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Understanding the structure and function of catalases: clues from molecular evolution and in vitro mutagenesis

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Abstract

This review gives an overview about the structural organisation of different evolutionary lines of all enzymes capable of efficient dismutation of hydrogen peroxide. Major potential applications in biotechnology and clinical medicine justify further investigations. According to structural and functional similarities catalases can be divided in three subgroups. Typical catalases are homotetrameric haem proteins. The three-dimensional structure of six representatives has been resolved to atomic resolution. The central core of each subunit reveals a chracteristic "catalase fold", extremely well conserved among this group. In the native tetramer structure pairs of subunits tightly interact via exchange of their N-terminal arms. This pseudo-knot structures implies a highly ordered assembly pathway. A minor subgroup ("large catalases") possesses an extra flavodoxin-like C-terminal domain. A $\geq$25Å long channel leads from the enzyme surface to the deeply buried active site. It enables rapid and selective diffusion of the substrates to the active center. In several catalases NADPH is tightly bound close to the surface. This cofactor may prevent and reverse the formation of compound II, an inactive reaction intermediate. Bifunctional catalase-peroxidases are haem proteins which probably arose via gene duplication of an ancestral peroxidase gene. No detailed structural information is currently available. Even less is know about manganese catalases. Their di-manganese reaction centers may be evolutionary

Abbreviations: ANC: *Aspergillus niger* catalase, APX: ascorbate peroxidase, AT: 3-amino-1,2,4-triazole, BLC: bovine liver catalase, CcP: *Saccharomyces cerevisiae* cytochrome *c* peroxidase, HEC: horse erythrocyte catalase, HPII: *Escherichia coli* hydroperoxidase II, INH: isonicotinic acid hydrazide (isoniazid), MLC: *Micrococcus luteus* catalase, OEC: oxygen evolving center, ORF: open reading frame, PAGE: polyacrylamide electrophoresis, PDB: Brookhaven protein data bank, PEG: polyethylene glycol, PMC: *Proteus mirabilis_PR* catalase, PTS: peroxisomal targeting signal, PVC: *Penicillium vitale* catalase, SA: salicylic acid, SCC-A: *Saccharomyces cerevisiae* catalase A, SCC-T: *Saccharomyces cerevisiae* catalase T, TMV: tobacco mosaic virus.

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related to manganese centers in photosystem II. Current research in this field focuses mainly on stress-regulation of catalase expression, on the role of catalases in plant defense, and on in vivo and in vitro folding of catalases. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Typical catalase; Catalase-peroxidase; Manganese catalase; Oxidative stress; Protein folding

1. Introduction (historical overview)

The early history of catalases goes back to the 19th century when they became one of the first sources of valuable information about the nature and behaviour of enzymes. Thenard (1811), the discoverer of hydrogen peroxide, expected that its degradation in living tissue is performed by a special substance, which Schönbein (1863) identified as a certain kind of 'ferment'. Loew (1901) was the first who named this H_2O_2-degrading enzyme catalase, but it took further 22 years until Warburg (1923) demonstrated that the active center of catalase contains iron, as concluded from the characteristic inhibition with cyanide. In 1927, Wieland offered a simple explanation for the 'catalase function' in cells, according to which H_2O_2 was acting as a hydrogen donor for a hypothetical catalyst, which in hydrogenated form reduced another molecule of H_2O_2 to water (Wieland and Franke, 1927). Stern (1936) demonstrated that in all then known catalases protoporphyrin IX was the active group, and the first crystals of beef liver catalase (BLC) were obtained a year later (Sumner and Dounce, 1937).

In 1947, the pioneering work of Chance led to the discovery of the primary complex formed between catalase and hydrogen peroxide (compound I, Chance, 1947) and initiated a reexamination of the physiological role of catalase, proposed to be in fact peroxidatic by Keilin and Hartree (1945). Chance suggested that the behaviour of the enzyme depends only on the steady-state concentration of hydrogen peroxide; at low concentrations a peroxidatic function, at higher concentrations the catalatic function would be prevalent (Chance, 1951). In addition to compound I, Chance (1949) discovered a further derivative named compound II which was independently observed by other investigators (Lemberg and Foulkes, 1948). A few years later this spectrum of catalase intermediates was completed with the detection of an inactive form — compound III (Chance, 1952).

The first prokaryotic catalase was obtained from *Micrococcus luteus* by Herbert and Pinsent (1948). In the next decade more species became available for detailed analysis (Deutsch, 1951; Galston et al., 1952; Clayton, 1959). In the sixties the role of some key residues in the active center was elucidated and their importance for tertiary structure stabilisation was discussed (Nakatani, 1960, 1961). In the next decade numerous studies were carried out on the expression and cellular localisation of catalase in prokaryotes (Frank et al., 1972; Blaise and Armstrong, 1973; Hanker and Rabin, 1975; Chester, 1979) and lower eukaryotes, mainly in yeast (Perlman and Mahler, 1970; Volfová, 1975; Zimniak et al., 1976). The mechanism of import into peroxisomes, where the majority of eukaryotic catalases is found, was studied intensively (Parish, 1975; Veenhuis et al., 1976).

The first electron density maps were obtained by Vainshtein et al. (1980) and Vainshtein et al. (1981) for a catalase from a lower eukaryote (*Penicillium vitale*) at 3.5 Å. At the same time crystals of BLC were obtained by Eventoff et al. (1976), which were suitable for detailed X-ray

analysis at 2.5 Å resolution (Reid et al., 1981; Murthy et al., 1981). In 1984, Kirkman and Gaetani (1984) reported tightly bound NADPH in BLC, which then was also detected in the X-ray structure of BLC (Fita and Rossmann, 1985a). In the course of various sequencing projects a broad spectrum of catalase sequences from organisms of all kingdoms appeared in the public databases in the last 17 years (e.g. Schroeder et al., 1982; Hartig and Ruis, 1986; Cohen et al., 1988; von Ossowski et al., 1991; Buzy et al., 1995; Guan and Scandalios, 1995). From these data it became clear that this large group of oxidoreductases is not homogenous. Based on characteristic physical and biochemical properties Goldberg and Hochman (1989a) classified catalases into three subgroups (see Section 2). One prominent subgroup with enhanced peroxidatic activity was entitled catalase-peroxidases (Loewen et al., 1983; Nadler et al., 1986; Hochman and Shemesh, 1987; Goldberg and Hochman, 1989a). At about the same time non-haem catalases with manganese in the active center were detected (Kono and Fridovich, 1983; Allgood and Perry, 1986; Barynin and Grebenko, 1986). The possible evolution of catalase-peroxidases was first addressed by Welinder (1991), and that of true catalases by von Ossowski et al. (1993).

Many attempts to engineer this protein can be attributed to the potential application of catalases in medicine and industry (Shaked and Wolfe, 1988). Due to their rather low structural stability most of the earlier work was devoted to increase this stability. Crosslinking with various bifunctional reagents led to immobilised catalase with modified activity (Schejter and Bar-Eli, 1970; Tarhan and Telefoncu, 1992). Attachment to PEG increased the half life of catalase in circulation and decreased its sensitivity to proteolysis (Abuchowski et al., 1977; Beckman et al., 1988).

The last 10 years have seen the application of DNA manipulation strategies on catalase genes. The first engineered catalase was constructed in 1990 (the yeast peroxisomal species with a His-tag to allow affinity purification) and opened a new era of molecular biology investigations on catalase (Binder et al., 1991).

Currently (April 1998) the sequences of about 80 true catalases, 16 catalase-peroxidases and one manganese catalase are available in public databases and six three-dimensional structures of catalases are solved to high resolution. With this broad range of species in hand one can systematically analyse the structure–function relationships and follow the evolutionary history of hydrogen peroxide dismutating enzymes.

The aim of this review is to give a detailed survey on the increasing field of enzymes with pronounced catalytic activity with special emphasis on phylogeny and structural peculiarities. We will not discuss the mechanism of the catalytic action in detail, but we try to rationalise the structural basis of the distinct enzymatic properties of the major classes of hydroperoxidases. After classification of all currently known catalases in three large subgroups (Section 2), a detailed characterisation of the unique catalase fold is presented (Section 3). Structural similarities of catalases with other protein classes are also discussed in this chapter. In Section 4, the functional meaning of channels leading to the active site of typical catalases is discussed, and Section 5 summarises what is known about the presumptive function of the cofactor NADPH in the respective subfamily of catalases. Section 6 summarises the state of knowledge about translocation of eukaryotic catalases to peroxisomes, and some future perspectives in this field are outlined in Section 7.

2. Classification of catalases based on evolutionary relationships

(Haem)catalase is one of few enzymes whose prosthetic group alone (ferriprotoporphyrin IX) catalyses the same reaction as the holoenzyme, though many orders of magnitude less efficiently (Kremer, 1965, Jones et al., 1973). Various substituted porphyrins were synthesised which provide even better models of hydroperoxidases, the most striking examples being Fe(III) tetraphenylporphyrins (Bruice, 1991). Not surprisingly then, in addition to catalases a whole range of other haem proteins is capable of heterolytic decomposition of H_2O_2 to O_2 and water. In most cases, however, the corresponding catalytic activity is negligible (methaemoglobin, metmyoglobin (Keilin and Hartree, 1950), cytochrome oxidase (Bickar et al., 1982), chloroperoxidase (Hewson and Hager, 1979), ascorbate peroxidase (Dalton, 1991)) and these proteins will not be subject of this review.

The assessment of bromoperoxidases is less clear. In many microorganisms this enzyme is involved in the biosynthesis of chlorinated products (Neidleman and Geigert, 1986). One subgroup, haem-containing bromoperoxidases, besides being able to brominate, also show considerable catalatic and peroxidatic activity. Enzymes were isolated from a variety of strains of *Streptomyces* and *Pseudomonas* (van Pee and Lingens, 1985a,b; Wiesner et al., 1985; Knoch et al., 1989). They contain ferriprotoporphyrin IX (sometimes less than stoichiometric amounts), are inactivated by dithionite, and apparently are homodimers, with subunits ranging from 61 to 77 kDa. These properties might indicate a relationship to catalase-peroxidases. Recently, the gene coding for the enzyme from *Streptomyces venezuelae* ISP5230 has been sequenced (Facey et al., 1996), revealing clear sequence homology with typical haem catalases. Taking into account the known properties of the purified protein (Knoch et al., 1989), this protein represents a unique member of the family of typical catalases, obviously forming stable dimers with brominating capability. However, it cannot be ruled out that these two reports are dealing with two different proteins. This view may be strengthened by the significantly lower subunit size calculated from the ORF (54 kDa) as compared with the size observed in SDS-PAGE (61 kDa), and the fact that disruption of this gene did not impair chloramphenicol biosynthesis. More data about other haem-containing bromoperoxidases are required to settle this issue.

A first attempt to classify the various enzymes with significant catalatic activity was made by Goldberg and Hochman, who suggested the division of all existing catalases into three subgroups: typical, atypical and catalase-peroxidases (Goldberg and Hochman, 1989a). It should be noted that this classification relied basically on selected physical and chemical properties of isolated species, which were not always purified to homogeneity. Major problems concern the group of so called atypical catalases, since examples are known of typical catalases erroneously classified as atypical due to some isolation artefacts (e.g. the atypical yeast catalase, Seah and Kaplan, 1973). On the other hand, with an ever increasing number of complete sequences available, distinct homologies were detected, which gave rise to an evolutionary based classification (von Ossowski et al., 1993; Mayfield and Duval, 1996). Currently more than 90 catalase sequences appear to belong to one of three distinct subgroups: two of them are haem containing, namely typical (sometimes known as true) catalases and catalase-peroxidases. The third group are (non-haem) manganese catalases. At present it appears not necessary to include a further subgroup of atypical catalases, because most of the

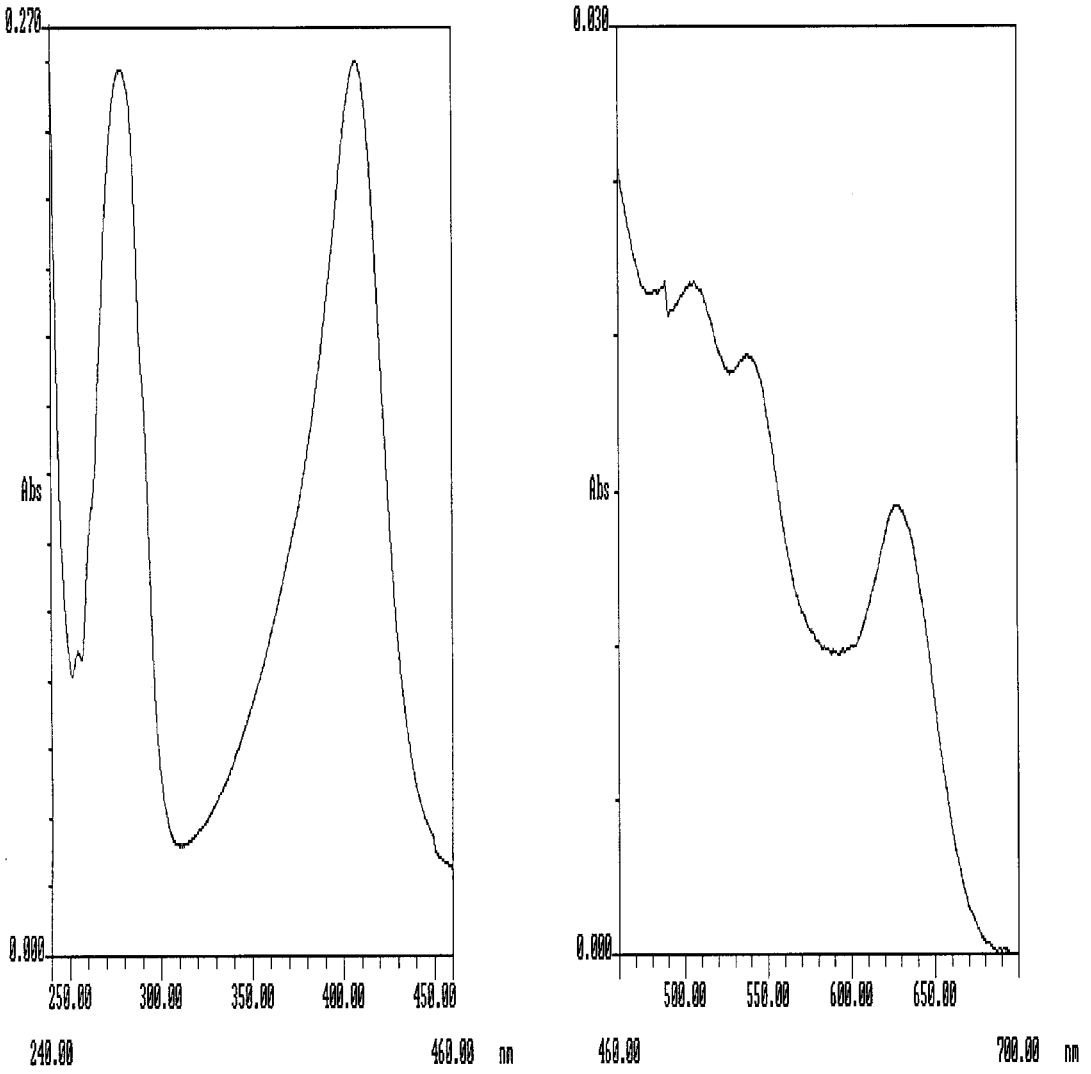

Fig. 1. The electronic spectrum of *Saccharomyces cerevisiae* catalase A purified to homogeneity. Like other typical catalases this enzyme is characterised by a rather strong absorbance in the Soret band (406 nm) and $R_z \approx 1$ (i.e. ratio A_{406}/A_{280}).

species previously called 'atypical' (e.g. chlorin-bearing catalases; Goldberg and Hochman, 1989b) clearly belong to one of the above-mentioned evolutionary-based groups. The general characteristics of these H_2O_2 cleaving isoenzymes will be summarised in the next three sections.

