A Double Residue Substitution in the Coenzyme-binding Site Accounts for the Different Kinetic Properties between Yeast and Human Formaldehyde Dehydrogenases*  

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Glutathione-dependent formaldehyde dehydrogenase (FALDH) is the main enzymatic system for formaldehyde detoxification in all eukaryotic and many prokaryotic organisms. The enzyme of yeasts and some bacteria exhibits about 10-fold higher $k_{cat}$ and $K_m$ values than those of the enzyme from animals and plants. Typically Thr-269 and Glu-267 are found in the coenzyme-binding site of yeast FALDH, but Ile-269 and Asp-267 are present in the FALDH of animals. By site-directed mutagenesis we have prepared the T269I and the D267E mutants and the D267E/T269I double mutant of Saccharomyces cerevisiae FALDH with the aim of investigating the role of these residues in the kinetics. The T269I and the D267E mutants have identical kinetic properties as compared with the wild-type enzyme, although T269I is highly unstable. In contrast, the D267E/T269I double mutant is stable and shows low $K_m$ (2.5 μM) and low $k_{cat}$ (285 min$^{-1}$) values with S-hydroxymethylglutathione, similar to those of the human enzyme. Therefore, the simultaneous exchange at both residues is the structural basis of the two distinct FALDH kinetic types. The local structural perturbations imposed by the substitutions are suggested by molecular modeling studies. Finally, we have studied the effect of FALDH deletion and overexpression on the growth of S. cerevisiae. It is concluded that the FALDH gene is not essential but enhances the resistance against formaldehyde (0.3–1 mM). Moreover, the wild-type enzyme (with high $k_{cat}$ and $K_m$) provides more resistance than the double mutant (with low $k_{cat}$ and $K_m$).

Formaldehyde is a highly reactive compound that is present in the environment as a result of natural processes and human industrial activity. Moreover, formaldehyde is also generated endogenously by several metabolic pathways (1–5). In humans the level of formaldehyde in blood has been estimated as 0.46–2.81 μM (6). The elimination of formaldehyde in eukaryotic cells is mainly carried out by glutathione-dependent formaldehyde dehydrogenase (FALDH) (7). Formaldehyde reacts spontaneously with glutathione (GSH) to form the S-hydroxymethylglutathione (S-HMGSH) adduct which, in the presence of NAD$^+$, is oxidized to S-formylglutathione by FALDH (8). S-Formylglutathione is irreversibly hydrolyzed by S-formylglutathione dehydrogenase to formate and GSH (8). In addition to the glutathione-dependent formaldehyde dehydrogenase activity, FALDH is also active with long chain alcohols, particularly with ω-hydroxylated fatty acids such as 12-hydroxydocosanoic acid (9–12). Interestingly FALDH is capable of catalyzing efficiently the NADH-dependent degradation of S-nitrosothiol glutathione derived from the glutathione nitrosation by nitric oxide (13). FALDH is a dimeric enzyme with subunits of 40 kDa, containing two zinc atoms per subunit, and it appears to be ubiquitous in eukaryotic organisms (14). FALDH is a member of the medium chain alcohol dehydrogenase (ADH) family, and it represents the ADH class (class III) most closely related to the ancestral line that originated by successive gene duplications the ADH enzymes now found in animals and plants (15). X-ray diffraction analysis reveals that human FALDH shares a similar overall structure with the known class I ADH three-dimensional structures (16).

Although there is a high degree of sequence identity (63%) between the human and yeast enzyme, the $K_m$ and $k_{cat}$ values for the Saccharomyces cerevisiae enzyme (20 μM and 3100 min$^{-1}$, respectively) are significantly higher than those for the human FALDH (4 μM and 200 min$^{-1}$) (17, 18). In general, $K_m$ values for S-HMGSH are higher for all yeast and bacterial FALDH known (25–260 μM) (14, 19, 20) than for the animal and plant FALDH (4–9 μM) (7, 18, 21–23). Therefore, the existence of two FALDH kinetic types is apparent as follows: the microorganism type with high $K_m$ and $k_{cat}$ values, and the multicellular organism type with both low $K_m$ and $k_{cat}$ values. Here we report site-directed mutagenesis performed on the S. cerevisiae enzyme in order to identify residues responsible for the different kinetic characteristics of the two distinct FALDH types. In addition, we study the effect of FALDH deletion and overexpression on the growth of S. cerevisiae with the aim of elucidating the metabolic importance of FALDH in yeast.

