A Glutathione Redox Effect on Photosynthetic Membrane Expression in *Rhodospirillum rubrum*

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Running title: Glutathione in *Rhodospirillum rubrum*

Keywords: *Rhodospirillum rubrum*, glutathione, redox signaling, photosynthetic bacteria

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The formation of intracytoplasmic photosynthetic membranes by facultative anoxygenic photosynthetic bacteria has become a prime example for exploring redox control of gene expression in response to oxygen and light. While a number of redox-responsive sensor proteins and transcription factors have been characterized in several species during the last years in some detail, the overall understanding of the metabolic events that determine the cellular redox environment and initiate redox signaling is still poor. In the present contribution we demonstrate that in *Rhodospirillum rubrum*, the amount of photosynthetic membranes can be drastically elevated by external supplementation of the growth medium with the low-molecular-weight thiol glutathione. Neither the widely employed reductant DTT nor oxidized glutathione did cause the same response, suggesting that the effect was specific for reduced glutathione. By determination of the extracellular and intracellular glutathione levels, we correlate the GSH/GSSG redox potential to the expression level of photosynthetic membranes. Possible regulatory interactions with periplasmic, membrane and cytosolic proteins are discussed. Furthermore, we found that *R. rubrum* cultures excrete substantial amounts of glutathione to the environment.
INTRODUCTION

It is well-established that the thiol-containing tripeptide glutathione (γ-Glu-Cys-Gly, GSH) is the most abundant redox buffer in all eukaryotic and in many prokaryotic cells and that GSH is critically important for the defense of oxidative stress which inevitably is associated with respiratory life. Various functions and mechanisms where GSH is involved in bacteria are summarized in a recent review (22). During many of these reactions, two molecules of GSH are oxidized to glutathione disulfide (GSSG) and the reduced form has to be recycled by the enzyme glutathione reductase at the expense of NADPH. A signaling role for GSH, however, is so far not readily established. In bacteria, the reversible formation of intramolecular or intermolecular disulfide bonds of cysteines of regulatory proteins appears to be a common motif in molecular redox switches. For example, the ArcB sensor of *E. coli* or the PpsR and RegB-type regulators in facultative anoxygenic photosynthetic bacteria undergo reversible thiol/disulfide switches when exposed to different redox conditions in vitro (21, 32). The latter group of bacteria (Rhodospirillaceae) has contributed significantly to the recent paradigms of redox signaling and control of gene expression due to its facultative phototrophic lifestyle which allows to adapt their energy metabolism to the redox conditions of the environment. Under conditions of limiting oxygen, the Rhodospirillaceae induce the biosynthesis of an extensive system of pigmented intracytoplasmic membranes (ICM) which harbor photosynthetic reaction centers and light-harvesting complexes. The major environmental factor that determines the amount of ICM is the availability of oxygen.
since aerobic growth conditions repress the transcription of photosynthetic genes (*puf*, *puc*, *bchl*, *crt* and others) and the formation of ICM (for a review see (2)).

In *Rhodobacter capsulatus*, the membrane-bound histidine kinase RegB is part of a global redox-responding two-component regulatory system which is involved in controlling gene expression for ICM biosynthesis and many other genes related to energy metabolism when oxygen becomes limiting (6). The redox signal for silencing the kinase activity of RegB has been identified to originate from the membranous ubiquinone (UQ) pool (33). In addition, a reversible intermolecular thiol/disulfide switch within the cytoplasmic region of RegB is critical for activity and implicates perception of cytosolic redox signals in addition to membrane-localized quinones (32). In these molecular aspects, RegB is similar to the global redox sensor ArcB of *E. coli* which was the first two-component histidine kinase where a regulatory effect of UQ was demonstrated (10) and where the oxidation of critical Cys residues by UQ to an intramolecular disulfide bond was suggested based on *in vitro* experiments (21). In both cases, however, the cellular reductant that reconverts the thiol/disulfide switch to produce the active kinase conformation under low (anaerobic or microaerobic) oxygen conditions, has not been identified yet.