2.1. Monofunctional haem catalases (typical catalases)

Members of this largest subgroup are found in almost all aerobically respiring organisms, both prokaryotes and eukaryotes, but so far no typical catalase was found in the domain of

A) distal side of the prosthetic haem group

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Bacsu        35_HLLEKLAHFNRERVPERVVHAKGAGAHGYFEVTNDVTKYTKAAFLSEVGKRTPLFIRFS
EcoHPII     109_ILREKITHFDHERIPERIVHARGSAAHGYFQPYKSLSDITKADFLSDPNKITPVFVRFS
Haein        44_WLNEKLADFVREVIPERRMHAKGSGAFGTFTVTHDITKYTRAKIFSEVGKKTEMFARFT
Promi        35_WFLEKLAHFDREVIPERRMHAKGSGAFGTFTVTHDITKYTRAKIFSEVGKKTEMFARFS
Aspng        86_IFRQKLQRFDHERVPERVVHARGAGAYGTFKSYADWSNVTAADFLSANDKETPMFCRFS
Penja        45_LFTEIIFAFDRERVPERAVHARGTGAHGTFLSYEDWSNLTAASFLSAEGKFTPEMTRFS
Sacce-a      51_NLIDSLAHFNRENIPQRNPHAHGSGAFGYFEVTDDITDICGSAMFSKIGKRTKCLTRFS
Sacce-t      56_HLLENIASFDRERVPERVVHAKGGGCRLEFELTDSLSDITYAAPYQNVGYKCPGLVRFS
Arath        46_HLVEKLANFDRERIPERVVHARGASAKGFFEVTHDISNLTCADFLRAPGVQTPVIVRFS
Bovin        55_VFTDEMAHFDRERIPERVVHAKGAGAFGYFEVTHDITRYSKAKVFEHIGKRTPIAVRFS
Br-perox     35_LLLEKLAHFNRERIPERVVHARGAGAYGTFTLTRDVSRWTRAAFLSEVGKRTETFLRFS
consensus          ek a fdreriPervvHa g  a g F  t d    t  a     g  tp  vRFs

Bacsu        94_TVAGELGSADTVRDPRGFAVKFYTEEGNYDIVGNNTPVFFIRDAIKFPDF--IHTQKRDPK
EcoHPII     168_TVQGGAGSADTVRDIRGFATKFYTEEGIFDLVGNNTPIFFIQDAHKFPDF--VHAVKPEPH
Haein       103_TVAGERGAADAERDIRGFALKFYTEEGNWDLVGNNTPVFFLRDPRKFPDL--NKAVKRDPR
Promi        94_TVAGERGAADAERDIRGFALKFYTEEGNWDMVGNNTPVFYLRDPLKFPDL--NHIVKRDPR
Aspng       145_TVVGFRGSVDTARDVHGHACRFYTDEGNYDIVGINFAPFFIQDAIQFPDL--VHAIKPMPN
Penja       104_TVSGARGSADTARDVHGFATRFYVDEGNFDIVGNNIPVFFIWDVIIEPTLMALHAQKPNPR
Sacce-a     110_TVGGDKGSADTVRDPRGFATKFYTEEGNLDWVYNNTPVFFIRDPSKFPHF--IHTQKRNPQ
Sacce-t     115_TVGGESGTPDTARDPRGVSFKFYTEWGNHDWVFNNTPVFFLRDAIKFPVF--IHSQKRDPQ
Arath       105_TVIHERGSPETLRDPRGFAVKFYTREGNFDLVGNNFPVFFIRDGMKFPDM--VHALKPNPK
Bovin       114_TVAGESGSADTVRDPRGFAVKFYTEDGNWDLVGNNTPIFFIRDALLFPSF--IHSQKRNPQ
Br-perox     94_TVAGSLGAADAVRDPRGWALKFYTEEGNYDLVGNNTPVFFIKDAIKFPDF--IHTQKRDPY
consensus      tV  e gs  t RD rGfa kfyt  G  D vgnn pvFf rD   Fpd    h  k  p
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B) proximal side of the prosthetic haem group

