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The oxidant-responsive diaphorase of *Rhodobacter capsulatus* is a ferredoxin (flavodoxin)-NADP(H) reductase

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Abstract Challenge of *Rhodobacter capsulatus* cells with the superoxide propagator methyl viologen resulted in the induction of a diaphorase activity identified as a member of the ferredoxin (flavodoxin)-(reduced) nicotinamide adenine dinucleotide phosphate (NADP(H)) reductase (FPR) family by N-terminal sequencing. The gene coding for *Rhodobacter* FPR was cloned and expressed in *Escherichia coli*. Both native and recombinant forms of the enzyme were purified to homogeneity rendering monomeric products of ~30 kDa with essentially the same spectroscopic and kinetic properties. They were able to bind and reduce *Rhodobacter* flavodoxin (NifF) and to mediate typical FPR activities such as the NADPH-driven diaphorase and cytochrome *c* reductase.

Key words: Ferredoxin (flavodoxin)-(reduced) nicotinamide adenine dinucleotide phosphate reductase; Ferredoxin; Flavodoxin; Oxidative stress; *Rhodobacter capsulatus*

1. Introduction

Rhodobacter capsulatus is a Gram-negative purple phototrophic bacterium that can adapt its metabolism to grow under various environmental conditions. When illuminated in anaerobiosis, these microorganisms adopt a photosynthetic lifestyle, generating adenosine triphosphate (ATP) through an anoxygenic electron transport around a single photosystem [1]. On exposure to air, they shift to a respiratory metabolism after de novo synthesis of terminal oxidases and dehydrogenases [2], whereas expression of the pigment binding proteins encoded by the *puf* and *puc* operons is repressed, leading to progressive disappearance of reaction centers and antennae [3].

The onset of respiration confronts the growing bacteria with still another challenge, as the reducing equivalents delivered by the catabolic routes proceed through a series of intermediates along the respiratory chain. Although molecular oxygen acts as the terminal acceptor, it may also subtract electrons from intermediate carrier components promoting the synthesis of toxic derivatives collectively known as reactive oxygen species (ROS), including the superoxide anion radical, hydrogen peroxide and the hydroxyl radical [4]. Along the path of evolution, aerotolerant organisms have developed a series of enzymatic and non-enzymatic devices to ameliorate the hazards of ROS production and accumulation [4,5]. When the build-up of toxic species cannot be restrained by the activity of these protectors, the outcome is oxidative stress and eventually, damage.

Little is known on the nature of the antioxidants recruited during the transition from anoxygenic photosynthesis to respiration in phototrophic bacteria. In *R. capsulatus*, a peroxidase, a catalase-peroxidase, a cambialistic superoxide dismutase and an oxygen-responsive thioredoxin were implicated in the defense against ROS toxicity [6–9]. Many antioxidant responses also induce the expression of oxidoreductase and dehydrogenase activities. At least five loci involved in the superoxide response of *Escherichia coli* are related to oxidoreductive processes and/or electron transfer including the *fpr* gene encoding a ferredoxin (flavodoxin)-(reduced) nicotinamide adenine dinucleotide phosphate (NADP(H)) reductase (FPR). These flavin adenine dinucleotide (FAD)-containing enzymes catalyze the reversible electron transfer between NADP(H) and obligatory one-electron carriers such as the iron-sulfur protein ferredoxin (Fd) or the flavoprotein flavodoxin (Fld) [10]. In *E. coli*, FPR levels increase ~20-fold on exposure of the bacteria to methyl viologen (MV), a redox-cycling compound that initiates superoxide propagation [11,12]. The paradox of a diaphorase with potential superoxide propagating properties being recruited during the superoxide response of several bacterial species has been addressed by us in a former article [13]. Tolerance to MV toxicity correlates with the cellular contents of this reductase, even beyond physiological levels of FPR expression and induction [12,14]. Although the mechanism of protection remains unclear, the involvement of FPR in oxidative stress responses has also been demonstrated in *Azotobacter vinelandii* [15] and *Salmonella enterica* [16].

In the framework of a systematic effort toward understanding the antioxidant defense systems of phototrophic bacteria, we report here the isolation and characterization of the FPR of *R. capsulatus*. We also show that expression of this flavoprotein is induced under oxidative stress conditions, suggesting that FPR might be a member of the antioxidant response in these microorganisms.

