Site-directed mutagenesis of the lower parts of the major substrate channel of yeast catalase A leads to highly increased peroxidatic activity

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Abstract Five single replacement mutants of catalase A from *Saccharomyces cerevisiae* were prepared (F148V, F149V, F156V, F159V, and V111A). The exchanges were expected to relieve steric constraints in the lowest part of the major substrate channel. The overall stability of the isolated enzymes is unaffected by the respective amino acid exchanges, but some modifications lead to decreased protohaem binding. All isolated mutants (most pronounced the V111A-species) show decreased catalatic and markedly increased peroxidatic activity, both with aliphatic and aromatic substrates. These effects can in part be explained by steric effects, but also reveal destabilisation of compound I.

Key words: Catalase; Protein engineering; Site-directed mutagenesis; Substrate channel; Peroxidase; *Saccharomyces cerevisiae*

1. Introduction

This investigation is part of our current project to study the structure-function relationships in catalase A (hydrogen peroxide:hydrogen peroxide oxidoreductase, EC 1.11.1.6) from *Saccharomyces cerevisiae*.

Aerobic organisms have evolved different systems to destroy potentially harmful reactive oxygen species [1,2]. Typical catalases and general peroxidases, the two major groups of haem-containing hydroperoxidases, are among the most important examples. Members of these two families show closely related reaction schemes [3,4]. In either case the first step involves activation of the ferric enzyme by H_2O_2 or aliphatic hydroperoxides, leading to a ferryl-radical known as compound I. Compound I then either oxidises a second molecule of H_2O_2 or aliphatic alcohols in a two-electron step, or a wide variety of organic compounds via two subsequent one-electron steps, thus returning the enzyme to its ferric state [5,6]. The former type of reaction is typical for catalases, the latter for peroxidases. Catalatic reactivity is, however, to a very low extent also revealed by some peroxidases (as well as by several other haem proteins like metmyoglobin and cytochrome oxidase), and vice versa [7–9]. Therefore, differences in the various protein moieties of these enzymes obviously are responsible for the relative rates of both types of catalytic action.

Extensive studies with non-enzymatic models, as well as detailed biochemical and X-ray structural data of members of both subfamilies gave rise to several assumptions to explain the overwhelming preference of the catalatic pathway in catalases. One of these approaches is based on the characteristic location of the active sites of catalases. The haem groups of peroxidases are in a polar environment and accessible to solvent (e.g. [10,11]). On the other hand, X-ray structures of haem-containing catalases from a wide range of sources (*Micrococcus lysodeikticus* [12], *Penicillium vitale* [13], and bovine liver [14]) reveal very similar spatial arrangements around their active sites. In these enzymes the haem groups are deeply buried in non-polar pockets, being connected with the surface by rather narrow channels. The dimensions of these substrate channels as well as their polarity may be responsible for the extremely slow oxidation of typical peroxidase substrates.

We tried to evaluate this theory by engineering the substrate channel of catalase A from *Saccharomyces cerevisae*. Single or multiple amino acid-replacements were performed by site-directed mutagenesis of the corresponding gene *CTA1* to obtain additional information about their functional roles. So far, this technique has been applied to modify the essential histidine and asparagine residues of hydroperoxidase I (HPI) of *Escherichia coli*, demonstrating their requirement for the oxidation of protohaem IX to haem d [15]. The modifications reported here concentrate on the lowest – and sterically most restricted – portion of the major substrate channel. Several large apolar side chains were replaced by similar smaller ones to make the active sites of the enzyme more accessible for large organic substrates. According to this hypothesis the exchanges should lead to an increase in the rate of peroxidatic reactions concomitant with the partial loss of the catalatic activity, finally leading to a hybrid catalase/peroxidase.

2. Materials and methods

2.1. In vitro-mutagenesis

Oligonucleotide-directed mutagenesis of the gene *CTA1* cloned with its own promoter in the shuttle vector YEp 352 [16] was performed following standard PCR protocols [17] with the DNA polymerase from *Thermus brockianus* (Finnzymes OY, Espoo, Finland).

2.2. Isolation of catalase A

CTA1 gene was overexpressed from a multicopy vector (YEp352) in *S. cerevisiae* (ctt1$^-$, cta1$^-$, pep4$^-$) grown at 30°C for 48 h in a medium containing 1% (w/v) yeast extract, 2% (w/v) meat peptone, and 3% (v/v) ethanol (YPE). The purification of the enzyme will be described elsewhere in detail. Shortly, crude extracts obtained by homogenisation with a Braun cell homogeniser in the presence of 1 mM PMSF, 1 μg/ml

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Abbreviations: PMSF, phenylmethylsulfonyl fluoride; MES, 2-(*N*-morpholino)-ethanesulfonic acid; GdmCl, guanidinium chloride; ABTS, 2,2′-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid); BLC, bovine liver catalase; WT modC, wild type catalase A with histidine-tagged C-terminal part.

