A Disorder to Order Transition Accompanies Catalysis in Retinaldehyde Dehydrogenase Type II*

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Retinaldehyde dehydrogenase II (RalDH2) converts retinal to the transcriptional regulator retinoic acid in the developing embryo. The x-ray structure of the enzyme revealed an important structural difference between this protein and other aldehyde dehydrogenases of the same enzyme superfamily; a 20-amino acid span in the substrate access channel in retinaldehyde dehydrogenase II is disordered, whereas in other aldehyde dehydrogenases this region forms a well defined wall of the substrate access channel. We asked whether this disordered loop might order during the course of catalysis and provide a means for an enzyme that requires a large substrate access channel to restrict access to the catalytic machinery by smaller compounds that might potentially enter the active site and be metabolized. Our experiments, a combination of kinetic, spectroscopic, and crystallographic techniques, suggest that a disorder to order transition is linked to catalytic activity.

In the developing embryo the generation of retinoic acid by metabolism of the inactive precursor retinol must be finely regulated. The consequences of deregulation of this process are tragic; the teratogenic effects of retinoic acid analogs used to treat cystic acne are thought to be a result of aberrant retinoic acid concentrations during morphogenesis (1). In their studies of the developing spinal cord, McCaffery and Drager (2) observed retinaldehyde dehydrogenase activity with the same spatio-temporal distribution as that of retinoic acid and subsequently identified the enzyme retinaldehyde dehydrogenase type II (RalDH2) in their examination of the effect of retinoic acid on the developing spinal cord, McCaffery and Drager (2) observed retinaldehyde dehydrogenase activity with the same spatio-temporal distribution as that of retinoic acid and subsequently identified the enzyme retinaldehyde dehydrogenase type II (RalDH2) in their examination of the effect of retinoic acid signaling in the formation of the neural axis (3). Similar studies suggested a role for RalDH2 in the developing mammalian heart (4). The validity of the hypothesis that RalDH2 produces retinoic acid in the developing embryo was confirmed by the transgenic knock-out mice results of Chambon and co-workers (5). RalDH2−/− embryos exhibit the spectrum of defects associated with developmental processes previously shown to be retinoic acid-dependent and die at mid-gestation. Near full rescue is observed when retinoic acid is administered to the mothers (5). These results provide a striking contrast to those of similar experiments in which the different retinoic acid receptor genes were mutated in mice. Six genes code for two classes of retinoic acid receptors in the mammalian genome, and the inactivation of a single gene for a retinoic acid receptor results in only mild abnormalities in developmental phenotypes (6, 7). However, RalDH2 activity is indispensable in morphogenesis (5). Prior to this work, RalDH2 was cloned by the Napoli laboratory as a result of their efforts to identify enzymes that produced retinoic acid in the testes (8). Retinoic acid is abundant in the testis, and the use of that tissue avoided the complications of using liver as a source for cDNA. The liver has many more abundant aldehyde dehydrogenases, such as the phenobarbital-inducible dehydrogenases, which would complicate the identification of the retinaldehyde-specific dehydrogenase. In the adult animal RalDH2 is expressed in brain and lung as well as the testis (8).

RalDH2 is a member of the NAD-dependent aldehyde dehydrogenase (ALDH) superfamily for which there are several structures available in addition to that of RalDH2 (9–13). Those structures of the enzyme from bovine mitochondria and sheep liver have the highest sequence identity with RalDH2 (66 and 73% identity, respectively). A structure for the highly homologous lens protein γ-crystallin (70% identity) has been described recently (14) as well. Aldehyde dehydrogenases utilize a conserved Cys (Cys-302 in the bovine mitochondrial enzyme) in the oxidation of aldehyde to carboxylic acid with the concomitant reduction of NAD to NADH. RalDH2 is able to process retinal free or as it is found in the cell bound to its carrier protein cellular retinol/retinal binding protein (8). Many aldehyde dehydrogenases are classified as detoxification enzymes as the enzymes selectively eliminate aldehydes generated in the metabolism of xenobiotics and endogenous compounds. However, the knock-out experiments described above clearly demonstrate that RalDH2 is the source of retinoic acid required for normal development and consequently indicate a pivotal role for the enzyme in the production of the transcriptional regulator retinoic acid.