A second mechanism of control of photosynthetic gene expression is mediated by conserved cytosolic repressor proteins designated as PpsR in *Rhodobacter sphaeroides* (7) and CrtJ in *Rhodobacter capsulatus* (28). Here, the importance of intramolecular disulfide bond formation as redox-sensing mechanism is somewhat controversial. Masuda et al., (23) determined the midpoint potential (*E_m*) for the disulfide bond formation of CrtJ to be -180 mV (at pH 7.0) by redox titration with GSH/GSSG and also
demonstrated the intramolecular disulfide bond occurs only in aerobic but not in anaerobic cells. In contrast however, Cho et al., (4) reported that in *R. sphaeroides* cells, both critical cysteines of PpsR constantly exist in the reduced thiol form irrespective of the oxygen and light conditions. Strikingly, the *E_m* value of PpsR, determined by Kim et al., (18) is much more negative than CrtJ with a value of -320 mV. Although the cellular reductant for reconverting the above mentioned disulfide proteins to the thiol conformation has so far not been addressed explicitly, an involvement of the GSH-glutaredoxin system has been shown in *R. capsulatus* (19). However, the effects on other cellular redox systems or a quantitative estimation of the cellular redox potential of the cytoplasm has not been conducted in that study, and it is still a compelling question how the occurrence of thiol/disulfide switches can function in the reducing environment of the cytoplasm. In the present study we determined the cellular [GSH]/[GSSG] ratio as a measure of the cellular redox environment in *Rhodospirillum rubrum*. The starting point was a series of ICM induction experiments closely following the study of Malpica et al., (21) for the ArcB redox sensor of *E. coli* which could be activated by externally supplied dithiothreitol (DTT) but not by GSH. A similar pattern would provide more evidence about the existence of analogous sensors controlling photosynthetic gene expression in *R. rubrum*. In *R. rubrum* it is particularly interesting that the amount of ICM is dramatically affected by the nature of the carbon growth substrate, a phenomenon still not completely understood at the metabolic level. A growth medium containing succinate and fructose as carbon sources (M2SF medium) results in strongly elevated expression of ICM under
semiaerobic dark conditions, reaching ICM levels which are equivalent to phototrophic cultures grown at low light intensities (11).

A homologue of the cytosolic PpsR repressor has been identified in *R. rubrum* in a previous study (1). However, the midpoint potential of this protein and its importance for redox regulation in *R. rubrum* has up to now not been experimentally resolved. In addition, for the different growth media, a correlation of UQ redox states and expression levels of ICM has been demonstrated previously (13), which suggests that a quinone-sensing signaling systems might be present also in *R. rubrum*. 
MATERIAL AND METHODS

Bacterial organism and growth conditions. Experiments with *R. rubrum* were performed with strain ATCC 11170. *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* were employed for comparison (see supplemental material). For growth experiments, M2S and M2SF media as described in (11) were used. M2S contains succinate as sole carbon source; M2SF offers two carbon substrates, fructose and succinate. Bacteria were grown in shaker flasks with 3 baffles on a Certomat BS1 rotary shaker (Sartorius, Goettingen, Germany). The oxygen supply was set by varying the ratio between surface and culture volume. For semiaerobic cultures, flasks were filled with half of their maximal volume, for aerobic growth only 1/5 of total volume was used. Photosynthetic cultures were grown in filled Pyrex flasks illuminated with tungsten light bulbs as described previously (12).

Quantification of cell growth and cell volume. Cell density was monitored at 660 nm and the production of photosynthetic membranes by measuring the absorbance of photosynthetic reaction centers and light-harvesting complexes at 880 nm with a UV/Vis spectrophotometer (V-670, Jasco, Tokyo, Japan) in a 1-cm-path-length cuvette. For determination of the cellular dry weight (CDW), 10 ml culture broth were harvested, washed with 0.98 % NaCl (w/v) then centrifuged for 10 min at 5,000 × g, and the cell pellet subsequently lyophilized in a freeze-dryer (Christ, Osterode am Harz, Germany) to dryness and weighed afterwards. From the measurements a conversion factor was derived where an optical density of $A_{660} = 1.0$ corresponds to 0.35 g CDW.
The application of the Nernst equation (see below) requires that concentrations of conjugate redox pairs are considered as molar concentrations. For this purpose the cell volume was estimated by determining the dry weight of 5 ml wet cell paste after freeze drying overnight in a lyophilizator (Christ). From the measurements a conversion factor was derived where 1 g cell dry weight (CDW) corresponded to 2.29 ml cell volume (average of three independent determinations with a standard deviation of 0.006. No correction was made for interstitial water).

Determination of GSH and GSSG. A modification of the glutathione reductase cycling assay (34) was applied for determination of reduced GSH and oxidized GSSG. Samples for GSH determination were obtained by centrifugation of 2 ml culture broth at 4 °C and 5,000 × g for 10 min. The cell pellets were washed two times with NaCl (0.98 %, w/v), then frozen and stored at -80 °C. For the extraction of intracellular GSH, 1.25 ml of 10 % (w/v) sulfosalicylic acid (SSA) were added and after resuspension, three freeze and thaw cycles followed. After another centrifugation step (as above), the supernatant was stored at -80 °C until measurement, and the pellets were extracted again to ensure, all GSH was extracted. For the measurement of GSH, acid extracts were diluted with assay buffer (see below) to achieve a final concentration of 1 % (w/v) SSA. For reduced GSH a simple colorimetric assay was used, which can be completed to a cycling assay by NADPH and glutathione reductase to determine also GSSG. Assay mixtures included: assay buffer (100 µl 0.1 M HEPES, 5 mM EDTA pH 7.5), 10 µl 5,5'-dithiobis(2-nitro-benzoic acid) (DTNB), 60 µl sample, standard or blank (assay buffer). After thoroughly mixing the absorbance was measured at 412 nm with a PowerWave microplate spectrophotometer (Biotek, Winooski, VT, USA). This value determines the
amount of reduced GSH in the samples. To determine the total amount of GSH and
GSSG, 30 µl of 10 mM NADPH including 10 U L\(^{-1}\) glutathione reductase in assay buffer
was added and the reaction kinetics was recorded. The slope of the kinetics correlates
with the total amount of glutathione in the sample. To calculate the amount of GSSG, the
amount of GSH has to be subtracted from total glutathione and divided by 2.