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Bacsu       321_GIDVSPDKMLQGRLFAYHDAHRYRVG-ANHQALPIN
EcoHPII     399_GLDFTNDPLLQGRLFSYTDTQISRLGGPNFHEIPIN
Haein       330_GIGASPDRMLQARLFNYADAQRYRLG-VNYRQIPVN
Promi       321_GISFSPDKMLQGRLFSYGDAHRYRLG-VNHHQIPVN
Aspng       376_GIDFTDDPLLQGRLFSYLDTQLTRHGGPNFEQIPVN
Penja       337_GVDFTEDPLLQGRLFSYLDTQLNRHG-PNIQQLGFN
Sacce-a     339_YQEASADPVLQARLFSYADAHRYRLG-PNFHQIPVN
Sacce-t     366_GIKPSNDSVLQARLFSYPDTQRHRLG-ANYQQLPVN
Arath       332_GIHYSDDKLLQTRVFSYADTQRHRLG-PNYLQLPVN
Bovin       341_GIEPSPDKMLQGRLFAYPDTHRHRLG-PNYLQIPVN
Br-perox    321_GIGPSPDKMLQGRLFAYGDAHRYRVG-INADHLPVN
consensus      g   s D mlq R f Y Da r R G  n   lPvN
```

Fig. 2. Multiple sequence alignment of typical catalases in the areas of the distal (a) and proximal (b) HAEM ligand, respectively. The numbers indicate the position of each segment in the corresponding sequence. Functionally important residues, discussed in chapter III are in black boxes. The *consensus* sequence below the alignment contains the invariantly conserved residues (uppercase) as well as residues conserved at least in 75% of all known catalases (lowercase). 'Br-perox' denotes the bromoperoxidase from *Streptomyces venezuelae* (Facey et al., 1996), which, according to its sequence belongs to the group of typical catalases. The abbreviations are listed in Table 1 (partly modified from Zámocký et al., 1997a).

Table 1
Sources of typical catalases included in this study

Abbreviation	Organism	Specification	Accession #
Bacsu	*Bacillus subtilis*		P26901
Br-perox[a]	*Streptomyces violaceus*		P33569
EcoHPII	*Escherichia coli*	HPII	P21179
Haein	*Haemophilus influenzae*		P44390
Promi	*Proteus mirabilis*		P42321
Aspng	*Aspergillus niger*		P55303
Penja	*Penicillium janthinellum*		P81138
Sacce_a	*Saccharomyces cerevisiae*	catalase A	P15202
Sacce_t	*Saccharomyces cerevisiae*	catalase T	P06115
Arath	*Arabidopsis thaliana*		P25819
Bovin	*Bos taurus*	bovine, liver	P00432

[a] "Br_perox" denotes the bromoperoxidase-catalase (Facey et al., 1996) which sequence clearly belongs to the group of typical catalases.

Archae. Numerous organisms — mainly plants — even have two or more isoforms. Most of these hydroperoxidases are homotetramers, 200–340 kDa in size with four prosthetic haem groups. In the majority of cases ferric protoporphyrin IX was found, but in some representatives haem d resides in the active center, formed autocatalytically from protohaem IX (Bravo et al., 1997). Characteristic physical features include their distinct electronic spectrum with rather strong absorbance in the Soret band, reflected by R_Z values around 1 (i.e. ratio $A_{406\ nm}/A_{280\ nm}$, typical example in Fig. 1), irreversible inhibition by the suicide inhibitor 3-amino-1,2,4-triazole (AT), and the fact that their ferric haem group cannot readily be reduced with sodium dithionite. In addition to a very efficient catalatic reaction mode ($k_{cat} = 4\times10^7\ M^{-1}\ s^{-1}$, for human erythrocyte catalase, Schonbaum and Chance, 1976) they can also catalyse 2-electron peroxidations of short-chain aliphatic alcohols at reasonable rates (Sichak and Dounce, 1986). Genes coding for typical catalases were isolated and sequenced from more than 75 organisms. In some cases the tight binding of an additional cofactor, NADPH, to each subunit has been demonstrated (Kirkman et al., 1987), but the same may be true for the majority of these enzymes. The presumptive functions of this cofactor will be discussed in some detail in Section 5. The currently best analysed representatives come from: bovine liver (Schroeder et al., 1982), *Saccharomyces cerevisiae* (Cohen et al., 1988), *Escherichia coli* (von Ossowski et al., 1991), maize (Guan and Scandalios, 1995) and *Proteus mirabilis* (Buzy et al., 1995).

The conserved core of typical catalases comprises about 390 amino acids (from residue 70 to 460 — numbering for yeast catalase A; Klotz et al., 1997) spanning four structural domains. The highest degree of homology is found in the area around the essential distal histidine, and around the proximal haem-ligand, tyrosine, respectively, as demonstrated in Fig. 2 (Table 1) (Zámocký et al., 1997c). The tertiary structure of the β-barrel, and to some extent also the wrapping domain, which harbour the above-mentioned essential residues with their surroundings is highly conserved in all currently resolved structures of typical catalases (cf. Section 3 for details).

Fig. 3. Multiple sequence alignment of catalase-peroxidases with ascorbate peroxidases and *Saccharomyces cerevisiae* mitochondrial cytochrome *c* peroxidase in areas adjacent to the distal and proximal haem ligands, respectively. Functionally important residues are printed in black boxes. The numbers indicate the start of each segment in the corresponding sequence. The *consensus* sequence below the alignment contains the invariantly conserved residues (uppercase) as well as residues conserved in at least 75% of all known sequences of the Class I of the peroxidase superfamily (lowercase). The abbreviations are listed in Table 2.

2.2. Catalase-peroxidases

The second distinct group is called catalase-peroxidases. Members of this subgroup are found in all three living kingdoms, although in eukaryots they were detected only in fungi until now (Fraaije et al., 1996; Levy et al., 1992). One may speculate that these hydroperoxidases are successors of the first ancestral H_2O_2-degrading enzymes in the biosphere. Their molecular weight varies from 120 to 340 kDa and in general they are homodimers (Obinger et al., 1997b; Nagy et al., 1997a), although homotetramers were also reported (Hochman and Shemesh, 1987; Hochman and Goldberg, 1991). Furthermore, and in marked contrast with typical catalases, reversible dimer–tetramer association of catalase-peroxidases has been reported (*Mycobacterium smegmatis*, Billman-Jacobe et al., 1996). A relatively large momomeric unit comprising more than 700 amino acids arose probably from duplication of an ancestral gene

Table 2
Sources of catalase-peroxidases included in this study

Abbreviation	Organism (strain)	Enzyme specification	Accession #
Arcfu-CP	*Archaeoglobus fulgidus*	catalase-peroxidase	AE000951
Bacst-CP	*Bacillus stearothermophilus*	catalase I	M29876
Ecoli-HPI	*Escherichia coli*	catalase HPI	P13029
Mycin-CP	*Mycobacterium intracellulare*	catalase-peroxidase	Q04657
Mycin-CP	*Mycobacterium tuberculosis*	catalase-peroxidase	Q08129
Scosp-CP	*Synechococcus sp.* (PCC7942)	catalase-peroxidase	D61378
Atcy-APX	*Arabidopsis thaliana*	ascorbate peroxidase	Q05431
Pissa-APX	*Pisum sativum*	ascorbate peroxidase	P48534
Sacce-CCP	*Saccharomyces cerevisiae*	cytochrome *c* peroxidase	P00431

(Welinder, 1991). Fourteen prokaryotic and two archaebacterial representatives were sequenced until now (no sequence from eukaryots is available) and these genes exhibit remarkable homology. Furthermore, they show much higher sequence homology with haem peroxidases than with typical catalases. Fig. 3 (Table 2) demonstrates the high homology of the distal and proximal haem ligand surroundings of catalase-peroxidases to the corresponding regions in ascorbate peroxidases and yeast cytochrome *c* peroxidase. All enzymes mentioned in this figure are evolutionary classified as class I of the superfamily of plant, fungal and bacterial peroxidases (Welinder, 1992).

Obviously the most characteristic feature of catalases-peroxidases is their bifunctional catalytic behaviour. The maximal catalatic turnover of catalase-peroxidases is two or three orders of magnitude lower than that of typical catalases (Marcinkeviciene et al., 1995, Burner and Obinger, 1997). On the other hand, their K_M value for hydrogen peroxide is in the millimolar range (Kobayashi et al., 1997; Obinger et al., 1997a), one or two orders of magnitude below the corresponding values for typical catalases (Ogura, 1955; Kinoshita et al., 1994; Latyshko and Gudkova, 1996). Thus, the overall rate (k_{cat}/K_M) at low substrate concentrations is only about one order of magnitude below that of typical catalases, i.e. they are fairly equivalent under physiological conditions. Generally catalase-peroxidases will accept a broad range of organic substrates in peroxidation, reminiscent of the classical secretory plant peroxidases of class III. With few exceptions, however, the maximal rates are far beyond those obtained with, e.g. horseradish peroxidase. One can conclude that in vivo a second molecule of H_2O_2 rather than a peroxidase substrate will serve as electron donor to recover the resting ferric form of these enzymes.

All known catalase-peroxidases exhibit unusual haem spectra with rather low R_z values, which can be explained by nonstoichiometric haem binding (corresponding to 0,4–1 haem/subunit). In contrast to typical haem catalases their catalatic activity shows a sharp pH optimum around 6.5. Catalase-peroxidases also are significantly more sensitive to inactivation by higher temperatures, pH and H_2O_2 than typical catalases (Goldberg and Hochman, 1989b).

Unlike typical catalases, the enzymes of this subtype are not inhibited by AT, but their haem iron can be readily reduced with sodium dithionite. From experiments with *Mycobacterium tuberculosis* a new and specific type of inhibitor was observed — isoniazid (INH, Johnsson et

al., 1997). Catalase-peroxidase is able to activate this drug which after peroxidation becomes toxic for the cells. Mycobacterial strains bearing a mutated form of *katG* (the gene coding for catalase-peroxidase), which produce catalytically inactive hydroperoxidase, are able to survive treatment with INH (Haas et al., 1997). Interestingly, catalase-peroxidase from *M. smegmatis* oxidises INH in a peroxide-independent way involving an oxyferrous form of the enzyme generated by reduction of the ferrihaem with hydrazine (Magliozzo and Marcinkeviciene, 1996).

2.3. Manganese catalases

Until now only three manganese catalases are known, one from lactic acid bacteria (Beyer and Fridovich, 1985) and two from thermophilic organisms (Barynin and Grebenko, 1986, Kagawa et al., 1997). These enzymes are sometimes referred to as pseudocatalases (Kono and Fridovich, 1983, Allgood and Perry, 1986) due to the fact that they utilise manganese ions (instead of ferric haem) in their active sites. The species from *Lactobacillus plantarum* was cloned and sequenced (Igarashi et al., 1996). Its subunit comprises 266 amino acids. Very recently, also the sequence of the thermostable manganese catalase from *Thermus sp.* YS 8–13 was reported which shares 34% identity with the enzyme from *Lactobacillus* (Kagawa et al., 1997). The X-ray structure of catalase from *Thermus thermophilus* has been determined at low resolution, but unfortunately the amino acid sequence of this species remains unknown (Vainshtein et al., 1985). The absence of significant homology to any other protein group indicates that these enzymes may form an unlinked evolutionary group. In contrast to haem containing species, manganese catalases are not inhibited by CN^- and N_3^-. The molecular weight of the known native Mn catalases ranges from 170 to 210 kDa, and they may form unusual oligomeric structures (homopentamers or homohexamers, Beyer and Fridovich, 1985; Barynin and Grebenko, 1986).

3. Structural features of prokaryotic and eukaryotic catalases

3.1. Typical catalases

The crystal structure of six typical haem catalases was determined at almost atomic resolution. They include three prokaryotic representatives, namely: *M. luteus* catalase (MLC; Murshudov et al., 1992), *P. mirabilis* catalase (PMC; Protein Data Bank codes: 1CAE, 1CAF, Gouet et al., 1995), *E. coli* hydroperoxidase II (HPII; PDB code: 1IPH, Bravo et al., 1995) and three eukaryotic species: *P. vitale* catalase (PVC; code: 4CAT, Vainshtein et al., 1981; Melik-Adamyan et al., 1986), *S. cerevisiae* catalase A (SCC-A; code: 1A4E, Berthet et al., 1997, Mate et al., in preparation) and beef liver catalase (BLC; codes: 7CAT, 8CAT, Murthy et al., 1981; Fita et al., 1986). Although some important differences in the primary structure are found between these six proteins (Fig. 2), the three-dimensional structure appears well conserved. All of them are homotetramers with 222 molecular symmetry. The subunit forms a characteristic globule with an extended N-terminal arm. Fig. 4 shows a monomer of catalase A from the yeast *Saccahromyces cerevisiae* as representative example.

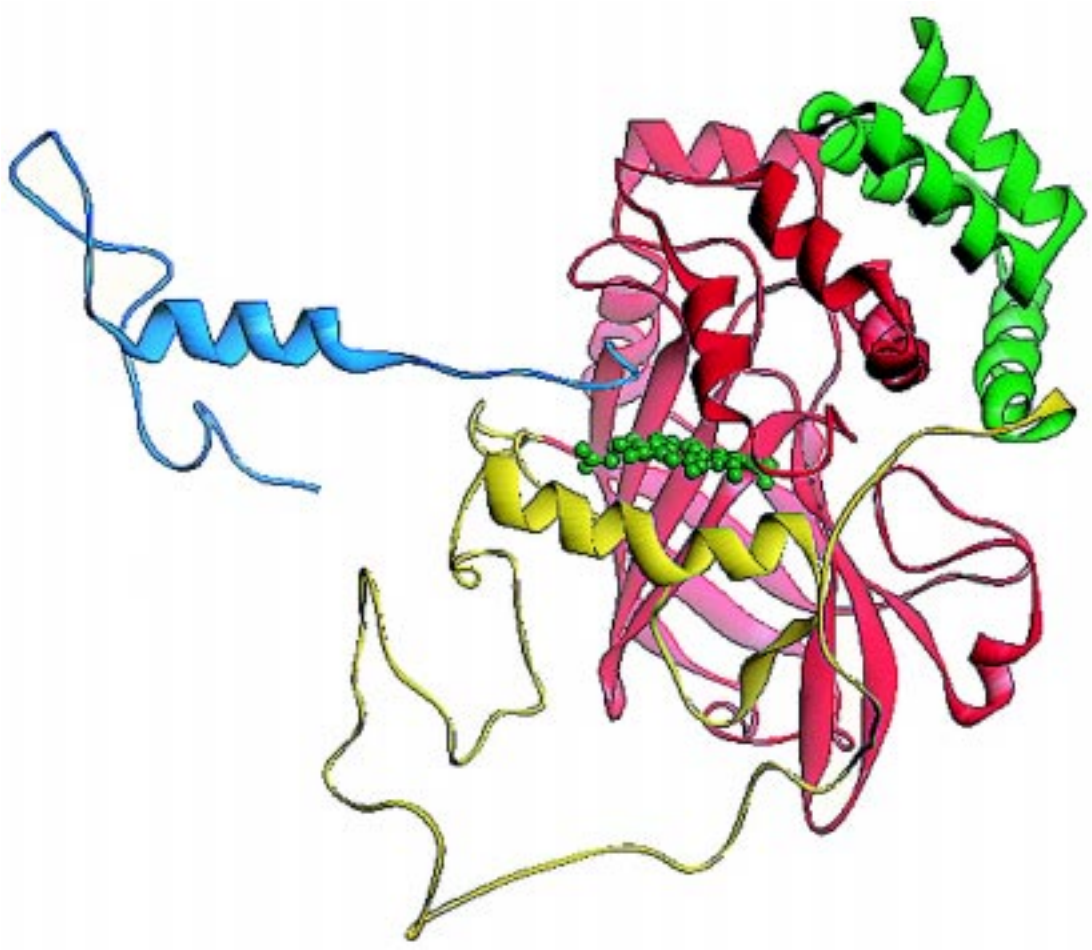

Fig. 4. Ribbon presentation of one monomer of yeast catalase A. The prosthetic haem group is localised at the center of the globular structure. The N-terminal arm extends from the β-barrel region of the subunit and forms a hairpin-like structure. Colour scheme: dark green — haem group, cyan — N-terminal arm, red — β-barrel domain, yellow — domain connection, green — α-helical domain.

In each subunit four distinct structural regions can be identified: the N-terminal arm comprising the first 70 amino acids, the β-barrel domain (positions 72–318, if not otherwise stated, the numbering in this chapter refers to SCC-A), the domain connection, sometimes referred to as 'wrapping domain' (residues 319–439) and the α-helical domain (positions 440–503). In a minor subgroup (representing 9% of all currently sequenced typical catalases) an extra domain exists in the C-terminal region, approx. 150 residues long (Fig. 5). According to its fold it is often addressed as 'flavodoxin-like' domain. Whereas the β-barrel domain is extremely well conserved from lower prokaryotes to higher eukaryotes, there is slightly more variability in the domain connection and in the α-helical domain, but the highest degree of divergences occurs in the C- and N-terminal areas, respectively. A short characterisation of the above-mentioned structural parts follows.

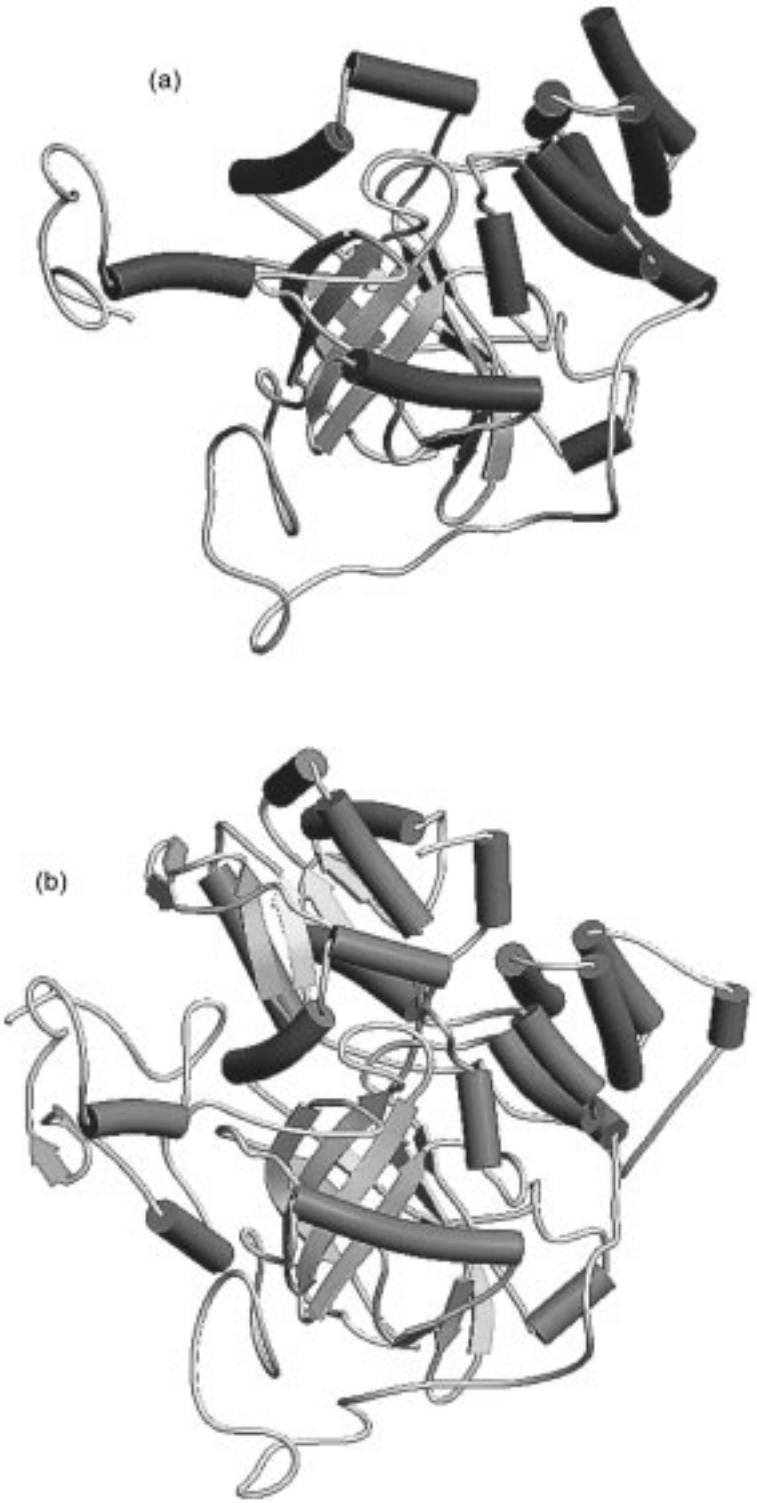

Fig. 5. Comparison of the overall fold of (a) SCC-A and (b) HPII monomers. Prominent differences between these two catalases are found mainly in the N-terminal arm and in the domain connection. Additionally, HPII exhibits an extra α,β-('flavodoxin-like') domain; which slightly displaces the α-helical domain relative to the respective position in SCC-A.

3.1.1. The N-terminal arm

The N-terminal region of a typical catalase forms an arm which participates in a remarkable intersubunit knot-like structure (Melik-Adamyan et al., 1986). This arm extends from the globular (β-barrel) region of one subunit, but at about half of its length bends back in an 'elbow' like structure. Most of the N-terminal arm is buried between neighbouring subunits; the region around the elbow penetrates the wrapping loops of both the *Q*- and *R*-related subunits (nomenclature of the molecular twofold axes according to Murthy et al., 1981; cf. Fig. 6). It also seals the haem pocket of the *R*-related subunit, interacting with the side-chains of pyrrole I and IV. The extended N-terminal arms of HPII and BLC partly close the lateral entrance to the major substrate channel described for BLC (Bravo et al., 1995), whereas in PMC and SCC-A, which lack the first α-helix in this region, the opening of the substrate channel to the solvent is larger (Mate et al., in preparation). However, this 'upper substrate groove' (Buzy et al., 1995) does not lead to increased peroxidatic activity of SCC-A or PMC, and the significance of these structural differences remains unknown.

The essential distal histidine is present in all haem catalases, allowing the proper binding and reduction of a peroxide molecule. It is invariantly situated at the C-terminus of the N-terminal arm and its reactivity is controlled by the highly conserved side chain architecture of residues from the β-barrel domain, which allows the formation of a specific hydrogen bonding network.

Interestingly, a methione sulphone (Met53*) was found on the distal side of PMC, near the essential histidine (Gouet et al., 1995). There is biochemical evidence that the oxidation of Met53 to sulphone results from a posttranslational modification (Buzy et al., 1995). Its orientation and hydrogen bonding with Asn144 makes the entry to the active site more polar in this species than in other catalases, where a valine is found in this position. Together with the bulkiness of the sulphone moiety this may explain the unusually weak inhibition of PMC by AT (Jouve et al., 1983).

3.1.2. The β-barrel domain

This largest domain is well defined in all refined structures of typical catalases. According to sequence alignments it has nearly identical length in all species, and so clearly is the most highly conserved part of the molecule, bearing many catalytically and structurally important residues in a characteristic three-dimensional organisation.

