. It was found that E_3K had only about 50% of the activity of E_4 enzyme, indicating again that K-subunit

Table 2: Estimation of the Specific Activity of Heterotetrameric Aldehyde Dehydrogenases

		activity (%)			
			dominance[c]		
hetero-tetramer	type of dimer[a]	no dominance[b]	Model I	Model II	measured
E_4	E−E (100%)	100	100	100	100
E_3K	E−E (50%) E−K (50%)	76	100	52	48
E_2K_2[d]	E−E (17%) K−K (17%) E−K (66%)	52	36	20	12
EK_3	E−K (50%) K−K (50%)	28	4	4	5
K_4	K−K (100%)	4	4	4	3.9

[a] Refers to the percent of a dimer pair in the tetramer. [b] Assumes the E-subunit functions independently of the K-subunit. [c] Active based on the models shown in Figure 5 where the K-subunit is dominant over the E-subunit. [d] E_2K_2 could exist in two tetrameric forms, (E−E + K−K) and (EK + EK), in a ratio of 1:2. The distribution shown is for a mixture of these two forms when the expressions of E- and K-subunits are equal.

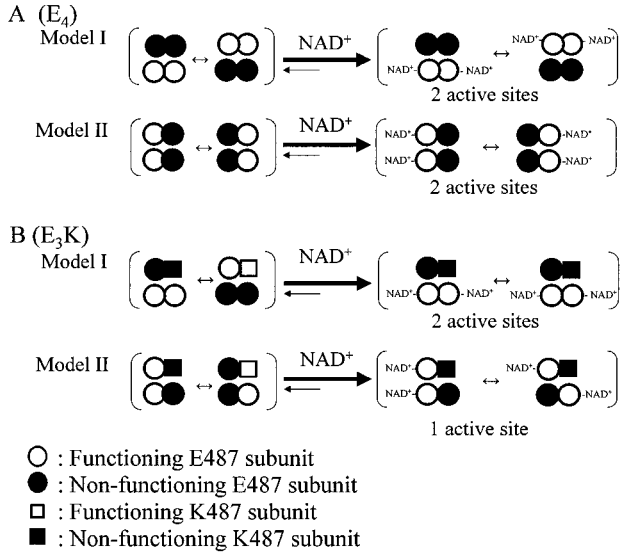


FIGURE 5: Models to explain half-of-the-site reactivity. In each model, it is assumed that an equilibrium exists between active and inactive subunits in the enzyme since the enzyme exhibits half-of-the-site reactivity. (A) Model I shows an equilibrium between a pair of active subunits and a pair of inactive subunits. Model II shows an equilibrium between an active and an inactive subunit in each dimer pair. For the E_4 enzyme, NAD^+ would bind to just one dimer pair in Model I but would bind to one subunit in each dimer for Model II. Either model would predict the half-of-the-site reactivity. (B) Illustrated is an example using E_3K to explain the half-of-the-site reactivity. Because of the high K_M for NAD^+ to the K-subunit, NAD^+ will bind to the E-subunit preferentially. The inactive dimer pair with two E487 subunits in Model I would bind NAD^+ and become an active dimer pair. Thus, the activity of E_3K enzyme would be the same as the E_4 enzyme and have a burst of 2 mol of NADH per mole of enzyme just like the E_4 enzyme for, in the tetramer, there are only two functioning subunits. For Model II, NAD^+ would only bind to one E-subunit in each dimer pair. The E−K dimer, though, has essentially no activity, because the K-subunit is dominant over the E-subunit. The remaining E−E dimer would have only one functioning active site. Then, the E_3K heterotetramer would have only half the activity of the E_4 enzyme. Finding that the specific activity and burst magnitude of E_3K were essentially half those of the E_4 enzyme (Table 1) is consistent with Model II being operative.

could inactivate the E-subunit in the same dimer pair. The E-subunits in the other dimer pair were not affected because, if they were, the activity of E_3K would have been less than 50%. E_2K_2 contained two combinations of heterotetrameric ALDH (E−E + K−K and E−K + E−K) in a ratio of 1:2. We were unable to further separate the E_2K_2 combinations; however, in the E_2K_2 heterotetramer, only one-sixth of the total dimer pairs would be the highly active E−E dimer. One could expect, then, that the maximum activity of the E_2K_2 form would be 20% of that of E_4 enzyme. E_2K_2 was found to possess about 12% of the activity, which is consistent with the above discussion. We cannot explain why the value was lower than 20%. It is possible that some small dimer−dimer interaction exists, which was not detected in the E_3K form.

It was previously shown that the human ALDH (*14*), like other mammalian mitochondrial ALDHs, exhibited half-of-the-site reactivity (*3*). That is, in the tetramer, the subunits were functioning as two separate pairs and not as four independent subunits. Even though ALDH is composed of a dimer of dimers (*1, 2*), it is still not clear whether in the tetramer, one dimer pair is active and the other is inactive (Model I). Alternatively, it is possible that both dimer pairs were active but only one subunit in each dimer pair is functioning (Model II) (Figure 5).