The structures of aldehyde dehydrogenases involved in the metabolism of acetaldehyde have a long (∼18 Å) substrate access channel into which acetaldehyde can readily diffuse. However, retinal dehydrogenase differs in that it has a disordered loop within the substrate access channel, and it was suggested that it is precisely this disorder (13) that is key to the fact that substrate preference of RalDH2 is for long aliphatic aldehydes (8). A substrate-induced disorder to order transition was proposed to mediate substrate recognition. Hurley et al. (15) subsequently reported a disorder to order transition induced by the binding of the co-factor to the bovine mitochondrial ALDH2: R475Q mutant that involves the same region of that enzyme, but in RalDH2 with co-factor bound this region remains disordered as indicated by the lack of any interpretable electron

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The abbreviations used are: RalDH2, retinaldehyde dehydrogenase type II; ALDH, aldehyde dehydrogenase.
density in an otherwise clear electron density map. We report here a series of experiments designed to reveal the functional basis for the lack of a well formed substrate access channel in RalDH2. We asked whether access channel disorder might provide a mechanism for the enzyme, which requires a large substrate binding channel for its 20-carbon biological substrate, to restrict access to catalytic machinery by smaller aldehydes. This mechanism could be a result of the linking of a disorder to order transition to catalytic activity.

We reasoned that regions of a protein that are disordered and do not participate directly in catalysis or substrate binding are insensitive to amino acid substitutions. If we introduce mutations into a region of the disordered loop and observe a reduced catalytic activity that cannot be explained by a loss in substrate binding affinity, then we can infer that the segment of the polypeptide in which the mutation was made makes a specific intramolecular interaction during the catalytic cycle, i.e., a disorder to order transition occurs. This is precisely what we observe; mutation of the disordered segment results in a reduced apparent $V_{\text{max}}$ for the enzymatic reaction catalyzed by RalDH2.

Fluorescence data support our interpretation of the experimental results. In the region of the disordered loop there are two Trp residues (Trp-176 and Trp-451). If the loop adopts a conformation similar to the corresponding region of the homologous aldehyde dehydrogenases, these tryptophans would be shielded from solvent. An additional Trp (Trp-167) packs against the ribose ring in the NADH binding site. We observed a reduced fluorescence resonance energy transfer between Trp and NADH that accompanies catalysis in RalDH2 in which amino acids substitutions are introduced in the disordered loop region. Such an observation would be expected if the loop mutant were incapable of ordering in the access channel and shielding Trp-176 and Trp-451 from solvent, or if the position or environment of the nicotinamide ring were affected. Steady state kinetic data indicate that the affinity for a series of model substrates is not significantly affected by the amino acids substitutions. In contrast, the $K_m$ for the co-factor NAD is appreciably higher in the mutant enzyme despite the fact that the substitution was made in the access channel and removed from amino acids that directly interact with co-factor. These observations suggest non-local structural consequences of the mutation, consistent with a disorder to order transition.