It is essentially an eight-stranded antiparallel β-barrel with six α-helical insertions in the turns between the strands (Fita and Rossmann, 1985b; Melik-Adamyan et al., 1986). One part of the barrel is oriented towards the surface allowing intersubunit contacts, whereas the internal parts harbour several essential amino acid residues (His70, Ser109, Asn143). Together with bulky residues of the fifth section of the substrate channel they constitute the typical and highly conserved distal cavity accommodating the prosthetic haem group. Rapid diffusion of polar substrates through this hydrophobic environment essentially determines the very effective turnover of small peroxides by typical catalases.

The distal haem pocket implies the orientation of the imidazole ring of the essential histidine (His70) parallel to the plane of the prosthetic haem group. This orientation favours intensive π–π interactions between the essential histidine and the porphyrin. In all other known haem protein structures, including haem peroxidases, the imidazole ring of the essential histidine is perpendicular to the plane of the macrocycle with its imidazole nitrogen pointing towards the

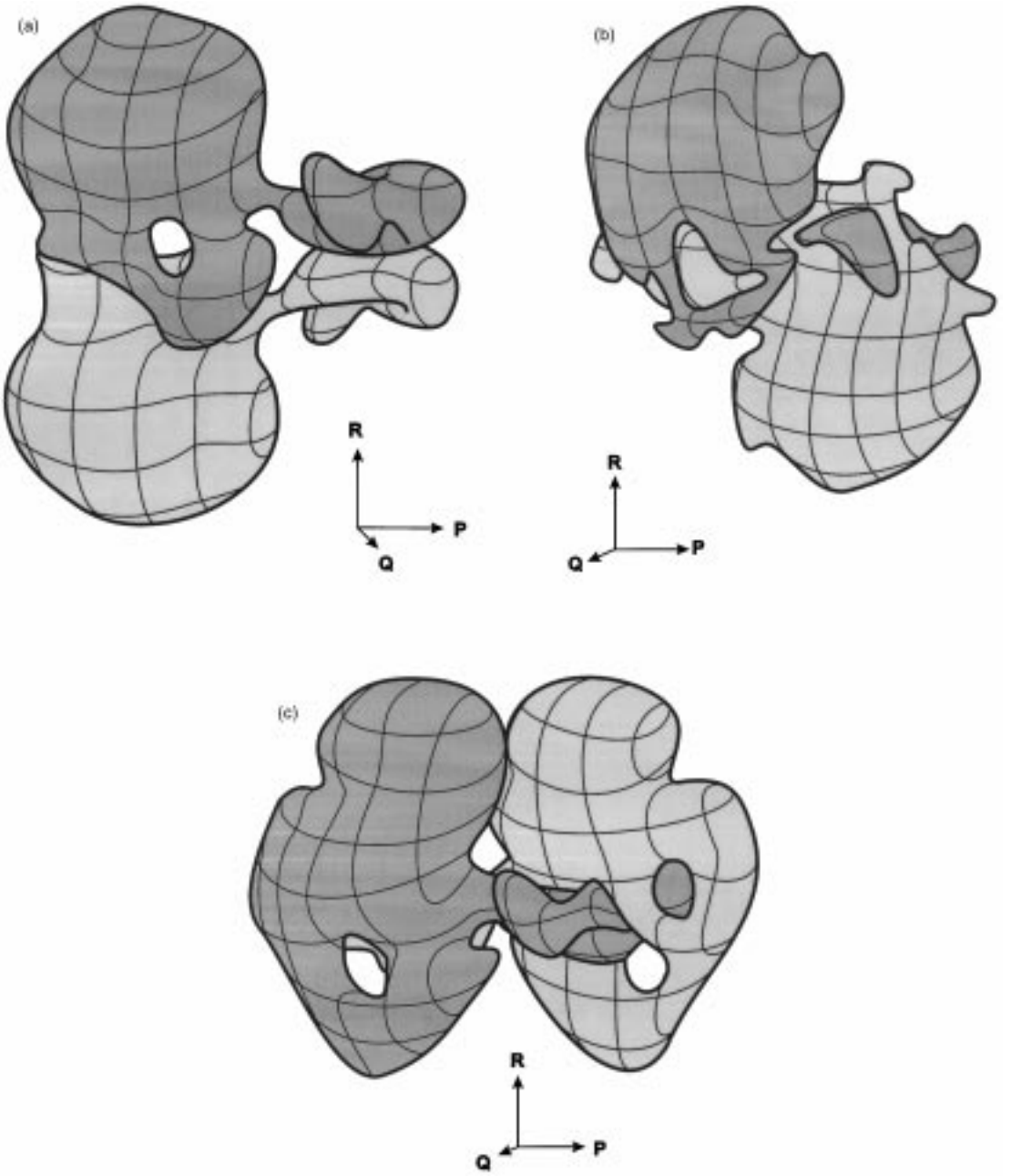

Fig. 6. Diagrammatic presentation of all dimers of yeast catalase A revealing characteristic intersubunit contacts (a) *P*-related, (b) *Q*-related and (c) *R*-related subunits (definition of axes according to Murthy et al. (1981)). The characteristic intersubunit connection is formed by the N-terminal arm of one polypeptide chain which penetrates the wrapping loops of both *Q*- and *R*-related subunits. The reference subunit is shown in grey, the respective second subunit in light grey.

central iron. This energetically disfavoured orientation presumably contributes to the increased reactivity of compound I in peroxidases. Ser109 is the nearest neighbour of His70, together with a water molecule providing a network of hydrogen bonds typical for catalases. Obviously a serine in this position is essential for maintaining the proper orientation and the nucleophilic character of the essential histidine, and thus stabilises the distal haem pocket structure. In fact it is conserved in 98% of the known sequences. With the exception of the serine–threonine replacement (1.8% residual activity), site-directed mutagenesis of this residue in SCC-A always leads to a total loss of activity (Herzog, 1996).

The coessential asparagine (Asn143) is another characteristic feature of typical catalases, whereas the equivalent position is occupied by an arginine in peroxidases. Its significance in proper orientation of the peroxide substrate and its influence on the redox potential of haem iron is supported by mutagenesis experiments (for SCC-A Herzog and Koller (1995) and for HPII Obinger et al. (1997a).

The main substrate channel represents the most distinct peculiarity of this domain. This structural motif, so far detected only in typical catalases, forms a connection from the molecular surface to the haem group. The hydrophobic and narrow character of the lower part of this channel allows only small substrates to reach the buried haem groups (Reid et al., 1981). We give a detailed discussion of the structure and function of the main channel in Section 4, supported by results from site-directed mutagenesis.

The minor channel connects the molecular surface next to the cleft which harbours the adenine moiety of bound NADPH (between Lys234 and Gln302) with the lower third of the major channel (Fita et al., 1986). It is quite narrow throughout its length (4–7 Å), but most restricted at its mouth to the major channel between residues Pro124 and Leu196. When NADPH is bound in the respective subclass of catalases, the entrance of this channel is almost completely blocked, so diffusion of substrates/products does not appear likely through this path.

Parts of the surface of the β-barrel domain also contribute to the tight binding of NADPH in the respective subgroup of typical catalases, essentially residues of the helix α5 and of strand β6 (Zámocký et al., 1997a). The most important residues are Ile195 and Arg200, which cooperate in binding of the adenine moiety of NADPH, whereas Gln302 interacts with the nicotinamide part of the cofactor.

3.1.2.1. Structural similarities of the β-barrel domain with members of the calycin family. An all-against-all comparison of a database of protein domains (Holm and Sander, 1993) reveals significant similarity between a major part of the β-barrel domain of typical catalases and the 3-D structure of calycins. This is a group of proteins having no significant sequence similarity, but which adopt a common antiparallel β-barrel structure. Members of the calycin family with known 3-D structure (including streptavidin, PDB code 1SRI) possess a characteristic pocket in their structure which corresponds to a similar, though partially covered pocket in the β-barrel domain of catalase, formed by Phe80, Phe131 and Trp140. It has been suggested (Russell and Sternberg, 1996) that this pocket can serve as a potential binding site for inhibitors of catalase (e.g. salicylic acid). However, no experimental support exists for this hypothesis. The areas of structural similarity between typical catalases and calycins are highlighted in Table 3.

Table 3
Structural similarity between typical catalases and the members of the calycin superfamily detected with the Dali method (Version 2.0; Holm and Sander, 1993). The following notation is used for data columns: STRID1/STRID2, PDB identifiers of search structure and aligned structure; *Z*, *Z*-score, i.e. strength of structural similarity in standard deviations above expexted. The matched structures are sorted by *Z*-score; RMSD, positional root mean square deviation of superimposed CA atoms in Angstroms; LALI, total number of equivalenced residues; LSEQ2, length of the entire chain of the equivalenced structure; %IDE, percentage of sequence identity over equivalenced positions; NFRAG, total number of equivalenced fragments; PROTEIN, description of the aligned structure

STRID1	STRID2	Z	RMSD	LALI	LSEQ2	%IDE	NFRAG	Protein
1a4e	1a4e	66.8	0.0	489	489	100	1	catalase A from *Saccharomyces cerevisiae*
1a4e	8cat	54.1	1.2	465	498	51	15	beef liver catalase resolution 2.5 Å
1a4e	7cat	54.1	1.2	465	498	51	15	beef liver catalase resolution 2.5 Å
1a4e	2caf	52.8	1.4	466	475	45	13	catalase from *Proteus mirabilis* — compound I
1a4e	2cah	52.7	1.4	466	475	45	13	catalase from *Proteus mirabilis* complexed with NADPH
1a4e	2cae	52.7	1.5	466	475	45	13	catalase from *Proteus mirabilis* (without NADPH)
1a4e	2cag	52.3	1.5	466	475	45	13	catalase from *Proteus mirabilis* — compound II
1a4e	1iph	35.1	1.4	474	727	39	15	catalase HPII from *Escherichia coli*
1a4e	1obp	4.3	4.3	109	158	9	17	odorant-binding protein from *Bos taurus*
1a4e	1sri	3.9	3.8	94	118	10	10	streptavidin (complexed with 3′,5′-dimethyl-haba)
1a4e	1epa	3.7	3.1	90	151	7	11	epididymal retinoic acid-binding protein
1a4e	1beb	3.7	3.4	95	156	11	14	β-lactoglobulin from *Bos taurus*
1a4e	1mup	3.6	3.2	94	157	11	13	major urinary protein (complex with 2-(sec-butyl)thiazoline)
1a4e	1aqb	3.4	4.1	100	175	9	12	retinol-binding protein from pig plasma
1a4e	1bbp	2.6	3.8	99	173	5	14	bilin binding protein from *Pieris brassicae*
1a4e	1hmt	2.3	3.3	87	131	7	12	fatty acid binding protein from human muscle

3.1.3. The domain connection

The first third of this part of the molecule, including the so-called 'essential helix' α9, is embedded into a large groove at the bottom of the β-barrel domain. At the end of helix α9, the polypeptide chain forms a large loop which loosely wraps around half of the β-barrel domain, firmly attaching to the globular structure only at its very end. With the exception of the 18 residues-long α-helix it lacks discernible secondary structure. This element is functionally the most important and most highly conserved part on the haem's proximal side. The proximal haem-iron ligand resides in this helix, a unique tyrosine (Tyr355) only found in typical catalases. In catalase-peroxidases, like in most peroxidases, this position is occupied by a

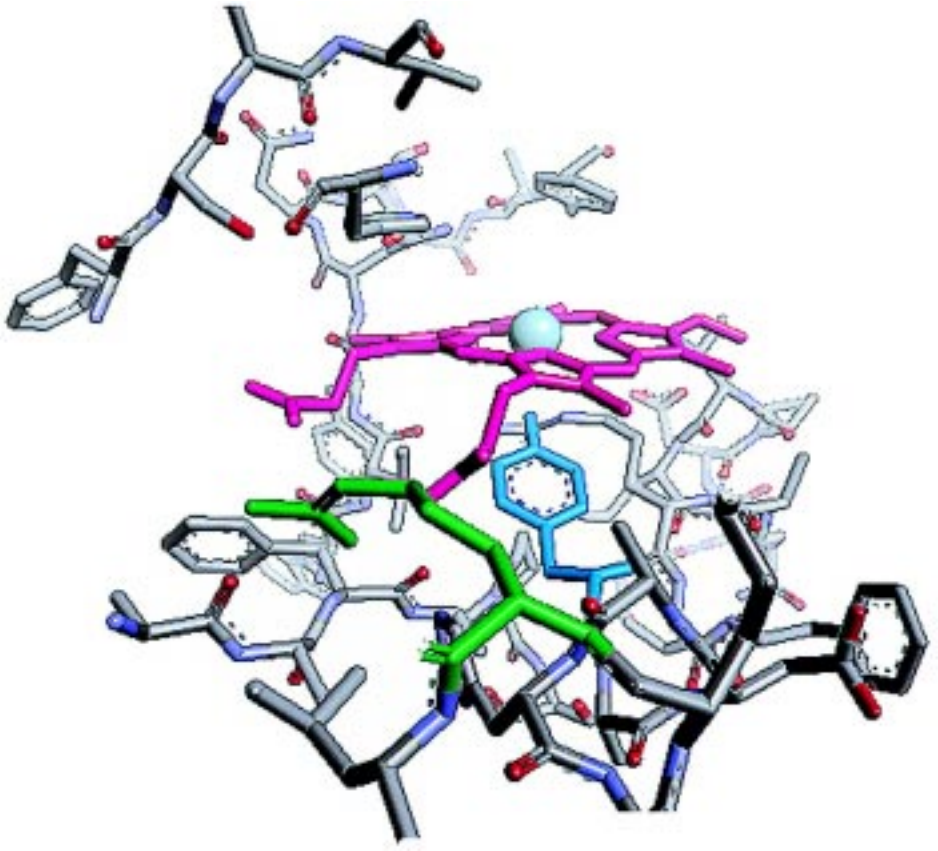

Fig. 7. The structure of the domain connection around the proximal haem ligand (Tyr355) in catalase A from *Saccharomyces cerevisiae*. The highly conserved orientation of the tyrosine phenyl ring (coloured blue) and the distance between the phenolic oxygen and the haem iron (coloured cyan) are peculiarities of typical catalases. Arg362 (coloured green) involved in charge interaction with the propionate of pyrrole ring IV of the haem is also highly conserved among typical catalases.

histidine. The orientation of the tyrosine phenyl ring and the distance between the phenolic oxygen and the metal ion are virtually identical in BLC, MLC, SCC-A and HPII, respectively (Fig. 7). An outstanding feature present only in HPII is a covalent bond between Cβ of the essential Tyr415 and the imidazole ring of a neighbour histidine residue (His392). The eventual meaning of this cross-link is not clear, but there exists a relationship between this His–Tyr linkage and the self-catalysed haem conversion from b to d (Bravo et al., 1997).

Some parts of the 'wrapping loop' dock to the outer parts of the β-barrel domain, but two eye-shaped regions (392–412 and 425–434, respectively) form open loops. Upon tetramer assembly, the N-terminal arm of the *Q*-related subunit threads through the larger of these loops, whereas the outermost part of the amino-terminal arm of the *R*-related subunit penetrates the smaller one (Fig. 6).

3.1.3.1. The haem group in typical catalases. The haem pocket is defined by residues from the β-barrel (distal side and along the edges of pyrrole rings I, II and III), from the domain connection (proximal side) and from the N-terminal arm of the *R*-related subunit (lining the edges of pyrrole rings I and IV). The propionic side chains of the porphyrin exhibit intensive ionic interactions with Arg67 and with a short, evolutionary conserved segment of the wrapping loop around Arg362 (Gouet et al., 1995). Whereas in isolated subunits parts of the distal side of the prosthetic group remains uncovered — quite similar to the situation in CcP and HRP, the haem groups are well buried inside the tetramer (Murthy et al., 1981; Melik-Adamyan et al., 1986; Gouet et al., 1995).

In BLC up to 50% of its haem groups are degraded to biliverdin and bilirubin (Brindle et al., 1986), through breakage of the sterically most accessible bridge between pyrrole rings II and III. A similar situation is seen with PMC (35% degradation, Jouve et al., 1983), whereas no such modification is found in MLC and SCC-A.

HPII and PVC contain a chlorin, i.e. a partially saturated porphyrin macrocycle, rather than protoporphyrin IX (haem b) as prosthetic groups (Chaga et al., 1992). This modified porphyrin, termed haem d, incorporates a *cis*-hydroxy γ-spirolactone at the saturated pyrrole ring III, leading to their characteristic electronic spectrum (Murshudov et al., 1996). Interestingly, a mixture of haem b and haem d is found in HPII isolated from anaerobically grown *E. coli*. Any residual haem b is converted to haem d when the isolated protein is treated with H_2O_2 (Loewen et al., 1993). This strongly supports the hypothesis that protohaem is first bound to the apoprotein of HPII, followed by tetramerisation, and eventually hydroxylation of the haem groups utilising one of the first H_2O_2 molecules bound to the enzyme (Timkovich and Bondoc, 1990). The functional requirements for chlorin formation in HPII were extensively studied by the group of P.C. Loewen by site-directed mutagenesis. The essential histidine (128 in HPII) is strictly required. The mutants His128Ala and His128Asn both are completely inactive and incorporate haem b (Loewen et al., 1993). In both respects the coessential asparagine (201) is not an absolute requirement. The engineered variants Asn201Ala, Asn201Asp and Asn201Gln reveal reduced catalytic activity down to 0.4% of the wild type, but still incorporate haem d. The properties of the mutant Asn201His proved most interesting: as isolated it incorporated protohaem, and showed $<1\%$ residual activity; when treated with a continuous flow of hydrogen peroxide a reversible conversion to a haem d-like species of increased reactivity occurred via compound I formation (Obinger et al., 1997a). Interestingly, replacement of the corresponding residue in SCC-A reveals completely different results. With the exception of a glutamine, replacement of Asn143 by any residue led to inactive, haem-less proteins. Asn143Gln, however, incorporates a mixture of protohaem and a yet unidentified chlorin (Herzog and Koller, 1995). This species shows reduced catalatic activity and reflects an increased tendency of compound II formation, concomitant with largely increased rates of 1-electron peroxidations (Herzog, 1996).

In addition to the covalent modification, the prosthetic groups of the two large catalases for the most part are inverted with respect to the orientation found in BLC, MLC, PMC and SCC-A (Bravo et al., 1995; Murshudov et al., 1996). From the data cited above it is clear that this flipping is unrelated with the cyclisation at pyrrole ring III. Given the tight embedding of the prosthetic groups in the fully assembled holotetramer one can safely assume that the prosthetic group is already incorporated in this orientation upon holomonomer formation. Due

to this 'flipping' the γ-spirolactone and the hydroxy substituent of the haem d groups of PVC and HPII (mainly at pyrrole ring III) are opposite to the essential distal histidine (Chiu et al., 1989). Furthermore, they are on the proximal side, raising the question about the mechanism of this hydroxylation (Loewen, 1996). A model has been proposed to explain both, the formation of haem d, and the formation of the covalent bond between Tyr415 and His392 mentioned above (3.1.3.). It involves formation first of compound I, binding of a molecule of H_2O_2 on the proximal side and an anion close to the imidazole of His392, acting as general base catalyst (Bravo et al., 1997).

The structural basis for the inversed configuration of the porpyrin macrocyle in PVC and HPII is not full understood. In these enzymes the protoporphyrin ring is held in its position by the contacts of its methyl and vinyl groups with residues Ile41, Val209, Pro291, Leu342 (numbering for PVC) which are conserved in HPII but show marked differences to haem b containing catalases like BLC (corresponding residues Met60, Ser216, Leu298, Met349). Additional hydrogen bonding of the hydroxy group of haem d and the O-γ oxygen of Ser349 stabilises the modified prosthetic group (Murshudov et al., 1996). In BLC and MLC the equivalent position is occupied by an alanine (Ala356 in BLC). In PVC a second oxygen of haem d can form a hydrogen bond with the guanidinium group of proximal Arg357. Nonetheless, the haem group could also be incorporated in the 'normal' orientation without major problems.

In both large catalases two oxygens of haem d, O1D and OND, are close to the aromatic ring of the axial ligand tyrosine with very short interatomic distances. These polar interactions (Burley and Petsko, 1988) could change the distribution of π-electrons in the phenol group and thus influence the catalytic properties of haem d catalases (Bravo et al., 1997). This may be related to the rare formation of compound II in this subclass, actually never demonstrated in HPII. However, a similar resistance to compound II formation was also found in Aspergillus niger catalase (ANC), which obviously binds protohaem (Kikuchi-Torii et al., 1982). It was suggested that the high carbohydrate content (12%) of ANC might stabilise compound I of this enzyme (Kikuchi-Torii et al., 1992).

Recently crystals of the complex of PVC with AT, and of compound I and compound II of PMC have been prepared, allowing X-ray analysis. Resolution of the PVC–AT complex at 1.8 Å resolution confirmed earlier data about the covalent binding of this inhibitor to the essential distal histidine (W.R. Melik-Adamyan, personal communication). Obviously a hydrogen bond between the inhibitor and the coessential asparagine is formed, rather than the expected coordination with the haem-iron. The analysis of the structure of the two oxidised reaction intermediates of PMC, using time-resolved X-ray diffraction, revealed no gross conformational differences compared to the native enzyme (Gouet et al., 1996). In both compounds the haem iron is slightly above the respective position in the resting enzyme (displaced towards the distal ligand), and the ferryl-oxygen is clearly visible. In the structure of compound I (but not in compound II) binding of an anion was observed about 18 Å below the haem-iron, with so far unknown function (Jouve et al., 1997).

3.1.4. The α-helical domain

This region docks to the surface of the β-barrel domain, interacting with three helices (α3, α4 and α5) which separate the two halves of the β-barrel. It is moderately conserved among