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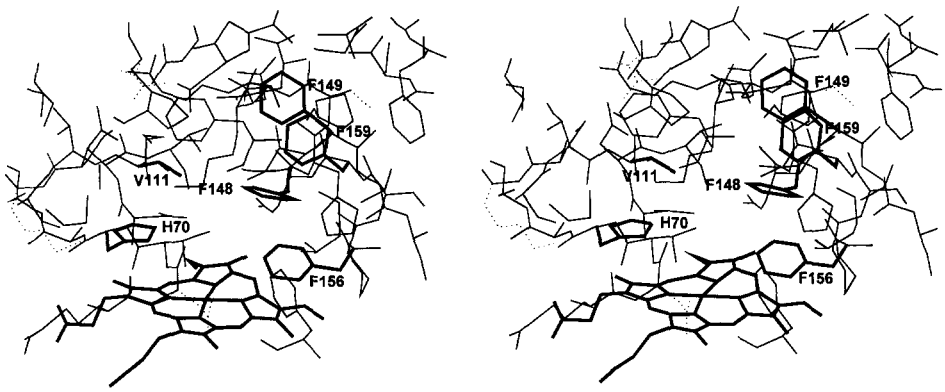

Fig. 1. Stereo view of the haem's distal side in catalase A as modelled to the structure of BLC [14]. The lowest part of the major substrate channel is visible, including the side chains of the amino acids exchanged in this work. Residues not identical in BLC and catalase A are shown in dotted lines.

each of pepstatin A and leupeptin, were fractionated by precipitation first with ethanol/$CHCl_3$, 1:1 (v/v), then twice with ammonium sulphate, followed by consecutive chromatography steps on Zn^{2+}-loaded Chelating Sepharose, Sepharose 6B-CL, and hydroxylapatite (HTP, Bio-Gel).

2.3. Determination of enzymatic activities

Catalatic activities were determined at 240 nm according to Roggenkamp et al. [18]. One unit was defined as the amount of enzyme catalysing the conversion of 1 μmol of H_2O_2 min^{-1} at an initial concentration of 15.67 mM at 22°C. The oxidation of aliphatic alcohols was based on the continuous slow generation of H_2O_2 by the reaction of glucose oxidase (from *Aspergillus niger*, Boehringer Mannheim, Germany) and detected by means of a coupled enzymatic assay with yeast aldehyde dehydrogenase (Boehringer Mannheim) [19]. The same system of H_2O_2-generation was applied in the oxidation of ABTS (2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)) by catalase (250 to 1,000 catalatic units), which was followed for 1 to 5 min at 30°C at 660 nm [20]. Identical conditions were applied to determine the rates of oxidation of guaiacol.

2.4. Unfolding of purified proteins

Solutions of highly purified catalase A in 0.2 M potassium phosphate, pH 7.5 or 50 mM MES, pH 6.0 were mixed with the appropriate volumes of buffer and a 10 M urea or 8 M guanidinium chloride (GdmCl) in the same buffer to obtain final concentrations of the denaturant between 0.25 and 7.5 M at constant protein concentrations between 1.0 and 2.5 mg/ml. The stock solutions of urea and GdmCl, respectively, were freshly prepared according to Nozaki [21] and Pace [22]. Unfolding at 0°C or 25°C was followed by measuring the intrinsic fluorescence (excitation at 290 nm, emission at 350 nm) against appropriate blanks without protein. The midpoints of the unfolding curves obtained from these data were determined by fitting to a simple exponential function.

2.5. Further methods

Protein was determined according to Bradford [23] with bovine serum albumin as standard. Pyridine haemochrome was prepared according to the modification reported by Takaichi and Morita [24].