In addition, we determined the crystal structure of the mutant enzyme in the presence of NAD and absence of substrate and show that it is essentially unchanged from the native structure; the “disordered loop” of the access channel is disordered in the presence of NAD and absence of substrate just as it is in the wild-type enzyme. Thus an inadvertent ordering of a reduced fluorescence resonance energy transfer between Trp and NADH was added to a final concentration of 0.5 mM to induce RalDH2 expression. The culture was then grown for an additional 1 h after induction under the same conditions followed by an additional 48 h of incubation carried out at room temperature with constant agitation (220 rpm). Cells were harvested by centrifugation at 6,000 × g for 10 min at 4 °C and were subsequently resuspended in low salt buffer (25 mM KCl, 1 mM EDTA, 2 mM β-mercaptoethanol, and 20 mM HEPES, pH 8.4) at a concentration of 35 ml per 1 liter of bacterial culture and then frozen at −80 °C. Cells were lysed with three cycles of freezing and thawing of the cell suspension. DNase and MgCl2 were added at a final concentration of 40 Kunitz units and 5 mM, respectively, per 35 ml of cell suspension to degrade chromosomal DNA. Protease inhibitors pepstatin and leupeptin were also added to the lysate to help prevent proteolytic cleavage of RalDH2. The crude lysate was centrifuged at 25,000 × g for 1 h at 4 °C. All further protein purification steps were conducted at 4 °C. The supernatant was removed and diluted 2-fold with low salt buffer and then applied to a column containing 70 ml of Affi-Gel Blue Gel in which RalDH2 was able to bind to the column via its nucleotide binding domain. The column was thoroughly washed with a NaCl step gradient (25 mM, 500 mM, and 1 mM NaCl) using 3 column volumes of low salt buffer supplemented with the corresponding amount of NaCl. RalDH2 was then eluted with 2 column volumes of low salt buffer containing 2 mM NaCl. The eluant was concentrated to 5% w/v and analyzed by SDS-PAGE for protein purity before dropwise flash freezing in liquid nitrogen and storage at −80 °C.

Crystallization—Crystals were grown in hanging drop vapor diffusion experiments. Drops were 2 μl of RalDH2:Li6EGFrNa450K (7.6 mg/ml) in the presence of excess NAD (2 mM) and retinol (at 1.8 times the concentration of enzyme) plus an equal volume of well solution (7% PEG 6000, 2% 2-methyl-2,4-pentanediol, and 0.1 M Tris, pH 7.5). The crystals belong to space group P212121 and have unit cell dimensions of $a = 83.32$, $b = 142.03$, $c = 85.00$, $β = 93.88$. There is one tetramer in the asymmetric unit.

Data Collection and Structure Determination—Crystals of the RalDH2 loop mutant were transferred directly to mother liquor supplemented with 20% glycerol and frozen in the stream of an Oxford Cryojet. X-ray data were collected with a Mar345 image plate scanner mounted on a Nonius FR591 generator fitted with Osmic mirrors. A total of 177 frames of 1° oscillations were collected to give a 3 Å resolution data set with a $R_{merge}$ of 14.9%. The data set is complete (72% in the highest shell) with 3.2-fold redundancy. At this resolution the overall $I/σ$ is 9.5 (1.8 in the highest resolution shell). The structure was solved by the method of molecular replacement. A tetramer of the native structure of rat RalDH2 served as a search model (13). The resulting 3.3 Å resolution structure was refined with 4-fold non-crystallographic symmetry constraints to an $R_s$ of 22.9% (0.304) with no $c$ cutoff applied to the reflections. The model includes 14,571 protein atoms, 108 co-factor atoms (only the adenine dinucleotide phosphate is positioned), and 39 water molecules. Average protein, co-factor, and water $B$-factors are 61, 45, and 65 Å², respectively. Diffraction data were processed with Denzo and Scalepack (16). All other crystallographic calculations were performed with CNS (17). Maps were interpreted with the program O (18).

Kinetic Measurements—Enzyme assays for the determination of the $K_m$ values of the substrates were conducted over at least six substrate concentrations (4 to 250 μM for hexanal, octanal, and decanal and 1 mM for 142.03 Å, $c = 85.00$ Å, $β = 93.88°$. There is one tetramer in the asymmetric unit.
to 128 nm for acetaldehyde) in an Applied Photophysics stopped-flow reaction analyzer by monitoring NADH fluorescence (excitation wavelength 340 nm, emission cutoff filter 420 nm) with a 0.2-cm path length. These experiments were performed at 25 °C. Enzyme activity was quantitated for each set of experiments by monitoring the absorbance of NADH at 340 nm in the stopped-flow. Measurements were performed at either 230 or 460 nm enzyme in the presence of 1 to 4 mM NAD, 10 mM HEPES, pH 8.5, 150 mM KCl, 1 mM EDTA, and 2 mM β-mercaptoethanol. These concentrations are those in the observation cell. Each experimental trace in fluorescence mode is the average of three consecutive observations. For absorbance mode data, at least eight traces were averaged.