species with a relatively high α-helical content (four contiguous α-helices, α10 to α13, within a 70-residues long area). Residues from α10 line up a major fraction of the groove harbouring the adenine moiety of bound NADPH in those species which bind this cofactor (SCC-A, BLC and PMC). The corresponding residues (Phe445, Val449, Leu450 in BLC) are not conserved among all (typical) catalases. The characteristic sequence pattern in regions responsible for interaction with NADPH divides all known typical catalases in three subgroups: those with tight NADPH-binding, those with moderate binding of the cofactor, and those which are not capable of binding the NADPH (Zámocký et al., in preparation).

Interestingly, there is distinct structural homology between this domain and the so-called Armadillo repeat region of the murine adhesive junction protein β-catenin (3BCT, Huber et al., 1997). This superhelix of helices mediates binding of cadherins and transcription factors, forming complexes which themselves are linked to the actin filament network (Hoschuetzky et al., 1994; Orsulic and Peifer, 1996). As in the case of the Armadillo motif, α-helical domains of typical catalases accumulate positively charged residues on the solvent-exposed surface. At the moment there is no indication, however, of any functional correspondence to this structural relationship. The same applies to the structural similarity between the solvent-exposed parts of the α-helical domain of BLC and the corresponding region of sperm-whale myoglobin, reported by Cockcroft and Osguthorpe (1991) based on relative-residue surface-accessibility patterns.

3.1.5. The 'flavodoxin-like' domain

The two largest members of the group of haem catalases with known structure (HPII and PVC) reveal an extra carboxy-terminal domain, called 'flavodoxin-like' domain (Melik-Adamyan et al., 1986; Bravo et al., 1995). Similar structural motifs of about 150 residues can be deduced from sequences of five additional typical catalases. In addition to this extra domain, these catalases also show extended N-terminal regions (50–70 residues long), and both extensions obviously contribute to the increased chemical and thermal stability of these large-subunit catalases. It is tempting to speculate that there is some mechanistic connection between the increased size of these large catalases and some functional peculiarities, including the conversion of haem b to d, the absence of bound NADPH and the low or even absent tendency of compound II formation (Loewen, 1997). Interestingly, members of this subgroup are found both in bacteria and fungi. This situation could be explained by horizontal gene transfer between evolutionary independent organism sharing a common habitat, but an endosymbiotic origin of peroxisomes is also discussed (Klotz et al., 1997).

In HPII this domain spans from Gly600 to Ala753. It is characterised by a high degree of well-defined secondary structure elements (Fig. 5). Four α-helices and eight β-strands can be distinguished in HPII; six of these strands form a parallel β-sheet, the last pair an antiparallel β-sheet. There are some insertions and deletions between HPII and PVC, and the antiparallel β-sheet is absent in PVC. The connection between the α-helical domain and the flavodoxin-like domain lies in the crevice between the α-helical and the β-barrel domain, exactly at the site where the adenine-moiety of NADPH binds to BLC.

The role of this extra domain remains unknown. The central part of this domain (including the parallel β-sheet) belongs to a large group of structurally related proteins, including D-glyceraldehyde 3-phosphate dehydrogenase, 3-phosphoglycerate kinase, glycogen phosporylase

b, succinyl CoA synthetase, flavodoxin, D-ribose-binding protein and Ni–Fe hydrogenase. Paradoxically, although containing a Rossmann fold (typical for nucleotide binding in other proteins) no indication of bound NADPH has been detected. The structural similarity between flavodoxin from *Clostridium MP* and the respective domain of PVC is of the same order as the similarity between the NAD-binding domains of different dehydrogenases. Therefore it seems plausible that flavodoxins and the flavodoxin-like domain of catalases diverged from a common ancestor, although convergence to a stable fold cannot be ruled out (Melik-Adamyan et al., 1986).

3.1.6. Quaternary structure of typical catalases

Typical catalases are tetramers, with molecular 222 symmetry (Eventoff et al., 1976; Vainshtein et al., 1979). In plants usually different types of monomers encoded by a small gene family are expressed. Native plant catalases can be formed from identical as well as different monomers (Ni and Trelease, 1991; Scandalios, 1994), and thus in plants generally various isoforms exist. Different isomers isolated from one plant may have nonidentical catalytic properties (Havir and McHale, 1990), and in some cases even the general protein properties differ from those of typical catalases. Hirasawa et al. (1989) reported an apparently dimeric form of spinach catalase, containing protohaem as well as a novel haem (most likely an iron-chlorin), which may be built up from monomers similar or identical to those found in the 'normal' tetrameric enzyme. A dimeric catalase (subunit size ~63 kDa) associated with PSII-membranes, but with otherwise typical properties has been isolated from the same source (Sheptovitsky and Brudvig, 1996).

Though built up from only one type of subunit, BLC and other mammalian catalases also exist in multiple forms (Heidrich and Hannig, 1968; Holmes, 1972; Aebi et al., 1974), which can be interconverted in vitro. In the case of BLC five isoforms exist; according to small angle X-ray scattering they differ with respect to packing and mobility of the polypeptide chains (Kunz et al., 1978). There are also frequent reports of dimers or higher aggregates of the native tetramer (Takeda and Samejima, 1977a,b; Miyahara and Samejima, 1981). In preparations of porcine erythrocyte catalase covalently-(disulfide-) linked dimers and tetramers of tetramers were found.

The catalase tetramer is a rather compact arrangements of subunits, revealing an extensive and complex pattern of interactions (Fig. 8). Some of these interactions virtually are incompatible with a simple way of tetramer assembly by combining four prefolded monomers (Vainshtein et al., 1986). Almost the entire N-terminal arm of each subunit is deeply buried between two neighbouring subunits, and major parts of the domain connection ('wrapping domain') are covered by residues of neighbouring subunits in the tetramer (Figs. 6 and 8). The most intriguing structural feature of the assembled protein is the pseudo-knot formed by parts of the N-terminal arm and the connecting loops of the *Q*- and *R*-related subunits. In oligomeric proteins intersubunit interfaces tend to be roughly planar (Jones and Thornton, 1995), but in a minority of cases arms (N-terminal more often than C-terminal arms) fold in a way to clasp two subunits together. This arm exchange (e.g. dogfish lactase dehydrogenase, Abad-Zapatero et al., 1987; ribulosebisphosphate carboxylase, Paul et al., 1991) or even domain swapping (e.g. diphtheria toxin, Bennett et al., 1994; Schlunegger et al., 1997) provides a significant, though flexible contribution to the stability of the oligomeric structure.

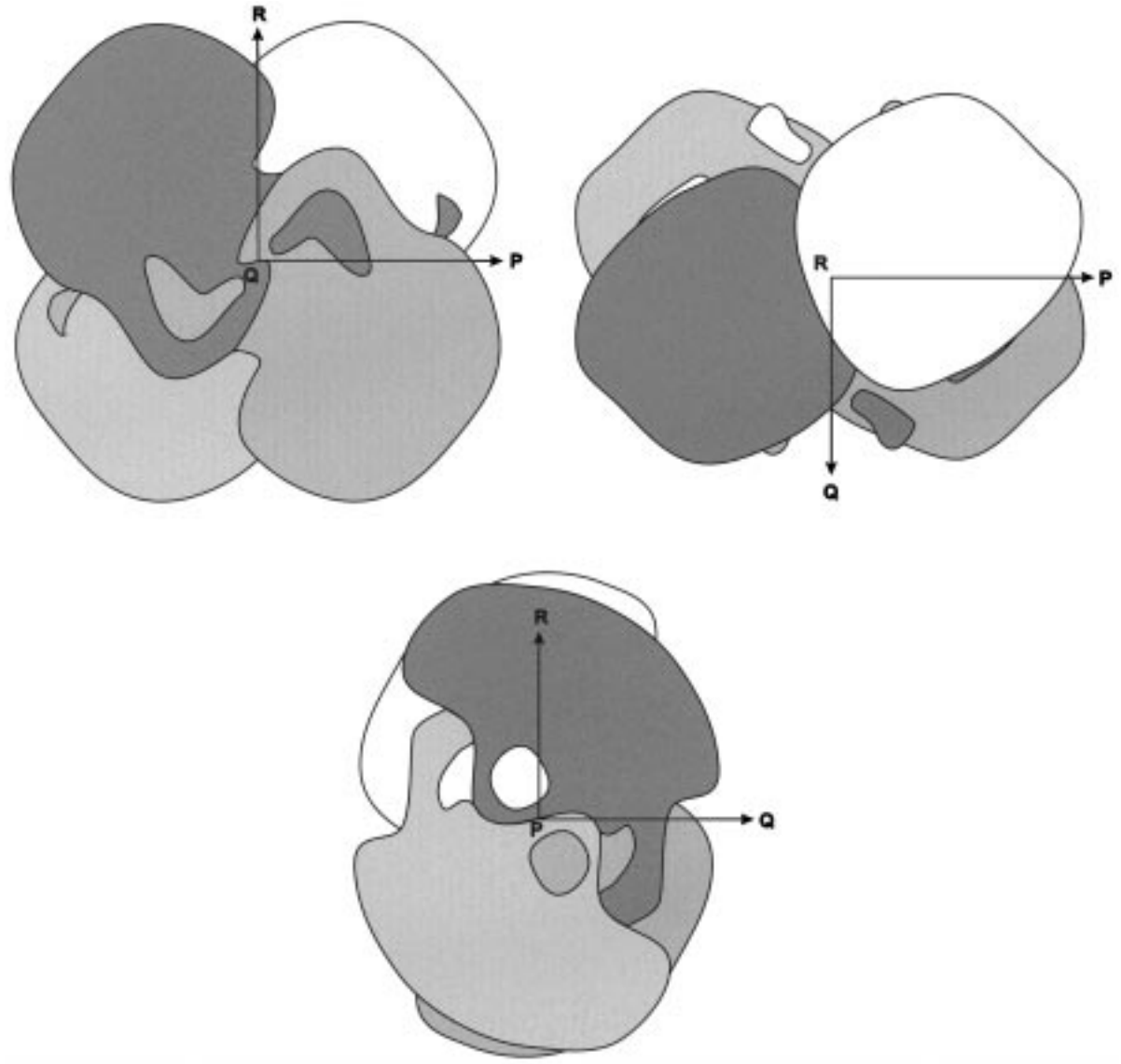

Fig. 8. Schematic presentation of a SCC-A tetramer viewed along each of the three symmetry axes. The pseudo-knot connection between pairs of subunits is clearly visible.

Sometimes this is achieved by twisting the N-terminal arms of two subunits around each other (e.g. isocitrate dehydrogenase, Hurley et al., 1989; 5-aminolaevulinate dehydratase, Erskine et al., 1997), but to our knowledge no other example of threading through narrow holes has been reported.

In PMC about 35% of all residues are involved in close ($\leq$4 Å) intersubunit contacts (Gouet et al., 1995). In PVC and HPII, the 'flavodoxin-like' C-terminal domains also contribute to intersubunit interactions across the *R*-axis, thereby stabilising the quaternary structure (Vainshtein et al., 1986). In HPII the exceptional length of the N-terminal arm further

increases the contact area between subunits (Bravo et al., 1995). Despite the large number of intersubunit contacts in the tetramer, replacement of even single residues in the respective segment of the N-terminal arm may effectively interfere with normal tetramer assembly. This obviously is the case in the Csb acatalasemia mouse mutant, bearing the Gln11His exchange (Shaffer and Preston, 1990). The same is true for two mutants of SCC-A with exchanges in helix α1 (His58Tyr) and at the 'elbow' of the N-terminal domain (His42Arg) (C. Herzog, personal communication).

The four haem groups are well hidden in the tetramer. The shortest distance of the haem irons to the protein surface is between 16 and 18 Å, and about 23 Å to the center of the molecule. The distances between pairs of haem iron atoms range from ~31 to ~46 Å. There exists, however, a complex network of hydrogen bonds linking the vicinity of *P*-related haem groups, including Asn60, Arg61, and Asp357 of the *R*-related subunit and a conserved water. This arrangement could provide the structural basis for cooperativity of the corresponding active sites, but so far there is no convincing experimental evidence for cooperativity of sites in haem catalases.

All known structures of catalases contain several cavities. The largest is located around the intersection of the molecular axes ('central cavity'), the second largest arranges parallel to the *P*-axis, but neither of them has any obvious connection to the molecular surface, so most likely they are nonfunctional. Besides the major and minor substrate (or haem) channels, which are built up by each monomer on its own, no further channels are detectable in the assembled tetramer.

3.1.7. Assembly and disassembly of native catalases

Folding and assembly of typical catalases must be a highly coordinated process to avoid formation of nonproductive intermediates. The assembly most likely follows the path: formation of holomonomers → holodimers → holotetramers. This model is based on the observation that only holotetramers are formed, even when the biosynthesis of monomers exceeds haem synthesis (Ruis, 1979), and on the formation of a single ellipsoidal dimer species upon dissociation of mammalian catalases (Tanford and Lovrien, 1962). The major challenge obviously is the insertion of a large portion of the N-terminal extension into the loop formed by the domain connection of the *Q*-related subunit. The *R*-related dimer has been suggested as viable intermediate in tetramer formation (Murthy et al., 1981), but the *Q*-axis-related dimer appears better qualified for this role, since in both other cases threading of the N-terminal arms of each subunit through the hole formed by the wrapping loops of the corresponding subunit would be required upon dimerisation of these dimers, which does not appear feasible at that assembly stage. Two sequences describing the formation of *Q*-axis-related dimers are in discussion: (i) the respective wrapping loop could dock to the β-barrel domain of the same subunit prior to insertion of the arm of the second subunit and (ii) this arm might interact first with the β-barrel domain, followed and thereby stabilised by wrapping of the domain connection around this initial dimer (I. Fita, personal communication). Irrespective of the pathway of assembly, the subunit interaction has to occur before the arm folds back to its own chain. As in several other proteins involving arm exchange, a proline (Pro65 in SCC-A, extremely well conserved in evolution) situated at the hinge of the N-terminal arm may be

essential to achieve the extended conformation required for dimerisation (Bergdoll et al., 1997). Both hypothesis are currently tested by site-directed mutagenesis in SCC-A.

Dissociation of typical catalases is usually obtained at extremes of pH (Samejima and Yang, 1963; Sund et al., 1967) and on freeze-drying (Deisseroth and Dounce, 1967; Potier et al., 1994), but there are considerable differences in the stability of the native conformation among catalases. For example, the rate of dissociation at acid pH and in SDS is much faster for BLC than for MLC (Jones et al., 1982). Additionally, thermal inactivation of MLC involves reversible dissociation into monomers (Jones and Suggett, 1968). Dissociation is also induced by succinylation (Kiselev et al., 1968) and acetylation (Furuta et al., 1974), which may also lead to rather stable dimers. In most cases, however, monomers are formed upon dissociation, usually followed by release of the haem (Tanford and Lovrien, 1962).

Dissociation, and, hence, inactivation of tetrameric catalases even occurs at otherwise physiological conditions upon severe dilution (first observed by George, 1949). For the comparatively stable enzyme from *M. luteus* K_d was estimated as 10^{-22} M^2 at pH 7.0 and 21°C (Jones and Suggett, 1968). For SCC-A and BLC this parameter is several orders of magnitude larger, and it is further increased upon release of bound NADPH (Zámocký and Koller, unpublished results). Preparations of human erythrocyte catalase usually contain small amounts of dimers and even monomers (Wiemer et al., 1992). This tendency is dramatically increased in Swiss-type acatalasemia (Aebi et al., 1976). It should be noted, however, that mammalian catalases may bind to microsomal membranes in vivo. Mouse liver catalase associates with a variety of subcellular membranes, leading to enhanced stability and enhanced catalatic activity (Pegg et al., 1989).

Under certain conditions, including treatment at very high pH (≥11.0), subunits are obtained which possess much higher peroxidatic activity than the respective native enzyme (Inada et al., 1961; Caravaca and May, 1964). This situation resembles the increased rates of 1-electron-peroxidations observed after partial digestion of catalases (Anan, 1958), a situation similar to the well-known formation of 'microperoxidase' from cytochrome *c* (Aron et al., 1986). In all these cases the haem groups become accessible for bulky 1-electron donors following the respective treatment of the parent enzyme. However, Sichak and Dounce (1986) demonstrated that the peroxidatic activity observed following alkaline-treatment of BLC is no true enzymatic activity, but rather goes back to haem groups nonspecifically attached to the partially unfolded catalase monomers.

So far renaturation of typical catalases has proven virtually impossible. Samejima and Yang (1963) reported up to 50% recovery of catalytic activity of acid-denatured BLC upon rapid dilution, but the yield of correct reassembly very rapidly decreased with prolonged times of denaturation. Molecular chaperones like α-crystallin, which significantly protects catalase against thermal inactivation (Hook and Harding, 1997) and GroEL/ES slightly increase the yield of renaturation of SCC-A, but the problem of in vitro folding and assembly of tetrameric catalases still remains unsolved (Zámocký and Koller, 1997).

3.2. Catalase peroxidases

At the moment high-resolution crystal structures are not available for this group. Due to the pronounced sequence homology with other Class I peroxidases (yeast cytochrome *c* peroxidase

(CcP) and plant ascorbate peroxidases (APX)) catalase-peroxidases can be modelled into these known structures. One can safely predict that at least the N-terminal half of catalase-peroxidase monomers will have the same overall helical fold as CcP and APX. For some members of this group this may be true also for the C-terminal half of each polypeptide. The general arrangement of these two halves, and of the two or more subunits remains to be determined. The only information available comes from small-angle X-ray scattering which has been undertaken with the enzyme from *M. tuberculosis* (Nagy et al., 1997b). This protein appears to assemble as a dimer, with head-to-head topology. More detailed data about different catalase-peroxidases are required to elucidate whether some peculiarities in the primary structures, or the differences in tertiary and quaternary structure are responsible for the marked differences in catalytic behaviour between catalase-peroxidases and monofunctional peroxidases.

3.3. Manganese catalases

Little information is available about this catalase subgroup, since only three members have been characterised to some degree. Subunits with molecular weights around 30 kDa are organised as tetramers or hexamers, and are remarkably stable at high temperatures (Allgood and Perry, 1986). The crystal structure of the enzyme from *T. thermophilus* has been resolved at moderate resolution (Barynin et al., 1986). The enzyme is a hexamer with 32 molecular symmetry, revealing an all-α-fold (seven helices per subunit). The central α4-core structure, which contains the di-manganese reaction center, reflects structural relationship with haemerythrin, cytochrome c', cytochrome b_{562}, apoferrtin and the coat protein of TMV. Interestingly, the structure of these active sites is similar to half of the tetranuclear manganese complexes of the oxygen-evolving center (OEC) of photosystem II (Ananyev and Dismukes, 1996). Since these centers evolve O_2 from H_2O_2 in the dark (Frasch and Mei, 1987), one may speculate that manganese catalases and manganese OEC's could have a common ancestor (Blankenship and Hartman, 1998).

