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Site-directed mutagenesis studies of the metal-binding center of the iron-dependent propanediol oxidoreductase from *Escherichia coli*

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The amino acid residues involved in the metal-binding site in the iron-containing dehydrogenase family were characterized by the site-directed mutagenesis of selected candidate residues of propanediol oxidoreductase from *Escherichia coli*. Based on the findings that mutations H263R, H267A and H277A resulted in iron-deficient propanediol oxidoreductases without catalytic activity, we identified three conserved His residues as iron ligands, which also bind zinc. The Cys362, a residue highly conserved among these dehydrogenases, was considered another possible ligand by comparison with the sequences of the medium-chain dehydrogenases. Mutation of Cys362 to Ile, resulted in an active enzyme that was still able to bind iron, with minor changes in the K_m values and decreased thermal stability. Furthermore, in an attempt to produce an enzyme specific only for the zinc ion, three mutations were designed to mimic the catalytic zinc-binding site of the medium-chain dehydrogenases: (1) V262C produced an enzyme with altered kinetic parameters which nevertheless retained a significant ability to bind both metals, (2) the double mutant V262C−M265D was inactive and too unstable to allow purification, and (3) the insertion of a cysteine at position 263 resulted in a catalytically inactive enzyme without iron-binding capacity, while retaining the ability to bind zinc. This mutation could represent a conceivable model of one of the steps in the evolution from iron to zinc-dependent dehydrogenases.

Keywords: alcohol dehydrogenase; metal-binding site; site-directed mutagenesis; *Escherichia coli*; iron; zinc.

The oxidoreductases catalyzing the interconversion of alcohols, aldehydes and ketones can be divided into three major categories: (1) NAD(P)-dependent alcohol dehydrogenases (ADHs), (2) NAD(P)-independent ADHs and (3) FAD-dependent alcohol oxidases. The first category can, in turn, be divided into three groups that have a possible common ancestor with reference to the coenzyme-binding site [1, 2]: group I medium-chain zinc-dependent dehydrogenases, group II short-chain zinc-independent dehydrogenases, and group III 'iron-activated' dehydrogenases.

To date, all members of the iron-activated dehydrogenase family are microbial and have been identified on the basis of primary structure homology. It includes ADHs with subunit size around 40 kDa, such as ADH II from *Zymomonas mobilis*, ADH IV from *Saccharomyces cerevisiae*, methanol dehydrogenase (MDH) from *Bacillus methanolicus*, two butanol dehydrogenases (BDH A and BDH B) from *Clostridium acetobutilicum*, and propanediol oxidoreductase (POR) from *Escherichia coli* [2]. Three multifunctional dehydrogenases, ADH Es from *E. coli* and from *C. acetobutilicum* [2] and ADH 2 from *Entamoeba hystolitica*, with a molecular mass of 96 kDa, are also included in this group [3, 4].

There is no current information about the three-dimensional structure for any of the enzymes of this group. A putative iron-binding motif was proposed by Bairoch [5] and Cabiscol et al. [6]. In both cases, these authors involved a 15-amino-acid stretch, which includes three of the four strictly conserved His residues. Two of them are in an HXXXH motif, situated in an α-helix, which has two correctly positioned imidazole rings that might chelate a metal ion [7]. Similar motifs have been described in other iron-containing enzymes [8] and in zinc-containing enzymes [9, 10].

In *E. coli*, the anaerobic metabolism of fucose requires the activity of POR, the product of *fucO* gene. The physiological role of POR is to reduce the L-lactaldehyde to L-1,2-propanediol, a fermentation product, using NADH as a cofactor [11]. The enzyme, which is induced by the methylpentose, regardless of the respiratory conditions of the culture, remains fully active in the absence of oxygen. In the presence of oxygen, POR becomes oxidatively inactivated by an iron-catalyzed mechanism [12, 13].

In order to identify the amino acid residues involved in the metal-binding site of the iron-containing dehydrogenase family, we used site-directed mutagenesis of the *fucO* gene. The mutant enzymes obtained were purified and their enzyme kinetic properties were characterized. We also examined their metal content and assessed how this affected the catalytic activity.