The activity of the dual mutant enzyme for retinal was compared with that of the wild-type enzyme by monitoring fluorescence changes under identical assay conditions. When retinal (which absorbs at 340 nm) is the substrate, care is taken to avoid quenching of the fluorescence by performing the measurements at concentrations of retinal with an absorbance of less than 0.1. We found that the mutant had 34% of the activity of the wild-type enzyme under identical assay conditions, but given the spectral properties of retinal we were unable to determine whether the apparent reduction in the rate of retinal oxidation could not be overcome at higher substrate concentrations as the absorbance properties of retinal and retinoic acid interfered with our assay at higher substrate concentrations.

The data for the NADK, determinations were obtained by monitoring the absorbance of NADH with an Agilent diode array spectrophotometer with octan (50 μM) as the substrate. Data were obtained for six concentrations (in triplicate, 0.125–4 mM NAD for the mutant). Data Analysis—Values for K, and Vmax were determined by nonlinear regression analysis of a plot of velocity [s] versus substrate (or NAD) concentration data to the Michaelis-Menten equation. For the acetaldehyde, data from the mutant enzyme the data fit best to equation (1), where V1 and V2 are maximal velocities, K1 and K2 are Michaelis constants, and A is the concentration of substrate.

\[ v = V_1[A/(K_1 + A)] + V_2[A/(K_2 + A)] \]  
(Eq. 1)

Fluorescence Spectra—Fluorescence emission spectra of protein-bound (in the absence of substrate or product) and free NADH were recorded at excitation wavelengths of 295 nm and 340 nm in a Jasco FP-620 spectrofluorimeter. For these spectra, enzyme was incubated with an excess of NADH and desalted with Sephadex G-25 prior to the measurements.

Fluorescence Changes in the Pre-steady State Regime—The change in fluorescence that occurs upon mixing of substrate and enzyme as above was monitored by excitation at 295 nm in an Applied Photophysics stopped-flow reaction analyzer. The cutoff filter used for these measurements was 420 nm. The observed NADH fluorescence has two components: fluorescence that is caused by absorbance by NADH at 295 nm, and that emission occurs as a result of energy transfer from the excited Trp residues within the Förster distance of NADH. Experiments were performed with a catalytically inactive mutant (RalDH2-C301A) as well as the catalytically active mutant enzyme. No signal is observed for either 230 or 460 nm enzyme in the presence of NAD and the absence of substrate or substrate analog. Amino acids – 457–477 were not visible in the electron density maps (13) and unlike their counterparts in the previous ALDH structures, which were also determined in the presence of co-factor but absence of substrate (9–12, 15) were disordered. (Alternatively, these regions may have more than one possible conformation, and these conformations could be randomly distributed in the tetramer of the asymmetric unit.) In contrast to what is observed for ALDH2 and ALDH3, retinaldehyde dehydrogenase type II shows preference for aldehydes with long hydrocarbon chains in vitro and does not efficiently oxidize acetaldehyde (8). These data led us to propose a model for how disorder in this region might determine substrate specificity; the disordered loop provides a mechanism to link substrate binding to catalysis. We reasoned that the binding of a large hydrophobic substrate such as retinal induces a conformational change that orders the loop and enables catalysis. Larger aldehydes are unable to promote this conformational change because they lack sufficient surface for productive interaction. Implicit in this model is the idea that the channel must order (no matter what the substrate) for optimal catalytic activity. To test this theory we chose to introduce two mutations in the mobile loop: residues Leu-458 and Asn-459 were replaced by Phe and Gly, respectively, their counterparts in the highly homologous bovine mitochondrial dehydrogenase (10). Studies with this enzyme by others have provided an enormous amount of mechanistic information that we can utilize to guide our interpretation of the data obtained for RalDH2 (10, 19–26).