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. coli strains DH5α and BL21(DE3)pLys (Novagen) were grown in Luria–Bertani (LB) medium [17] supplemented, when required, with 100 μg/ml ampicillin, 34 μg/ml chloramphenicol and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). *R. capsulatus* strain

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37b4 (DSM938) was cultured at 32°C in malate mineral medium under the aeration conditions described in [7].

2.2. Cloning procedures

Recombinant DNA techniques were carried out by following established procedures [17]. The *Rhodobacter fpr* gene was obtained by polymerase chain reaction (PCR) amplification using chromosomal DNA as a template. The forward (5′-ATTGCCATGGCGAAAGTCCTGC-3′) and reverse (5′-CTGCCGAAGCTGCGAAACT-3′) primers were designed on the basis of the N-terminal sequence determined for the *fpr* gene product and the DNA sequence reported in the *R. capsulatus* Genome Project (http://www.rhodol.uchicago.edu). The amplified fragment carrying a *Nco*I site at the 10th codon of the *fpr* gene, was ligated into pGEM-T-easy (Promega) to yield pGEM-fpr-*Nco*, digested with *Nco*I and *Sac*I and inserted into compatible sites of pET-32a (Novagen) to produce pET32-FPR. The Fld gene *nif*F [18] was also cloned after PCR amplification of chromosomal DNA, using primers 5′-GGGACACCATGGCGAAGATCG-3′ and 5′-GCAGCACGGCCAGAACAT-3′. The forward primer introduced a *Nco*I site that allowed cloning into pET-32a, rendering plasmid pET32-Fld. Both recombinant plasmids were finally introduced into *E. coli* BL21(DE3)pLys.

2.3. Protein purification

Rhodobacter cells were cultured, collected and disrupted as described [8]. Lysates were cleared by centrifugation (60 min at 140 000 × *g*), and subjected to ammonium sulfate fractionation. The sediment obtained between 30 and 70% of saturation was dissolved in 50 mM Tris–HCl pH 7.4, 10% (v/v) glycerol, desalted (HiPrep Desalting 26/10, Amersham) and applied on an 8-ml Q Sepharose high performance column (Amersham) equilibrated with the same buffer and developed with a 0–500 mM NaCl linear gradient. Fractions containing FPR were re-chromatographed at pH 9.0 and finally purified through a Mono-Q HR 5/5 column (Amersham) developed with a pH gradient from 7.4 to 4.5. FPR was monitored by measuring diaphorase activity and the absorbance at 450 nm (A_{450}).

To purify recombinant FPR and Fld, cleared lysates from transformed *E. coli* cells were applied onto a Ni-nitrilotriacetic acid (NTA) fast-flow affinity column (Qiagen). The fusion products were eluted with 300 mM imidazole in 50 mM Tris–HCl pH 7.0, digested with enterokinase and purified as recommended by the supplier (pET System, Novagen). Protein concentration was estimated as described [19]. Samples of both *R. capsulatus* and *E. coli* preparations were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [20] and stained with Coomassie brilliant blue.

2.4. Amino-terminal sequencing

An aliquot of the 30–70% ammonium sulfate fraction was resolved by non-denaturing PAGE using the buffer system described by Laemmli [20] without SDS, and assayed for diaphorase activity in situ. A lane from this gel was subjected to a two-dimensional SDS–tricine PAGE, and electroblotted onto a polyvinylidene fluoride (PVDF) membrane. Protein bands displaying activity in the first dimension were excised, and their N-terminal sequences determined using a Procise 494 automatic sequencer.

2.5. Spectroscopic measurements

Absorption spectra were recorded in a Shimadzu UV-2450 spectrophotometer and the FPR extinction coefficient at 452 nm (ε_{452}) was determined by quantitating the FAD released from the apoprotein after heat denaturation (70°C, 5 min). Circular dichroism (CD) spectra were determined using a Jasco J-810 spectropolarimeter. Fluorescence quenching measurements were carried out on desalted samples using a Kontron SFM 25A spectrofluorometer.

2.6. Enzyme assays

Diaphorase and Fd- or Fld-dependent cytochrome *c* reductase activities were measured according to [21]. The reported K_m and V_{max} values are the means of four independent determinations using 100 nM FPR. Activity staining of native polyacrylamide gels was carried out by incubation in 50 mM Tris–HCl pH 8.0, 0.3 mM $NADP^+$, 3 mM glucose-6-phosphate, 1 unit/ml glucose-6-phosphate dehydrogenase and 7.5 μg/ml nitroblue tetrazolium. Activity bands were visualized by the formation of a violet formazan precipitate.