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Abbreviations. POR, propanediol oxidoreductase; ADH, alcohol dehydrogenase; MDH, methanol dehydrogenase; BDH, butanol dehydrogenase.

Enzymes. Escherichia coli propanediol oxidoreductase (EC 1.1.1.77); restriction endonucleases *Eco*RI, *Hin*dIII (EC 3.1.21.4); *Taq* polymerase (EC 2.7.7.7); *Escherichia coli* alcohol dehydrogenase E (EC 1.2.1.10); *Zymomonas mobilis* alcohol dehydrogenase I and II (EC 1.1.1.1).

Note. Both Núria Obradors and Elisa Cabiscol contributed equally to this article.

Table 1. Primers used for site-directed mutagenesis. Mutated nucleotides are in boldface type. *Eco*RI and *Bam*HI restriction sites are underlined.

Name	Nucleotide sequence	Plasmid	Amino acid exchange	Denomination
A	5′-GGGAATTCATCAGCGCATTTACCAG-3′			
B	5′-GTTGGATCCTTCGTAAAGCAACAAGG-3′			
C1	5′-GGGTTAGGGTTG**TGC**CATGGTAT-3′	pNO136	Val262→Cys	V262C
D1	5′-G**GCA**CAACCCTAACCCAACATTC-3′			
C2	5′-TGGATGATGTT**AT**TACCGGTGGCA-3′	pNO139	Cys362→Ile	C362I
D2	5′-A**AT**AACATCATCCAGTGCCGCCT-3′			
C3	5′-GTTAGGGTTGGTG**TGC**CATGGTAT-3′	pNO115	Val262→Cys→His263	C263ins
D3	5′-**GCA**CACCAACCCTAACCCAACAT-3′			
C4	5′-TTAGGGTTGGTGC**G**TGGTATGGC-3′	pNO151	His263→Arg	H263R
D4	5′-A**C**GCACCAACCCTAACCCAACAT-3′			
C5	5′-TGCATGGTATGGCG**GC**TCCACTG-3′	pNO5	His267→Ala	H267A
D5	5′-A**GC**CGCCATACCATGCACCAACC-3′			
C6	5′-TATAACACTCCA**GCA**GGTGTTGCGAACGCC-3′	pNO13	His277→Ala	H277A
D6	5′-AACACC**TGC**TGGAGTGTTATAAAAGCG-3′			
C7	5′-**TGC**CATGGT**GAT**GCGCATCCACT-3′	pNO16	Met265→Asp	V262C−M265D
D7	5′-ATGCGC**ATC**ACCATG**GCA**CAAC-3′		Val262→Cys	

MATERIALS AND METHODS

Organisms and plasmids. The strain used for expression of *fucO* wild-type and mutant gene products was *E. coli* K38 [14]. Antibodies were obtained using strain ECL1 [15] as a source of POR enzyme. Plasmids used were: (1) pGP1−2, which contains T7 RNA polymerase gene under the control of the inducible λ PL promoter, the gene for heat-sensitive λ repressor cI857, the kanamycin-resistance gene (*Kan*^{R}) and the P15A origin [16]; and (2) pT7−6, which is a colE1-based plasmid which contains the T7 RNA polymerase promoter of ϕ10 in front of a polylinker sequence, and the β-lactamase gene in reverse configuration [16]. The strain K38 and plasmids were provided by S. Tabor (Harvard Medical School, Boston).

Site-directed mutagenesis by PCR amplification. Site-directed mutagenesis was performed by overlap PCR as described previously [17]. The primers used for each mutation are listed in Table 1. Primers A and B, flanking the complete *fucO* gene, had *Eco*RI and *Bam*HI restriction sites, respectively, in their 5′ ends to give the desired orientation when ligated into the expression vector. The internal overlapping primers C and D had the desired mutation in their matching sequence. Two separate PCRs were carried out on the template gene to give products AC and DB. These products have the mutation incorporated at their ends. The final product AB was made by hybridizing the overlapping strands from the two fragments and extending this overlap with DNA polymerase.

