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Involvement of Conserved Glycine Residues, 229 and 234, of *Vibrio harveyi* Aldehyde Dehydrogenase in Activity and Nucleotide Binding

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The involvement of two conserved glycine residues (Gly229 and Gly234) in activity and nucleotide binding in *Vibrio harveyi* aldehyde dehydrogenase (ALDH) have been investigated. Each of the glycine residues has been mutated to alanine and the mutant ALDHs have been expressed in *Escherichia coli* and specifically labelled with [^{35}S]methionine. The G229A mutant was inactive with either NADP$^+$ or NAD$^+$ as coenzyme and did not bind to 2′,5′-ADP Sepharose, indicating a complete loss of nucleotide affinity. In contrast, the G234A mutant showed a high affinity for 2′,5′-ADP Sepharose. Purified G234A mutant showed similar kinetic properties to the native enzyme including a pre-steady-state burst of NADPH; however, the Michaelis constants for NAD$^+$ and NADP$^+$ were increased by 3- to 9-fold, showing that the mutation had an effect on saturation of the enzyme with NAD(P)$^+$. These data are consistent with the structure for the nucleotide binding domain of Vh.ALDH being similar to that of class 3 or class 2 mammalian ALDHs which differ from the classical nucleotide binding domain found in most dehydrogenases.

Most NAD(P)$^+$-dependent enzymes have a similar tertiary structure for the nucleotide binding domain. The core topology for the dinucleotide binding region contains up to 6 parallel β strands(# 1 - 6) connected by α-helices(A-E) as part of a complete $\beta_1\alpha_A\beta_2\alpha_B\beta_3$ unit and a second complete or partial $\beta_4\alpha_D\beta_5\alpha_E\beta_6$ unit (1) containing at least the β_4 strand (2). The structure for the dinucleotide binding region was originally based on the structures for lactate dehydrogenase,malate dehydrogenase, alcohol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase and contained two complete $\beta\alpha\beta\alpha\beta$ structures involved in AMP and nicotinamide binding,respectively, with the AMP site having distinct features that could be readily identified (3, 4, 5, 6). In particular, the initial 30-35 residues comprising the $\beta_1\alpha_A\beta_2$ fold served as a fingerprint region with three major characteristics; 1) a glycine rich turn with a consensus sequence of $G_1X_2G_3X_4X_5G_6$ between β_1 and α_A in close proximity to the pyrophosphate moiety of the dinucleotide, 2) six specific positions occupied by small neutral or hydrophobic residues involved in hydrophobic interactions and 3) a conserved negatively charged residue at the end of the β_2 strand hydrogen bonded to the 2′ and 3′ hydroxyls of the ribose of the AMP moiety (7). Recently, a positively charged residue (Arg or Lys) at the beginning of β_1 has been suggested as a fourth characteristic property (2).

Although most NAD(P)$^+$-dependent enzymes have a very similar structure for the nucleotide binding site (3), a different pattern for hydrogen bonding to the dinucleotide has been observed for a few dehydrogenases and reductases. Among these enzymes are glutamate dehydrogenase from *Clostridium symbiosum* (8) and glutathione reductase from *E. coli* (9) and human erythrocytes (10, 11) in which the last glycine residue in the GXGXXG consensus sequence is replaced with an alanine. In these enzymes the glycine rich turn (GXGXXA) is directly involved in hydrogen bonding with the ribose of AMP and the negatively charged residue at the end of β_2 is replaced by a neutral residue (12).

In aldehyde dehydrogenases(ALDHs) the classic GXGXXG consensus is absent, however, a closely related GXXXXG motif is clearly conserved. Only 19 residues are absolutely conserved and 12 residues nearly conserved(>87%) in ALDHs including 13 glycines (13, 14). The GXXXXG motif recognized in the three major classes(class 1,2 and 3) of mammalian ALDHs contains two of these conserved glycines as the first and last residues (G_{245} and G_{250} in class 1 and 2 human liver ALDH and G_{187} and G_{192} in class 3 rat liver ALDH, respectively) and was proposed to be located in a $\beta\alpha\beta$

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fold between the first β strand and the α helix. A negatively charged residue(E_{268} in class 1 and 2 and E_{209} in class 3 ALDHs) at the end of the second β strand as well as hydrophobic residues at the expected positions are conserved in the putative $\beta\alpha\beta$ fold (7, 14).