3. Results and discussion

3.1. Identification of an oxidant-responsive FPR from R. capsulatus

Soluble extracts from *R. capsulatus* cells contain three distinct NADPH-dependent diaphorases, as revealed by activity staining of non-denaturing polyacrylamide gels (Fig. 1A). Although this pattern remained unaltered when cells were shifted from anaerobic illuminated cultures to O_2-saturated media (Fig. 1A), incubation with 100 μM MV resulted in a 2- to 3-fold increase in the levels of the diaphorase displaying the higher electrophoretic mobility (Fig. 1B). The enzymes were further separated by a two-dimensional SDS–PAGE (Fig. 1C), and the N-terminal sequences of the two major species were determined after blotting onto PVDF membranes. A search in gene databases revealed that the diaphorase of intermediate mobility was homologous to bacterial

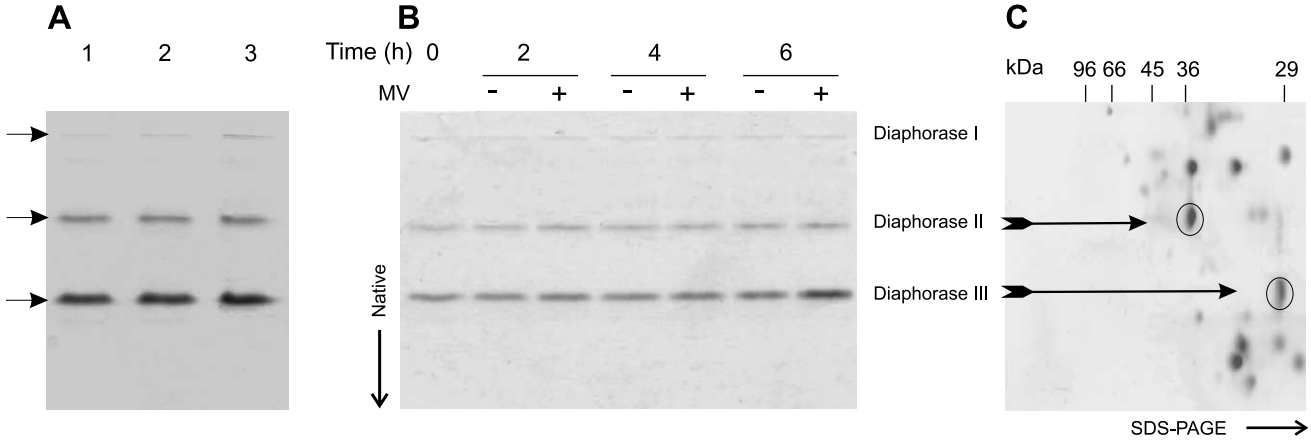

Fig. 1. *R. capsulatus* possesses a diaphorase that responds to MV. Cleared lysates corresponding to 15 μg (gel A) or 10 μg (gel B) of soluble protein were resolved by non-denaturing PAGE and stained in situ for diaphorase activity. A: Patterns of activity found in aerobic (lane 1), semi-aerobic (lane 2) or phototrophic (lane 3) cultures. B: Aerobic cultures were exposed to 100 μM MV and aliquots were withdrawn at 2, 4 and 6 h, for PAGE and activity staining. C: A partially purified extract (100 μg protein) was subjected to PAGE and in situ activity staining. Proteins were then resolved on a second dimension by SDS–PAGE, transferred to PVDF and stained. Molecular masses are given on top of the gel. The two major diaphorases are indicated by arrows.

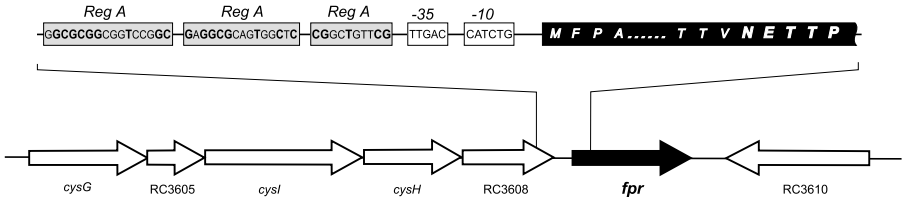

Fig. 2. The *R. capsulatus* genomic region around the *fpr* gene. Arrows indicate direction of transcription. The *fpr* coding sequence (in black) is located downstream of a gene cluster which is homologous to a bacterial operon involved in cysteine biosynthesis [39]. The N-terminal sequence determined on the purified FPR is highlighted. The 5′ untranslated region contains canonical −10 and −35 consensus sequences, and three RegA boxes centered at −80, −128 and −188 (consensus sequences are bold).