For the six single mutants, the first two separate PCRs were carried out using the cloned wild-type *fucO* gene as a template. For the double mutant, DNA from the mutant pNO136 was used as a template.

To obtain the mutated products AC and DB, PCR reactions were performed in a final volume of 100 µl, containing 25 ng template, 100 pmol each primer, 200 µM dNTPs, 1.5 mM magnesium chloride and 2.5 U *Taq* polymerase (BRL). Samples were first kept at 94.0 °C for 120 s to ensure initial denaturation and then subjected to 20 cycles of 45 s at 94.0 °C (denaturation), 60 s at 60.0 °C (annealing), and 120 s at 72.0 °C (elongation). After the completion of the 20 cycles, the mixture was incubated for 5 min at 72.0 °C. The products were purified by means of agarose gel electrophoresis.

Overlap extension was performed including the flanking primers to amplify the recombinant products as well as the non-productive strands [17]. The conditions for the overlap PCR were similar to the first PCR except for the annealing temperature, which was different in each reaction, depending on the overlapping region. The mixture contained, in a final volume of 100 µl: 100 pmol each of the primers A and B, 200 µM dNTPs, 1.5 mM magnesium chloride, 1.25 U *Taq* polymerase (BRL) and equivalent DNA molecules of products AC and DB. The products of the overlapping extension were purified by agarose gel electrophoresis, digested with *Bam*HI and *Eco*RI and cloned in Bluescript SK$^-$. The resulting plasmids were sequenced by the dideoxy chain-termination method (T7-sequencing kit, Pharmacia Biotech.) to verify the substitutions introduced and to confirm the absence of additional changes in the coding sequence.

Cloning and expression of POR in *E. coli*. A system consisting of two compatible plasmids, pGP1−2 and pT7−6, was used to express the *fucO* gene. The Bluescript SK$^-$ plasmid containing an insert of 1.2 kb, encoding either the wild-type or the different mutated genes was digested with *Eco*RI and *Bam*HI, purified by agarose gel electrophoresis and cloned in the expression vector pT7−6. The resulting plasmid was used for transformation of the K38 strain containing plasmid pGP1−2 which provides for the expression of T7 RNA polymerase after heat induction.

Transformed cells were grown aerobically at 30.0 °C up to A_{590} = 1.5 in an induction medium consisting of 20 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl, 2 g/l glycerol, and 6.8 g/l KH_2PO_4 (the pH was adjusted to 7.2 with NaOH), plus 100 µg/ml ampicillin and 50 µg/ml kanamycin. At this point, cultures were heated to 42.0 °C for 30−45 min and then cooled to 30.0 °C and harvested after 3 h. Cell extracts were prepared as described previously [6] in 50 mM Tris/HCl, pH 7.5, containing 2.5 mM NAD. Protein concentrations were measured according to Bradford [18] using BSA as standard.

Protein expression quantification. Quantification of POR expression was carried out by means of Laurell rocket immunoelectrophoresis [19], according to a calibration curve (not shown) derived using POR purified from strain ECL1. Anti-POR antibodies were obtained as described previously [20].

Enzyme purification. POR (wild-type and mutants) was purified by the method of Cabiscol et al. [6], except that the ammonium-sulfate-precipitation step was omitted. Monitoring of inactive enzymes was performed by Laurell rocket immuno-

electrophoresis. Enzyme purity was assessed by SDS/electrophoresis performed according to Laemmli [21], using 10% acrylamide as resolving gel. Proteins were stained with Coomassie brilliant blue R-250 [22].

Kinetic measurements. POR activity was measured by its NADH-dependent L-lactaldehyde reduction to propanediol using a molar absorption coefficient of $6.22\times10^{-3}\,M^{-1}\,cm^{-1}$ at 340 nm [20], and glycolaldehyde as an alternative substrate for the enzyme [23]. Kinetic parameters given are mean values from at least three different measurements.