Very recently the first crystal structure of an ALDH (class 3 ALDH from rat liver) has been reported along with the location of the NAD^+ binding site (15). The GXXXXG motif was indeed found in a $\beta\alpha\beta$ fold[16], however, it was located between β_4 and α_D rather than as expected between β_1 and α_A and was in close contact with the nicotinamide ring and not with the pyrophosphate bridge (15). Although the glycine-rich GXXXXG turn for rat liver ALDH is in a critical location for nucleotide interaction, its specific role may be different than the GXGXXG fingerprint of the nucleotide binding site of other $NAD(P)^+$-dependent dehydrogenases. The GXXXXG motif in a class 2 mammalian ALDH was also found at the N-terminal of the α_D helix, although the nicotinamide mononucleotide portion of NAD^+ was bound in a different conformation to the class 2 ALDH than to class 3 ALDH (17).

The ALDH from *V. harveyi* is a homodimer of 55 kDa subunits with an unique preference for $NADP^+$ as a coenzyme. The specificity for $NADP^+$ is reflected in a low K_m for $NADP^+$ with the k_{cat}/K_m for $NADP^+$ being 40 fold higher than that with NAD^+ (13). Vh.ALDH has a high affinity for 2′,5′ ADP-Sepharose[18] and easily can be purified to homogeneity by one step purification on this support (19, 20). Alignment of its amino acid sequence with other ALDHs shows that Vh.ALDH contains the characteristic GXXXXG motif. In this study, the conserved glycines in the GXXXXG fingerprint of Vh.ALDH have been mutated to determine their role in activity and nucleotide interaction and whether the functional properties of these mutants are different than that found for mutants of the conserved glycine rich turn(GXGXXG) found in other dehydrogenases.

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                        β            α          β
                      -----      ---------   ------
                     XOXOXGXGXXGXXXOXXOXXXXXXXXXXOXOX@X
                        ##*#    #                  #  *
VhALDH              KAVGFTGSVGGGRALFNLAHERPEPI-PFYG-ELG  255
Cl1hu               DKVAFTGSTEVGKLIKEAA-GKSNLK-RVTL-ELG  270
Cl2hu               DKVAFTGSTEIGRVIQVAA-GSSNLK-RVTL-ELG  270
Cl3rt               DHIMYTGSTAVGKIVMAAA--AKHLT-PVTL-ELG  211
Cl3mrt              DHILYTGNTAVGKIVMEAA--AKHLT-PVTL-ELG  209
Colialdh            DAIAFTGSTRTGKQLLKDA-GDSNMK-RVWL-EAG  269
Spbtaldh            DKIAFTGSSATGSKVMASA--AQLVK-PVTL-ELG  262
Fothfaldh           RKIGFTGSTEVGKHIMKSC-ALSNVK-KVSL-ELG  675
Ohmucsaldh          NAITFTGETRTGEAIMRAA--AKGVR-PVSF-ELG  256
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FIG. 1. Amino acid sequence comparison of Vh.ALDH with human liver cytosolic ALDH (27), Cl1hu, human liver mitochondrial ALDH (28), Cl2hu, 2,3,7,8,-tetrachlorodibenzo-p-dioxin induced rat liver ALDH (29), Cl3rt, rat liver microsomal ALDH (30), Cl3mrt, *E. coli* ALDH (31), Colialdh, betaine ALDH from spinach (32), Spbtaldh, formyl tetrahydrofolate dehydrogenase from rat liver (33), Fothfaldh, hydroxymuconic semialdehyde dehydrogenase from Pseudomonas (34), Ohmucsaldh. O, @, G, X, β and α stand for hydrophobic residue, acidic residue, glycine, "any residue", β strand and α helix respectively. * and # indicate absolutely conserved and nearly conserved residues, respectively.

MATERIALS AND METHODS

Materials. [^{35}S] Methionine (800 Ci/mmol) and [^{35}S] dATP (1400Ci/mmol) were obtained from Du Pont- New England Nuclear. $NADP^+$ and NAD^+ were purchased from Sigma Chemical Co. Restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Labs or Pharmacia. Aldehydes were from Aldrich. 2′,5′ ADP Sepharose was from Pharmacia Biotech.

DNA sequencing. DNA sequencing was performed using dideoxy chain termination method (21).

Site directed mutagenesis. The Vh.ALDH in M13 was mutated based on the phosphothiorate method (22, 23) using the Sculptor mutagenesis kit from Amersham Int. The codons for glycine residues 229 and 234 (GGA) were altered to GCA (alanine). The mutations in the DNA were confirmed by nucleotide sequencing.

Protein concentration. Protein concentration was determined using the Bio-Rad protein determination kit with bovine serum albumin as a standard.