EXPERIMENTAL PROCEDURES

Materials—Yeast nitrogen base without amino acids (Wickerham formula) was purchased from U. S. Biochemical Corp. Bacto-agar was from Difco. Zymolyase-100T was from ICN Biomedicals. β-NAD$^+$ (grade III), NADH, glutathione, paraformaldehyde, glass beads (0.4–0.6 mm), DEAE-Sepharose, glucose, and medium supplements (amino acids, ad-
enine hemisulfate, and uracil) were from Sigma. Blue-Phosphorose CL-6B, Superdex 200 HR 10/30, PD10 columns, PhastGel native buffer strips, and Native PhastGels were supplied by Amersham Pharmacia Biotech. A 20% solution of \( \text{H}_2\text{O} \) formaldehyde (98% isotopic enrichment) in \( \text{H}_2\text{O} \) was obtained from Medical Isotopes Inc. Protein-purify Q5 HR was from GeneElute. The template plasmid was the pBluescript, previously digested with BglII and BclI and cloned into FALDH-pBluescript, previously digested with the same restriction enzymes, substituting the wild-type fragment with the mutagenized fragment. Since the introduction of T269I mutation resulted in a lineal fragment containing the FALDH gene was then digested with NcoI/ClaI, subcloned into the Yeplac181 vector, and expressed in the same way as the wild-type enzyme. To insert the mutation D267E, the mutagenic primers B’, 5’-TTTGAATCTGGAATATCGAAACCATTATG-3’, and C’, 5’-ACCAGATACATGAAAATGAAATAC-3’, were used. The primer C’ was designed in order to insert not only mutation D267E but also a silent mutation that creates a new XbaI restriction site (underlined). In the first round two amplifications were performed, one with primers A and B’ and another with primers C’ and D, both with 100 ng of FALDH-pBluescript as a template, 1 \( \mu \)M each of primer, 200 \( \mu \)M of each dNTP, 3 mM MgSO\(_4\), and 1 unit of Vent DNA polymerase. Amplifications were performed with 30 amplification cycles of 1 min at 95 °C, 1 min at 54.9 °C, and 40 s at 72 °C. In a second round the products from the first round were used as templates together with primers A and D in 30 amplification cycles of 1 min at 95 °C, 1 min at 54.9 °C, and 40 s at 72 °C. The amplification product was digested with BglII and BclI and subcloned into FALDH-Yeplac181 previously digested with the same restriction enzymes, substituting the wild-type fragment between these restriction sites with the mutagenized fragment. The presence of mutation and the correct fragment orientation was confirmed by digestion with Xbal. All mutated DNA was completely sequenced, prior to expression, to ensure that no unwanted mutations were present.

The mutant enzymes were expressed and purified using a similar procedure as the one described for the wild-type enzyme with some differences. For the T269I mutant, an unstable enzyme, purification was performed with yeast grown to exponential phase (in order to avoid the high protease levels found in the stationary phase), and all steps were carried out in presence of 30% glycerol and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 1 \( \mu \)g/ml pepstatin A). For the D267E/T269I enzyme, due to the low activity detected in the initial lysate, it was necessary to start the purification procedure immediately after growth of culture that were grown in a bioreactor (Biotest-UB, B braun Biotech), and the subsequent chromatographic steps were scaled accordingly.