Metal analysis. Metal content of the homogeneous proteins, including Fe, Zn, Cu, Co, Ni, Sr and Mn, was determined by inductively coupled plasma-MS with a Perkin-Elmer Elan-6000. In all cases prior to metal analyses, samples were applied to a Sephadex G-25 PD-10 column (Pharmacia) equilibrated with MilliQ water (resistivity greater that 18 MΩ) in order to eliminate reagents and metals not bound to the enzyme. Fractions were collected in metal-free polypropylene tubes. Water blank samples, representing the flow-through collected in the void volume of the column, were also analyzed without significant detectable metal levels. In order to evaluate the enzyme metal-binding capacity, purified proteins (1 mg/ml) were incubated with 10 mM *o*-phenantroline to remove metal ions from the enzymes. This treatment caused inactivation due to iron depletion. After gel-filtration on a PD-10 column, samples were incubated with either 150 μM ferrous sulfate or 150 μM zinc chloride for 30 min; and gel-filtrated again in a PD-10 column to remove unbound metal. POR activity and metal content were determined in the resulting samples. In all cases, to avoid adsorption of the metalloproteins to the tube walls, samples were sonicated for 5 min before being digested with 1% nitric acid. Solutions to be tested were adjusted to a range of metal concentrations of 20–200 ppb and carried an internal standard of 500 ppb of rhodium.

RESULTS

Generation of specific POR mutants. The amino acid sequence of POR contains three His residues located between position 250 and position 280 [24, 25], postulated to be involved in metal binding [5, 6] and strictly conserved among all members of this family. Fig. 1 shows the amino acid alignment of this region with other members of the family and is compared with the well-known catalytic zinc-binding region of several dehydrogenases of the medium-chain, zinc-dependent family, where one such His distribution is also present.

To further investigate the metal-binding on POR, selected amino acid residues were changed by site-directed mutagenesis. Of the three conserved His residues (263, 267 and 277) believed to be metal-ion ligands, His263 (coded by CAT) was replaced by Arg (CGT), a residue found frequently in the metal-binding consensus of many zinc dehydrogenases and denominated H263R. His267 (CAT) was replaced by Ala (GCT) and His277 (CAC) by Ala (GCA); these are referred to as H267A and H277A, respectively. Both substituting residues (Arg and Ala) are generally considered non-metal-liganding. On the basis of the conserved residues involved in the metal binding of zinc-dependent dehydrogenases [1, 2], three mutations were introduced to mimic this metal-binding site. Val262 (coded by GTG) was replaced by Cys (TGC), denominated V262C, and a Cys residue (coded by TGC) was inserted at position 263, and referred to as C263ins. A double mutant was also obtained by introducing into mutant V262C another substitution consisting of the replacement of Met265 (ATG) by Asp (GAT), and the resulting mutant was denominated V262C−M265D. Finally, Cys362 (coded by TGT), with unknown function and strictly conserved among all members of this family, was replaced by Ile (coded by ATT) and denominated C362I. This residue could

Group III Iron-activated DH	260	261	262	263	264	265	266	267	268	...	276	277	278	279	280	281	282	283	284	285	...	361	362	363
E. coli POR	G	L	V	**H**	G	M	A	**H**	P		P	**H**	**G**	V	A	N	A	I	L	L		V	**C**	T
Z. mobilis ADHII	G	Y	V	**H**	A	M	A	**H**	Q		P	**H**	**G**	V	C	N	A	V	L	L		A	**C**	A
S.cerevisiae ADHIV	G	Y	V	**H**	A	L	A	**H**	Q		P	**H**	**G**	V	C	N	A	V	L	L		A	**C**	H
E.coli ADHE	G	V	C	**H**	S	M	A	**H**	K		P	**H**	**G**	L	A	N	A	L	L	I		Q	**C**	T