The mutated amino acids (458–459) are located at the amino-terminal end of the disordered loop region and in the homologous structures form a surface at the base of the substrate access channel. The catalytic Cys (Cys-301 in RalDH2) is between the wall of the access channel that Phe-459 provides and the NAD binding site. The three-dimensional context of the mutation is highly conserved between RalDH2 and ALDH2, and in the latter enzyme the corresponding Phe participates in an aromatic cluster with amino acids found in RalDH2 as well (Trp-177 and Phe-465; ALDH2 numbering). Multiple reasons are behind the choice of this dual substitution (1). This sequence difference between RalDH2 and ALDH2 (LN versus FG) could conceivably be one of the reasons that the channel is disordered in the former but ordered in the latter enzyme. Both Phe and Gly could contribute to stabilization of the loop in ALDH2 as Phe participates in the aromatic cluster referred to above, and Gly appears to be required for its lack of a side chain. In such a case the introduction of the substitutions might order the channel, and this result would allow us to determine the consequence of a “permanent” ordering (versus “permanent” disordering) of the loop on catalysis (2). It is important to emphasize here that the disordered loop is not fully surfaced-exposed but confined in the volume of the access channel. Thus in its disordered state, the loop region may represent a mobile barrier that smaller substrates must circumnavigate to reach the catalytic cysteine. A dual substitution was required to ensure no net gain in the volume of amino acids that must be accommodated in the channel (3). The mutated positions are removed in sequence from Ser-470, an invariant amino acid (Ser-471 in ALDH2 numbering) that has been shown to be important in this superfamily of enzymes. A substitution of an immediate neighbor of this amino acid could conceivably interfere directly with enzyme activity. In Fig 1a, the structure of ALDH2 with NADH bound as reported by Perez-Miller and Hurley (27) is provided to illustrate the above points. Amino acids that are identical in the two enzymes are shown in green.
It appears that the substitution of Phe-Gly for Leu-Asn in the disordered loop prevents this peptide from adopting a fully functional conformation in the three-dimensional structure. Thus, the introduction of amino acid changes that do not form part of the catalytic machinery results in impaired catalytic activity, and a disorder to order transition must occur for effective catalysis. The substrate channel must become ordered for optimal catalysis (even for substrates that do not fill the access channel), and the mutations prevent the loop from making this disorder to order transition. In both the mutant and wild-type enzymes, the catalytic residues are mobile as inferred from the fact that the electron density for these amino acids is poor. If ordering of the loop has non-local structural consequence (as we show below with respect to the NAD binding site) then ordering of the loop could be linked to stabilization of the functional conformation of the catalytic residues.

As described above, acetaldehyde is a very poor substrate for RalDH2. It is straightforward to reconcile this low activity with our suggestion that a substrate induced disorder to order transition is required for optimal activity; the two-carbon substrate is just too small to invoke the conformational change necessary to optimally order the catalytic machinery. The mutant enzyme RalDH2:L458F/N459G has 66% of the activity for acetaldehyde of the wild-type enzyme over a concentration range of 0.250–32 mM substrate. However, the data for the mutant enzyme exhibit negative cooperativity; the data fit best to a double rectangular hyperbola based of the fact that the average least squares of the residuals were the lowest for Equation 1. It is interesting to note that $V_{\text{max}}$ (1) for the mutant enzyme is equivalent to the $V_{\text{max}}$ of the wild-type enzyme. This might

