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Detlef P. Terzenbach · Michael Blaut

Purification and characterization of a catalase from the nonsulfur phototrophic bacterium *Rhodobacter sphaeroides* ATH 2.4.1 and its role in the oxidative stress response

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Abstract When challenged with reactive oxidants, the nonsulfur phototrophic bacterium *Rhodobacter sphaeroides* ATH 2.4.1 exhibited an oxidative stress response during both phototrophic and chemotrophic growth. Upon preincubation with 100 μM H_2O_2, catalase activity increased fivefold. Catalase was also induced by other forms of oxidative stress, heat-shock, ethanol treatment, and stationary-phase conditions. Only one band of catalase activity was detected after native and denaturing PAGE. The enzyme was purified 304-fold with a yield of 7%. The purified enzyme displayed a heterodimeric structure with subunits of 75 and 68 kDa, corresponding to a molecular mass of approximately 150 kDa for the native enzyme. The subunits had almost identical amino-terminal peptide sequences, sharing substantial similarity with other bacterial catalases. The enzyme exhibited an apparent K_m of 40 mM and a V_{max} of 285,000 U (mg protein)$^{-1}$. Spectroscopic analysis indicated the presence of protoheme IX. The heme content calculated from pyridine hemochrome spectra was 0.43 mol per mol of enzyme. The enzyme had a broad pH optimum and was inhibited by cyanide, azide, hydroxylamine, 2-mercaptoethanol, and sodium dithionite. These data indicate that this catalase belongs to the class of monofunctional catalases.

Key words *Rhodobacter sphaeroides* · Catalase · Enzyme purification · Induction · Oxidative stress

D. P. Terzenbach · M. Blaut[1]
Institut für Mikrobiologie, Georg August Universität,
Grisebachstrasse 8, D-37077 Göttingen, Germany

Present address:
[1] (✉) Gastrointestinale Mikrobiologie,
Deutsches Institut für Ernährungsforschung Potsdam-Rehbrücke,
Arthur-Scheunert-Allee 114-116,
D-14558 Bergholz-Rehbrücke, Germany
e-mail: blaut@www.dife.de
Fax +49-33200-88407

Abbreviation *ABTS* (2,2′-azono-di[3-ethyl-benzothiazolin-(6)-sulfonate]

Introduction

Rhodobacter sphaeroides, a member of the nonsulfur purple bacteria (Rhodospirillaceae), is known for its outstanding metabolic versatility. Current research on representatives of this family deals with nitrogen fixation, CO_2 fixation, and regulation of photosynthesis, all of which are regulated by oxygen (Eraso and Kaplan 1994; McEwan 1994). Moreover, during aerobic growth, facultative anaerobes such as the Rhodospirillaceae require a defense system to neutralize the toxic side effects of oxygen and its reduced derivatives, O_2^- and H_2O_2. Important elements in the oxidative stress response of aerobic organisms are superoxide dismutase and catalase. While the regulation of the oxidative stress response is quite well understood in the Enterobacteriaceae, only little is known about the regulation of any kind of stress in the Rhodospirillaceae. The chaperonines Cpn 10 and Cpn 60 from *R. sphaeroides* (Terlesky and Tabita 1991) may be elements of stress response in this organism. Preliminary studies in this laboratory showed that *R. sphaeroides* can be rendered more tolerant to oxidative stress by previous treatment with heat, starvation, or ethanol. This suggests that catalase was an important element in the observed stress response.

There are two major classes of bacterial catalases: (1) the typical monofunctional catalases (HPII), which are homologous to the catalases of eukaryotes, and (2) the bifunctional catalase-peroxidases (HPI), which to date have been found only in bacteria. The monofunctional catalases share a number of characteristic features such as resistance to chloroform/ethanol treatment and a broad pH optimum. They exist as homotetramers or homohexamers with subunits of 60–90 kDa. The catalase-peroxidases have catalytic and peroxidatic activity with a strong dependence on pH, are not inhibited by 1,2,4-amino-triazole, and are generally less stable than catalases. They have been found in *Klebsiella pneumoniae* (Hochmann

and Goldberg 1991), *Bacillus* (Hicks 1995), *Chromatium* (Nadler et al. 1986), and the closely related *Rhodobacter capsulatus* (Hochman and Shemesh 1987). Both classes of catalase contain protoheme IX with the exception of the HPII catalase from *Escherichia coli*, which contains a chlorine instead (Loewen and Switala 1986).