Group I Medium-chain zinc-dependent DH	44	45	46	47	48	49	50	51	52	...	60	61	62	63	64	65	66	67	68	69	...	173	174	175
Horse	G	I	**C**	R	S	**D**	D	H	V		P	L	P	V	I	A	**G**	**H**	**E**	A		G	**C**	G
Human α	G	I	**C**	G	T	**D**	D	H	V		P	L	P	V	I	L	**G**	**H**	**E**	A		G	**C**	G
Human β	G	I	**C**	R	T	**D**	D	H	V		P	L	P	V	I	L	**G**	**H**	**E**	A		G	**C**	G
Rat	G	V	**C**	R	S	**D**	D	H	A		P	L	P	V	A	L	**G**	**H**	**E**	G		G	**C**	G
Maize 1	S	L	**C**	H	T	**D**	V	Y	F		V	F	P	R	I	F	**G**	**H**	**E**	A		S	**C**	G
Arabidopsis	S	L	**C**	H	T	**D**	V	Y	F		L	F	P	R	I	F	**G**	**H**	**E**	A		S	**C**	G
Aspergillus	G	V	**C**	H	T	**D**	L	H	A		K	H	P	L	I	G	**G**	**H**	**E**	A		L	**C**	A
S. cerevisiae 1	G	V	**C**	H	T	**D**	L	H	A		K	L	P	L	V	G	**G**	**H**	**E**	G		L	**C**	A
S. cerevisiae 2	G	V	**C**	H	T	**D**	L	H	A		K	L	P	V	L	G	**G**	**H**	**E**	G		L	**C**	A
B. stearothermophilus	G	V	**C**	H	T	**D**	L	H	A		K	L	P	L	I	P	**G**	**H**	**E**	G		F	**C**	A
Z. mobilis (ADH I)	G	V	**C**	H	T	**D**	L	H	V				G	R	I	T	**G**	**H**	**E**	G		F	**C**	A

Fig. 1. Comparison of metal-binding regions of several dehydrogenases. Amino acid alignment of proposed metal-binding site present in the microbial group III 'iron-activated' NAD(P)-dependent dehydrogenases, [2, 42] and comparison with the eukaryotic zinc-containing medium-chain NAD(P)-dependent dehydrogenases [1, 43]. All entries of the second group are alcohol dehydrogenases. Both groups showed a 30% similarity. Residue numbering system follows that of the POR in iron-activated dehydrogenase family, and that of the horse enzyme in medium-chain zinc-dependent dehydrogenase family (the catalytic zinc atom is bound by Cys46, His67 and Cys174). Strictly conserved residues among all members of each family are in boldface type. Residues mutated in this work are underlined.

Table 2. POR protein and specific activity in crude extracts of wild-type and mutant strains. Cells were grown and induced as described in Materials and Methods and cell extracts obtained. POR activity was measured and amount of POR obtained by Laurell rocket immunoelectrophoresis. ND, not detected.

Mutation	POR amount	Specific activity	
	mg POR/mg total protein	U/mg total protein	U/mg POR
Wild-type	0.044	1.02	23.2
V262C	0.040	0.58	14.5
C362I	0.041	0.23	5.6
C263ins	0.038	ND	ND
H263R	0.044	ND	ND
H277A	0.046	ND	ND
H267A	0.047	ND	ND
V262C−M265D	0.022	ND	ND

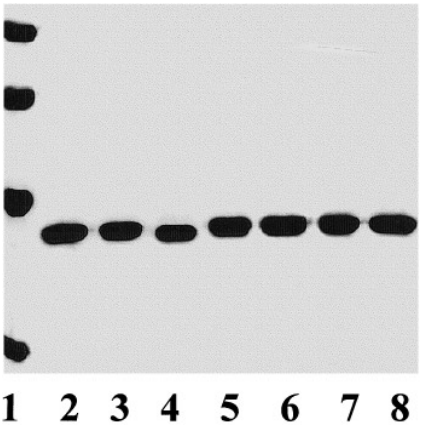

Fig. 2. SDS/PAGE of purified wild-type and mutant enzymes. Purified proteins (3 μg) were separated by means of SDS/PAGE (10% acrylamide) followed by staining with Coomassie brilliant blue. Lane 1, protein standard (M_r: 92 400, 66 200, 42 700 and 31 000 Da); lane 2, wild-type; lane 3, V262C; lane 4, C362I; lane 5, C263ins; lane 6, H263R; lane 7, H277A; lane 8, H267A.

be compared with the zinc ligand Cys174 from medium-chain, zinc-dependent dehydrogenases.