thioredoxin reductases. On the other hand, the sequence of the MV-inducible enzyme had a perfect match in the genomic sequence of *R. capsulatus* strain SB1003, 28 residues downstream of the initial methionine in an open reading frame displaying 52 and 35% identity with *A. vinelandii* and *E. coli* FPRs, respectively (Fig. 2). This observation suggests that post-translational processing must involve removal of an unusually long stretch at the N-terminus, or that synthesis might begin at the valine residue located immediately upstream of the initial glutamine found in the mature protein. The gene was tentatively named *fpr*, and could be mapped to a region of the SB1003 chromosome encoding other redox-linked enzymes involved in cysteine biosynthesis (Fig. 2).

Using the *Rhodobacter* SB1003 genomic information, we amplified the putative *fpr* gene of strain 37b4 and determined its nucleotide sequence (EMBL/GenBank accession number AF232063). The deduced polypeptides were identical in the two strains except for conservative substitutions at Thr153 and Thr196 that were replaced by alanine in the 37b4 sequence. Analysis of the upstream region (Fig. 2) revealed the presence of −10 and −35 promoter consensus sequences for the housekeeping RNA polymerase [22]. Interestingly, three sequences separated by 40 nucleotides are recognizable as putative RegA binding regions (Fig. 2), and could be involved in regulation of *fpr* expression in response to the cell redox status [23].

3.2. *Purification and characterization of* R. capsulatus *FPR*

Rhodobacter FPR was purified ~400-fold, and the molecular mass deduced after SDS–PAGE (~30 kDa, Fig. 3A) agrees well with that predicted by the nucleotide sequence (30 138 Da). Size exclusion chromatography on a Superdex G 200 HR 10/30 column indicated that the native enzyme is a monomer (data not shown). A protein with essentially the same mass was isolated from *E. coli* cells expressing the *Rhodobacter fpr* gene under control of the T_7 promoter. The electronic and CD spectra of the air-oxidized reductases purified from either source were also identical. The absorption spectrum was typical of flavin-containing proteins, with bands centered at 372 and 452 nm in the visible region and 272

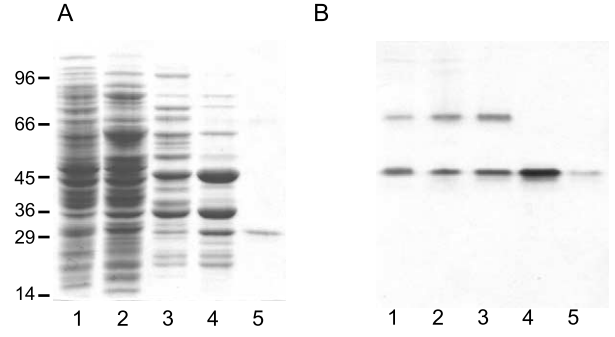

Fig. 3. Purification of FPR from *R. capsulatus*. Soluble fractions obtained at various purification stages were resolved by SDS–PAGE and stained for protein (A), or by non-denaturing PAGE and stained for activity (B). The amounts of loaded protein are given in parentheses. Lanes: 1, cleared cell lysates (30 μg); 2, 30–70% ammonium sulfate fraction (30 μg); 3, eluate from Q-Sepharose at pH 7.4 (8 μg); 4, eluate from Q-Sepharose at pH 9.0 (8 μg); 5, purified FPR after Mono-Q chromatography (0.5 μg). Molecular masses of standards are given on the left.