Many bacteria express various forms of catalase and peroxidase and regulate them individually: *K. pneumoniae* contains three different types of catalase (Hochmann und Goldberg 1991), and a *Streptomyces coelicolor* strain is known to produce six catalases depending on growth rate (Kim et al. 1994). Also, the closely related *R. capsulatus* is known to contain two different peroxidases and a catalase (Hochman und Shemesh 1987). Their expression is regulated in response to pH [*Bacillus* (Yumoto et al. 1990)] or growth phase [*K. pneumoniae* (Hochmann and Goldberg 1991) and *S. coelicolor* (Kim et al. 1994)]. It has also been shown that catalases play a role in the general stress response of *Bacillus subtilis* (Völker et al. 1994). The purpose of this study was to characterize the oxidative stress response in *R. sphaeroides* and to elucidate the role of catalase activity in this process. The catalase was purified and compared to a catalase partially purified by Clayton (1959). The amino-terminal peptide sequences of the subunits were determined and compared to those of other bacterial catalases.

Materials and methods

Growth of bacteria

R. sphaeroides ATH 2.4.1 (DSM 158) was routinely grown chemoheterotrophically at 30 °C in AT medium (Imhof and Trüper 1976) containing succinic acid as the sole source of carbon and energy. For challenge experiments, 50-ml cultures in Erlenmeyer flasks with baffles were incubated on a rotary shaker and grown to an OD_{578} of 0.35–0.4. At this point, the cultures were divided into aliquots that were subsequently treated with agents causing oxidative or other kinds of stress. Phototrophic cultures were grown in the same medium in completely filled 100-ml bottles illuminated with 1,000 lx.

Enzyme activities and protein determination

Catalase was assayed according to the method of Rorth and Jensen (1967) by following the release of oxygen from H_2O_2 with a Clark oxygen electrode. The reaction chamber (2 ml) was connected to a water bath kept at 30 °C. The H_2O_2 concentration was 5 mM unless stated otherwise. Only the first 1–2 min of linear activity were used for calculations. Activity staining of catalase followed the method of Clare et al. (1984). Peroxidase was measured by the method of Hochman and Goldberg (1991). The amount of protein was measured by the method of Smith et al. (1985) using bicinchoninic acid with bovine serum albumin as the standard.