**HPLC analyses.** Concentration of succinate in the culture supernatants were
determined by HPLC at 210 nm using a quaternary Agilent 1100 high pressure liquid
chromatography (HPLC) system (Agilent, Palo Alto, CA, USA) equipped with a
photodiode array detector. 10 µl of the sample were injected and separated by reversed-
phase chromatography on an Inertsil ODS, 5 µm, 250 × 4.6 mm column (GL Sciences,
Torrance, CA, USA) and isocratic elution with 0.1 M ammonium dihydrogen phosphate
(pH 2.6) at a flow rate of 1 mL min\(^{-1}\). Ubiquinone-10 and ubiquinol-10 were determined
by HPLC as described previously (13).

**Calculations.** The redox potential at pH 7.0, \(E'\) (mV) of the cellular GSH/GSSG pool
was calculated according to the Nernst equation: \(E' = E^{0'} + \frac{RT}{nF} \ln Q\), where \(Q\) denotes
the ratio of \([GSH]^{stc}/[GSSG]/[GSSG]^{stc}/[GSH]^2\) with \([GSH]^{stc}\) and \([GSSG]^{stc}\) being 1 M
concentrations (standard conditions). Note that the explicit consideration of \([GSH]^{stc}\) and
\([GSSG]^{stc}\) results in consistency of the units. \(E^{0'}\) denotes the standard redox potential at
pH 7.0 (-240 mV for the GSH/GSSG couple (29)); \(R\) is the gas constant (8.31 J K\(^{-1}\) mol\(^{-1}\)
1); \(T\) is temperature (303 K); \(F\) is the Faraday constant (9.65 x 10\(^{4}\) C mol\(^{-1}\)), and \(n\) is the
number of electron transferred (\(n = 2\) for GSH/GSSG). Gibbs free energy for the
reduction of GSSG by NADPH was calculated from results of the present study and
published data (13) according to
\[ \Delta G' = \Delta G_0' + RT \ln \left( \frac{[\text{NADP}^+][\text{GSH}]}{[\text{NADPH}^+H^+][\text{GSSG}]} \right) \]

with \( \Delta G_0' = n F \Delta E_0' \).
RESULTS AND DISCUSSION

Effects of redox-active agents on photosynthetic membrane production. The starting point of the present study was to get more evidence about the molecular basis for the strongly elevated ICM expression when *R. rubrum* is grown in M2SF medium under semiaerobic dark conditions (11, 12, 13). We thus examined the effects of externally supplied redox compounds on *R. rubrum*, following the experiments performed by Malpica et al., (21) for characterizing the redox-sensing mechanism of ArcB in *E. coli*. In their study, stimulation with externally supplied reductants, resulted in different transcription levels of reporter genes with an ArcB/ArcA regulated promoter system. Application of the membrane permeating reductant DTT strongly increased reporter gene expression even at aerobic conditions. In contrast, reduced GSH had almost no effect, a results which was explained by the inability of the lipophobic GSH molecule to permeate through the *E. coli* cytoplasmic membrane, thus being incapable to interact with the cytosolic Cys residues of ArcB.

It therefore was very surprising that in our experiments, the response of ICM expression in *R. rubrum* to a similar treatment resulted in a pattern that was different to the previous results for ArcB. Fig. 1 shows the effects of four different redox-active agents on ICM formation in a series of cultivations with *R. rubrum*. Cells were grown under dark conditions with succinate (M2S medium) or succinate/fructose (M2SF medium) as carbon source(s). In all cultures, during the initial aerobic growth phase, the high ICM levels of the semiaerobically grown inoculum cultures declined to low basal levels due to oxygen repression. When diffusion of molecular oxygen to the growing...
culture became limiting, the semiaerobic expression of ICM was induced, indicated by now increasing $A_{\text{880}}/A_{\text{660}}$ ratios. Generally, with succinate as carbon source (M2S medium), the semiaerobic dark expression of ICM yields only low basal levels ($A_{\text{880}}/A_{\text{660}} = 0.7 - 0.8$), whereas the combination of succinate and fructose (M2SF medium) results in much higher ICM levels ($A_{\text{880}}/A_{\text{660}} = 0.9 - 1.1$) which when using conventional media are only observed in phototrophically-grown cultures. As illustrated in Fig. 1, the addition of 1 mM GSH to a culture grown with succinate at semiaerobic conditions resulted in a strong increase in the biosynthesis of ICM while growth was nearly unaffected. The elevated ICM expression was identical for applied GSH concentrations of 1 mM - 5 mM (data not shown). In contrast, the addition of GSSG had no effect (data not shown). Fig. 1 also shows that the effect of DTT was different than GSH. Cells exposed to 0.5 - 2 mM DTT were severely distorted in growth. In contrast to what we expected, and also in contrast to GSH treatment, ICM levels immediately heavily dropped upon administration of DTT. The $A_{\text{880}}/A_{\text{660}}$ curves in Fig 1. both suggests that DTT exerted some kind of thiol stress on *R. rubrum* which however had a transient course. After approx. 20 h, ICM expression recovered and 0.5 mM DTT yielded identical levels to the GSH-stimulated cells. Cells exposed to 2 mM DTT, maintained the lowered ICM levels for the rest of the cultivation. The administration of 1 mM L-Cys did not alter growth or ICM production. In addition to L-Cys, the two other amino acids L-Gly and L-Glu which compose the GSH tripeptide, when applied either as single compounds (1 mM) or all three amino acids together, had no effect (data not shown) thus indicating that the observed elevation of ICM was specific for GSH.
In a complementary experiment, the glutathione-depleting agent diethylmaleate (DEM) was added. DEM is conjugated with GSH by GSH-S-transferase (3), thus depleting the cellular pool of GSH but not of GSSG. Since we wanted to see whether DEM reduces ICM expression, we tested this compound with M2SF medium which normally produces high levels of ICM at semiaerobic dark conditions. Administration of 1 mM and 4 mM DEM resulted in a pronounced inhibition of growth, and ICM formation was significantly lowered to basal levels. Treatment with H$_2$O$_2$ or diamide which are expected to oxidize the GSH pool resulted in a similar decrease in ICM levels (data not shown).