Kinetic Studies—Enzyme activity was determined spectrophotometrically at 25 °C by monitoring the production of NADH at 340 nm \( \varepsilon_{340} = 6.22 \text{ mm}^{-1} \text{ cm}^{-1} \) with a Hewlett-Packard 8452A diode array spectrophotometer. One unit of activity corresponds to 1 \( \mu \)M of NADH produced per min. For determination was performed from 0 to 0.5 \( \mu \)M NAD\(^+\) and 0.2 \( \mu \)M FALDH in 0.1 M sodium phosphate, pH 8.0, with S-HMGSH (formed by mixing formaldehyde and glutathione) (7). The standard assay mixture contained 1 mM formaldehyde, 1 mM glutathione, 2.4 mM NAD\(^+\) in 0.1 M glycine/NaOH, pH 10. Formaldehyde dehydrogenase activity was measured in 0.1 M sodium phosphate, pH 8.0, with S-HMGSH (formed by mixing formaldehyde and glutathione) (7). The standard assay mixture contained 1 mM formaldehyde, 1 mM glutathione, 2.4 mM NAD\(^+\) in 0.1 M sodium phosphate, pH 8.0. For the kinetic studies concentration of S-HMGSH was established by adjusting the total concentrations of glutathione and formaldehyde in order to maintain the concentration of free glutathione at 1 mM, on the basis of the dissociation constant of S-HMGSH. Dissociation constant values of 1.5 mM (7) and 1.3 mM (30) were used for diprotio- and S-hydroxy-[\(^3\)H]methylgluthathione, respectively. Formaldehyde (20%) was obtained by hydrolysis of paraformaldehyde during 16 h at 100 °C. Stock solutions of formaldehyde were standardized as reported (17). Kinetic constants were calculated by using the non-linear regression program Enzfitter (Elsevier Biosoft). \( K_a \) and \( K_{NAD^+} \) values were calculated with the COMP program (31). Values are expressed as the mean of at least three separate experiments.

Native Polyacrylamide Gel Electrophoresis and Western Blot Analysis—Native gel electrophoresis was performed in a PhastSystem instru-ment (Amersham Pharmacia Biotech) but only one gel for each sample. 12.5% polyacrylamide gels with PhastGel native buffer strips and sub-sequent silver staining. For Western blot analysis, proteins were separated by SDS-polyacrylamide gel electrophoresis as described (32) and subsequently transferred to a polypropylene membrane (Selex 20) by electroblothing in 25 mM Tris, 200 mM glycine, pH 8.5, containing 20% methanol. The membrane was blocked against nonspecific protein bind-
ing by overnight treatment in Tris-buffered saline (Tris/NaCl) containing 10 mM Tris/HCl, 150 mM NaCl, pH 8.0, with the addition of 1% (w/v) bovine serum albumin (BSA). The membrane was then incubated for 2 h with rabbit anti-(tritium class III ADH) antisera diluted 1:1000 in Tris/NaCl/BSA, washed in Tris/NaCl/BSA for 5 min, incubated for 1.5 h with goat anti-rabbit IgG alkaline phosphatase-conjugated, and washed five times in Tris/NaCl/BSA for 5 min each time. The membrane was further developed by incubation in 100 mM Tris–HCl, 100 mM NaCl, 5 mM MgCl₂, pH 9.5, containing 16.5% (w/v) 5-bromo-4-chloro-3-indolyl phosphate and 0.33% (w/v) nitro blue tetrazolium. When sufficiently developed, the reaction was stopped by washing in water.

**Molecular Modeling**—A three-dimensional model of the 260–280-residue region, in the coenzyme binding domain of yeast formaldehyde dehydrogenase, was obtained by adopting its amino acid sequence into the known fold of the human FALDH subunit. For residues 266–274, main chain angles (\(\phi\) and \(\psi\)), were free, whereas main chain angles, \(\phi\) and \(\psi\), were kept fixed in order to preserve the whole loop shape (avoiding any backbone deviation). For residues 266–274, main chain angles (\(\phi\) and \(\psi\)) and side chain angles (\(\chi_i\)) were reached to an optimal conformation. After iterative combined geometry and energy optimization and adjustments of polar hydrogen positions, the whole 260–280-residue region was subject for a biased Monte Carlo procedure (33) (1000 iterations for every Monte Carlo step and \(5 \times 10^6\) total steps). The temperature of the simulation was 1500 K, in order to avoid any local minimum energy. The same procedure was used with each mutant studied. The calculations were performed in a Silicon Graphics Indigo 2 R10000 workstation.