Purification of catalase

Approximately 10–12 g (wet wt.) of frozen chemoheterotrophically grown cells was thawed in 40 ml 50 mM Tris-HCl buffer (pH 7.5) and washed twice. Cells were broken by four to five passages through a French pressure cell at 130 MPa. Lysis was checked microscopically. After centrifugation at 120,000 × *g* for 1 h, the supernatant (cytoplasmic fraction) contained all the catalytic activity. The supernatant was vigorously mixed with chloroform/ethanol (10 : 5 : 3, v/v) for at least 10 min at room temperature. After centrifugation for 20 min at 8,000 × *g* and 4 °C, the upper phase was subjected to a two-step ammonium sulfate precipitation. First, ammonium sulfate was brought to 65% by addition of saturated ammonium sulfate in 50 mM Tris-HCl (pH 7.5), and the supernatant was discarded. The pellet was resuspended in 50 mM Tris-HCl (pH 7.5), brought to 33% $(NH_4)_2SO_4$, and centrifuged at 8,000 × *g* for 10 min. The pellet was discarded, and the supernatant was applied to a phenyl Sepharose column (2.6-cm i.d. × 30 cm; 30-ml bed volume) equilibrated with 5 mM K^+-phosphate (pH 7) containing 0.5 M ammonium sulfate. The column was washed with a decreasing step gradient of 0.5, 0.25, and 0 M ammonium sulfate (110, 44, and 33 ml, respectively) in 5 mM K^+-phosphate buffer (pH 7) at a flow rate of 1.1 ml/min. The column was subsequently eluted with an increasing step gradient of 15 and 50% (v/v) ethylene glycol in 5 mM K^+-phosphate buffer (pH 7) (44 and 55 ml, respectively). Active fractions, which eluted at 50% (v/v) ethylene glycol, were pooled and applied to a DEAE-Sephacel column equilibrated with 5 mM K^+-phosphate buffer (pH 7). The column was eluted with an increasing step gradient of NaCl (0, 0.2, 0.4, 0.6, 0.8, and 1.0 M) in 5 mM K^+-phosphate buffer (pH 7) at a flow rate of 1.1 ml/min. Catalase eluted at 200 mM NaCl. Active fractions were pooled, diluted twofold with H_2O, and applied to a Mono Q column [1-ml bed volume; equilibrated with 5 mM K^+-phosphate buffer (pH 7)] connected to an Fast Protein Liquid Chromatography (FPLC) system (Pharmacia, Freiburg, Germany). The enzyme was eluted with 25 ml of a linear 0–500 mM NaCl gradient in 5 mM K^+-phosphate buffer (pH 7). The active fractions were pooled, diluted twofold with H_2O, and applied again to the Mono Q column, which was subsequently washed with 195 mM NaCl in 5 mM K^+-phosphate buffer (pH 7). Catalase was eluted with a linear 195–300 mM NaCl gradient in K^+-phosphate buffer (pH 7). The active fractions contained the purified catalase.

Microsequence analysis

Purified enzyme was heated to 95 °C for 5 min in denaturing buffer [50 mM Tris-HCl (pH 6.8), 0.4% SDS, 2.5% (v/v) mercaptoethanol; final concentrations], and the subunits were separated by SDS-PAGE (13% polyacrylamide) according to Laemmli (1970). Approximately 500 pmol of each subunit was transferred onto Polyvinylidendifluorid (PVDF) membrane for amino-terminal sequence analysis. Sequence determinations were performed by B. Schmidt (Institute for Biochemistry, University of Göttingen, Göttingen, Germany) and by H. Hobein (Institute for Hygiene, University of Göttingen, Göttingen, Germany).

Results and discussion

Oxidative stress response

To determine whether *R. sphaeroides* induces an oxidative stress response, catalase activity was measured in cell-free extracts of phototrophically or chemotrophically grown cells of *R. sphaeroides*. Oxidative stress was applied by incubating exponentially growing cultures with 250 μM H_2O_2 or 150 μM benzyl viologen for 30 min or by shifting a phototrophic culture to oxic conditions, i.e., by transferring the culture into an Erlenmeyer flask with baffles and shaking vigorously. H_2O_2 induced the catalase activity fivefold in chemotrophically growing and in phototrophically growing cells, whereas benzyl viologen induced catalase fourfold in chemotrophically growing and twofold in phototrophically growing cells. The cells shifted from anoxic to oxic conditions also responded

Table 1 Purification of catalase from *Rhodobacter sphaeroides*

Purification step	Total protein (mg)	Total activity (units)	Specific activity [units (mg protein)$^{-1}$]	Yield (%)	Purification (-fold)
Cytoplasm	1,125.0	207,110	184	100.0	1
Ethanol/chloroform	192.0	135,475	706	65.3	3.8
Ammonium sulfate	40.8	102,405	2,511	49.4	13.6
Phenyl-Sepharose	18.2	83,834	4,606	40.4	25
DEAE-Sephacel	2.44	45,107	18,471	21.8	100
Mono Q 2	0.26	14,429	55,926	6.9	304

with a fivefold increase in catalase activity. Crude extracts analyzed by native PAGE revealed only one activity-stained band of catalase corresponding to an apparent molecular mass of 150 kDa (not shown).