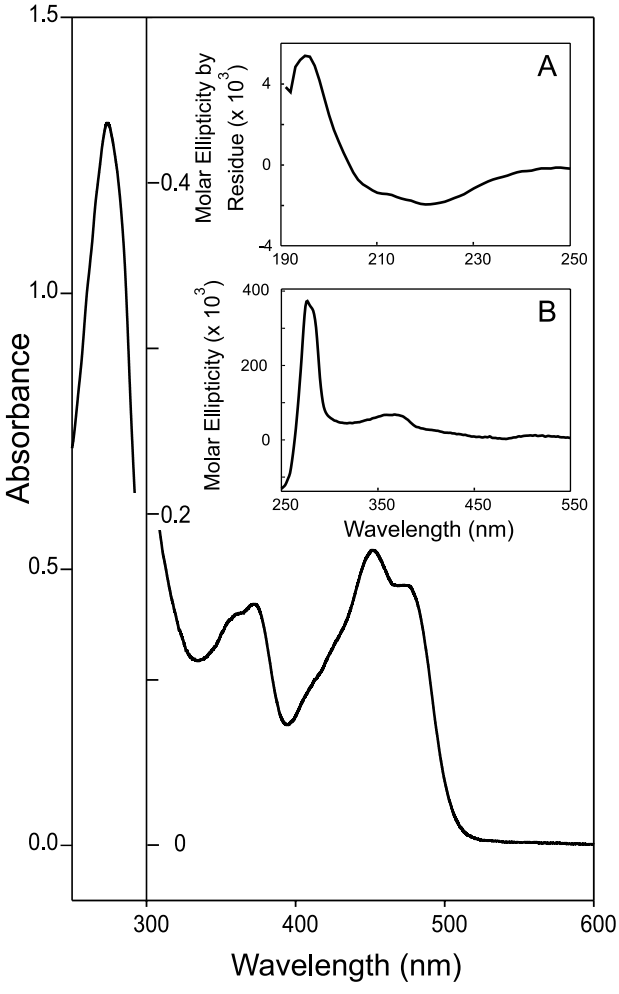

Fig. 4. Spectroscopic characterization of *R. capsulatus* FPR. For the spectrum at wavelengths below 300 nm, enzyme was diluted 3-fold. The insets display CD spectra of 0.45 μM FPR (A) or 9 μM FPR (B) in 5 mM phosphate buffer pH 7.0.

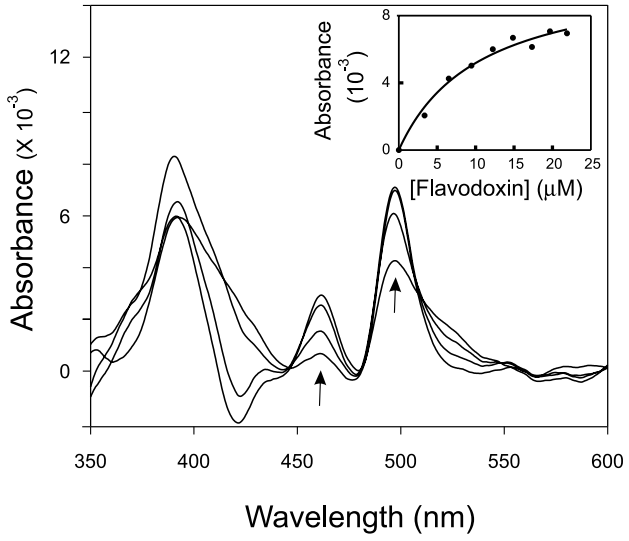

Fig. 5. Difference absorption spectra elicited by binding of *R. capsulatus* Fld to FPR. The titration was performed at 25°C by adding Fld to a 30 μM FPR solution in 5 mM Tris–HCl pH 7.2. The spectra were recorded at the following molar ratios: 0.11, 0.31, 0.57, and 0.70. The inset shows the plot of the absorbance at 498 nm as a function of Fld concentration. Experimental data (filled circles) were fit to the theoretical equation for 1:1 stoichiometry by means of non-linear regression.

nm in the ultraviolet region (Fig. 4). The A_{272}/A_{452} ratio was 7.33.

Heat denaturation of the reductase released the bound cofactor allowing an estimation of $\varepsilon_{452} = 10\,893\ M^{-1}\ cm^{-1}$. The intrinsic flavin fluorescence increased about 20 times upon unfolding of the holoprotein, an indication of the extensive isoalloxazine quenching characteristic of FPR proteins. The prosthetic group was shown to be FAD as its fluorescence increased 10 times after phosphodiesterase treatment [24,25].