The elevated ICM levels in GSH-stimulated cells, are strongly reminiscent to the high $A_{580}/A_{660}$ ratio achieved by using the M2SF cultivation medium. When this medium was employed the already high amounts of ICM could be increased only marginally by additional treatment with GSH. Furthermore, at aerobic growth conditions, GSH administration to a M2SF culture induced basal ICM biosynthesis even in the presence of normally inhibitory concentrations of molecular oxygen (data not shown).

In conclusion, the data presented in Fig. 1 demonstrate that externally added GSH enhanced ICM production in *R. rubrum* whereas treatment with GSH-depleting agents resulted in attenuated ICM production.

**Determination of extracellular and intracellular GSH.** The effect of GSH supplementation raises the question about the cellular targets and molecular mechanisms underlying the observed stimulation of ICM expression. Taken the impermeability of the inner cytoplasmic membrane for GSH into account, as argued by Malpica et al., (21), it seems possible that the effect may be caused by interactions with periplasmic...
components and subsequent transmembrane redox signal transduction. In *E. coli* a periplasmically located dithiol oxidase DsbA, catalyzes the transfer of electrons from the periplasmic compartment to the membranous UQ pool via the membrane protein DsbB during oxidative protein folding (16). In the *R. rubrum* genome sequence, four DsbA oxidoreductase genes and one DsbB homolog are present (see Table S1 in the supplemental material for gene annotations). Consequently, it appears possible that reduction of DsbA by externally supplied GSH would allow to activate sensor kinases of the RegB/ArcB type indirectly by reduction of the UQ pool. We therefore determined the redox state of UQ\textsubscript{10} in cell extracts by HPLC-MS as previously described (13) after GSH-treatment of the culture. In the control cultures the total pool of UQ\textsubscript{10} was 42.0 % reduced whereas upon administration of 5 mM GSH, the UQ pool was in fact more than 60 % reduced. We note that GSH was not able to reduce UQ\textsubscript{10} directly in isolated chromatophores, due to the inaccessibility of the polar GSH molecule to the lipophilic membrane compartment (data not shown). The above finding would be in agreement with externally applied GSH leading to increased UQ sensor kinase activity by partial reduction of the UQ pool with transfer of reducing equivalents via known periplasmic disulfide bond formation proteins. The scenario also would not require GSH to enter the cytosolic space, thus satisfying the previous argument of Malpica et al., (21) for ArcB in *E. coli*. It has, however, to be noted that a number of studies have demonstrated the occurrence of GSH uptake by different transport systems (ABC transporters (CydDC) (27), *ybiK/spt* gene products (25), *yli* gene products (31)) during the last years, which allow to actively import, as well as to excrete GSH from *E. coli* cells. A protein BLAST search revealed the presence of homologous transport systems also in the *R. rubrum*
genome sequence (see Table S1 of the supplemental material). We therefore had to consider additional mechanisms with a cytosolic mode of action of GSH, and quantitatively analyzed the cellular GSH/GSSG pool after exposure to external GSH, as well as in untreated control cultures. The data summarized in Table 1 were obtained with untreated cells as a reference dataset of variations of the cellular GSH/GSSG redox status in response to three different growth conditions: aerobic cultures (M2S medium, no ICM biosynthesis), semi-aerobic cultures (M2SF medium, high-level expression of ICM), and anaerobic photosynthetic cells (M2S medium). Samples were taken from batch cultures at late exponential phase, assumed to represent pseudo-steady-state conditions. It was found that the total intracellular concentration of GSH (which is \([\text{GSH}] + 2[\text{GSSG}]\)) varied, depending on the growth conditions, between 6.7 \(\mu\text{mol g}^{-1}\text{CDW}^{-1}\) and 7.48 \(\mu\text{mol g}^{-1}\text{CDW}^{-1}\) (Table 1), thus confirming the range of a previous determination by Fahey et al., (9) for \(R. \text{rubrum}\), who however did not determine reduced and oxidized forms separately. In our cultivations we were able to detect oxidized GSSG only when M2S medium and aerobic growth conditions were applied. The ratio of \([\text{GSH}] / [\text{GSSG}]\) was 32.2, meaning that about 94% of the total amount of GSH was in the reduced thiol form even under aerobic conditions where ICM biosynthesis was repressed by oxygen.