**RESULTS**

**Identification of Functional Residues and Site-directed Mutagenesis**—Yeast FALDH must exhibit structural differences from the group of animal and plant FALDHs, which should explain its distinct kinetic properties. Comparison of the yeast (*S. cerevisiae* and *Candida maltosa*) and animal and plant FALDH sequences shows that, in general, those residues that participate in the substrate binding, coenzyme binding, and subunit-subunit interactions are conserved. There is one relevant change in the coenzyme-binding pocket: a Thr at position 269 in yeast instead of Ile found in the human enzyme, used as an example of the animal and plant FALDH type. The presence of a polar residue at position 269 may affect coenzyme binding and could contribute to the kinetic differences between the human and yeast FALDH. In order to check for this hypothesis the residue Thr-269 in *S. cerevisiae* FALDH was changed to Ile (T269I mutant). The comparison between the FALDH primary structures also revealed that the presence of Thr at position 269 was associated with Asp at position 267 in yeast, whereas the presence of Ile at position 269 was associated with Gln at position 267 in human as well as in all animal and plant FALDHs. Thus, we also decided to change Asp-267 to Glu (D267E mutation) and the double mutation Asp-267 to Glu and Thr-269 to Ile (D267E/T269I).

**Enzyme Expression, Purification, and Stability**—Wild-type and mutant FALDHs were expressed in the FALDH yeast, and the stability of each protein was analyzed prior to purification. The wild-type enzyme did not lose any activity in the crude extract after 3 days at 4 °C, and it was purified to homogeneity in three chromatographic steps. From 25 g of yeast cells, 240 mg of pure enzyme were obtained with a 24% yield and a specific activity of 32 units/mg. The D267E mutant was also stable in the crude extract, and it was purified to homogeneity with an 11% yield and a specific activity of 25 units/mg. From 37 g of yeast, 44 mg of homogeneous protein were obtained. In contrast, the T269I mutant showed a complete inactivation in the crude extract, after less than 24 h at 4 °C. The loss of activity was correlated with the loss of FALDH protein by Western blot analysis (not shown), suggesting digestion by proteases. Different agents for stabilizing the T269I enzyme were tested. Thus, after 12 h at 4 °C, activity was 87% of the initial value in the presence of 1 mM EDTA, 13% in the presence of protease inhibitors, and 90% in the presence of 30% glycerol. This unstable protein was, therefore, purified to homogeneity in the presence of 30% glycerol and protease inhibitors in all chromatographic steps. From 37 g of yeast, 410 mg of purified enzyme were obtained with a 31% yield and a specific activity of 29 units/mg. The NAD⁺ (2.4 mM) added to the enzymatic assay prevented the inactivation during the activity determination in the absence of glycerol.

After purification, the T269I mutant was still unstable. Thus, when glycerol concentration was reduced from 30 to 10%, there was an immediate decrease in FALDH activity by 50%, and when glycerol was reduced to 0.6% the mutant enzyme was completely inactivated. The T269I mutant did not change its activity when it was incubated in the presence of 30% glycerol and 1 mM EDTA, but it lost completely its activity in 10% glycerol plus 1 mM EDTA. In contrast, the wild-type enzyme kept 100% activity in 1 mM EDTA, even in the absence of glycerol. Analysis by SDS-polyacrylamide gel electrophoresis, electroblotting, and immunodetection of the inactivated mutant enzyme showed a single band with the same mobility and intensity than that of the active enzyme, which demonstrates absence of protein degradation (not shown). Native polyacrylamide gel electrophoresis showed that the samples that conserved activity exhibited a band around 80 kDa, at the same level to that of the wild-type enzyme (Fig. 1). However a smear was detected in all T269I samples with activity, possibly indicating partial unfolding and aggregation of the protein while the sample was entering the polyacrylamide gel, due to glycerol dilution. On the other hand, neither the T269I sample inactivated by incubation in EDTA with 10% glycerol, nor the denatured wild-type enzyme showed any band (Fig. 1). This could be due to protein aggregation in either case that did not allow the protein to penetrate the gel. Taken together, these data indicate that mutant T269I was highly unstable because it rapidly unfolded in the absence of a suitable concentration of stabilizing agents. Moreover, it was also more sensitive to the chelating effect of EDTA. Surprisingly, the addition of a single methylene group in the side chain of residue 267 stabilized the T269I enzyme. Thus, the D267E/T269I double mutant showed no loss of activity after 3 days at 4 °C. From 393 g of yeast, 16 mg of D267E/T269I protein were obtained with a final yield of 41% and a specific activity of 2.9 units/mg.
Kinetic properties of yeast wild-type and mutant FALDHs compared with mammalian FALDH