3. Results

Since the X-ray structure of catalase A is not yet available, this work is based on the crystal structure of the bovine liver enzyme (BLC), which has been resolved at 2.5 Å resolution [14]. The homology between the two enzymes is high within the complete haem-containing domain, and very high in the close vicinity of the active sites [25], therefore this approach appears safe. In BLC, substrates have to pass through a more than 20 Å long channel to allow interaction with the haem iron and the essential residues on the haem's distal side [26]. The lower half of this channel is rather narrow and includes a nick caused by the side chain of V111 in catalase A. Its walls are almost exclusively formed by apolar side chains (V111, G112, D123, P124, F148, F149, F156, F159, I160, V193, L196, and CH_2-stretches of Q163 and K164). At the location corresponding to G112 an alanine is found in BLC, all further residues are conserved in the mammalian enzyme. Fig. 1 illustrates the general arrangement of the lowest part of the haem channel of catalase A, as obtained by computer graphics modelling to the known structure of BLC (INSIGHT II, Biosym Technol.). As shown in Fig. 1, the bulky side chains of F148, F149, and F159 may in fact be responsible for the exclusion of larger substrate molecules from the active site. Similarly, the isopropyl side chain of V111 limits the passage of substrates and forces them to swerve around it.

We tried to relieve this tension by means of site-directed mutagenesis. The gene *CTA1*, inserted in the shuttle vector

Table 1
Expression of wild type and mutant catalase A in *S. cerevisiae*

	Total protein (mg/ml) in crude extract	Catalase in % of total protein	Specific activity in crude extract (U/mg)	Specific activity of purified enzyme (U/mg)
Wild type	23–44	5.2–7.4	3,740–5,320	71,400
WT modC	15–37	7.1–8.8	4,890–6,170	70,100
V111A	19–46	5.9–10.7	770–1,180	10,900
F148V	8.6	n.d.	≈10	n.d.
F149V	18–52	2.5–3.1	530–1,190	21,050
F156V	78	6.4	82	1,180
F159V	16–44	4.4–4.7	1,160–1,230	26,000

Table 2
Unfolding stability of catalase A

Denaturant, time of equilibration	Unfolding followed by	Wild type	WT modC	V111A
GdmCl, 20 min	catalatic activity	1.27	1.12	1.18
Urea, 20 min	fluorescence intensity (290/350 nm)	3.28	3.60	n.d.
Urea, 60 min	catalatic activity	1.65	1.51	1.70
Urea, ≥ 120 min	fluorescence intensity (290/350 nm)	1.65	1.56	1.85

Midpoint values ($D_{1/2}$ in mol/l of denaturant) of unfolding curves at 0°C and pH 7.5.

YEp352 under the control of its authentic promoter, was mutagenised by PCR. We prepared single mutants by replacing each of the phenylalanines cited above by valines. Furthermore, we replaced V111 by an alanine, and F156 again by valine. The latter residue has its side chain close to and arranged parallel to the tetrapyrrole system, so it is assumed to be involved in haem packing too.

The active-site related mutations were performed in an already engineered gene. To allow a faster and more efficient purification than following the established protocol [27], the C-terminal region of the protein was modified. The terminal sequence -S-K-F.OH was replaced by -S-R-H-H-H-F.OH to allow purification by metal chelate affinity chromatography (MCAC). The protein isolated from this strain (H550) is indicated as wild type (WT) modC in the following.

The mutated genes were expressed in the yeast strain H578 (cta^-, ctt^-, $pep4^-$) grown on YPE for 24 to 50 h. Table 1 shows the respective yields, revealing that in most cases the mutated proteins are overexpressed to a similar degree as the corresponding wild type enzyme.

Wild type catalase A, the modified wild type, and one of the single site mutants were tested for conformational stability in detail. As shown in Table 2, the differences in the relative ease of chemical denaturation are almost identical in these cases. The differences in unfolding stabilities of closely related proteins can be quite accurately determined from the corresponding $D_{1/2}$-values, which are directly obtained from denaturation isotherms, even though there may be some uncertainties in the determination of the respective stabilities in the absence of denaturant. As calculated from the differences in the midpoint values and the steepness of the corresponding unfolding isotherms, upon modification of its C-terminal residues the wild type becomes less stable by about 0.61 kJ/mol, whereas the V111A mutant is even slightly more stable than the unmodified enzyme (−0.12 kJ/mol). Preliminary data (not shown here) allow similar conclusions for the three reasonably active single F⇒V mutants. These differences are at the lower edge of comparable data with other proteins [28]. This supports the assumption that the overall structure is unaffected by the incorporated exchanges, and corresponds well with the observed accumulation of the overexpressed mutated proteins.