![Fig. 1. The structure co-factor binding site of ALDH2 and a comparison of RalDH2 and ALDH2 structures.](image-url)
suggest that the catalytic machinery is disordered in both enzymes, as one might predict from a substrate induced order to disorder transition that must be induced by the binding of a large hydrophobic substrate. Neither the mutant nor wild-type enzyme is able to achieve an ordered structure of the loop with such a small substrate. However, the mutant enzyme displays negative cooperativity, indicative of communication between subunits, whereas the wild-type enzyme does not. Note that in Fig. 1b, it is clear that if the loop is positioned as it is in the homologous ALDH2 it lies at the dimer interface and loop conformation in one protomer could have structural consequences in its mate, consistent with the need for intersubunit communication. Our data do not rule out a more trivial explanation for the apparent difference. These experiments are done at high concentrations of acetaldehyde (mM), and the mutant enzyme may simply be more susceptible to inactivation by acetaldehyde covalent modification. It is interesting to note that the loop region has been implicated in intersubunit communication in terms of the dominant negative effect of a mutation of R475Q in ALDH2 (15, 28). Our steady state kinetic data indicate that the mutant enzyme has a reduced catalytic rate of product release. Accordingly, we obtained pre-steady state data by measuring the absorbance of NADH produced for both the release. Consequently two values for both \( V_{\text{max}} \) and \( K_m \) are reported.

| Substrate       | Wild type |              |              | RaLDH2:L458F/N459G |              |              |
|-----------------|-----------|--------------|--------------|-------------------|--------------|--------------|
|                 | \( V_{\text{max}} \) | \( K_m \) | \( V_{\text{max}} \) | \( K_m \)       | \( V_{\text{max}} \) | \( K_m \)       |
| Decanal         | 960 ± 25  | 1 ± 0.2 \( \mu \text{M} \) | 510 ± 120  | 1 ± 0.2 \( \mu \text{M} \) |               |              |
| Octanal         | 1080 ± 130 | 10 ± 0.6 \( \mu \text{M} \) | 390 ± 20  | 5 ± 0.6 \( \mu \text{M} \) |               |              |
| Hexanal         | 820 ± 20  | 5 ± 0.3 \( \mu \text{M} \) | 690 ± 30  | 8 ± 0.7 \( \mu \text{M} \) |               |              |
| Acetaldehyde    | 650 ± 140 | 14 ± 6 \( \mu \text{M} \) | 630 ± 85  | 31 ± 5 \( \mu \text{M} \) | 31 ± 5 \( \mu \text{M} \) | 0.14 ± 0.07 \( \mu \text{M} \) |
| NAD             | 67 ± 10 \( \mu \text{M} \) |              | 390 ± 36 \( \mu \text{M} \) |              |              |

**Fig. 2.** Enzyme activity monitored by the production of NADH. A linear rate of NADH production is observed when the absorbance of NADH is monitored at 340 nm (A340) (left panel, wild-type enzyme; right panel, RaLDH2:L458F/N459G). These data correspond to the standard reaction conditions of 460 nM enzyme, 4 mM NAD in assay buffer (see under “Experimental Procedures”). The reaction is initiated with the mixing of substrate (in this case 250 \( \mu \text{M} \) hexanal).

Therefore what we observe is not a consequence of a lowered rate of product release. Fluorescence spectroscopy provides experimental data to support a disorder to order conformational change. RaLDH2 has six Trps per monomer, and three are in the immediate vicinity of the nicotinamide ring as positioned by Perez-Miller and Hurley in ALDH2 (27). Of particular interest are Trp-176 and Trp-451 in RaLDH2, which in our structure with the mobile loop are exposed to solvent in the access channel. In the homologous enzyme ALDH2, solvent access to the corresponding tryptophans is restricted by the now ordered loop that covers them. In ALDH2, the counterpart for Trp-176 and Trp-451 forms part of an aromatic cluster with Phe-458 (Phe-459 in ALDH2 numbering) (Fig. 1a) and is \( -8 \) \( \AA \) from the nicotinamide ring as positioned in ALDH2, whereas the counterpart for Trp-451 is wedged between a Val and a Leu and \( -10 \) \( \AA \) from the co-factor. Assuming a similar loop conformation is adopted in RaLDH2, we looked to see if changes in Trp fluorescence might accompany substrate binding. In addition to the tryptophans in the vicinity of the mobile loop, there is an interaction of Trp-167 with the ribose ring of the NAD co-factor. The three tryptophan (Trp-167, Trp-176, and Trp-451) NADH fluorescence might accompany substrate binding. In addition to the tryptophans in the vicinity of the mobile loop, there is an interaction of Trp-167 with the ribose ring of the NAD co-factor. The three tryptophan (Trp-167, Trp-176, and Trp-451) NADH distances are all within the Förster distance of 25 \( \AA \) (29) for resonance energy transfer for this donor (Trp) and acceptor (NADH) pair to occur.