Activity with S-HMGSH was measured in 0.1 sodium phosphate, pH 8.0, with 2.4 mM NAD\(^+\). \(K_m\) was measured with 10–500 \(\mu\)M NAD\(^+\) and 2–300 \(\mu\)M S-HMGSH. \(K_{m(NADH)}\) was determined with 20–1000 \(\mu\)M NAD\(^+\) and 0–100 \(\mu\)M NADH at a fixed concentration of 150 \(\mu\)M S-HMGSH, except for the double mutant where 20 \(\mu\)M S-HMGSH was used.

| FALDH          | S-HMGSH | NAD\(^+\) | NADH, \(K_i\) |
|----------------|---------|-----------|-------------|
|                | \(K_m\) | \(k_{cat}\) | \(k_{cat}/K_m\) | \(K_m\) | \(k_{cat}\) | \(k_{cat}/K_m\) |
| \(S. cerevisiae\) |         |           |             |        |         |             |
| Wild type      | 20      | 3100      | 155,000     | 45     | 86      | 19          |
| D267E mutant   | 14      | 2100      | 150,800     | 20     | 93      | 12          |
| T269I mutant   | 15 \(a\) | 2200      | 147,900     | 9 \(a\) | ND \(a\) | ND          |
| D267E/T269I mutant | 2.5  | 285       | 113,600     | 25     | 180     | 14          |
| Human          | 4.0 \(b\) | 200 \(b\) | 50,000 \(b\) | 9 \(c\) | ND \(c\) | ND          |
| Rat            | 0.9 \(d\) | 216 \(d\) | 235,000 \(d\) | 3 \(c\) | ND \(c\) | ND          |

\(a\) The kinetic constants for coenzymes could not be properly determined because the T269I mutant is unstable at low coenzyme concentrations.

\(b\) Data from Ref. 12
\(c\) Data from Ref. 14

Functional Residues of Formaldehyde Dehydrogenase

Kinetic Characteristics of the FALDH Mutants—The steady-state kinetic properties of D267E and T269I mutants are similar to those of the wild-type enzyme (Table I). Interestingly, the D267E/T269I double mutation strongly affects the kinetic parameters. Thus, the double mutant exhibits \(k_{cat}\) and \(K_m\) values for S-HMGSH approximately 11-fold lower and 8-fold lower, respectively, than those of the wild-type enzyme. The \(k_{cat}\) and \(K_m\) also decreased approximately 5- and 12-fold, respectively, for 12-hydroxydodecanoic acid (not shown). As a result of the kinetic changes, the D267E/T269I mutant shows kinetic characteristics similar to those of the human and in general to those of the all animal and plant FALDHs. The enzymes of these organisms contain Ile-269 and Glu-267 like general to those of the all animal and plant FALDHs. The kinetic characteristics similar to those of the human and in general to those of the all animal and plant FALDHs. The enzymes of these organisms contain Ile-269 and Glu-267 like general to those of the all animal and plant FALDHs.

With the aim of investigating whether the interconversion of the ternary complex is the limiting step in the enzyme mechanism and whether this is affected by mutation, the deuterium isotope effects on the kinetic constants of yeast FALDH were measured (Table II). The primary deuterium kinetic isotope effects on the macroscopic kinetic constants (\(k_{cat}\) and \(k_{cat}/K_m\)) are small for the wild-type and the double mutant FALDHs (Table II), indicating that the isotope-sensitive hydride transfer step is not significantly rate-limiting during catalysis in both cases.