With the exception of the species with the exchange F148V, which is an almost inactive, rather unstable protein, all catalase A variants could be purified to apparent homogeneity. The amount of haem incorporated in the isolated protein was determined as pyridine haemochrome. Based on a tetramer with M_r ca. 235 kDa, the wild type as well as the modified wild type enzyme contain one molecule of protohaem IX per subunit, though some preparations were below this value. Most preparations of the mutated enzymes reported here failed to come to this stoichiometry, though in most cases the deficiency in haem content is not very pronounced (Table 3). According to polyacrylamide gradient gels (not shown here), only tetramers appear to be present in these preparations, either smaller oligomers or larger aggregates being not detectable. From these data and the recorded electronic absorption spectra the respective extinction coefficients at the Soret band region were calculated, which are also given in Table 3. For all mutants these coefficients are in the range of the corresponding wild type value with most likely insignificant deviations. From the data presented in Table 3 it is clear that the designation catalase A (for atypical) is misleading. The R_z-value (A_{406}/A_{280}) perfectly matches that of other, 'typical' yeast catalases. In our opinion the enzyme sample originally prepared and described by Seah et al. [29] largely consisted {145}of compound II rather then the ferricatalase species. In our hands, the R_z-value decreased to about 0.5 within 2 or 3 weeks (and within several days with some active-site mutants), even in the presence of ethanol or NADPH, so catalase A is certainly somewhat less stable to autoxidation than related enzymes from other sources.

As seen from Table 4, none of the modified catalases came close to the parent polypeptide with respect to catalatic activity. When calculated per haem, i.e. comparing intact active sites only, 40% or less of the wild type activity are found. At low concentrations of H_2O_2, short primary aliphatic alcohols are oxidised faster than a second molecule of hydrogen peroxide [30]. With ethanol the wild type enzyme reacts still markedly faster than the mutated forms, but generally less pronounced

Table 3
Haem characteristics of catalases from yeast

	Stoichiometry haem/subunit	A_{406}/A_{280}	ε_{406} ($cm^{-1} \cdot M^{-1}$, $\times 10^{-5}$)	ε_{406}/haem ($cm^{-1} \cdot M^{-1}$, $\times 10^{-5}$)
Catalase T (from [27])	n.d.	0.930	2.02	n.d.
Catalase A (from [29])	n.d.	0.190	0.36	n.d.
Catalase A (this work)	0.99	1.012	2.59	0.65
Wild type modC	0.98	1.011	2.54	0.65
V111A	0.76	0.884	2.00	0.67
F149V	0.91	0.918	2.26	0.62
F156V	0.085	0.086	0.20	0.59
F159V	0.92	1.002	2.50	0.68

than with H_2O_2. With longer chain alcohols, however, the mutants are increasingly more effective than the wild type. When applying ≥ 5-carbon alcohols no reaction is detectable with the latter, whereas some mutants are capable of catalysing the oxidation of these substrates at reasonable rates (data not shown).

A similar picture is seen when comparing the effectiveness of the various catalase variants in catalysing true peroxidations, i.e. the oxidation of large organic substrates by single electron transfers. The potential to oxidise ABTS is at least several fold increased by any of the respective amino acid exchanges; the same is true with guaiacol as substrate. The corresponding data are presented as relative ratios of reactivities in Fig. 2.

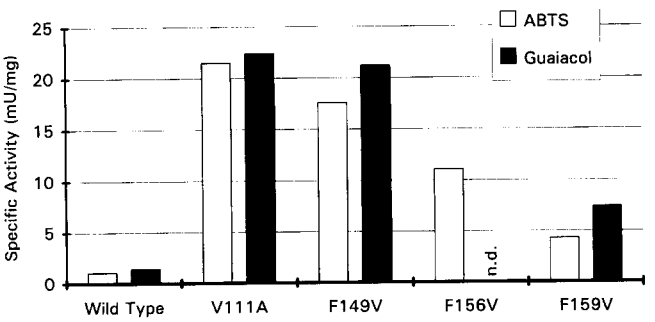

Fig. 2. Specific activities of wild type catalase A, and of 4 single-replacement mutants as indicated, with 1.33 mM ABTS and 1,33 mM guaiacol, respectively, as substrates at 30°C. The enzyme variant WT modC did not significantly differ from the wild type behaviour. All data are average values from 2 or 3 different preparations, and are corrected for < 100% haem saturation.

4. Discussion

Site-directed mutagenesis is a very powerful tool to evaluate the relative importance of individual amino acid side chains for maintaining the particular functions of distinct proteins [31,32]. We report here some biochemical and catalytical properties of several mutants of the yeast catalase A, prepared with the intention to allow higher peroxidatic reaction rates than the negligible activities of the parent enzyme.

One of the major problems in studying structure function relationships of proteins by site-directed mutagenesis is the obvious difficulty of establishing that the exchanges, though affecting the molecule on a local scale, don't have any more dispersed effects on the conformation and thus the behaviour of the entire molecule [33]. Ideally, the structures of both, the parent and the mutated protein should be available at high resolution. Being too large to make n.m.r. techniques workable in this case, X-ray analysis of the C-modified wild type as well as of the V111A mutant is in progress, but not yet available. In the mean time we have to rely on spectroscopic data and on the determination of the general stability of the catalase mutants.