To elucidate the role of catalase as an element in the general stress response, catalase activity was determined in response to a number of well-known inducers of oxidative stress. The redox cycling agents methyl viologen and benzyl viologen led to an increase in catalase activity, whereas menadione, which is also considered to act as a redox cycling agent, had no inducing effect. Benzyl viologen at 10 mM killed approximately 99% of the cells after treatment for 30 min, while menadione treatment did not change viable counts even at concentrations of 50 mM. All other oxidizing agents had equally little effect on catalase activity. Stationary-phase cells exhibited a twofold higher catalase activity than did noninduced, exponential-phase cells. The finding that heat shock and preincubation with ethanol induced catalase activity supports the idea that catalase is part of a general stress response in *R. sphaeroides*. Again, native and denaturing PAGE revealed the presence of only one band of catalase activity.

In contrast to *R. capsulatus*, cell extracts of *R. sphaeroides* displayed only low peroxidatic activity. This activity could not be visualized by activity staining after native PAGE. Therefore, it has to be concluded that the induction patterns of catalase observed in *R. sphaeroides* are due to one single enzyme. In order to study the regulation of catalase expression, we purified the enzyme. The results of a typical purification of the catalase from *R. sphaeroides* is given in Table 1.

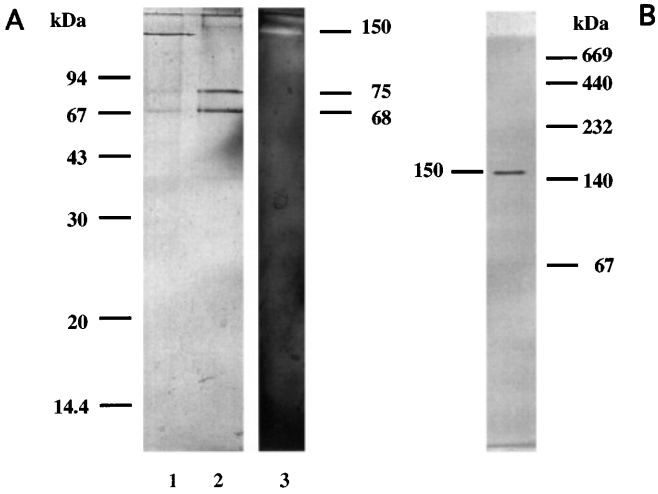

Fig. 1 **A** Denaturing SDS-PAGE (13% polyacrylamide) (Laemmli 1970) and **B** native gradient PAGE (8–20% polyacrylamide) of purified catalase (4.7 μg per lane) from *Rhodobacter sphaeroides*. **A** *Lane 1* untreated catalase stained for protein with silver (Blum et al. 1987), *lane 2* catalase heated to 95 °C for 5 min in denaturing buffer [50 mM Tris-HCl (pH 6.8), 0.4% SDS, and 2.5% (v/v) mercaptoethanol; final concentrations] and stained for protein with silver, and *lane 3* untreated catalase stained for activity with nitroblue tetrazolium. **B** Untreated catalase stained for protein with silver. The HMW and LMW protein standards (Pharmacia, Heidelberg, Germany) for estimating the molecular mass of the native enzyme and of the subunits are indicated

Physicochemical properties of the purified catalase

Native gradient PAGE of the purified catalase indicated a molecular mass of 150 kDa (Fig. 1 B). Upon heating to 80 °C, the enzyme formed two bands in an SDS polyacrylamite gel corresponding to apparent molecular masses of 75 kDa (subunit A) and 68 kDa (subunit B), respectively (Fig. 1 A, lane 2). Extended heating to 95 °C for up to 20 min did not change this pattern. Without heating, the enzyme did not dissociate into its subunits (Fig. 1 A, lane 1) even when preincubated with 7 M urea, 10% SDS, 1 M dithioerythritol, and 10% 2-mercaptoethanol. A similar stability at room temperature with respect to urea and SDS was also reported for the catalase HPII from *E. coli* (Loewen and Switala 1986).