Growth Kinetics of FALDH-deleted and FALDH-overexpressed Strains—With the aim to test whether FALDH is essential for cell life, an \(S. cerevisiae\) haploid strain was constructed such that the gene coding for FALDH was replaced by the \(U R A 3\) gene. This resulted in a deletion of the \(F A L D H\) gene, and, therefore, in the absence of FALDH activity in the homogenate. Both the null mutant (FALDH\(^{-}\)) and the wild-type strain (FALDH\(^{+}\)) could grow equally well in a rich medium, demonstrating that the deletion is not lethal. The physiological importance of the enzyme is, however, supported by the experiments shown in Fig. 2. FALDH\(^{-}\) grew very slowly in the presence of 0.6 mM formaldehyde in the culture medium with a long lag phase and a long generation time (25 h). In contrast, FALDH\(^{+}\) grew with a much shorter generation time (11 h) under these conditions. In the presence of 0.3 mM formaldehyde, FALDH\(^{-}\) could grow much faster (generation time of 8 h) than at 0.6 mM but also at a lower rate than the FALDH\(^{+}\) strain (generation time of 4 h), demonstrating that FALDH is an effective enzymatic system for formaldehyde detoxification in \(S. cerevisiae\).

To obtain a high level of FALDH expression, the \(NcoI/Clai\) fragment was subcloned into the episomal plasmid Yeplac181 and transformed into the FALDH\(^{-}\) strain. This increased the FALDH activity approximately by 10-fold in the homogenate. The yeast strain overexpressing FALDH\(^{+}\) was able to grow in the presence of 1 mM formaldehyde in the culture medium, whereas the FALDH\(^{-}\) and FALDH\(^{-}\) strains did not grow under these conditions after 32 h (Fig. 3). The growth of the FALDH\(^{-}\) yeast was accompanied by a decrease of formaldehyde concentration in the medium, demonstrating an active metabolism of formaldehyde in this strain.

Yeast overexpressing wild-type FALDH (FALDH\(^{+}\)) grew faster than the yeast that overexpresses the D267E/T269I mu-
found in human FALDH makes the yeast enzyme kinetically similar to the human FALDH, with low $K_m$ and $k_{\text{cat}}$ values for S-HMGSH (Table I). Remarkably, each individual substitution, D267E or T269I, separately, does not significantly affect the enzyme kinetics. The T269I change needs to be accompanied by the substitution of Asp-267 with Glu, as found in the human enzyme, in order to transform the yeast enzyme kinetics in a mammalian FALDH kinetic type (Table I). Moreover this double mutant is as stable as the wild-type enzyme, demonstrating that the instability introduced by the single T269I mutation is counteracted by the D267E change.

The decrease in $k_{\text{cat}}$ observed for the double mutant suggests that the structural changes have affected the rate-limiting step of the catalyzed reaction. The primary deuterium kinetic isotope effects on the kinetic constants reveal that the hydride transfer is not significantly rate-limiting during catalysis, in both the wild-type enzyme and the D267E/T269I double mutant, suggesting that product(s) release is the rate-limiting step. This is also the case for bovine FALDH (30) and for other medium chain alcohol dehydrogenases (40). We can conclude that the double mutation probably has decreased the $k_{\text{cat}}$ by decreasing the rate of product(s) release. That dissociation of NADH is limiting is suggested by the finding that $k_{\text{cat}}$ for the wild-type enzyme shows a 4-fold increase when 3-acetylpyridine adenine dinucleotide (acetyl-NAD$^+$) is used as cofactor. Similarly, increases in activity were found in FALDHs from other species when acetyl-NAD$^+$ substituted NAD$^+$ (30), suggesting that the acetyl function in the nicotinamide ring increases the rate of dissociation of the coenzyme. Similarly $k_{\text{cat}}$ for the double mutant increased by a factor of 7 when acetyl-NAD$^+$ substituted NAD$^+$. This difference of a 4–7-fold increase in $k_{\text{cat}}$ may indicate that in the double mutant the release of NADH is more limiting. In conclusion, the lower $k_{\text{cat}}$ values for S-HMGSH in the D267E/T269I is probably the consequence of a slower NADH release. However, no difference was found for $K_{\text{cat}}$ (NADH) between the wild-type and double mutant enzymes. This may be explained by a similar effect of mutation on both product release ($k_{\text{cat}}$) and association ($k_{\text{on}}$) constants and therefore without significant change of $K_{\text{cat}}$(NADH.$^+$). Interestingly, the substitution of Ile-269 with Ser in the horse class I ADH was described to increase the ethanol oxidation 26-fold due to a faster release of NADH (40), although in that case an increase in the NADH dissociation constant was observed. The different effect of the substitution on the cofactor constants may be a consequence of differences in the kinetic mechanism, which is ordered for class I ADH and probably random for FALDH (41, 42).