In addition to the steady-state values of Table 1, the time course of \([\text{GSH}]\) and [GSSG] was monitored during semiaerobic batch cultivations with M2S and M2SF media (Fig. 2, Fig. 3). In the control cultivations not exposed to external GSH, the intracellular GSH pool sizes stayed between 5 - 8 \(\mu\text{mol g}^{-1}\text{CDW}^{-1}\) throughout the cultivations in both media compositions (Fig. 2D, Fig3D) which is in accordance with the data of Table 1. GSSG constantly was below the detection limit, indicating a completely (>99%) reduced...
intracellular GSH pool. Interestingly, starting at the exponential growth phase, GSH was found to accumulate in the extracellular growth medium, reaching a final concentration of 6 µM in M2S medium (Fig. 2C) and even 12.6 µM in M2SF medium (Fig. 3C), respectively. Although it has been described before that some bacteria excrete GSH in substantial amounts into the environment, we are not aware that this feature has hitherto been noticed in \( R. \ rubrum \) or any other photosynthetic bacterium. Eser et al., (8), found about 30 % of total GSH outside the cell in \( E. \ coli \) cultures and high concentrations (up to 10 µM) of GSH accumulated in supernatants of logarithmically growing \( E. \ coli \) and \( Salmonella \ typhimurium \) according to Owens and Hartman, (24). In all cases, the biological function of GSH excretion has so far remained enigmatic.

Fig. 2 shows data obtained with M2S media, where 1 mM GSH was added. However, due to partial oxidation of the GSH preparation, the effective concentration of GSH was only about 0.6 mM and the stimulation of ICM expression was therefore weaker than in Fig. 1. It does however not affect the major results of Fig. 2. In both cultivations depicted in Fig. 2 and Fig. 3, the administered GSH was depleted from the culture media accompanied by rapidly rising intracellular GSH levels and by the induction of ICM expression. With M2SF medium, the intracellular concentration of GSH increased from about 10 µmol g\(^{-1}\) CDW\(^{-1}\) to 50 µmol g\(^{-1}\) CDW\(^{-1}\). (Fig. 3D). In contrast to untreated cells, GSSG was now detectable in cell extracts at about constant levels with 1.6 – 1.9 µmol g\(^{-1}\) CDW\(^{-1}\) throughout the cultivation. It is interesting that with M2SF medium, the onset of GSH uptake and concomitant increase in the intracellular GSH and GSSG was initiated only after fructose was consumed almost completely from the growth medium, while succinate was still present (Fig. 3C).
This finding suggests that during the initial growth period, fructose degradation yielded sufficient reducing equivalents to lower the cellular redox potential, thus inducing high ICM production and preventing GSH to enter the cell. After the consumption of fructose, the low redox potential required for high level production of ICM was maintained by the influx of GSH.

In conclusion, the depletion of administered extracellular GSH in combination with the more than 5-fold increase in intracellular GSH, is a clear indication that the cells were capable to take up GSH by active transport mechanisms. From the data, a specific GSH uptake rate of 0.041 mM h\(^{-1}\) g\(^{-1}\) CDW\(^{-1}\) was calculated for the experiment with M2SF medium (Fig. 3). Although the more than 5-fold increase of the intracellular GSH concentration after external GSH addition, the direct uptake of GSH however cannot account for the nearly complete depletion of GSH from the cultivation medium. While the intracellular increase of GSH was about 40 µmol g\(^{-1}\) CDW\(^{-1}\), a much larger amount of more than 13 mmol g\(^{-1}\) CDW\(^{-1}\) vanished from the culture supernatant during the same time period (M2SF medium, Fig. 3). We do not know what happened to the majority of the added GSH; extracellular oxidation to GSSG however was not detected. One possibility would be that a large portion of the supplemented GSH was degraded by extracellular and/or intracellular enzymes such as γ-glutamylpeptidases (30) to fuel salvage pathways for amino acids. The successive uptake of fructose and GSH could therefore also be interpreted as a diauxic growth phenomenon where GSH is used as a growth substrate after the preferential consumption of fructose. However, the possible role as a source for carbon or amino acids is unlikely to account for the elevated ICM production because as mentioned above the admistration of the individual amino acids of
GSH did not affect growth or ICM levels. It is also important to note that in a cultivation where GSH (20 mM) was employed as sole carbon source, *R. rubrum* was unable to grow (data not shown). Furthermore, the supplementation with GSH did not increase growth rate or final cell densities (Figs. 1-3). It is therefore unlikely that the putative utilization of GSH as a source of amino acids did account for the elevation of photosynthetic gene expression. The results rather suggest that the accumulation of GSH in the cytosolic compartment of *R. rubrum* alters the cellular redox potential thus elevating redox-dependent ICM expression. Consequently, direct interactions of GSH with soluble redox-regulatory proteins have to be considered in addition to membrane or periplasmic signal transduction.