4. Significance of the main substrate channel for the reaction profile of typical catalases

In aerobic organisms, catalases have evolved to a characteristic group of enzymes which very efficiently decompose toxic peroxides to prevent their accumulation in the cell. With very few exceptions they harbor haem in their reaction centers. There is a close relationship to peroxidases, another large family of haem proteins, both with respect to function and their different reaction cycles. On the other hand, the relative reaction rates (catalatic reaction versus peroxidatic reaction) dramatically differ between true catalases, catalase-peroxidases and plant peroxidases. Unlike compound I of catalases, the corresponding intermediate of peroxidases reacts preferentially in two subsequent 1-electron oxidation steps via compound II (Schonbaum, 1982). Peroxidases are at least four orders of magnitude less effective in 2-electron reduction of compound I (catalatic reaction mode and one step peroxidation, Claiborne et al., 1979).

Several hypotheses have been raised to rationalise the characteristic reactivity of catalases. Important differences with respect to the distal and proximal haem ligands and their reactivity, which distinguish the two major classes of hydroperoxidases, were mentioned in Section 3. Here we focus on a hypothesis, which offers an explanation for the very low efficiency of typical catalases in 1-electron peroxidations (Srivastava and Ansari, 1980). According to this concept, sterical constraints should play the major role in the decreased turnover of large peroxides and substrates like phenols and aromatic amines by catalases. In typical catalases, substrates have to enter the active site via the main substrate channel, which in its lowest sections is very narrow and hydrophobic, therefore allowing only diffusion of small molecules with low polarity. Thus, the rate of 1-electron reduction of catalase compound I should be limited by the very low rate of diffusion of bulky organic substrates through the lower half of the entrance channel. This is in marked contrast with the situation in peroxidases (and catalase-peroxidases as well), with reaction centres in close proximity to the molecular surface. In the majority of haem peroxidases the distal haempocket is easily accessible for all potential substrates (Jespersen et al., 1997). The long, narrow and hydrophobic channel of typical catalases presumably not only prevents ready access to the active site for bulky substrates, but may also line up several molecules of H_2O_2 to form a molecular chain, thus contributing to the extremely fast turnover (I. Fita, personal communication).

4.1. Cavities and channels in proteins

In the last few years a wealth of structural data has accumulated, which clearly demonstrates the importance of cavities and channel-like structures for the function of a broad spectrum of proteins. In terms of function the large group of membrane proteins forming solvent-filled and ion channels in e.g. porins (Schulz, 1996), hydrophilic and charged funnels (e.g. canavalin, Ko et al., 1993) and central cavities in multisubunit assemblies (e.g. proteasomes; Lowe et al., 1995), are not related with the major substrate channel of catalases, and therefore will not be discussed in this review. Still, a handful of proteins exist with a similar architecture, positioning the active site at the end of a diffusion channel or cylinder-like structure. In two cases we can find a deeply buried active site at the bottom of a narrow channel: methylmalonyl CoA mutase (Thoma and Leadlay, 1996) and L-arginine:glycine amidinotransferase (Humm et al., 1997). Furthermore, a 'substrate access channel' exists in cytochrome P450 (Poulos et al., 1986; Raag et al., 1993), and for prostaglandin H2 synthase-1 access to the cyclooxygenase active site is attained via a rather long channel with a wide mouth and a narrow gate on the way (Picot et al., 1994).

However, the very long and narrow channel forming a connection between the molecular surface and the prosthetic haem group appears to be a unique feature of typical catalases. The central and lower parts of the main substrate channel are very highly conserved in this enzyme subgroup, pointing to its significance for proper catalytic function. Site-directed mutagenesis of the gene coding for catalase A, discussed later in this chapter, produced catalases with altered structure of the channel, relieving sterical constraints for larger substrates. Comparison of the engineered enzymes with wild-type SCC-A has demonstrated the importance of this characteristic structure for the stability and reaction specificity of the whole enzyme.

4.2. The structure of the main substrate channel

The main substrate channel is funnel shaped, with a large cross-section at the molecular surface and a narrow entrance to the haem cavity (Fita and Rossmann, 1985b). In SCC-A it is 26 Å long, having a large entrance of 17 Å diameter which closes to 5.5 to 4.5 Å in the middle and lower parts. PMC exhibits similar parameters (length 30 Å, narrow parts down to 5 Å, Gouet et al., 1995). The channel can be divided in five sections, which contain residues of different polarity, the general pattern being well conserved among species.

Whereas the shape of the main substrate channel itself is almost identical, the situation at its opening to the solvent is rather distinct in catalases from different sources. The opening is lined by residues from helices of the β-barrel domain and of the helical domain. Access to this opening is best in PMC and SCC-A, which lack helix α1 and thus have rather short N-terminal arms. In BLC, and even more pronounced so, in HPII, the markedly longer N-terminal

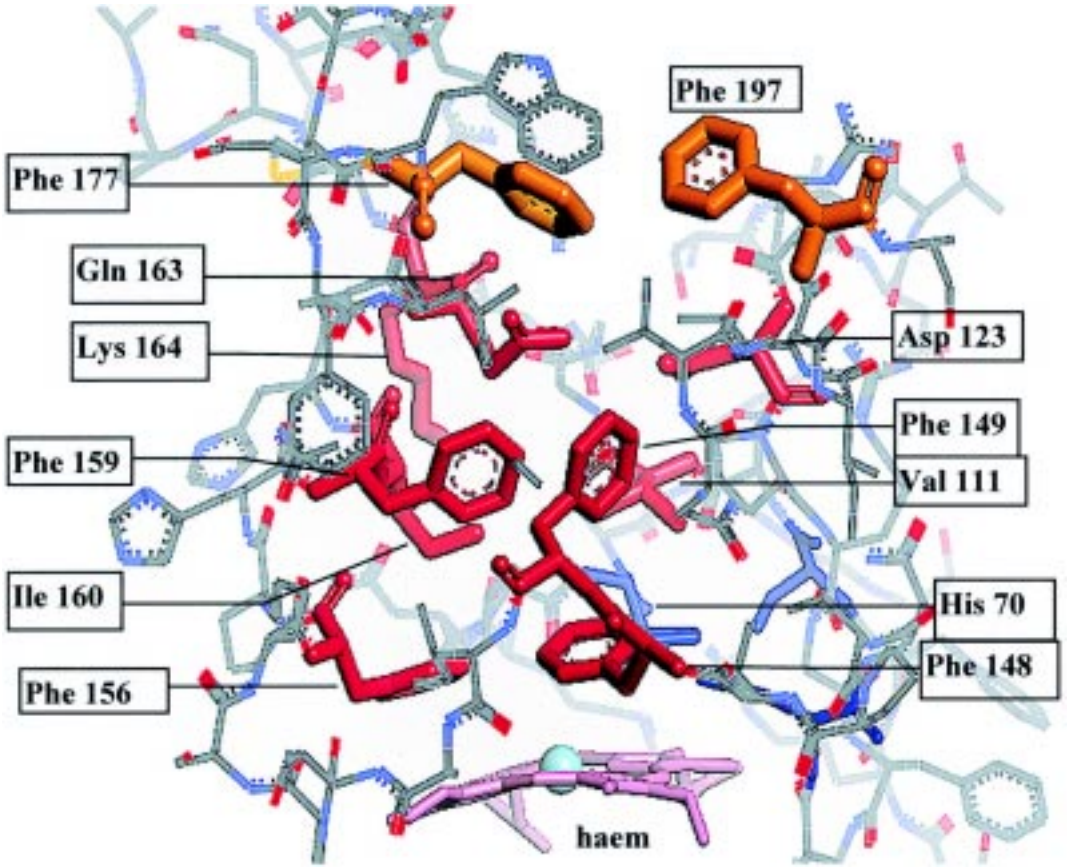

Fig. 9. Three dimensional representation of the fourth and fifth section of the main substrate channel in SCC-A. Catalytically important residues are coloured in blue, and bulky residues forming the walls of the fifth (lowest) section of the channel in red. Two bulky residues from the fourth section of this channel (Phe177, Phe197) are coloured in orange.

Table 4
Overview of the enzymatic activity of wild type catalase A from *Saccharomyces cerevisiae* and various engineered variants

Catalase Species	Catalatic activity[a]	1-Electron peroxidation[b] with ABTS[d]	2-Electron peroxidation[b] with ethanol	2-Electron peroxidation[b] with 1-propanol
Wild type	89,750	1	6330	100
V111A	23,650	46	3680	1610
V111A + F95L	8300	26	1400	380
F148V	10[c]	0	0	0
F149V + P184S	36,170	18	2350	30
F156V	970	11	930	1390
F159V	26,000	4	3040	60
I160A	20,370	15	180	n.d.

[a] In U/mg of purified protein.[b]In mU/mg of purified protein.[c]Yeast strain grown at 22°C.[d]2,2′-azino-bis-(3-ethyl-benzthiazoline-6-sulphonic acid).

portions of the *R*-related subunit partly block the lateral entrance to the upper channel section. However, no significant difference in the rate of 1-electron peroxidations was detected between these 4 catalases. Obviously the 'upper substrate groove' present in PMC and SCC-A has no influence on the diffusion of larger substrates to the prosthetic haem group (Gouet et al., 1995). In HPII, a major fraction of the channel opening is shielded by the C-terminal domain of the *R*-related subunit. Presumably this contributes to the lower overall rate of hydrogen peroxide dismutation (15% of the rate for BLC) and the outstanding low rate of ethanol peroxidation (less than 1% of BLC) in this species (Obinger et al., 1997a). The interior part of the channel (sections two to five) are deeply buried in the globule of each subunit and the sequential and structural conservation among species increases towards the lower parts. The second section is rather polar, with four water molecules located at this level in BLC. The third segment consists mainly of hydrophobic residues. The two *R*-related subunits are interconnected at this level which permits the *R*-dimer to share substrates between two active centers. The fourth section presents an entrance to the haem cavity and bulky apolar residues

Table 5
Rate constants for 1- and 2-electron peroxidations of wild type yeast catalase A, variants with single-residue replacements in the area of the major substrate channel, and of bovine liver catalase (from Zámocký, 1995)

Catalase Species	k_4 for 1-propanol	k_4 for 1-butanol	K_M for ABTS (μM)	k_{cat} for ABTS (s^{-1})	k_7 for ABTS ($mol^{-1}\ dm^3\ s^{-1}$)
SCC-A wild type	2.00	0.15	41.4	2.88×10^{-3}	69.5
F 149 V + P184S	0.68	0.17	56.6	1.59×10^{-2}	310.2
F 159 V	0.10	–	20.3	9.81×10^{-5}	4.9
V 111 A	7.48	4.73	144.6	1.01×10^{-1}	800.2
F 156 V	–	–	31.4	7.18×10^{-3}	2682.3
BLC	5.68	0.32	170.3	8.54×10^{-2}	669.6

(Phe177 and Phe197 for SCC-A, conserved in 60% of known sequences) present serious sterical constraints for large substrates. The final (fifth) section is lined up by bulky apolar amino acid residues, forming a narrow neck (Fig. 9). The minimum diameter of the neck restricts passage to molecules with van der Waals diameter not exceeding 3.5 Å (Fita and Rossmann, 1985b). This steric restriction probably also helps to preorient the substrate by interactions of its hydrophobic parts with bulky aromatic and aliphatic residues. Therefore, the peroxide (polar) part of organic hydroperoxides enters the haem cavity first. Experiments with substituted alcohols and the isotope effect on alcohol peroxidation support the view that the substrate has to adopt a correct orientation for successful peroxidation (Schonbaum and Chance, 1976). Substrates have to move around Val111 which covers the essential distal histidine before reacting. Landing on the haem iron is also restricted by residues Phe148, Phe149, Phe156, Phe159 and Ile160. Although the detailed mode of reaction remains to be elucidated, the known kinetic parameters of the reaction with small peroxides and aliphatic alcohols support this opinion about the significance of the channel structure on the reaction specificity and high turnover number of typical catalases.

4.3. Function of the main channel studied by site-directed mutagenesis

Site-directed mutagenesis certainly is one of the most powerful tools to evaluate the relative importance of individual residues for the reaction mechanism of a particular enzyme. In the region of the major substrate channel several single or double replacement mutants have been reported, mainly from yeast peroxisomal catalase A. All mutants bear single amino acid exchanges in the lower parts of the main channel (Nykyri et al., 1993; Zámocký et al., 1995). The crystal structure of one of them (Val111Ala) has been resolved (Mate et al., in preparation). The enzymatic properties of these single-exchange mutants are summarised in Table 4. In general, the catalatic activity was slightly or moderately decreased, but 1- and 2-electron peroxidations were significantly increased, up to about 50-fold. A more detailed

Table 6
Influence of bound NADPH on the unfolding stability of engineered yeast catalases. Presented are midpoint values (in M of denaturant) for the unfolding with urea at pH 7.5 (from Zámocký and Koller, 1997)

Catalase species	Catalatic activity	Haeme exposure to solvent $A_{406}/A_{365\ nm}$	Trp fluorescence $I_{340\ nm}$
Wild type – NADPH	1.05	n.d.	1.2
Wild type + NADPH	1.2	1.5	1.1
F149V – NADPH	1.15	0.95	1.05 3.5
F149V + NADPH	0.85	0.75	0.85
F149W – NADPH	0.7	n.d.	0.7 2.5
V111A – NADPH	1.9	n.d.	1.7
V111A + NADPH	0.65	0.6	0.65 2.2
I160A – NADPH	1.3	n.d.	n.d.

analysis of the modified reactivity of selected species is possible with data summarised in Table 5, showing rate constants of single individual reaction steps. Almost any point mutation planned to make the region of substrate binding more accessible for large substrates led to an increase in both 2-electron peroxidations of aliphatic alcohols (with longer chains) and aromatic 1-electron donors. The ratio of these two respective activities varied among mutants. The overall stability of engineered catalases was only moderately affected (Table 6).

Replacement of Phe148 with a smaller residue led to completely inactive proteins with virtually no haem incorporated. In all catalases from prokaryotes to higher eukaryotes, a phenylalanine is found at this position; the orientation of its side chain is parallel to the haem macrocycle, at a distance of about 7 Å. These intensive van der Waals interactions obviously strongly contribute to haem binding in the course of holoenzyme assembly. Therefore it seems to be essential in this position, its replacement with valine causing destabilisation and, eventually, misfolding.

The side-chain of Phe156 lies in an almost perfectly parallel plane about 3.5 Å above the pyrrole ring I of the porphyrin, allowing strong π–π interaction with the prosthetic group and van der Waals interactions with the haem vinyl and methyl groups. Though a phenylalanine is strictly conserved at this position, it can be replaced with a similar, still bulky and hydrophobic residue without complete loss of function. The replacement by valine retains a fraction ($>10\%$) of the catalatic activity, but the engineered protein exhibits severe deficiency in haem binding and reduced stability against unfolding. The rate of both, 1- and 2-electron peroxidations is increased, indicating that a smaller residue in this position produces more space and may have an influence on the orientation of diffusing substrates.

Val111 is strictly conserved among all sequenced typical catalases, and thus appears to be an absolute requirement for this enzyme class. The exchange by a similar, but smaller residue (V111A) facilitates the passage of large substrates through the channel immediately above the essential histidine. In the mutant enzyme, the distal pocket appears solvent-accessible without serious restriction in any part of the channel (Mate et al., in preparation). This replacement may also affect the interaction of substrates with the essential histidine (70), and the cooperation between His70 and the coessential asparagine (143).

A phenylalanine at position 149 is highly conserved; in about 4% of all sequences it is replaced by a tyrosine or proline. The catalytic reactivity changed significantly upon replacement of Phe149 by valine. The rate of 2-electron peroxidations with aliphatic alcohols larger than ethanol was slightly increased, and a pronounced enhancement of 1-electron peroxidatic activity in comparison to wild type was observed. Detailed kinetic analysis revealed major effects of this substitution on the reactivity of compound I. Replacement with a tryptophane slightly destabilised the protein, and significantly decreased the rate of any 2-electron transfer reactions. On the other hand, the 1-electron peroxidatic activity far exceeded that of the wild type enzyme. One might speculate that in compound I of this mutant the unpaired electron is localised in this side chain quite close to the porphyrin macrocycle, but this remains to be confirmed by biophysical examination.

The Phe159Val-mutant enzyme very closely resembles the wild type protein. Its capability to catalyse 1-electron peroxidations (with ABTS) is identical with that of the wild type, and the reactivity towards hydrogen peroxide and aliphatic alcohols is only slightly reduced (Zámocký, 1995). It can be concluded that residues lining up the channel at this position can be replaced

with similar, smaller ones without significantly affecting the properties and reactivity of compoud I. In fact, similar residues (leucine, valine, methionine) are found at this position in several catalases, mostly from plants.

Position 160 is only moderately conserved in catalases from different species, but invariably is occupied by residues with bulky aliphatic side-chains (isoleucine in 49% of all cases, valine in 40%). Exchange by alanine (never occurring in nature) reveals slightly decreased catalatic activity, but a significant increase in 1-electron peroxidations and spectral and kinetic characteristics similar to the mutant Phe156Val. According to Rueffer et al. (1995), isoleucine in this position will seriously interfere with binding of salicylic acid to the haem iron, whereas valine, which is found in this position in the majority of plant catalases, can relieve this steric constraint in the proposed mode of salicylic acid binding.