Fluorescence emission spectra of NADH in the absence and presence of enzyme (in the absence of substrate and product) clearly reveal an enhanced NADH signal at the excitation wavelength for Trp (Fig. 3) when NADH is protein bound and thus indicate Trp to NADH fluorescence resonance energy transfer. Upon mixing enzyme/NADH with substrate, a large
increase in fluorescence at the excitation wavelength for Trp (295 nm) is observed for the wild-type enzyme (Fig. 4). This increase is twice that observed at the excitation wavelength of NADH. Similar experiments with RalDH2:L458F/N459G produce a signal ~50% of that for the wild type, but again the fluorescence recorded at the excitation wavelength of Trp is approximately twice that observed for the NADH excitation wavelength. The fluorescence signal we observe is that which passes through the 420-nm cutoff and can be attributed to emission by NADH as a result of resonance energy transfer from the excited Trp and/or emission by NADH caused by its absorbance properties at 295 nm. We performed the same measurements with a C301A mutant. The catalytic cysteine is invariant in this family of enzymes, and Farres et al. (19) have demonstrated that its mutation to alanine in a highly homologous ALDH yields an inactive enzyme that is able to bind co-factor. Our C301A binds co-factor as well, as during the purification it 1) binds a dye affinity column and is eluted with NAD and 2) elutes from a sizing column with bound NAD. RalDH2:L458F/N459G displays a dramatically reduced fluorescence signal, and C301A under identical assay conditions produces none. These observations may indicate that NADH is serving as an intrinsic fluorescent probe, and the decrease in the signal for the double mutant is a result of a conformational difference which results in altered fluorescent properties. No signal is observed for the C301A because no NADH is produced. There is ample precedent for exploitation of NADH fluorescence as an intrinsic probe (e.g. Ref. 30). If one assumes that the disordered loop adopts the conformation observed in homologous ALDHs, the nicotinamide ring of the NADH is 7 Å from the site of the mutations and could serve as a reporter group for conformational differences that affect the NADH binding site or the environment of any Trp residues within the range for fluorescence resonance energy transfer. Accordingly, we found that during catalysis Trp-NADH fluorescence resonance energy transfer is enhanced. Note that in Fig. 3 the protein bound NADH emission is greater at the excitation wavelength for NADH (340 nm), whereas when we observe NADH fluorescence from reduced co-factor generated in the course of the reaction (Fig. 4) the signal is significantly greater at the excitation wavelength of Trp. This may indicate a difference in the conformation of the enzyme in the ternary (enzyme-co-factor-substrate) versus binary (enzyme-NADH) complex. Furthermore, the fluorescence properties of RalDH2:L458F/N459G differ from that of the wild-type protein. Because the nicotinamide ring is a sensitive probe of its environment, this observation could be interpreted as a conformational difference between the double mutant and wild-type proteins. No such structural differences are observed in comparison of the crystal structures of the wild-type and loop mutant enzymes (both crystallized with bound co-factor) as the region in which the mutations were made is not apparent in either structure.