The mutated forms of POR were expressed from seven different plasmids, pNO136, pNO139, pNO115, pNO151, pNO13, pNO5, and pNO16, corresponding to the replacements V262C, C362I, C263ins, H263R, H277A, H267A, and V262C−M265D, respectively. In addition to these mutated forms, non-mutated wild-type POR was expressed from plasmid pNO121, which was used as a reference protein (Table 1). Specific activity and kinetic properties of POR from pNO121 were similar to those of the wild-type POR from *E. coli* ECL1 (not shown).

Expression and enzyme activity of mutated proteins. POR expression was induced as described in Materials and Methods. Values of enzyme activity and amount of POR, quantitated by Laurell rocket immunoelectrophoresis, were determined in cell extracts (Table 2). All single-mutated proteins were recovered in normal yield (approximately the same as the wild-type enzyme). The double mutant V262C−M265D yielded half of the POR protein due to its low stability, indicated by the extremely low protein recovery observed after the first gel-filtration step in the purification process. For this reason, no further analysis of this mutant protein could be pursued.

Only two (V262C and C362I) of the constructed mutant enzymes retained catalytic activity, although at lower level than wild-type POR. Neither of the proteins with mutated His residues believed to be the iron ligands, nor that containing the insertion of Cys at position 263, showed enzyme activity.

Metal-binding capacity and enzyme activity in wild-type and mutant purified enzymes. Proteins were purified by liquid chromatography as described in Materials and Methods, and their homogeneity was indicated by the pattern of bands obtained on SDS/PAGE (Fig. 2). Of all the metals tested (Cu, Co, Ni, Sr, Mn, Zn, and Fe), only the last two were detected at significant levels (Table 3). These results showed a decreasing amount of the native-bound iron in the mutants, which is responsible for the decreasing POR specific activity observed (wild-type > V262C > C362I). No significant binding of iron was detected in the rest of the mutants. However, when the native-bound zinc was recorded, a similar pattern of metal content was found for all mutants except H267A which, although demonstrated negligible binding of iron, bound more zinc than wild-type. It is worth noting that the content of iron and zinc ions found in pure preparations of native POR are similar to that found in ADH II from *Z. mobilis*, another enzyme of this group.

Table 3. Specific activity and metal content of purified wild-type and mutant enzymes. Purified enzymes were treated as described in Materials and Methods, enzymatic activity was recorded and submitted to metal analysis. ND, not detected.

Mutation	Specific activity	Metal content	
		Fe	Zn
	U/mg	atom/subunit	
Wild-type	19	0.45	0.50
V262C	14	0.27	0.34
C362I	8	0.13	0.25
C263ins	ND	0.04	0.07
H263R	ND	0.02	0.08
H277A	ND	0.02	0.07
H267A	ND	0.09	0.68

In this latter case, the result was explained by the higher value of the apparent dissociation constant for zinc than that for iron and the presence of iron and zinc in the complex media used to grow the cells [41]. This could also apply to POR enzyme.

To evaluate the full capacity of these enzymes to bind iron or zinc and test the effect of this binding on POR activity, these preparations were first depleted of metal by chelation with *o*-phenantroline (data not shown). Since iron is necessary for the catalytic activity of the enzyme, this treatment completely abolished POR activity. *o*-Phenantroline was removed by gel filtration, and either Fe^{2+} (as ferrous sulfate) or Zn^{2+} (as zinc chloride) was added to the enzyme in excess. After 30 min at room temperature, free metal was eliminated by gel filtration in MilliQ water. The enzymatic activity was determined and protein-bound metal was quantified (Table 4). In these conditions, mutants (V262C and C362I) showed amounts of iron bound close to those of wild-type enzyme, with similar levels of enzyme activity. As expected, no significant iron was bound to the rest of the mutants, except for H267A, which bound a similar amount of iron to wild-type enzyme, but displayed less than one-tenth of its specific activity. A parallel pattern of metal content was observed when the POR samples were loaded with zinc; nevertheless, despite its inability to bind Fe, the mutant bearing the inser-

Table 4. *In vitro* loading of purified wild-type and mutant enzymes. Metal bound to purified enzymes (10 μM) were removed by treatment with *o*-phenantroline and ferrous sulfate or zinc chloride added (150 μM). After 30 min incubation, metal not bound to the enzyme was removed and enzymatic activity and metal analysis were performed. ND, not detected.