**Assessment of the redox environment in the light of the cellular GSH pool and its implication for redox control.** The ratio of [GSH]/[GSSG] has frequently been employed as quantitative measure for the cellular redox state and its reducing capacity can be expressed in terms of redox potential using the Nernst equation as a tool. On a Nernst scale, the concentrations given in Table 1 for aerobic growth correspond to a GSH redox potential $E'_G$ of -209 mV. Applying more reducing growth conditions (semiaerobic growth and anaerobic photosynthetic growth, respectively) resulted in even higher levels for GSH and the failure to detect GSSG in cell extracts. These results strongly suggest that the [GSH]/[GSSG] ratio was shifted towards the reduced state in these cells, and GSSG levels were below the detection limit of the applied analytical method. Comparison with published data of GSH redox states in other bacteria shows that generally the ratio of [GSH]/[GSSG] covers a range from 50 to 300 (8, 14, 15). Applying a lowest ratio of 300 to our semiaerobic and anaerobic photosynthetic cultures, would
yield theoretical ambient redox potentials of -236 mV and -252 mV, respectively. This rough estimation would mean that the total GSH/GSSG pool size is more than 99% reduced in semiaerobic and anaerobic cells, consistent with published data for other bacteria, e.g. *R. sphaeroides* (14). In *R. capsulatus*, [GSH]/[GSSG] ratios according to (23) were virtually unaffected by the oxygen conditions and corresponded to -222 mV aerobically and -224 mV anaerobically.

The intracellular GSH/GSSG redox potential calculated from the data presented in Fig. 3, covers a span of 35 mV from the highest value of -182 mV in aerobic cells (calculated at t = 18 h) where no ICM were expressed, down to -217 mV in cells with induced ICM production after 45 h cultivation time. An important consequence of applying the Nernst equation for estimating the cellular redox environment is that, while the half cell potential \( E_h \) of most redox couples e.g. NAD(P)H/NAD(P)^+, UQH_2/UQ is determined exclusively by the ratio of the reduced and oxidized species, the redox potential of the GSH/GSSG couple depends on both the reduced/oxidized ratio as well as the absolute concentration of GSH. The reason for this difference is the change in molarity when on GSSG is converted to 2 GSH with the implication that GSH enters the Nernst equation as a squared term ([GSSG]/[GSH]^2) (29). An important consequence for the role of GSH as cellular redox buffer is that comparatively small alterations of the redox status of the GSH pool result in larger changes in the overall reducing capacity than would occur with other (equimolar) conjugate redox pairs. For example, the 35 mV decrease in \( E_h \) after stimulation with GSH calculated from the data of Fig. 3, was accompanied with an about 8-fold increase in the [GSH]/[GSSG] ratio. To achieve the
equivalent 35 mV change in the reducing capacity, the \([\text{NADH}^+ + \text{H}^+] / [\text{NAD}^+]\) ratio for instance would have to change more than 15-fold instead.

A correlation of ICM content and the redox status of the GSH pool is apparent from the data presented in Table 1, Fig. 2 and Fig. 3. Redox states of -209 mV (Table 1) and -182 mV (calculated from Fig. 3) were found to correspond with ICM biosynthesis being repressed by oxygen (ratio \(A_{880}/A_{660} = 0.55\) in virtually unpigmented cells). Since on the other hand, high levels of ICM were produced at -217 mV (Fig. 3), we conclude that the critical cytosolic redox potential for inducing photosynthetic gene expression is at about -210 mV on a GSH/GSSG scale.

The cellular redox potential in \(R.\ rubrum\) has been found previously to be poised around 310 – 320 mV for the NADH/NAD\(^+\) couple, and 330 – 335 mV for NADPH/NADP\(^+\) (13). Comparison of the redox potential of the GSH/GSSG pool with these data hence demonstrates that both redox systems are not in equilibrium under the conditions of living cells. Since in \(R.\ rubrum\) no glucose-6-phosphate dehydrogenase as major cellular source for providing NADPH + H\(^+\) is present (12), glutathione reductase probably receives its NADPH substrate mainly by pyridine nucleotide transhydrogenase (17) and hence ultimately oxidizes NADH by reduction of GSSG. For semiaerobic conditions this pattern can be supported now by experimental data where the extent of reduction increases in the order NADH (46.5 % reduced (13)) < NADPH (73.4 % reduced (13)) < GSH (more than 99.9 % reduced (this study). With the now available data it can be calculated that the reduction of the GSH pool at the expense of NAD(P)H is thermodynamically favorable with a \(\Delta G^\prime\) value of -13 kJ Mol\(^{-1}\) which is crucial for keeping the GSH/GSSG pool in the reduced state.
In summary, the data presented above demonstrate that the amount of expressed ICM responds specifically to the external supplementation with GSH, and is correlated with the redox status and concentration of the cytosolic GSH/GSSG pool.