Expression of native and mutant ALDHs in E. coli using the T7 RNA polymerase/promoter system. *E. coli* K38 cells (*E.coli* transfected with the pGP1-2 plasmid coding for T7 RNA polymerase under control of a temperature sensitive repressor (24) were transformed with the pT7-3 plasmid containing recombinant DNA. [^{35}S] methionine labelling and expression was performed as described before (13, 20).

ALDH activity assay. Aldehyde dehydrogenase activities were measured by monitoring NAD(P)H production in 50 mM phosphate buffer, pH 8, at 25°C using fluorescence emission at 460 nm after excitation at 340 nm on a Hitachi F-3010 fluorometer. Kinetic constants were determined at saturating concentrations of NAD^+ (10 mM) or $NADP^+$ (1 mM) and dodecanal (100 μM).

Secondary structure prediction. Prediction of the secondary structure for the Vh.ALDH amino acid sequence is based on the method of Chou and Fasman,1978 (25).

RESULTS AND DISCUSSION

Based on amino acid sequence homology, G_{229} and G_{234} of *V. harveyi* aldehyde dehydrogenase (Vh.ALDH) correspond to G_{245} and G_{250} of class 1 and class 2 human liver (G_{187} and G_{192} of class 3) ALDHs (Fig.1). Secondary structure prediction shows that the sequence encompassing these glycines(Fig. 1) forms a $\beta\alpha\beta$ fold with G_{229} and G_{234} being part of a glycine rich turn at the beginning of the α helix. These characteristics along with the presence of the conserved hydrophobic residues in the $\beta\alpha\beta$ fold and a charged residue after the second β strand(E_{253}) indicated that this sequence could fulfil the same function as suggested for the $\beta_1\alpha_A\beta_2$ fold found in the nucleotide binding fingerprint for other dehydrogenases (7) (Fig.1).

The two glycine residues, G_{229} and G_{234}, were mutated to alanine and expressed in *E. coli* K38. Although ALDH activity was not detectable in lysates of G229A with either NAD^+ or $NADP^+$ as coenzyme, the mutant protein could be detected by ^{35}S-methionine labelling

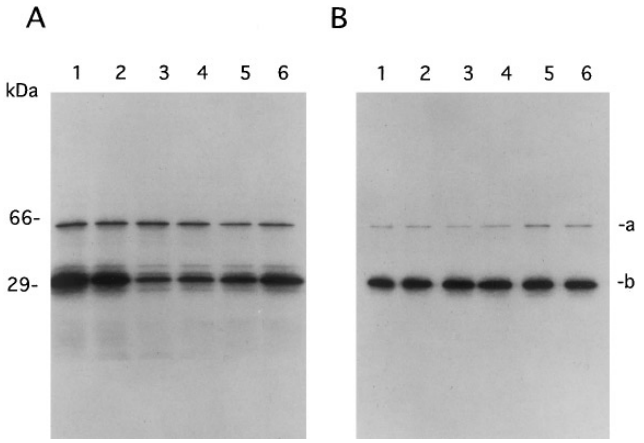

FIG. 2. Expression of G229A mutant ALDH in *E. coli* and extraction of labelled G229A mutant by sonication. G229A mutant was expressed and labelled with ^{35}S-methionine in *E. coli* K38 as described in Material and Methods. The K38 cells were centrifuged and resuspended in 50 mM phosphate buffer and 10 mM β-mercaptoethanol and were sonicated for 20 seconds. An aliquot of the lysate was centrifuged. Buffer containing SDS was added to the pellet and the supernatant and the samples resolved by SDS-PAGE. The sonication-centrifugation cycle was repeated 6 times. Lanes 1 to 6 are the pellet (A) and supernatant (B) of the lysate after a total of 20, 40, 60, 80, 100 and 120 seconds, respectively of sonication. Expressed proteins are labelled as (a) G229A mutant and (b) β-lactamase.

(Fig.2). The amount of G229A in the lysate supernatant increased with time of sonication showing that the inactive protein can be extracted in a soluble form.

To test for a change in affinity of the mutant G229A for 2′,5′ ADP, the lysate containing labelled G229A was mixed with a small amount of lysate containing recombinant Vh.ALDH and loaded onto a 2′,5′ ADP-Sepharose column. Figure 3 shows that the labelled G229A protein did not bind to the 2′,5′ ADP Sepharose