From Fig. 1a it is evident that the mutated amino acids (459–460 in ALDH2 numbering) are not directly involved in the binding of co-factor in either its oxidized or reduced state. In addition, the crystal structure of RalDH2:L458F/N459G revealed a native-like structure with no structural aberrations in the co-factor binding domain. Even when the nicotinamide ring is positioned for hydride transfer the mutated amino acids are 7 Å from this tip of the outstretched co-factor. Furthermore, the nicotinamide ring is reported to be mobile in other members of the superfamily (e.g. Ref. 25). Yet, mutation of amino acids
outside the perimeter of the mobile nicotinamide ring subsite affects the $K_m$ of the enzyme for NAD; the $K_m$ for the mutant enzyme is $-6$ times that of the native enzyme (Table I). The conformation that the mutated loop cannot adopt has structural consequences in the NAD binding site. This observation is consistent with what has been reported by others for bovine mitochondrial ALDH2 for the corresponding region in that enzyme. Hurley et al. (15) have found that mutation of Arg-475 to disordered in the co-factor binding region through a "domino effect." And Sheikh et al. (22) observe that mutation of Ser-471 to an alanine results in a 50-fold increase in the $K_m$ for NAD. An even more striking effect of a distal mutation on the NAD binding site was described for the naturally occurring ALDH2:E487K mutation. The mutation, some 13 Å from the co-factor binding site, results in a $-150$-fold increase in the $K_m$ for NAD (24, 31). Thus, although a disorder to order transition may be unique to RalDH2, transmission of a conformational change from the catalytic domain to the nucleotide binding domain is likely a common feature in this enzyme family.

A Polyhistidine Tag May Affect Activity Assays—When we initiated these studies we found that wild-type RalDH2 has improved catalytic activity for its biological substrate when prepared without a polyhistidine tag. Previous kinetic data reported for RalDH2 were obtained from the histidine-tagged form (8). We chose to redesign the expression construct to produce the enzyme without the amino-terminal extension and purify the enzyme by the use of affinity resins for nucleotide binding proteins as well as ion-exchange and size exclusion chromatography. The kinetic data for its biologic substrate retinal obtained from the non-histidine tagged enzyme differs significantly from that of the enzyme with a histidine tag; our wild-type RalDH2 has a $V_{\text{max}}$ of $-200$ nmol/min/mg for retinal at 25°. This value is 10 times that reported by Wang et al. (8) and measured in our lab with the histidine-tagged form. It is also $-50$ times the activity reported for the enzyme when purified as a glutathione-transferase fusion protein and then cleaved (32). In contrast, the $V_{\text{max}}$ values for the aliphatic aldehydes are only modestly improved (e.g. 1080 versus $-800$ nmol/min/mg for octanal). The data described in this report were all obtained from wild-type and mutant enzymes purified by conventional chromatographic methods that exploit affinity chromatography resins for nucleotide binding proteins. It is not likely that differences in the enzyme assays utilized are the source of the discrepancy in the results, as we also see the rate reported by Wang et al. (8) for the histidine-tagged enzyme (data not shown) with our experimental approach of monitoring NADH production by fluorescence. Rather it is more likely that the difference is a direct result of the fact that the amino terminus of the protein is positioned near the entrance to the substrate access channel.

Concluding Remarks—As described above, the substrate access channel in retinaldehyde dehydrogenase type II is not ordered in the absence of substrate, and it was suggested that this disorder is functional; a disorder to order transition that can only be invoked by larger substrates enables substrate discrimination. We reasoned that such a mechanism could be tested by site-directed mutagenesis as a region of the polypeptide that remains disordered through the catalytic cycle should be insensitive to sequence changes if the amino acids mutated are not directly involved in substrate binding or catalytic activity. We found that when mutations are introduced in a disordered loop that is confined to the substrate access channel, enzyme activity is impaired. This observation indicates that during the catalytic cycle this region must make a specific intramolecular contact. We demonstrate that NADH fluorescence properties of the loop mutant and the wild-type enzymes are distinctly different and suggest that this difference reflects different environments of the Trp residues within the Förster distance for fluorescence resonance energy transfer. Thus, although the crystal structure of the mutant enzyme in the presence of co-factor is essentially identical to that of the native-NAD structure, a conformation of the enzyme that is required for effective catalysis is not as readily accessible to the RalDH2:L458F/N459G mutant form.

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