These data clearly indicate the presence of a heterodimeric catalase in *R. sphaeroides*. In contrast, Clayton (1959) has reported that the catalase of *R. sphaeroides* exhibits a molecular mass of 232 kDa and a homotetrameric structure. During our investigations, we never observed a catalase with such a molecular mass. Isoelectric focusing of the native enzyme resulted in two distinct spots in 2D-PAGE corresponding to an isoelectric point of 5 for both polypeptides (not shown).

Spectroscopic analysis

Absorption spectra of the purified enzyme showed features typical of ferric heme proteins (Fig. 2). The native enzyme had a Soret band at 406 nm. This band was shifted to 426 nm upon addition of cyanide. Similar shifts have also been described for other catalases (Hochman and Shemesh 1987; Goldberg and Hochman 1989). The

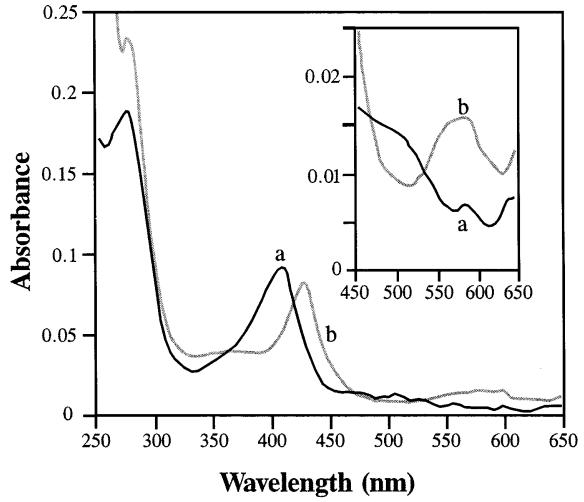

Fig. 2 Spectroscopic analysis of purified catalase. The spectra of the enzyme were recorded against a blank of identical buffer. **a** Native enzyme and **b** enzyme in the presence of 1 mM CN^-. The *insert* shows an enlargement of the spectra in the range of 500–650 nm. Absorption spectra were recorded with a Kontron Instruments model 930 spectrophotometer (Kontron Instruments, Neufahrn, Germany)

position of the major band in the visible region is located at 584 nm. However, the position of this band changes quite widely among the various ferric heme protein cyanide catalases (Hochman and Shemesh 1987). The A_{406}/A_{280} ratio of 0.513 is low for typical catalases, which usually exhibit ratios of approximately 1. Upon incubation of the enzyme with pyridine (25%, v/v) and 0.2 N NaOH, a typical pyridine-ferrohemochrome spectrum with maxima at 412, 538, and 551 nm was recorded, indicating the presence of protoheme IX (not shown). Using the extinction coefficient of 191.5 mM^{-1} cm^{-1} (Falk 1964), we calculated that 0.43 mol of protoheme IX was present per mol of native enzyme. This value is low for monofunctional catalases and may indicate that the heme was partly lost during the purification process.

Catalytic properties of the purified enzyme

The purified catalase from *R. sphaeroides* was not saturable with its substrate H_2O_2 (Fig. 3 A). Up to a concentration of 75 mM H_2O_2, the activity of the enzyme increased proportionally with respect to the substrate concentration. At concentrations exceeding 75 mM, the catalase was rapidly inactivated. This behavior is typical of catalases that follow first-order reaction kinetics (Aebi 1984). Nevertheless, using low concentrations of H_2O_2, Michaelis-Menten kinetics could be applied, resulting in an apparent K_M of 40 mM and a V_{max} of 285,000 U/mg. A more reliable parameter to describe first-order kinetics takes advantage of the reaction velocity constant k', which was calculated by dividing the first-order rate constant k by the enzyme concentration. For the purified catalase, k' was calculated to be 8.9 10^6 M^{-1} s^{-1}. This value is in the range reported for other catalases (10^7 M^{-1} s^{-1}; Aebi 1984). The enzyme did not show any peroxidatic activity with o-dianisidine, 3,3′-diaminobenzidine, or 2,2′-azino-di[3-ethyl-benzothiazolin-6-sulfonate]Na_2 (ABTS). Also, no degradation of the organic peroxides t-butyl-hydroperoxide and cumene-hydroperoxide was detectable.