Analysis of these effects of site-directed mutagenesis allows a validation of the theory about the significance of substrate diffusion for the reactivity of typical catalases, addressed at the beginning of this chapter. Obviously, sterical factors contribute to the very low 1-electron peroxidatic capability of typical catalases, virtually prohibiting direct access of aromatic substrates to the haem pocket. One crucial question which remains to be unambiguously answered is whether in the mutant species described above bulky single-electron donors (e.g. ABTS) really have access to the haem cavity. From competition experiments this may be the case in SCC-A Phe149Val, whereas in the wild type enzyme binding of these substrates appears restricted to the enzyme surface (F. Koller, unpublished results). These results require confirmation by e.g. cocrystallisation of this enzyme species with ABTS or guaiacol, which currently is under way.

Several single-site replacements affected the rates of peroxidation of 1-electron donors and of rather large 2-electron donors, respectively, in different ways. Obviously, the increased peroxidatic activity of SCC-A after replacement of bulky residues cannot be explained exclusively by steric factors, but also in terms of the destabilisation of compound I in the respective 'channel' mutants (Zámocký et al., 1995). Gouet et al. (1995) suggested that the cluster of highly conserved phenylalanine residues in the distal haem pocket may stabilise the π-cationic porphyryl radical in compound I by induction of a corresponding negative charge delocalised at the surface of this cluster (Süssman and Silman, 1992). Reduction of the size of this cluster by replacements with nonaromatic side chains then might enhance the readiness of compound I to accept single electrons. By applying rapid-mix freeze-quench techniques, Ivancich et al. (1997) recently showed that the reaction of BLC with peroxyacetic acid leads to a short-lived porphyryl radical, followed by a tyrosyl radical. While this transition is too slow to play any role in the catalatic reaction cycle, it may be related to the postulated protein radical intermediate compound II* (Hillar et al., 1994), a normal decay product of compound I under appropriate conditions. Even subtle changes in the haem vicinity may markedly affect the half-life of the porphyryl radical, and thus lead to a more pronounced peroxidatic reaction profile. This may also explain why the performed mutations of catalase A will increase the reactivity of compound I, but eventually lead to enhanced autooxidation of this enzyme (Zámocký et al., 1997b).

5. Role of NADPH in true catalases

An unexpected result was obtained when a search for proteins that bind NADPH in human erythrocytes was carried out in the course of studies on the regulation of the pentose phosphate pathway. The major reservoir of bound NADPH in these cells is catalase (Kirkman and Gaetani, 1984). With this information in hand, an area of previously uninterpreted electron density in the refined crystal structure of BLC (Murthy et al., 1981) could be ascribed to NADPH (Fita and Rossmann, 1985a). This extradensity was originally interpreted in terms of a hexapeptide (corresponding to peptides Th10 and Th11 frequently found in preparations of bovine erythrocyte catalase, Schroeder et al., 1982). This peptide, showing high homology with the corresponding parts of other mammalian catalases, obviously is cleaved off posttranslationally in BLC (and perhaps in all mammalian catalases), but it still may bind to empty NADPH binding sites. At the moment there is no obvious function for this presumptive interaction. Interestingly, this extra density also closely corresponds to the region connecting the extra C-terminal domain with the α-helical domain in PVC (Melik-Adamyan et al., 1986). Since that time the presence of nicotinamide adenine dinucleotide was also confirmed in PMC, MLC, SCC-A and *S. cerevisiae* catalase T (SCC-T) (Jouve et al., 1989; Murshudov et al., 1992; Hillar et al., 1994; Herzog et al., 1997). It can be concluded from sequence homologies that the majority of typical catalases in fact will bind this cofactor. Whether this also applies to plant catalases still remains open: catalase from potato was demonstrated to be NADPH-free (Beaumont et al., 1990), and most plant catalases are highly homologous to this species (in fact they represent an own evolutionary subgroup, Guan and Scandalios, 1996; Zámocký et al., 1997c). On the other hand, tobacco catalase obviously binds NADPH (Durner and Klessig, 1996), though binding appears less tight than in mammalian catalases. Most information about the occurrence and role of NADPH in typical catalases comes from studies on BLC, PMC, and more recently also on catalase A from the yeast *S. cerevisiae*. Due to the rather late discovery of NADPH its presumptive function is a matter of lively discussion.

5.1. Mode of NADPH-binding in typical catalases

The binding strength of NADPH varies considerably between different typical catalases. Tightest binding is reported for BLC, human erythrocyte catalase, and PMC. Whereas in these cases K_d is 10 nM or smaller (Kirkman et al., 1987), K_d for SCC-A was determined as 1.99 μM (Herzog et al., 1997). Accordingly, only partial occupancy of the respective sites is obtained in the crystal structure of this species (Mate et al., in preparation). A similar situation was found in the case of MLC (Murshudov et al., 1992), so only moderate strength of dinucleotide binding can be concluded for this enzyme too. It was also demonstrated that the strength of binding decreases in the order $NADPH > NADH \gg NADP^+ > NAD^+$ (Kirkman and Gaetani, 1984; Jouve et al., 1986).

Surprisingly, all attempts have failed to detect NADPH in the largest catalases (like HPII and PVC) containing the extra C-terminal domain, even though the βαβ Rossmann fold, which usually is characteristic for nucleotide-binding, forms part of this domain (Melik-Adamyan et al., 1986). In fact, the NADPH binding cavity of catalases is close to the enzyme surface, formed by residues from the β-barrel and the α-helical domain, and this part of the molecule is

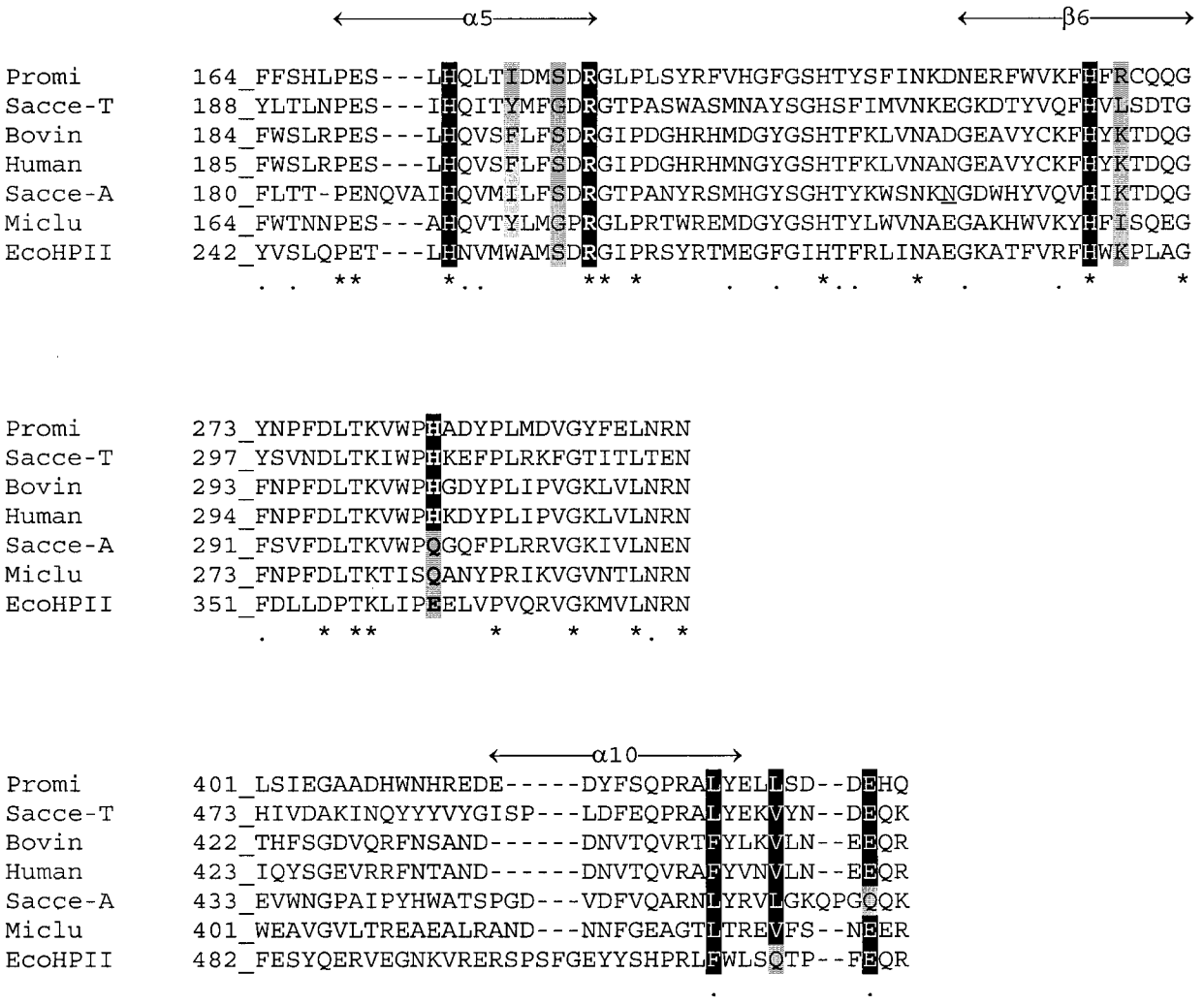

Fig. 10. Multiple sequence alignment of various typical catalases in sections responsible for the interaction with NADPH. *Proteus mirabilis-PR*, beef liver and human catalases are known to tightly bind this dinucleotide. According to sequence homology, also catalase T from *Saccharomyces cerevisiae* is expected to belong to this subgroup, whereas *Micrococcus luteus* catalase and catalase A from *S. cerevisiae* reveal only moderate NADPH binding. In contrast, HPII from *Escherichia coli* is unable to bind NADPH. All sequences and their abbreviations are from Table 1.

covered by the extra domain in both large-subunit catalases with resolved crystal structure. This is in agreement with the hypothesis of Schulz (1992), that nucleotide binding requires a particular local environment rather than a specific protein fold. The most important residues involved in binding are located on helices α5 and α10 of BLC. The bases of the cofactor are situated close to these helices, while the phosphates are farthest away from them. In contrast to nearly all other known structures of protein-bound dinucleotides, in BLC NADPH is folded into a right-handed helix (Fita and Rossmann, 1985a). The nicotinamide ring is roughly parallel to the adenine ribose with a closest approach of 3.6 Å.

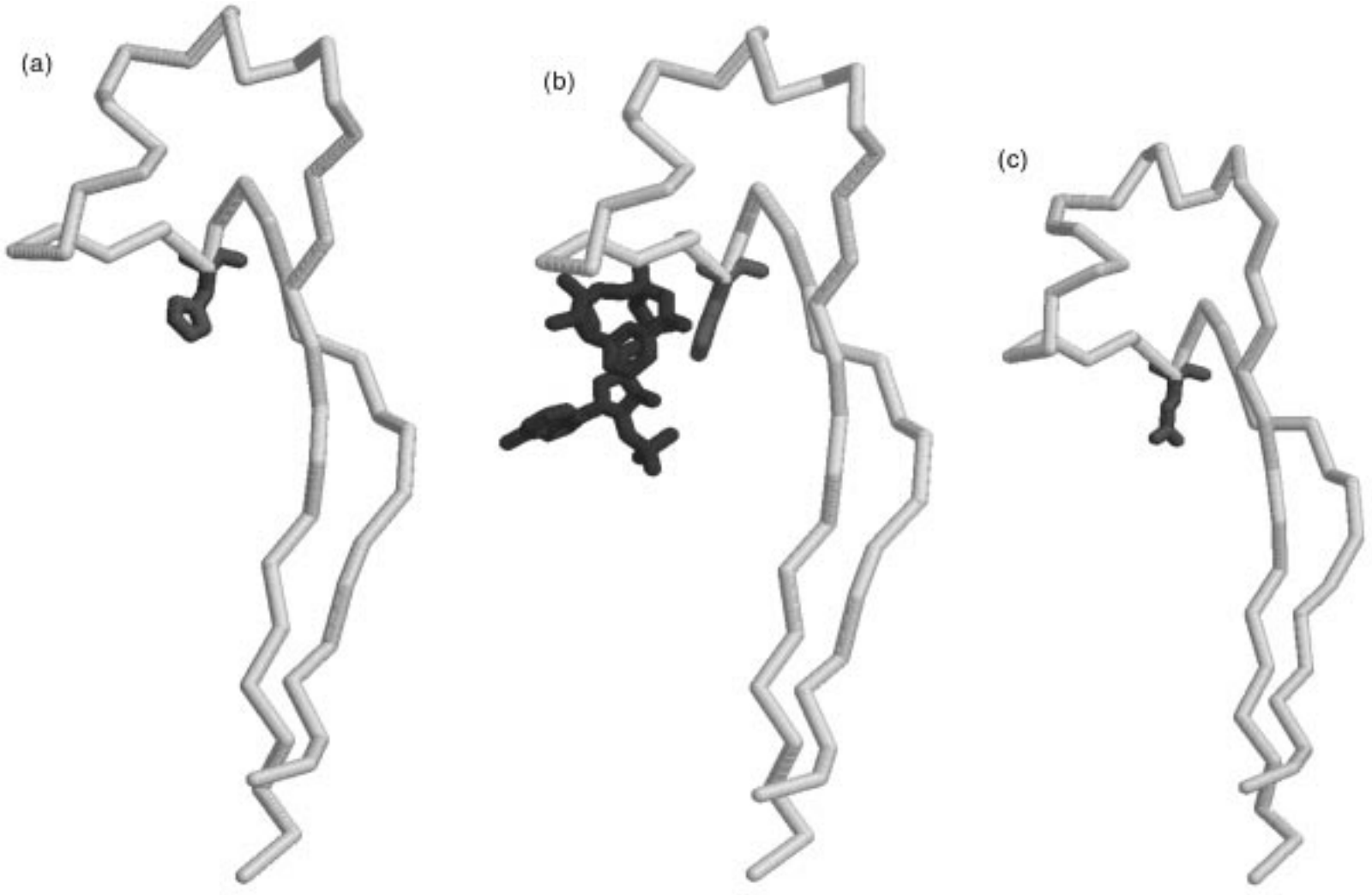

Fig. 11. Structural comparison of the loop connecting the strands β7 and β8 in (from left to right) PMC without NADPH, PMC with bound NADPH and SCC-A. The cofactor is coloured in dark grey. The sidechains of His284 (PMC) and Gln302 (SCC-A), respectively, involved in interactions with C4N of NADPH, are shown in light grey. The reorientation of the imidazole group of His284 upon binding of NADPH to PMC is clearly visible.

The sequence alignment of various NADPH-binding catalases in the above-mentioned areas supposed to interact with the dinucleotide is outlined in Fig. 10. Obviously, residues Phe197, Ser200, Arg202, Lys236 (numbering refers to BLC) are highly conserved. On the other hand, the positions Phe445, Val449 are only moderately conserved, but in NADPH-containing catalases conservative replacements are found (Phe to Leu, or Val to Leu). For Lys236 NADPH has to be present to achieve a charge balance in its environment. This lysine (together with Arg202 and Asp212) directly interacts with the O2′ of the cofactor. These three residues are considered responsible for the fact that in catalases NADP(H) binds better than NAD(H), and that the uncharged reduced forms are held much stronger than the charged oxidised cofactors. Asp212 has a different counterpart in PMC (His192), what accounts for a distinct orientation of the 2′ phosphate group (Gouet et al., 1995).

Several mutants of SCC-A have been produced to study the mode of NADPH-binding in this enzyme (Zámocký et al., 1997a). From homology modelling Arg200 (in the structure of PMC Arg182) is directly involved in binding of the adenine moiety of NADPH. Introducing the exchanges Arg200Asp and Arg200Asn into SCC-A lead to almost completely abolished NADPH binding, concomitant with a dramatic decrease of the stability and a very low catalatic activity. Gradual inactivation was observed during the isolation of Arg200Asp, and the isolated protein revealed severe haem deficiency. Similar results were obtained with the variant Arg200Asn. In BLC the side chain of Phe197 strongly contributes to the hydrophobic interaction of the protein with the adenine moiety of NADPH. The corresponding residue in SCC-A is Ile195. Surprisingly, the unique insertion of three residues in helix α5 (Gln187–Ala189, cf. Fig. 10) in SCC-A has no effect on the orientation of Ile195 and its interaction with the adenine moiety of the cofactor. Amino acid exchanges in this area, in particular of residue Ile195, are currently prepared and should assess its contribution to dinucleotide binding.

Another important residue is His304 (in BLC), with its side chain situated near C4 of the cofactor. It is supposed to interact strongly with the pyrophosphate group and the carboxyamide moiety of the nicotinamide. A water molecule occupies a pseudosubstrate site and performs hydrogen bond to this histidine, therefore influencing its polarity. This position may be crucial for the over-all binding strength, and the respective residue found in this position could allow to predict whether NADPH would tightly bind to a particular catalase or not. This position is occupied by glutamate in those enzymes not binding NADPH (e.g. PVC, HPII and numerous plant catalases). In catalases known for strong cofactor interaction always histidine is found (PMC, BLC, *Candida tropicalis* catalase), and a glutamine in weakly binding species like SCC-A. A glutamine is also found in MLC (Gln286; Murshudov et al., 1992), for which the 3-D structure at 1.5 Å resolution exists. An extra electron density was observed in the position corresponding to NADPH in BLC, but upon detailed analysis it appeared difficult to ascribe it to the dinucleotide. The situation is best interpreted by only partial occupancy of the NADPH sites, a situation very similar to that found in SCC-A (Mate et al., in preparation). Unfortunately no biochemical data about NADPH binding in MLC are available. In PMC the corresponding residue (His284) forms part of the loop between the strands β7 and β8, as illustrated in Fig. 11. The entire loop structure appears different in SCC-A and PMC. This can be a major reason for the rather weak interaction of NADPH with catalase A. Residues Leu449 and Leu453 from the α-helical domain show nonelectrostatic interactions with the adenine moiety of the cofactor.

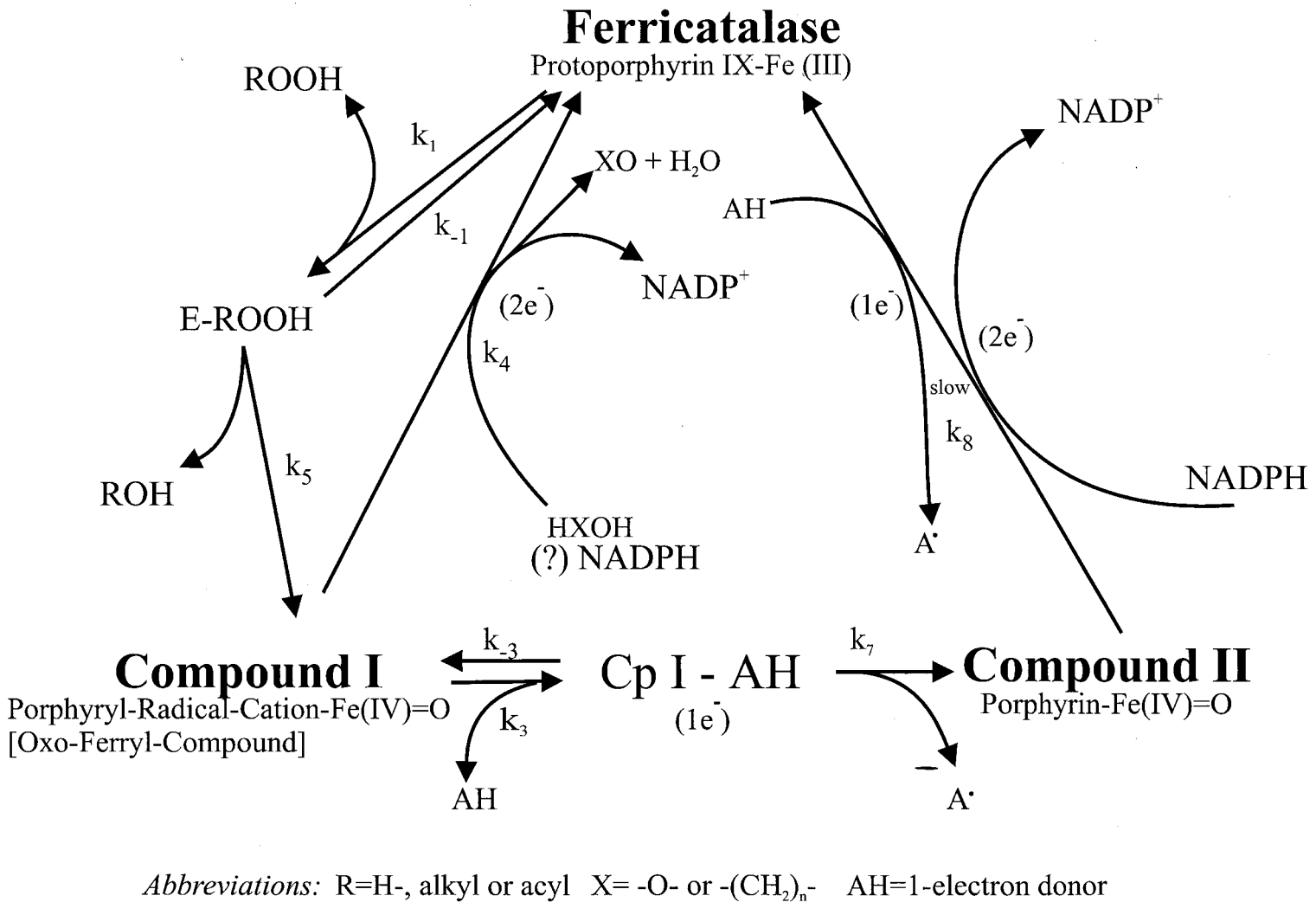

Fig. 12. Reaction scheme for typical catalases including the formation of intermediates (compound I and II) and the presumptive involvement of NADPH in the regeneration of the active ferricatalase form.

In PMC the contribution of the three most important residues (Arg182, His284 and Leu428) to the interaction with the dinucleotide was demonstrated by comparison of the structures obtained with and without the cofactor. Noticeable displacements of the above-mentioned residues were observed in the respective structures of PMC (Gouet et al., 1995).

5.2. Effects of NADPH on the reaction mechanism of catalase

The minimal distance between the haem group and NADPH is 13–14 Å in monomers of BLC and PMC (measured between C4N of the nicotinamide ring and the vinyl methyl group of pyrrole ring II). The transfer of electrons from NADPH to the haem is necessary to explain the main function of the nucleotide cofactor for the regeneration of the active enzyme, discussed below. However, electron transfer between groups separated more than 10 Å, with only weak electronic interaction between the redox sites, is unusual in proteins (Mayo et al., 1986). However, application of the Franck–Condon principle led to the development of the theory of electron-tunnelling (Marcus and Sutin, 1985; Canters and van de Kamp, 1992; Beratan et al., 1992), according to which electrons can flow from a donor to an acceptor through several jumps between the orbitals of the separating atoms. Jumps through covalently or hydrogen-bonded atoms are more likely than between nonbonded atoms. From the 3-D structures four possible paths were calculated from NADPH to the haem for BLC (Almarrson et al., 1993) and PMC (Bicout et al., 1995). The two with the best score lead through Ser196 (PMC), which can exist as seryl radical during electron tunnelling. This highly conserved residue actively supports the protective function of NADPH, which can thereby rescue the active ferricatalase at conditions otherwise favouring inactivation of the enzyme. The inactive form compound II (with Fe^{4+}), derived from compound I via 1-electron reduction, cannot be further reduced by 2-electron substrates like H_2O_2 or alcohols (Fig. 12). In vivo compound II may accumulate at very low concentrations of hydrogen peroxide. Its formation may present serious problems in vitro, which has to be circumvented e.g. by an extensive supply of NADPH, otherwise the inactivation of this enzyme limits all possible practical applications.

5.3. The proposed functions of NADPH

Under steady supply of hydrogen peroxide, compound II of BLC and human erythrocyte catalase disappear upon equimolar addition of NADPH (Kirkman et al., 1987). Cattani and Ferri (1994) demonstrated that NADPH-equipped BLC exhibited increased dismutation of peracetic acid. Although the involvement of NADPH in the 2-electron reduction of compound I to ferricatalase cannot be ruled out from all available experiments, most data support the hypothesis of Kirkman et al. (1987), that NADPH both prevents and reverses the accumulation of compound II.

Two hypothesis for the mechanism of the reaction of NADPH with compound II were proposed. According to the first one, a single-electron is transfered from NADPH to the haem group of compound II, reducing it to the resting ferric enzyme state (Kirkman et al., 1987). However, this would be a unique type of reaction for NADPH which in vivo is only known as 2-electron donor. Almarrson et al. (1993) tried to overcome this inconsistency by proposing a one-electron oxidation mechanism with the sequence NADPH/$NADPH^{\cdot +}$ (cation-radical)/

$NADP^{\cdot}/NADP^{+}$, with His234 functioning as general-base catalyst. The authors argued that one electron from NADPH is delivered to the haem iron, whereas the second electron is dissipated by the reaction with an exogenous oxidant such as dioxygen.

The second possible explanation suggests a 'normal' 2-electron mechanism of NADPH oxidation (Hillar and Nicholls, 1992; Hillar et al., 1994). This mechanism involves a protein radical intermediate named compound II^{*}, formed by electron transfer to the porphyrin group. The bound NADPH then actually reacts with compound II^{*}, a precursor of compound II, where the oxidising equivalent removed from the compound I radical is still located close to the haem. One of the two electrons transferred by NADPH serves to regenerate the radical residue to its neutral state, while the second one is transferred to the haem iron, reducing it to the resting ferric state. After a search in strand β5, forming a wall between the haem group and the site of NADPH binding, compound II^{*} was proposed to hold a tyrosyl radical, most probably Tyr214 in BLC (Hillar et al., 1994) which could accept both electrons from NADPH, transfering one to the prosthetic haem group. Similar radical migration has been described for both myoglobin and cytochrome *c* peroxidase (Wilks and Ortiz de Montellano, 1992). In the absence of NADPH, the radical would migrate elsewhere and eventually disappear by irreversibly oxidising some other electron donor, within or outside the protein. Alternatively, Bicout et al. (1995) suggest the localisation of the radical site in compound II^{*} at Ser196 as more likely to fulfil the electron transfer role instead of Tyr214. Finally, it should be noted that according to Mössbauer spectroscopy data the radical in compound I of BLC is partly delocalised onto the proximal tyrosine ligand (Weiss et al., 1996).