At present the cellular targets and mechanisms for GSH in *R. rubrum* are essentially unclear. A regulatory effect rather than only a metabolic role is however indicated by the above mentioned facts that GSH as sole growth substrate was not sufficient to support growth and that amino acids (Glu, Cys, Gly) did not affect growth or ICM levels in control experiments.

Considering published data in combination with genome sequence database analysis (supplemental material), a hypothetical network of interacting redox components of different cellular compartments emerges as proposed in Fig. 4. GSH enters the periplasmic space via outer membrane porins, and reducing equivalents are transferred from periplasmic thiol-proteins of the DsbA to the membranous UQ pool. The partial reduction of UQ subsequently releases the inhibition of UQ sensor kinases thus enhancing the expression of ICM components. The further transport of GSH into the cytosol subsequently lowers the cytosolic GSH/GSSG redox potential considerably (below -210 mV) enough to reduce regulatory disulfide proteins. Although, the midpoint potential of the PpsR homologue in *R. rubrum* has not yet been determined and its actual importance for the observed GSH effect remains speculative, the most simple and straightforward interpretation includes a direct response of the redox status of this protein. That GSH is the cellular reductant for conversion of CrtJ into the inactive thiol conformation has already been proposed by Masuda and co-workers (23) for *R. capsulatus*, based on the demonstration that GSH is capable to reduce CrtJ disulfide.
bonds in vitro. If we assume redox equilibrium between GSH/GSSG and the regulatory proteins included in Fig. 4, both RegB ($E_m = -294$ mV (32)) and PpsR ($E_m = -320$ mV (18)) would be more than 90% in the oxidized disulfide form over the 35 mV span of GSH/GSSG in Fig. 3. In contrast, because of its higher midpoint potential, a CrtJ-type repressor ($E_m = -180$ mV (24)) would be affected significantly and would switch from 76% being in the inactive reduced thiol form at aerobic GSH/GSSG values, to almost complete inactivation of the repressor with 98% being reduced under semiaerobic conditions.

It is important to note here that in both *R. capsulatus* as well as *R. sphaeroides*, the identical treatment with GSH did not result in elevated photosynthetic complexes (see supplemental material). In accordance, intracellular levels of total GSH did not accumulate after exposure to external GSH but maintained at about 4.5 µmol g$^{-1}$ CDW$^{-1}$ (standard deviation 0.06) in *R. sphaeroides*, and at about 4.2 µmol g$^{-1}$ CDW$^{-1}$ (standard deviation 0.3) in *R. capsulatus* (see supplemental material). It has to be assumed therefore that these species differ to *R. rubrum* with respect to the mechanisms for efficient uptake of GSH from the environment.

We are aware that the scenario outlined in Fig. 4 is probably much oversimplified and that further experimental verification is required, e.g. by investigating mutants where components of the redox network have been deleted. We also have ignored thioredoxins and glutaredoxins which clearly are important players in the thiol/disulfide network (19, 26) and which may be interconnected in the outlined signal transduction pathways.

In addition, it appears also possible that the GSH/GSSG redox potential acts by modulating the activity of metabolic enzymes alternatively or in parallel to transcriptional
effects. It is interesting in this respect that activation of the first enzyme of the tetrapyrrole biosynthetic pathway, δ-aminolevulinic acid synthase, was demonstrated to occur by reduction of several Cys residues using low molecular weight thiols in vitro (5). The *R. rubrum* enzyme in total contains 6 Cys residues (see supplemental material for gene annotation). A decrease of the cellular GSH/GSSG redox potential could therefore be important for the posttranslational regulation of bacteriochlorophyll biosynthesis including the stimulation of this enzyme.

The GSH effect is strongly reminiscent of the similar ICM elevation with M2SF medium which was also not found in these species (13). An intriguing possibility would be that in *R. rubrum* both growth conditions may essentially induce the same redox signaling pathways and lower the GSH/GSSG redox state either by reducing equivalents originating from fructose catabolism (via transhydrogenase and glutathione reductase) or directly from the accumulation of cellular GSH. The absence of the succinate/fructose (M2SF medium) effect in the Rhodobacter species, however, makes it likely that up to now unidentified regulatory proteins and circuits, not included in Fig. 4, are involved in *R. rubrum*. The identification of the target proteins of GSH in *R. rubrum* will be a subject of further studies.