From the crystal structure, we could not find evidence to suggest that differences in the NAD^+ binding domains existed in the apo enzyme. It is possible, though, that when one molecule of NAD^+ binds, the structure of the other dimer pair (Model I) or a subunit in the same dimer pair (Model II) is altered. The potential structural alteration could cause those subunits to become inactive. The crystallographers reported that two conformations of the nicotinamide ring can be found in the binary complex (*1, 2*). Having the heterotetramers of E_3K and EK_3 allowed us to determine which model is potentially operative. This was possible because under the assay conditions employed, only the E-subunit in the E−K dimer pair would be functioning. However, since the K-subunit renders the E-subunit essentially inactive, activity would primarily result from an E−E dimer pair in E_3K.

Both models predict half-of-the-site reactivity for the E_4 enzyme. From Model I, one can predict that E_3K, which contains an active E−E dimer pair, would be fully active in the presence of NAD^+ and would have E_4-like activity. From Model II, one can predict that just one of the two subunits in the E−E pair would be active so the enzyme would have half the activity of the homotetrameric E_4 enzyme. The activity of the E_3K form was 50%, as presented in Table 1. One could assume then, that a dimer pair in the tetrameric enzyme functioned as a unit with one active site, as presented in Model II.

The calculated activity of E_2K_2 would be 36% based on Model I and 20% based on Model II. The data presented in Table 1 show that the activity for the heterotetrameric form was more consistent with the predicted activities based on Model II. The specific activity of EK_3 would be the same for both models as there would only be one active subunit but its activity would be diminished by the presence of its K-partner.

We further tested the model by measuring the burst of NADH formation. Previous work in our laboratory showed that the E487 enzyme had a pre-steady-state burst of approximately 2 mol of NADH per mole of enzyme while

no burst was observed with K487 Oriental variant (*8*, *14*). The mutated E_4 and K_4 enzymes showed these same burst properties as did the parent enzyme. When we studied the burst of NADH formation with the heterotetramer, it was found that E_3K had a burst near 1 mol of NADH per mole of enzyme while EK_3 had no burst of NADH formation. Since the E-subunit was affected by the K-subunit in the same dimer pair, it was expected that EK_3 would have no burst. Finding no burst for EK_3 verifies that the E subunit in EK_3 was indeed affected by the K-subunit and it did not function like an E-subunit. In the E_3K heterotetramer, one could expect a burst magnitude of 2 if Model I were valid. The magnitude was 0.8, consistent with Model II where an E−E dimer only contains one functioning active site. Steady-state and burst data make it appear that the E-subunit was not acting independent of the K-subunits in the same dimer pair and the K-subunit would not affect E-subunits in the other dimer pair.

The presence of Lys at position 487 did not affect the subunits in the other dimer pair though it is located at the interface of the dimers. We do not have solid evidence to explain the mechanism of the half-of-the-site reactivity. However, based on the activity and burst magnitude of E_3K, we proposed that two dimer pairs in the tetrameric ALDH could be identical and each dimer pair could have only one functional active site.

The precise structural basis for the half-of-the-site reactivity or the dominance of the K-subunit over the E-subunit in heterotetrameric ALDH is not fully understood. Three distinct domains were found in each subunit: a coenzyme binding domain, a catalytic domain, and an oligomerization domain (*1*, *2*). The oligomerization domain is comprised of three-stranded antiparallel β-sheets, which is involved in subunit interactions in both the dimer and the tetramer. Residue Glu^{487}, located in the oligomerization domain, appears to be at a key interface between the subunits in the dimer pair. This glutamate is involved in two ion-pairing interactions, one to Arg^{264} from its own subunit and another to Arg^{475} contributed by the other subunit in the dimer pair (*1*). The presence of a lysine at position 487 could then affect not only its own subunit but also the other subunit. Arg^{475} is located near the nicotinamide binding site. The alternation of the interaction between the Glu^{487} and the Arg^{475} residues could cause a subtle change in the active site of the other subunit and interfere with catalysis. It was recently found though, that the mutation of Arg^{475} to a glutamine resulted in a 23-fold increase of the K_M for NAD^+ and converted the enzyme into one that exhibited positive cooperativity (*5*). The Arg^{264} is close to the Glu^{268} residue that functions as a general base (*14*). Structural perturbations near Arg^{264} and Arg^{475} could be responsible for adversely affecting the K_M for NAD^+ and for the decreased activity of both subunits in a dimer pair (*1*). The disruption of the two salt bonds by the mutation of residue 487 could be the reason that the K487 subunit possessed a dominant effect over the E487 subunit. However, restoring the salt bonds to the K487 enzyme did not convert the K487 enzyme's property to those of an E487 enzyme (*5*). The half-of-the-site reactivity could be related to the two conformations of the nicotinamide ring of NAD^+ found in the structure (*1*, *2*). Independent of the structural basis for the subunit interactions, this study showed that the effect of one subunit on the properties of another occurs between the subunits in the dimer pair and not between pairs of dimers. Though dimeric forms of ALDH are known (*18*), it has not been shown whether cooperativity in coenzyme binding or kinetics exists in these dimers.

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