Addition of NADPH to cofactor-free preparations of SCC-A significantly increases its catalatic activity, which partly may be accounted for by the tetramer stabilisation mentioned above, but also indicates an increase in the effective concentration of compound I. In the wild type protein, and more pronounced so in some single residue mutants (Asn143Gln, Val111Ala, Phe149Val) the rate of reduction of compound II^{*} was markedly enhanced. The first electron was efficiently transferred from NADPH to the putative protein radical site (leading to compound II), followed by a somewhat slower formation of ferricatalase. This mechanism was further supported by the analysis of overaged preparations of SCC-A (Herzog et al., 1997). After complete disappearance of the characteristic NADPH-linked fluorescence, new emission signals developed. Some of them may be attributed to the formation of dityrosines. In SCC-A two pairs of tyrosines (Tyr142/217 and Tyr132/376) are candidates for this radical process, which are currently tested by site-directed mutagenesis. In such samples addition of NADPH no longer shows any marked effects on the kinetics of the enzyme. From these experiments it was concluded that in SCC-A NADPH does not prevent the formation of compound II, but rather enhances its recycling.

In addition to its role in the recycling mechanism, experiments with SCC-A indicate that binding of NADPH stabilises the quaternary structure. Not only the reduced form, but to a lesser extent also the oxidised form of the dinucleotide (and, moderately, even NADH and NAD^{+}) contribute to the conformational stabilisation (Herzog et al., 1997). This is in agreement with Gouet et al. (1995), who propose that NADPH binding in PMC causes slight structural changes. Accordingly, these stabilising effects of the dinucleotide and its derivatives are more pronounced in mutants of SCC-A with decreased stability of the native conformation (Herzog, 1996).

One further function for NADPH in catalases was suggested from the binding experiments of rat catalase mRNA with BLC (Clerch et al., 1996). They found that only NADPH-depleted BLC could bind to the corresponding RNA section, whereas the enzyme saturated with the cofactor remained unbound to RNA. Homology of the dinucleotide binding structure of catalase and of RNA-binding proteins was suggested, supporting in particular the hypothesis that RNA-binding proteins may have evolved from (di)nucleotide binding systems (Hentze, 1994). It was concluded that NADPH could perform a function unrelated to the catalytic activity, namely to regulate the binding of the catalase to its own messenger (Clerch et al., 1996).

6. Import of eukaryotic catalases into peroxisomes

Peroxisomes, also known as microbodies, were discovered in the fifties by electron microscopy of mouse liver cells as (up to now) the last eukaryotic organelle. They are single membrane-lined organelles of 0.1–1 μm size, depending on the organism and growth conditions. They lack own DNA and ribosomes, but contain a great diversity of proteins, which have to be imported posttranslationally across the peroxisomal membrane (Lazarow and Fujiki, 1985). de Duve and Baudhuin (1966) formulated the peroxisome concept. Based on the analysis of cytochemical results, mainly enzyme distribution studies, they concluded the peroxisomal localisation of the physiologically closely associated H_2O_2-producing oxidases and H_2O_2-removing catalase (de Duve and Baudhuin, 1966). Later it was shown that the majority of eukaryotic catalases is located in the peroxisome (Subramani, 1993), in some conditions being the most abundant peroxisomal protein. It is located in the organelle matrix from where it readily leaks out if peroxisomes are osmotically damaged (e.g. rat liver catalase, Alexson et al., 1985).

The catalase suicide inhibitor-AT was intensively applied in screening for mutations in the peroxisomal assembly (van der Leij et al., 1992). After covalent attachment of AT to the enzyme hydrogen peroxide accumulates in the peroxisomes, causing immediate cellular death, whereas cells unable to accumulate H_2O_2 because of nonfunctional oxidases or because of their inability to assemble functional peroxisomes can survive and amplify.

During the last 10 years many investigation were performed to elucidate the consensus import sequences and folding state of proteins to be imported into microbodies, as well as receptors that perform the translocation process across the peroxisomal membrane. Two independent peroxisomal targeting signals (PTS) were observed, which show some variation among organisms but still can be identified in all peroxisomal proteins, particularly in catalases. PTS1 has been characterised in detail for a broad range of organisms. For mammalian catalases this motif was found approximately eight amino acid residues from the C-terminus (Trelease et al., 1996). In rat and mouse liver catalases the PTS1-sequence is SHI and SHM, respectively, and SHL in human kidney catalase (Bell et al., 1986). Its significance for successful peroxisomal import was demonstrated by deleting or modifying parts of PTS1. Both, the sequence motif itself and its correct location at the C-terminus are required for import (Trelease et al., 1996).

The PTS1 of plant catalases are markedly different from those found in mammals, but among them this sequence is highly conserved: it comprises the last three C-terminal residues with the sequence P–S/N/T–I/M. However, this targeting signal is functional only in the context with the adjacent C-terminal polypeptide, comprising at least six neighbouring residues (Mullen et al., 1997). In *Drosophila melanogaster* the PTS1 consists of the C-terminal tripeptide SKF, thus very homologous to yeast catalases (Orr et al., 1990).

Two independent targeting signals were found in catalase A from *S. cerevisiae* (Kragler et al., 1993). PTS1 of SCC-A, residing at the extreme C-terminus, is closely related with the corresponding motif of *Hansenula polymorpha* catalase (SKF, SKI, respectively). Interestingly, this signal is completely dispensable for peroxisomal import of catalase A. This was concluded from successful import of SCC-A with deleted C-terminal SKF-tripeptide, as well as of a his-tag modified SCC-A, with the C-terminal sequence changed to SHHHF (Binder et al., 1991).

The whole PTS1 has to be considered as consisting of the last six amino acids of *CTA1* (SSNSKF). After addition to import-incompetent hybrid proteins, these six residues were sufficient for their import into peroxisomes (Kragler et al., 1993). On the other hand, the addition of SKL alone, known as PTS1 consensus for a broad spectrum of import competent proteins, was not sufficient for a peroxisomal location of 6-dihydrofolate reductase in *S. cerevisiae*.

SCC-A contains another peroxisomal targeting signal in its amino terminal third (PTS2), sufficient to direct reporter proteins into microbodies. A protein devoid of the sequence between residues 126 and 143 could not be found inside peroxisomes. Furthermore, fusion proteins consisting of the N-terminal portion of catalase A (of 126–140 residues length) joined with subunit IV of cytochrome *c* oxidase were located inside the peroxisomes. Shorter N-terminal fragments of catalase A (<104 residues) were not sufficient to direct these constructs into microbodies. Kragler et al. (1993) conclude that the second peroxisomal targeting signal is situated most probably in the polypeptide segment between residues 104 and 126, but the area between 126 and 143 may also perform a significant contribution. This segment forms part of the strands β3 and β4, and the connecting loop, and obviously is essential for correct assembly of the first half of the antiparallel β-barrel structure. Consequently, one may conclude that PTS2 of SCC-A depends on this specific fold rather than on a distinct signal sequence present in this region. The protein bearing the 126–143 deletion could accumulate in a misfolded state, therefore not identified by the peroxisomal import apparatus.

Import across organelle membranes generally is believed to proceed via interaction of partially folded polypeptides with specific receptors followed by incorporation into the matrix of the target organelle (Neupert et al., 1990). According to this hypothesis, apocatalase subunits are exported from the cytoplasm into the peroxisomes by a receptor-mediated transport process, followed by (intraorganellar) association with haem and assembly of the functional protein (Bellion and Goodman, 1987; Subramani, 1993). However, the results of Walton et al. (1995) demonstrate that irreversibly folded proteins are also transported into peroxisomes; it appears therefore feasible that at least some proteins may fold in the cytosol, before they are imported into the peroxisome, and that protein unfolding is not a prerequisite for peroxisomal import. It was shown that in human skin fibroblasts catalase assembly to tetramers can take place in the cytosol (Middelkoop et al., 1993). On the other hand, catalase import into peroxisomes is inhibited by AT (Middelkoop et al., 1991). The existence of

functional tetramers in the cytosol appears a prerequesite for binding of this suicide inhibitor, which, however, also stabilises the assembled state. One might conclude that disassembly of native tetrameric catalase is required for successful peroxisomal import.

Two gene products in *S. cerevisiae*, PAS10 and PAS7 were shown to function as the receptors for the two signals PTS1 and PTS2, respectively. The translation product of PAS7 (PEX7p) was shown to interact with PTS2 of several peroxisomal proteins like e.g. thiolase (Erdmann and Kunau, 1992). Brocard et al. (1994) have shown that the tetratricopeptide repeat domain (TPR) of PAS10 (PEX5p) protein interacts with PTS1 in vivo. The part essential for this protein–protein interaction was shown to contain the complete tetratricopeptide repeat domain. The binding of PTS1 to this receptor is for catalase A, like for many other imported proteins highly specific, and the sequence upstream of PTS1 influences the binding affinity of PTS1 receptors.

Much less is known about the interaction of the second putative import signal of catalase A with peroxisomal receptors. However, other peroxisomal proteins like e.g. luciferase were reported to contain a large sequence or structure in their amino-terminal half, which upon modification abolishes import into peroxisomes (Gould et al., 1990). It would be interesting to compare the structural motifs of several other peroxisomal proteins with those of yeast catalase A to detect possible homologies directing this process.

7. Conclusions and future perspectives

As outlined in the previous chapters, a tremendous amount of research has been devoted to the field of typical catalases, covering structural aspects as well as their physiological functions. They have given us a rather complex understanding of this subgroup of hydrogen peroxide dismutating enzymes. Despite continuous intensive efforts to elucidate the physiological role of catalases, many important questions still remain to be answered. Now there is no doubt that the contribution of catalases to protect the organisms from damages caused by reactive oxygen species is essential. Hydrogen peroxide — a by-product of various oxidases and superoxide dismutases — is not only toxic by itself, but in a Fenton-type reaction can decompose to form the even more reactive hydroxyl radical. This radical is probably the most deleterious of the activated intermediates of oxygen, reacting with DNA, proteins and lipids in its proximity (Lindau-Shepard and Shaffer, 1993).

It was demonstrated that the expression of certain catalase isoenzymes in microorganisms is growth phase dependent (Chary and Netvis 1989; Loprasert et al., 1996). Some of them play an essential role in defence against oxidative stress (Orr and Sohal, 1992; Zheng et al., 1992; Rocha et al., 1996; Kawasaki et al., 1997). This protective function is not only directly achieved by rapid cleavage of hydrogen peroxide and small organic peroxides, but also concerning other cellular enzymes, mainly superoxide dismutases (Amstad et al., 1991; Michiels et al., 1994). Catalases are thought to protect superoxide dismutases against inactivation by higher levels of hydrogen peroxide (Fridovich, 1995).

An increasing number of investigations focus on the participation of catalases in plant defence, above all considering the regulation by substances like salicylic acid (SA). Although still controversial, it is suggested that plant catalases are regulated by this potential electron

donor in two ways. In healthy tissues, at low concentrations of H_2O_2 catalase is inhibited by SA, which causes a stimulation of defence-related genes. On the other hand, in tissues adjacent to necrotising cells, where high levels of H_2O_2 and other reactive oxygen species are produced, reasonable concentrations of SA will prevent inactivation of catalase (i.e. accumulation of the inactive compounds II and III) by serving as electron donor. Thus SA supports or substitutes for the protective function of catalase-bound NADPH (Durner and Klessig, 1996).

Since the residues considered responsible for the interaction with SA are highly conserved among typical catalases (Rueffer et al., 1995), site-directed mutagenesis of several haem-containing catalases in these areas can help to resolve the mechanism of SA inhibition and/or activation on a molecular basis.

Many questions are left open concerning the structure of the different classes of catalase, and the attempt to interpret these structures in terms of the known biochemical properties causes severe problems. Though catalases as well as peroxidases belong to the most thoroughly studied biomolecules, we still cannot readily define which structural elements are responsible for their particular pattern of reactivity. In many respects the reactive intermediates (compound I and II) of BLC and MLC on one side, and of CcP and HRP on the other side are closer related than one might expect (Chuang et al., 1989; Du and Loew, 1995), and the replacement of residues thought 'characteristic' for either class sometimes had only moderate effects (e.g. Adachi et al., 1993). There is no unequivocal explanation for the resistance of some catalases to form compound II, and it is not clear whether and how this may be linked with the extraordinary size of subunits (in HPII and ANC) or haem d formation, or haem inversion (HPII and PVC). We still do not understand the major substrate (haem) channel in typical catalases. At least at low concentrations of H_2O_2 the catalatic efficiency of catalase-peroxidases, which obviously don't share this structural element, is almost identical; on the other hand, in plant peroxidases the formation of compound I with H_2O_2 as substrate is about as rapid as in mammalian catalases; and, finally, some 'exit channel' has to be postulated to explain the very large turnover number of typical catalases at high substrate concentrations. Questions concerning the quaternary structure of typical catalases appear related to the former problem. Whereas peroxidases frequently are monomers and (at least most) catalase-peroxidases are dimers, typical catalases obviously strictly require a tetrameric assembly. Protein stabilisation, which frequently is the 'goal' of oligomerisation (Jaenicke, 1996) can be ruled out at least for catalases with 'normal' subunit size.

In any case the problem of catalase folding in vivo (and in vitro) is challenging. Until now spontaneous refolding of tetrameric catalases could not be achieved, even when all cofactors and active-site ligands were supplied. There is no indication of the participation of any molecular chaperone in catalase assembly in yeast, and the effects of heterologous chaperonins on in vitro refolding are only marginal. Still, the de novo design of a 'microcatalase', a stable and highly reactive counterpart to microperoxidases, remains one major objective of the research in this field, which eventually could find broad commercial application, e.g. in the field of biosensors.

Unlike typical catalases, the two remaining catalase subgroups, catalase-peroxidases and manganese catalases are only at the beginning of their comprehensive investigation. Nonetheless, their evolutionary origin, their relative simplicity, and their distinct modes of reaction will guarantee them numerous future applications.

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