3.3. Redox partners and catalytic properties

To test whether FPR could interact with physiological electron partners, the *Rhodobacter* Fld NifF was overexpressed in *E. coli*. Recombinant NifF displayed a typical Fld spectrum with maxima at 370 and 450 nm [26]. Complex formation between FPR and Fld was monitored by difference absorption spectroscopy. Spectral perturbations obtained by addition of NifF to an FPR solution at varying molar ratios were recorded (Fig. 5), and a K_d of 10.5 μM was calculated from the titration curve (Fig. 5, inset). The cytochrome *c* reductase activity of FPR was stimulated three times by the addition of NifF, to yield a turnover number of 0.25 s^{-1} and a K_m of 9 μM. Pea Fd also stimulated FPR-dependent cytochrome *c* reduction, although the K_m was 10 times higher. Diaphorase activity proceeded with a $k_{cat} = 7.2\ s^{-1}$ and a K_m for NADPH of about 80 μM, when potassium ferricyanide was employed as electron acceptor.

Reaction rates were comparable to those reported for the *E. coli* and *A. vinelandii* flavoenzymes, and 50-fold lower than the activities measured with the plant and cyanobacterial reductases [24,27–29]. The existence of large variations in turnover among enzymes that share extensive structural identity is intriguing. Interestingly, plant and bacterial reductases also differ in the conformation of the flavin cofactor. FAD binds in an extended form in chloroplast FPR, with the isoalloxazine ring system stacking between the aromatic side chains of two tyrosine residues [30]. In contrast, the adenosine of FAD in the *E. coli* and *A. vinelandii* reductases bends back from the diphosphate so that the adenine and isoalloxazine groups display extensive interaction [31,32].

Optimization for catalytic efficiency in *plant-type* FPR might be an adaptive response to the demands of the photosynthetic process that requires a high electron flow to sustain CO_2 fixation rates. In organisms growing on heterotrophic metabolisms or anoxygenic photosynthesis, FPR is involved in pathways that proceed at a much slower pace. In nitrogen-fixing bacteria such as *Azotobacter* and *Rhodobacter* species, Fds and Flds are the immediate electron donors to the nitrogenase [33–35]. Although the physiological donor(s) remain yet to be identified, and may even vary depending on growth conditions, several of these carrier proteins are able to efficiently reduce nitrogenase in vitro [33,36]. Two systems have been so far implied in Fd/Fld reduction, a pyruvate-dependent oxidoreductase [36] and a putative membrane-bound complex, funneling electrons from glucose metabolism and the photosynthetic apparatus, respectively [33,37]. We propose here a third device represented by FPR that might allow access to the abundant NADPH pool of the cell. Electron currents through nitrogenase occur at $\sim 0.1\ s^{-1}$ [36], a rate compatible with FPR turnover numbers.

Low levels of FPR can be found in *R. capsulatus* cells cultured under photosynthetic or respiratory conditions, but oxidative stress stimulates its expression by an as yet unknown mechanism (Fig. 1). Similar oxidant-dependent inductions occur in other aerobes [11,16,38], suggesting that these reductases play general and important antioxidant roles in changing environments.