Mutation	Fe added		Zn added
	activity	Fe	Zn
	U/mg	atom/subunit	
Wild-type	26	0.52	0.90
V262C	24	0.49	0.60
C362I	23	0.42	0.53
C263ins	ND	0.07	0.90
H263R	ND	0.05	0.07
H277A	ND	0.08	0.20
H267A	0.2	0.60	0.86

tion of the cysteine (C263ins) contained an average of 0.9 atoms of zinc per subunit.

Kinetic measurements. To determine whether these mutations produced changes in substrate and/or coenzyme affinities, kinetic parameters of wild-type and the two active mutants (V262C and C362I) were measured. As shown in Table 5, the K_m for glycolaldehyde and NADH were of the same order of magnitude in all three preparations, although mutant PORs showed higher values in both cases. Both V263C and C362I mutants presented a decrease in catalytic efficiency of threefold and tenfold, respectively.

Wild-type POR is known to be inactivated by zinc [26]. A plot of the apparent first-order rate constants for inactivation (k_{obs}) at various concentrations of $ZnCl_2$ allows the calculation of the second-order rate constant of inactivation, which was 9090 M^{-1} min^{-1} for wild-type and 1900 M^{-1} min^{-1} and 555 M^{-1} min^{-1} for V262C and C363I mutants, respectively, implying a destabilized metal-binding site with lower affinity for zinc in the mutant enzymes.

Thermal stability. With the exception of the double mutant, all other mutants were produced in yields comparable with the wild-type, which indicates their stability. However, changes in the pattern of thermal stability at higher temperature were observed when purified wild-type and V262C and C363I mutants were heated to 37.0 °C and their POR activity was measured at different times (Fig. 3). Mutant forms of POR were less stable than the wild-type, V262C being less stable than C363I.

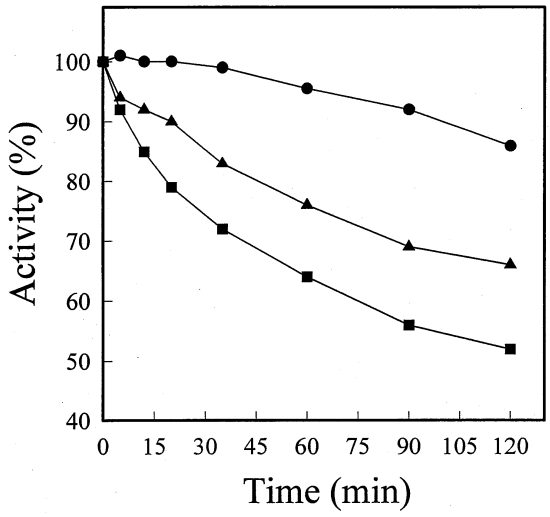

Fig. 3. Thermal stability of wild-type and active mutants. Pure preparations of wild-type and V262C and C362I mutants (0.8 mg/ml) in 50 mM Tris/HCl, pH 7.5, were kept at 4.0 °C. At zero time, 0.5 ml of each solution was heated to 37.0 °C and POR activity was measured at the plotted times. Wild-type (●), V262C (■), C362I (▲). The results are the average of two separate experiments.

DISCUSSION

POR belongs to the group of the NAD(P)-dependent, iron-activated dehydrogenases. None of these enzymes has a known three-dimensional structure and there is poor information about their metal-binding site. Indeed, the presence of iron has been clearly identified only in ADH II from *Z. mobilis* [27−29] and ADH E from *E. coli* [30], while other members of this family have been reported to bind metal ions other than iron [31−33]. In the case of POR, it was known that ferrous and manganous ions reactivate the enzyme, whereas zinc causes complete inactivation [26], although, at present no information on the enzyme-bound metal or the specific amino acid participation in the binding center has been reported.