Effect of pH, temperature, and inhibitors

The catalytic activity of the purified catalase was not affected by pH values between 6 and 10 (Fig. 3 B). Such a broad pH optimum is typical of catalases, while catalase-peroxidases usually have a distinct pH optimum around 6. The enzyme exhibited only a weak temperature dependence (Fig. 3 C). At 54 °C, the activity rose to 129% of the control at 30 °C. The stability of the enzyme decreased as a function of temperature, with a complete loss of activity at 80 °C. SDS-PAGE separated the subunits at this temperature. Incubation of the enzyme for 30 min at 30 °C caused a 50% loss of activity, and after 14 days at 4 °C approximately 90% of the activity was lost. Treatment of the enzyme with 5 µM each of cyanide, azide, and hydroxylamine decreased its activity by 72.5, 76.4, and 85.1%, respectively. 2-Mercaptoethanol (1 mM) was a less-potent inhibitor, reducing the activity to 36% of the control.

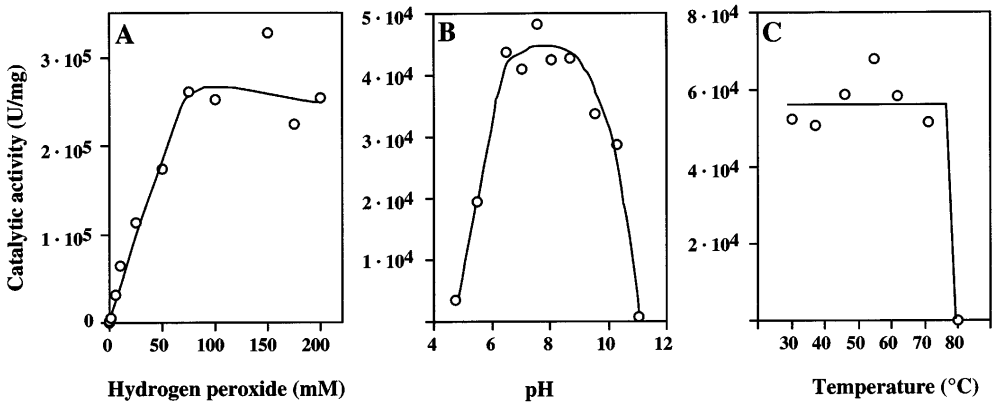

Fig. 3 Dependence of catalase activity on **A** H_2O_2 concentration, **B** pH, and **C** temperature. The following buffers (50 mM each) were used in **b**: citric acid (pK_1: 3.21, pK_2: 4.75, and pK_3: 6.40), Mes (pK_s: 6.1), Tes (pK_s: 7.4), Tris (pK_s: 8.08), and glycine (pK_s: 9.6). **C** The reaction mixture and the reaction chamber were preheated to the temperature indicated. Equilibration was allowed in the reaction chamber for 1 min. The reaction was started by addition of up to 20 µl diluted enzyme to a total volume of 2 ml

Table 2 Comparison of the N-terminal amino acid sequences of the two polypeptides KatA and KatB of the catalase from *Rhodobacter sphaeroides* with catalases from other organisms. – Amino acid not identified and • gap required for optimal sequence alignment