We finally mention that the present study has also two implications for biotechnology. First application of GSH to cultivation raises the amount of ICM and could be applied for enhancing ICM-associated products such as photosynthetic pigments or membrane proteins. Second, since GSH itself has become a product of industrial interest (20), the levels of the total pool sizes could be attractive in comparison to species which were already explored for this application. At present the fermentative production
of GSH uses predominantly *Saccharomyces cerevisiae* and a recent study reports that genetic engineering could improve GSH production with *S. cerevisiae* to final intracellular GSH concentrations of 80 mg L\(^{-1}\) (35). However, much higher product yields (up to 4,300 mg L\(^{-1}\)) are listed in a review on biotechnological GSH production (20). In combination with recently established high cell density cultivation of *R. rubrum* (36) the data of Table 1 can be extrapolated to a theoretical total yield of GSH of about 130 mg L\(^{-1}\) intracellularly and 221 mg L\(^{-1}\) excreted into the environment. Optimization of strains and media conditions might be used for further increasing the productivity.

**Acknowledgement.**

We thank Ruxandra Rehner and Melanie Säger for technical assistance. This study was supported by the FORSYS (research units in systems biology) initiative of the German Federal Ministry of Education and Research (grant No. 313922).
REFERENCES


TABLE 1. GSH/GSSG concentrations, redox potentials and levels of ICM in *R. rubrum* under different growth conditions. Aerobic cells were cultivated in M2S medium (no ICM expression), semiaerobic cells were from M2SF medium with (ICM expression induced). Phototrophic cells were cultivated with M2S medium.

<table>
<thead>
<tr>
<th></th>
<th>GSH (µmol/g CDW)</th>
<th>GSSG (µmol/g CDW)</th>
<th>[GSH]/[GSSG]</th>
<th>$E'_h$ (mV)</th>
<th>ICM ($A_{880}/A_{660}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic</td>
<td>6.30 ± 0.35</td>
<td>0.20 ± 0.04</td>
<td>32.18</td>
<td>-209</td>
<td>0.55</td>
</tr>
<tr>
<td>Semiaerobic</td>
<td>5.64 ± 0.23</td>
<td>$-0.02^*$</td>
<td>$-300^*$</td>
<td>$-236^*$</td>
<td>0.80</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>7.48 ± 0.96</td>
<td>$-0.01^*$</td>
<td>$-300^*$</td>
<td>$-252^*$</td>
<td>1.50</td>
</tr>
</tbody>
</table>

* concentrations of GSSG estimated by applying an assumed [GSH]/[GSSG] ratio of 300.
FIGURES LEGENDS

FIG. 1. Effects of externally supplied redox compounds on growth ($A_{660}$) and ICM expression ($A_{660}/A_{660}$) in *R. rubrum*. Symbols: GSH panels: $\Delta$, control; ■, 1 mM GSH. DTT panels: $\Delta$, control; ●, 0.5 mM DTT; ○, 1 mM DTT; ♦, 2 mM DTT. L-Cys panels: $\Delta$, control; ▲, 1 mM L-Cys. DEM panels: $\Delta$, control; ▲, 1 mM DEM; ◊, 4 mM DEM. The compounds were added to batch cultures at the time points indicated by black arrows. All cultivations were with M2S medium, except the DEM cultivations which were with M2SF medium to see an inhibiting effect of DEM on ICM production.

FIG. 2. GSH time courses in cell extracts (GSH$_{ic}$) and in the extracellular culture fluid (GSH$_{ec}$) during batch cultivations of *R. rubrum* with succinate (M2S) medium. White symbols: control cultivation without externally supplied GSH. Black symbols. GSH was added after 21 h. Squares, $A_{660}$; triangles, $A_{680}/A_{660}$; diamonds, GSH extracellular (GSH$_{ec}$); circles, GSH intracellular (GSH$_{ic}$); open diamonds, GSSG intracellular (GSSG$_{ic}$) in GSH-supplemented cells. Data points for GSH$_{ic}$ are mean values of three cell extracts with standard deviation indicated as error bars.

FIG. 3. GSH time courses in cell extracts (GSH$_{ic}$) and in the extracellular culture fluid (GSH$_{ec}$) during batch cultivations of *R. rubrum* with succinate/fructose (M2SF) medium. White symbols: control cultivation without externally supplied GSH. Black symbols: GSH was added after 21 h. Squares, $A_{660}$; triangles, $A_{680}/A_{660}$; diamonds, GSH
extracellular (GSH\textsubscript{ec}); circles, GSH intracellular (GSH\textsubscript{ic}); open diamonds, GSSG
intracellular (GSSG\textsubscript{ic}) in GSH-supplemented cells; asterisks, fructose; crosses, succinate.
Data points for GSH\textsubscript{ic} are mean values of three cell extracts with standard deviation
indicated as error bars.

FIG. 4. Hypothetical model of metabolic and regulatory interactions of externally applied
GSH, resulting in enhanced expression of ICM. DsbB omitted for clarity. THase, transhydrogenase; GR, glutathione reductase; PP, periplasmic space; CM, cytoplasmic
membrane; CS, cytosol.
FIG. 2.
FIG. 4.

GSH

THase

NADH+H+

NADP+

NADPH+H+

NAD+

DSbA

UQ / UQH₂

UQ-synthase (Reg Tpy)

HS

SH

CM

PP

PHOSPHOSYNTHETIC GENE EXPRESSION

(puf, beh, cri, ...)

GSH

GS S

HS

SH

PpsR

CS