**Molecular Model**—The x-ray structure of human FALDH (16) shows that Glu-267 and Ile-269 are located in the 267–271 loop at the coenzyme-binding site (Fig. 4, A and B). This loop is placed in the Rossmann fold of the medium chain ADHs, between the $\beta$D strand and the $\alpha$E helix; it is close to the adenine moiety, and it establishes several interactions with the bound coenzyme (43). The Ile-269 has its side chain directed toward the adenine ring, performing hydrophobic interaction with it, and its carbonyl oxygen is at hydrogen bond distance of the 3’-hydroxyl group of nicotinamide ribose. Residue Glu-267 makes hydrogen bonds with the main chain amino groups of amino acids 269, 270, and 271. As we have demonstrated, substitutions at residues 267 and 269 result in major changes in the kinetics and stability of the enzyme. Molecular models of the loop in the yeast FALDH and the mutated enzymes, based on the human FALDH structure (16), are presented in Fig. 4, C–F. The adenine ring is farther from Thr-269 in yeast FALDH (Fig. 4C) than from Ile-269 in human FALDH (Fig. 4B). This, together with the change of polarity, could affect the interac-

**Fig. 3. Growth kinetics of S. cerevisiae strains in the presence of 1 mM formaldehyde (A) and formaldehyde concentration in the corresponding culture medium (B). Open symbols indicate the growth of the strains in the absence of formaldehyde, and closed symbols indicate the growth in 1 mM formaldehyde. Triangles, FALDH$^-$; circles, FALDH$^+$; squares, strain overexpressing the FALDH gene (FALDH$^{+\text{cat}}$); diamonds, strain overexpressing the D267E/T269I mutant. The FALDH$^+$ and the double mutant strains have similar FALDH protein levels as estimated by enzyme activity and Western blot analysis of homogenates.

**DISCUSSION**

**Metabolic Importance of Yeast FALDH**—From FALDH gene dosage experiments we can conclude that FALDH is not essential for S. cerevisiae life under laboratory conditions, in agreement with a previous report (34). However, we can also conclude that the null mutant is more sensitive to the toxic effects of formaldehyde than the wild-type yeast and that the overexpression of FALDH enhances resistance to formaldehyde, indicating that FALDH is an effective enzymatic system for formaldehyde elimination in yeast. Probably the absence of FALDH in a mutant yeast strain would be disadvantageous in a natural situation where formaldehyde is a common environmental compound, and the defective strain would be selectively eliminated. The fact that FALDH is constitutively present in most animal and plant cells, and in many unicellular organisms (14, 17, 20, 23, 35–39), supports the notion that the enzyme is indispensable for the normal life of many species.

**Structure-Function Relationships**—Yeast FALDH has higher $K_m$ and $k_{\text{cat}}$ values than the corresponding mammalian enzyme (17). By using site-directed mutagenesis we have demonstrated that the double substitution of the yeast Thr-269 and Asp-267 with the corresponding residues Ile-269 and Glu-267 is detrimental for the normal life of many species.
tion of residue 269 with the coenzyme in the yeast enzyme. In addition, the distance of the Thr-269 carbonyl oxygen to the 3'-hydroxyl group of nicotinamide ribose increases slightly, probably impairing hydrogen bond formation (Fig. 4C). Moreover, in the yeast structure, Asp-267 performs hydrogen bond only with residue 269 but not with residues 270 and 271, which could indirectly affect the interaction of the loop with the coenzyme. These altered interactions imposed by the changes in residues 269 and 267 are probably responsible for the different kinetic properties of yeast FALDH. This is supported by the model of the mutated enzymes (Fig. 4, D–F). Substitution of Asp-267 by Glu does not affect the kinetic parameters of the