Organism		Sequence	Source
Rhodobacter sphaeroides KatA (75 kDa)		**TRMTTTAGAPII**	This work
Rhodobacter sphaeroides KatB (68 kDa)		R-**MTTTAGAPII**	This work
Brucella abortus	1	MTDRPI**MTTSAGAPI**P	U11439 (EMBL)
Streptomyces coelicolor	1	MPENNQKPL**TTVAGPPV**P	X96981 (EMBL)
Bacillus subtilis	1	MSSNKL**TTSWGAPV**G	M80796 (EMBL)
Streptomyces violaceus (venezuelae)	1	MTQGPL**TTEAGAPV**A	X74791 (EMBL)
Listeria seeligeri	1	MTDRRNL**TTNQGVPI**G	M75944 (EMBL)
Mycobacterium tuberculosis	1	MPEQHPPITE**TTT•GA**ASN	U06258 (EMBL)
Homo sapiens	21	AQKADVL**TTGAGNPV**G	P04040 (SwissProt)

Sodium dithionite was also inhibitory, but its inhibitory effect could not be quantitated by the catalase assay because of interference with the oxygen electrode.

Microsequence analysis

The N-termini of the two subunits of the purified catalase, designated KatA and KatB, were each sequenced twice by two independent laboratories. Ten of the first 12 amino acids of the two polypeptides were identical (Table 2). This finding and the fact that the molecular masses of the two polypepetides differed by 7 kDa may indicate that KatB is identical to KatA, but is shortened by a C-terminal peptide. The sequences obtained share substantial similarity with other bacterial catalases of the HPII type and with eukaryotic catalases such as the human enzyme.

Relation to and comparison with other bacterial catalases

The N-terminal amino acid sequence supports the classification of the catalase purified from *R. sphaeroides* as a member of the family of monofunctional catalases. Nevertheless, the enzyme differs from most other HPII catalases with respect to its molecular structure. It consists of two subunits of 75 and 68 kDa resulting in an apparent molecular mass of approximately 143 kDa, which to our knowledge is unique. Catalases of comparable size have been detected in *Streptomyces venezuelae* (Knoch et al. 1989), *Mycobacterium tuberculosis* (Wayne and Diaz 1986), and *K. pneumoniae* (Goldberg and Hochman 1989). However, all of these catalases are homodimers. A manganese-containing catalase from the obligate thermophile *Thermoleophilum album* (Allgood and Perry 1986) consists of four identical subunits of 34 kDa. The presence of two different subunits is known only in the case of the catalase-peroxidase of *Vitreoscilla* (Abrams and Webster 1990). Loewen and Switala (1986) have explained the appearance of the *E. coli* HPII catalase in the form of two subunits of 90 and 92 kDa with proteolytic degradation. This explanation cannot be valid for the catalase of *R. sphaeroides* described here for the following reasons: the catalase was stable as a dimer as long as the protein was not heated; heating to 80 °C for 30 s was sufficient to separate the subunits; extended heating to 95 °C for 20 min did not change the electrophoretic behavior of the protein; both subunits were always present in equal amounts; and at no time were we able to detect a small protein fragment in electrophoretic gels that would explain the difference in molecular mass between the two subunits.

Bacteria with more than one catalase regulate these enzymes individually. For example, the *E. coli* HPII catalase and the spore-specific catalase-2 from *B. subtilis* are expressed in the stationary phase (Loewen and Switala 1987). A strain of *S. coelicolor* is known to have six electrophoretically distinguishable catalases, all of which are regulated independently during the different phases of growth (Kim et al. 1994). Even though it appears that *R. sphaeroides* possesses only one constitutive catalase and no peroxidase or catalase-peroxidase, the former enzyme undergoes a regulation by various inducers such as H_2O_2 and the redox cycling agents benzyl viologen and methyl viologen. Redox cycling agents are generally considered to be effective through the formation of O_2^-, which may be converted further to the highly reactive hydroxyl radical. Since the induction of catalase by benzyl viologen was also detected in the absence of oxygen, it has to be assumed that benzyl viologen exerts additional effects. The inducibility of catalase by preincubation with ethanol or heat-shock treatment indicates that the catalase is an integral part of the general stress response.

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