As described above, the majority of our mutant proteins are isolated apparently as tetramers with to some degree less than stoichiometric amounts of haem bound. The question then is, whether this leads to major structural distortions of the molecule on the whole and, above all, of those subunits containing their prosthetic groups. To our opinion, there is no major difference between the structures of these 'hybrid' tetramers, containing apo- and holomonomers, and the corresponding holotetramers. This assumption is based:

(i) on the very similar unfolding profiles of wild type and mutants, respectively, both with respect to catalytic activity, a property clearly reflecting the conformational stability of the holosubunits, as well as to the intrinsic fluorescence, which, of course is contributed by all subunits;

(ii) on the virtually identical spectroscopic behaviour of the respective haem groups; the absorption spectra of the mutant enzymes appear 'normal', in the ferricatalase state as well as their compounds I and II. This also applies to the corresponding complexes with CN^-, HF, and the reactivities towards dithionite and 3-amino-1,2,4-triazol (data not shown here);

(iii) on observations with the V111A mutant, which has on one occasion been isolated containing 97% of the stoichiometric haem content; comparison with average preparations indicates that the specific activities *per haem* are virtually independent of the degree of haem saturation.

Table 4
Specific activities of catalase A mutants in 2-electron oxidations of different substrates

	H_2O_2 (U/mg)	Ethanol (mU/mg)	1-Propanol (mU/mg)	1-Butanol (mU/mg)
Wild type	71,400	6,330	104	2.7
WT modC	70,800	6,450	n.d.	n.d.
V111A	14,340	3,170	2,240	1,540
F149V	23,100	2,350	34	11.3
F156V	13,100	930	1,390	64
F159V	28,300	3,040	68	7.8

All data are corrected for < 100% haem saturation.

Almost any mutation planned to make the substrate binding region more accessible for large organic substrates was successful in terms of increased catalytic rates. Since 2-electron oxidations of long chain alcohols as well as 1-electron oxidations are both positively affected, steric factors are at least to some degree responsible for the relative inability of wild type catalases in true peroxidatic reactions. From a more detailed quantitative comparison of these increased, and from the concomitantly decreased catalatic rates, obviously the enzyme reactivity must have been also directly affected.

As seen from Table 4, the relative rates of 2-electron oxidations catalysed by catalase A dramatically decreases with increasing size of the substrate. This behaviour is well known for typical catalases, and classically steric constraints are held responsible [30], though obviously orientation factors are important too [30]. When compared with the corresponding wild type behaviour, this tendency is much less pronounced with the four mutants listed in Table 4. This effect is less impressive with the F149V and F159V species, and most obvious in the V111A mutant. In the latter case, the respective oxidation rates are at the same order of magnitude for aliphatic alcohols ranging from C_1 to C_5 (data for methanol and isoamyl alcohol not shown). At the same time, in absolute terms all mutants are less effective catalysts towards small 2-electron donors, including hydrogen peroxide. According to preliminary fast kinetic experiments and experiments with aliphatic hydroperoxides, the rate of formation of compound I is hardly affected by the incorporated exchanges. The decreased overall rates with H_2O_2 then reflect an altered reactivity of this intermediate. The very

bulky apolar side chains involved in formation of the substrate channel walls may also contribute in stabilising the orientation of the essential histidine's imidazole group (H70). An even partial reorientation of this side chain may interfere with its proposed role as general acid/base catalyst in the further reactions of compound I rather than with its formation [14]. The decreased catalatic activities may, however, also reflect inferior stability of compound I, i.e. an increased tendency to be reduced to compound II. The corresponding 1-electron donors could be extrinsic (substrates) as well as intrinsic ones. Both has been observed with our mutants, as shown in Fig. 2 and mentioned above.

So the changed pattern of reactivity of the mutated enzymes probably can be best explained by a combination of steric factors and an altered state of compound I. The former is presumably dominating in the case of the V111A mutant, conceivably because the side chain of this residue is critical for the bottle neck-like portion of the channel. The latter may be more important in the F149V species, which is almost as effective in 1-electron oxidations as the V111A mutant, but is much less so in the peroxidation of alcohols. Attempts to resolve the structures of these proteins, as well as extensive kinetic and biochemical studies are under way, and will eventually allow to elucidate this point. At the moment it is interesting to note that the mutants reported here represent already a large step towards the properties of another group of hydroperoxidases, the so-called catalase-peroxidases [35,36], which, however, obviously have evolved from general peroxidases [37].

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