**Fig. 4. Three-dimensional structure of the FALDH 267–271 loop.** Human FALDH structure, according to the crystallographic data (16), shows the loop in a general **ribbon** representation (A, in blue) and in a **stick** representation (B). Molecular models of the loop are also presented for the yeast FALDH wild-type (C), and for the D267E (D), T269I (E), and D267E/T269I (F) mutants. Molecular modeling was performed for the 260–280 region (see “Experimental Procedures”) although only the 267–271 loop is shown. NAD⁺ is represented in **magenta** and the catalytic zinc in gray. Hydrogen bonds are represented as **green dashed lines**. We considered 3.2 Å as the maximum hydrogen bond distance.
yeast enzyme, which is consistent with the lack of significant changes between the wild-type and D267E structures (Fig. 4, C and D). Substitution of Thr-269 with Ile makes the enzyme highly unstable but still catalytically similar to the wild-type enzyme. Molecular modeling of T269I indicates a rotation of residue 269 that makes Ile-269 separate from NAD\(^+\) (Fig. 4E). Interestingly, the effect of this mutation is on the stability of the enzyme but not on the kinetics. Probably the instability arises because the Ile of the mutant is directed toward an external hydrophilic area (AMP binding region) (Fig. 4). The stabilizing effect of NAD\(^+\) could be due to a direct interaction with the Ile-269 side chain, and/or to the conformational change induced by the coenzyme binding, that makes the active site less accessible to water. Molecular modeling of the D267E/T269I double mutant (Fig. 4F), a stable enzyme with similar kinetics to human FALDH, suggests that the Ile-269 side chain is directed toward the adenine ring, whereas the Ile carbonyl oxygen is at hydrogen bond distance from the 3'-hydroxyl group of the nicotinamide ribose, like in the human FALDH (Fig. 4B). The two substitutions are therefore necessary in order to have interactions of residue 269 with the coenzyme similar to those found in the human FALDH. The molecular modeling of the 260–280 region gives, therefore, an explanation for the structural perturbation imposed by substitutions at residues 267 and 269, which finally results in important kinetic and stability changes in the yeast FALDH.

A similar molecular explanation was suggested for the faster NADH release observed in the I269S mutant of class I ADH (40). In that case, evidence indicated that the substitution could affect the rate of conformational change of the enzyme-coenzyme complex (40). Likewise, the conformational change has been proposed as a limiting step for the FALDH-catalyzed reaction (30). Therefore, the double mutation of yeast FALDH could also affect the kinetic constants through an alteration of the rate of the conformational change induced by coenzyme binding.

In conclusion, the D267E/T269I mutations produce changes in the coenzyme binding region of yeast FALDH that result in an enzyme with low k\(_{cat}\) and K\(_m\) values. Apparently, natural selection has used these changes to generate the FALDH type found in animals and plants, organisms that are exposed to low formaldehyde concentrations. In contrast Asp-267 and Thr-269 are found in yeast FALDH, which consequently exhibits high k\(_{cat}\) and K\(_m\) values, as a metabolic adaptation (44) against the potentially high formaldehyde concentration in the yeast natural environment (Fig. 3) (17).

REFERENCES
1. Fujiwara, K., Okamura-Ikeda, K., and Motokawa, Y. (1984) J. Biol. Chem. 259, 10664–10668
2. Porter, D. H., Cook, R. J., and Wagner, C. (1985) Arch. Biochem. Biophys. 243, 396–407
3. Waydhas, C., Weigl, K., and Sies, H. (1978) Eur. J. Biochem. 89, 143–150
4. Shara, M. A., Dickson, P. H., Bagchi, D., and Stohs, S. J. (1992) J. Chromatogr. 576, 221–233
5. Yu, P. H., Lai, C.-T., and Zuo D.-M. (1997) Neurochem. Res. 22, 615–620
6. Hallier, E., Schröder, K. R., Asmuth, K., Dommermuth, A., Aust, B., and Goergens, H. W. (1994) Arch. Toxicol. 68, 425–427
7. Kuivusalo, L., and Kuivusalo, M. (1974) J. Biol. Chem. 249, 7653–7663
8. Parés, X., and Vallee, B. L. (1981) Biochem. Biophys. Res. Commun. 98, 122–130
9. Wagner, F. W., Parés, X., Holmquist, B., and Vallee, B. L. (1984) Biochemistry 23, 2193–2199
10. Fujiwara, K., Okamuraa-Ikeda, K., and Motokawa, Y. (1984) J. Biol. Chem. 259, 1509–1516
11. Gutiérrez, J. M. A., and Parés, X. (1995) Biochemistry 34, 4709–4713