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Review

Bacterial hemoglobins and flavohemoglobins: versatile proteins and their impact on microbiology and biotechnology

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Abstract

In response to oxygen limitation or oxidative and nitrosative stress, bacteria express three kinds of hemoglobin proteins: truncated hemoglobins (tr Hbs), hemoglobins (Hbs) and flavohemoglobins (flavo Hbs). The two latter groups share a high sequence homology and structural similarity in their globin domain. Flavohemoglobin proteins contain an additional reductase domain at their C-terminus and their expression is induced in the presence of reactive nitrogen and oxygen species. Flavohemoglobins detoxify NO in an aerobic process, termed nitric oxide dioxygenase reaction, which protects the host from various noxious nitrogen compounds. Only a small number of bacteria express hemoglobin proteins and the best studied of these is from *Vitreoscilla* sp. *Vitreoscilla* hemoglobin (VHb) has been expressed in various heterologous hosts under oxygen-limited conditions and has been shown to improve growth and productivity, rendering the protein interesting for biotechnology industry. The close interaction of VHb with the terminal oxidases has been shown and this interplay has been proposed to enhance respiratory activity and energy production by delivering oxygen, the ultimate result being an improvement in growth properties.

Keywords: Nitrosative stress; Nitric oxide detoxification; Pathogenic bacteria; Microaerobic growth; Biotechnological application; Protein engineering

Contents

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1. Introduction

The importance of oxygen for life relates to both its primary use as a substrate and its secondary effects on metabolism. Its use as a substrate allows cellular metabolism to work at optimal levels of substrate utilization and energy yield. Oxygen is required for the regulation of a variety of cellular functions, which are expressed in response to oxygen or to the presence of noxious byproducts that accumulate during the aerobic lifestyle. Oxygen and cognate oxygen radicals can directly or indirectly affect bacterial cells in at least two ways: altering the function of specific proteins or affecting the biosynthesis of specific sets of proteins. The latter effects are largely due to the effects of oxygen on the function of global transcription factors.

In humans, the protein molecule most closely associated with the utilization of oxygen is hemoglobin (Hb), the best-characterized oxygen-binding protein both at the functional and the molecular level. The presence of this oxygen-binding protein had long been thought to be restricted to mammals, but recent findings indicate an almost ubiquitous existence of Hbs in mammals, non-vertebrates, plants, and bacteria. This review will address the current state of knowledge concerning bacterial globin and their role in cellular metabolism.

2. Globin proteins: an overview

Globins are encountered in all five kingdoms of life and are assigned to vertebrate and non-vertebrate Hb classes. The latter group includes the Hbs present in plants, fungi and protozoa. The common characteristic of all Hb proteins is their ability to reversibly bind oxygen. Although the alignment of the amino acid sequences of globins from various sources reveals a highly variable or even almost random primary amino acid sequence, two key residues are conserved among all globin proteins encountered so far: Phe at position CD1 (topological position, for nomenclature see [1]) and His at position F8. Analysis of protein structures of globins reveals a typical tertiary structure, the classical globin-fold [2]. This highly conserved structure consists of six to eight α-helical segments that are connected by short intervening loops. The three-on-three helix structure forms a sandwich-like assembly and binds the heme moiety within a cavity surrounded by hydrophobic residues.

Vertebrate globins are encountered circulating in body fluids, where they deliver oxygen, or are expressed in muscle tissues (myoglobin), where they donate oxygen to the respiring cells. Very recently, two hexacoordinate Hbs have been discovered in mammals: a Hb present preferentially in the brain called neuroglobin, and an ubiquitously expressed Hb designated histoglobin or, alternatively, cytoglobin [3–5]. Neuroglobin has been suggested to protect neurons from hypoxic–ischemic injury [6]. Both proteins show less than 30% identity with either Hb or myoglobin.

Non-vertebrate globins exist in manifold variations (from monomeric to multisubunit structures) and are present in widely different anatomical sites, such as in the cytoplasm of specific tissues or freely dissolved in various body fluids. Non-vertebrate globins display a much higher variability in primary and tertiary structures, which might reflect their adaptations to specific functions compared to their vertebrate homologs (reviewed in [7]).