Mutation of any of the three conserved His residues (263, 267 and 277) involved in the putative metal-binding site to residues believed not to be metal-liganding amino acids (Ala or Arg) produced catalytically inactive and iron-deficient POR enzymes. This is consistent with the current knowledge that these residues are essential in binding the iron ion and, consequently, in maintaining catalytic activity. Our results indicate that His263 and His277 are strictly essential metal-ion ligands, because their mutation produced apoenzymes and, consequently, are without catalytic activity. However, replacement of His267 by Ala produced an enzyme that was unable to bind iron under physiological conditions although it retained the ability to bind zinc. When

Table 5. Kinetic parameters of wild-type and mutant enzymes.

Mutation	k_{cat}	Glycolaldehyde		NADH		Zn
		K_m	k_{cat}/K_m	K_m	k_{cat}/K_m	Inactivation constant [a]
	s^{-1}	mM	s^{-1} mM^{-1}	μM	s^{-1} μM^{-1}	M^{-1} min^{-1}
Wild-type	11.5 ± 0.8	0.48 ± 0.07	23.9	2.7 ± 0.3	4.4	9090
V262C	9.1 ± 0.6	1.05 ± 0.03	8.7	7 ± 0.5	1.3	1900
C362I	5.2 ± 0.6	2 ± 0.02	2.6	12 ± 2	0.43	555

[a] Second-order rate constant of inactivation.

this enzyme was loaded with iron *in vitro*, it remained catalytically inactive, suggesting an altered metal-binding site with very low affinity for iron, but maintaining similar affinity for zinc. It is likely that the bound iron is incorrectly positioned in the catalytic center of this mutant. Alternatively, one may consider the possibility of His267 acting as a catalytic residue, indirectly affecting the enzyme−substrate interaction, as proposed for His51 in horse liver ADH [34].

In contrast, Cys362 is not essential for the catalytic reaction, nor for metal binding, since addition of iron sulfate to the mutant C326I produced an enzyme as active as the wild-type with a similar iron content. Nevertheless, this substitution leads to protein destabilization, as shown by the decreased thermal stability. These considerations would also apply to the mutant V262C, designed to mimic the zinc ligand cysteine at position 46 of the zinc-dependent ADHs [35−37].

In these two mutants, changes in K_m for substrate or cofactor indicate that these mutations affected the structure of the active center. In addition, the concentration of the functional POR (iron-containing) was affected through modification of the metal affinity or occupancy of this active center. Consistently, changes in k_{cat} or catalytic efficiency in such mutants are a reflection of the proportion of the functional to total enzyme molecules.

Insertion of Cys at position 263, mimicking the zinc-binding site of medium-chain dehydrogenases, produced an apoenzyme which lost the iron-binding capacity, but was still able to bind zinc. It is likely that the shift produced by the insertion alters the orientation of the adjacent residues in the α-helix, thus affecting His residues 263 and 267, rendering them no longer available for iron ions. This hypothesis is based on the putative analogy to the tertiary structure of the segment Val14 to Arg79 of ADH I from *Z. mobilis*, which contains a Cys involved in zinc-binding, predicted using the Swiss-model program for automated comparative protein modeling [38, 39].

In the group of medium-chain zinc-dependent ADHs, the HXXXH motif, not involved in the metal binding, is present in the microbial enzymes, while it lacks the second His in plant ADHs and the first His in animal ADHs (Fig. 1). It can thus be hypothesized that the medium-chain zinc-dependent ADHs, and the iron-dependent dehydrogenases arose from a common ancestor, which had HXXXH as metal-binding motif. Before O_2 appeared in the atmosphere, Fe^{2+} was much more abundant than zinc [40]. Thus, the primitive dehydrogenases probably used Fe^{2+} as a catalytic metal. When O_2 appeared in the atmosphere, reactive oxygen species were produced and caused enzyme inactivation. In addition, iron was no longer easily accessible, because it became sequestered as insoluble Fe^{3+} compounds. In response to this situation, two evolutionary strategies appeared: enzymes working under aerobic conditions, such as the medium-chain dehydrogenases, developed high-affinity centers to bind antioxidant metals, e.g., zinc, and used them catalytically; as a counterpart, enzymes working anaerobically, whose aerobic inactivation would be useful to the cell, such as POR [12] or ADH II [41], maintained iron as a catalytic metal.

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