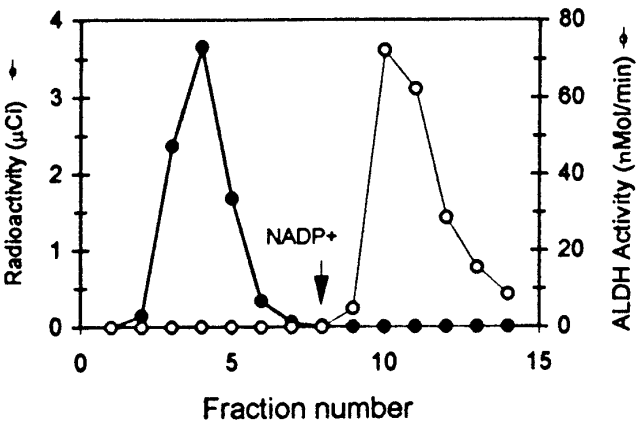

FIG. 3. Purification profile of recombinant Vh.ALDH and the G229A mutant ALDH on 2′,5′ ADP-Sepharose. A mixture of extracts of recombinant ALDH (0.3 U) and [^{35}S]methionine labelled G229A mutant ALDH were loaded onto a 2′,5′ADP-Sepharose column and then eluted with 50 mM phosphate, 10 mM β-mercaptoethanol, pH 7.0, followed by 100 μM NADP$^+$ in the same buffer.

TABLE 1

Kinetic Constants for Native and Mutant Vh.ALDHs

	K_m (μM)			k_{cat} (min^{-1})	
Enzyme	NAD$^+$	NADP$^+$	Dodecanal	NAD$^+$	NADP$^+$
G234A	3500	4.2	0.8	1037	164
Native	390	1.4	3	3600	510

whereas the wild type Vh.ALDH bound to the column and was eluted by NADP$^+$.

These results indicate that the conformation of the nucleotide binding pocket has been significantly changed in the G229A mutant and are consistent with the involvement of G_{229} as part of a sharp turn involved in nucleotide binding (7). Changing the first glycine residue in the GXGXXG fingerprint at the beginning of α_A in other nucleotide binding proteins has been demonstrated to cause a complete loss of nucleotide binding ability (26).

Labelling of a second mutant, G234A, with ^{35}S-methionine also confirmed that it was expressed in a soluble form in *E. coli*. However, in this case, ALDH activity could easily be detected in lysates using either NAD$^+$ or NADP$^+$ as coenzyme. The lysate containing G234A was loaded onto 2′,5′-ADP Sepharose and eluted in a homogeneous form with NADP$^+$ at the same positions as the native Vh.ALDH indicating similar nucleotide interactions with 2′,5′-ADP Sepharose. Table 1 shows that the specific activity of the purified G234A mutant was 3-fold lower than the native enzyme and the Michaelis constants for NAD$^+$ and NADP$^+$ had increased 3-9 fold. A presteady state burst of 2 mol NADPH per mol of dimeric protein was also measured showing that the rate limiting step is after hydride transfer and the same as that for the native Vh.ALDH.

These data show that the last glycine(G_{234}) in the $G_1X_2G_3X_4X_5G_6$ fingerprint is not absolutely critical for dinucleotide binding and that some flexibility exists in position 6 to allow substitution by alanine without major changes in activity and dinucleotide binding. Although this result could be consistent with the atypical structures observed for glutamate dehydrogenase (8), and glutathione reductase (9) with a fingerprint containing alanine rather than glycine as the sixth residue($G_1X_2G_3X_4X_5A_6$), these enzymes have a neutral rather than a charged residue at the end of the second β strand while Vh.ALDH has a negatively charged residue at this position (13). The structure for the glycine rich turn in Vh.ALDH would appear to more closely resemble that observed for the $G_{187}XXXXG_{192}$ turn in class 3 rat liver ALDH (15, 16). This turn is located between β_4 and α_D with the nucleotide binding motif involving the $\beta_4\alpha_D$ bend. This nucleotide binding motif is comparable to the $\beta_1\alpha_A\beta_2$ motif found in most other

dehydrogenases. The glycine rich turn in rat liver ALDH, however, is more closely associated with the nicotinamide moiety than the pyrophosphate moiety of NAD^+ with only the glycine at position 187 being close to the NAD^+ (G_{187} is 3.8 A^0 from the C4 position of the nicotinamide ring) (15). The position for the adenosine mononucleotide portion of NAD^+ for class 2 mitochondrial ALDH is similar to that for class 3 rat liver ALDH with the GXXXXG motif also located at the N-terminal of α_D. However, for class 2 mitochondrial ALDH, the nicotinamide mononucleotide portion is in a different conformation than in class 3 rat liver ALDH (17). The elimination of activity on mutation of the first glycine (G_{229}) in the GXXXXG motif of Vh.ALDH and the retention of activity on mutation of the last glycine residue (G_{234}) would be consistent with the structure found for rat liver ALDH where only the first glycine is in close contact with NAD^+ (15).

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