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References

[1] Drews, G. and Imhoff, J.F. (1991) in: Variations in Autotrophic

Life (Shively, J.M. and Barton, L.L., Eds.), pp. 51–97, Academic Press, New York.
[2] Dastoor, F.P., Forrest, M.E. and Beatty, J.T. (2001) J. Bacteriol. 179, 4559–4566.
[3] Gregor, J. and Klug, G. (1999) FEMS Microbiol. Lett. 179, 1–9.
[4] Storz, G. and Imlay, J.A. (1999) Curr. Opin. Microbiol. 2, 188–194.
[5] Pomposiello, P.J. and Demple, B. (2002) Adv. Microb. Physiol. 46, 319–341.
[6] Hochman, A., Figueredo, A. and Wall, J.D. (1992) J. Bacteriol. 174, 3386–3391.
[7] Cortez, N., Carrillo, N., Pasternak, C., Balzer, A. and Klug, G. (1998) J. Bacteriol. 180, 5413–5420.
[8] Tabares, L.C., Bittel, C., Carrillo, N., Bortolotti, A. and Cortez, N. (2003) J. Bacteriol. 185, 3223–3227.
[9] Li, K., Härtig, E. and Klug, G. (2003) Microbiology 149, 419–430.
[10] Arakaki, A.K., Ceccarelli, E.A. and Carrillo, N. (1997) FASEB J. 11, 133–140.
[11] Liochev, S.I., Hausladen, A., Beyer Jr., F. and Fridovich, I. (1994) Proc. Natl. Acad. Sci. USA 91, 1328–1331.
[12] Krapp, A.R., Rodriguez, R.E., Poli, H.O., Paladini, D.H., Palatnik, J.F. and Carrillo, N. (2002) J. Bacteriol. 184, 1474–1480.
[13] Krapp, A.R., Cortez, N., Palatnik, J.F., Tognetti, V.B., Valle, E.M. and Carrillo, N. (1997) in: Flavins and Flavoproteins (Stevenson, K.J., Massey, V. and Williams, C.H., Eds.), pp. 85–88, University of Calgary Press, Calgary.
[14] Bianchi, V., Haggard-Ljungquist, E., Pontis, E. and Reichard, P. (1995) J. Bacteriol. 177, 4528–4531.
[15] Yannone, S.M. and Burgess, B.K. (1998) J. Biol. Inorg. Chem. 3, 253–258.
[16] Pomposiello, P.J. and Demple, B. (2000) J. Bacteriol. 182, 23–29.
[17] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, 2nd Edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[18] Gennaro, G., Hubner, P., Sandmeier, U., Yakunin, A.F. and Hallenbeck, P.C. (1996) J. Bacteriol. 178, 3949–3952.
[19] Sedmak, J. and Grossberg, S. (1977) Anal. Biochem. 79, 544–552.
[20] Laemmli, U.K. (1970) Nature 227, 680–685.
[21] Orellano, E.G., Calcaterra, N.B., Carrillo, N. and Ceccarelli, E.A. (1993) J. Biol. Chem. 268, 19267–19273.
[22] Cullen, P.J., Kaufman, C.K., Bowman, W.C. and Kranz, R.G. (1997) J. Biol. Chem. 272, 27266–27273.
[23] Swem, L.R., Elsen, S., Bird, T.H., Swem, D.L., Koch, H.G., Myllykallio, H., Daldal, F. and Bauer, C.E. (2001) J. Mol. Biol. 309, 121–138.
[24] Isas, J.M. and Burgess, B.K. (1994) J. Biol. Chem. 269, 19404–19409.
[25] Fischer, F., Raimondi, D., Aliverti, A. and Zanetti, G. (2002) Eur. J. Biochem. 269, 3005–3013.
[26] Yakunin, A.F., Gennaro, G. and Hallenbeck, P.C. (1993) J. Bacteriol. 175, 6775–6780.
[27] Wan, J.T. and Jarret, J.T. (2002) Arch. Biochem. Biophys. 406, 116–126.
[28] Hurley, J.K., Morales, R., Martínez-Júlvez, M., Brodie, T.M., Medina, M., Gómez-Moreno, C. and Tollin, G. (2002) Biochim. Biophys. Acta 1554, 5–21.
[29] Carrillo, N. and Ceccarelli, E.A. (2003) Eur. J. Biochem. 270, 1900–1915.
[30] Bruns, C.M. and Karplus, P.A. (1995) J. Mol. Biol. 247, 125–145.
[31] Ingelman, M., Bianchi, V. and Eklund, H. (1997) J. Mol. Biol. 268, 147–157.
[32] Sridhar Prasad, G., Kresge, N., Muhlberg, A.B., Shaw, A., Jung, Y.S., Burgess, B.K. and Stout, C.D. (1998) Protein Sci. 7, 2541–2549.
[33] Masepohl, B. and Klipp, W. (1996) Arch. Microbiol. 165, 80–90.
[34] Jouanneau, Y., Meyer, C., Naud, I. and Klipp, W. (1995) Biochim. Biophys. Acta 1232, 33–42.
[35] Saeki, K., Suetsugu, Y., Tokuda, K., Miyatake, Y., Young, D.A., Marrs, B.L. and Matsubara, H. (1991) J. Biol. Chem. 266, 12889–12895.
[36] Yakunin, A.F. and Hallenbeck, P.C. (1998) Biochim. Biophys. Acta 1409, 39–49.
[37] Jeong, H.S. and Jouanneau, Y. (2000) J. Bacteriol. 182, 1208–1214.
[38] Yannone, S.M. and Burgess, B.K. (1997) J. Biol. Chem. 272, 14454–14458.
[39] Mansilla, M.C., Albanesi, D. and DeMendoza, D. (2000) J. Bacteriol. 182, 5885–5892.