Over the last dozen years, monomeric single domain globins have been found in symbiont-containing leguminous and non-leguminous plants, algae and a number of prokaryotes [8–12]. Chimeric globins consisting of an N-terminal globin domain and an adjacent, C-terminal redox-active protein domain, collectively called flavohemoglobins (flavoHbs), have been discovered in bacteria, yeasts and fungi [13–16]. Truncated globins (trHbs), which display a two-on-two helix architecture, are widely distributed in bacteria, unicellular eukaryotes and plants [17–20] (reviewed in [21]). TrHbs, which have previously also been designated as 'small Hbs' or myoglobin and first discovered in 1989 in *Paramecium caudatum*, have been suggested not to originate from the ancestral Hb, but to have evolved from a different protein [17,22].

3. Bacterial Hbs and flavoHbs

3.1. Identification of Hbs and flavoHbs

The obligate aerobic bacterium *Vitreoscilla* synthesizes a homodimeric Hb (VHb) [23]. Initially called cytochrome *o*, VHb was believed to be a soluble terminal oxidase in *Vitreoscilla* [23,24]. However, spectral, kinetic and structural properties proved the Hb character of this protein [8,24,25]. VHb is the best-characterized member of the group of bacterial Hb proteins. The *vhb* gene has been isolated and it encodes an oxygen-binding protein of 15.7 kDa [8,26,27]. Interestingly, when VHb was purified from *Vitreoscilla*, an NADH metHb reductase was co-purified [24,28]. Early work on VHb has been reviewed elsewhere and is therefore not described here [29].

By performing BLAST searches on finished and unfin-

Table 1
Compilation of bacterial Hb* and flavoHb genes[a]

Organism	Gene	Length (bp)	Reference/accession number
Aquifex aeolicus	*	420	AE000678.1
Campylobacter jejuni NCTC11168	*chb**	423	[30]; AL139079
Clostridium perfringens hyp27	Cp-*hb**	435	Farrés, J.[b]; AB028630
Novosphingobium aromaticivorans	*	450	NZ_AAAV01000175.1
Rhodopseudomonas palustris	*	426	NZ_AAAF01000001.1
Vitreoscilla sp.	*vhb**	441	[26,27]; M30794
Azotobacter vinelandii		1182	NZ_AAAD01000087.1
Bacillus anthracis A2012		1209	NC_003995.1
Bacillus halodurans C-125	*hmp*Bh	1236	Farrés, J.[b]; AB024563
Bacillus subtilis 168trpC2	*hmp*Bs	1200	[37]; D78189
Burkholderia fungorum		1245	NZ_AAAC01000178.1
Burkholderia sp. TH2		1182	AB035325.1
Corynebacterium glutamicum ATCC 13032		1161	NC_003450.2
Deinococcus radiodurans	*hmp*Dr	1212	[30]; AE001863
Erwinia chrysanthemi	*hmp*X	1188	[158]; X75893
Escherichia coli MG1655	*hmp*	1191	[34]; X58872
Magnetospirillum magnetotacticum		1242	NZ_AAAP01001552.1
Nostoc punctiforme		1320	NZ_AABC01000078.1
Oceanobacillus iheyensis		1221	NC_004193.1
Pseudomonas fluorescens		1182	NZ_AABA01000076.1
Pseudomonas aeruginosa PAO1	*hmp*Pa	1182	[30]; AE004695
Ralstonia eutropha	*fhp*	1212	[33]
Ralstonia metallidurans		1290	NZ_AAAI01000369.1
Ralstonia solanacearum		1206	NC_003295.1
Salmonella enterica serovar Typhi	*hmp*St	1191	[30]; AL627275
Salmonella enterica serovar Typhimurium	*hmp*Stm	1191	[35]; AF020388
Shigella flexneri 2a str. 301		1191	NC_004337.1
Sinorhizobium meliloti	*hmp*Sm	1212	Farrés, J.[b]; NC_003037
Staphylococcus aureus N315		1146	AP003129.2
Staphylococcus aureus subsp. aureus MW2		1146	NC_003923.1
Staphylococcus. aureus MU50		1146	NC_002758.1
Streptomyces coelicolor A3	*hmp*A2	1212	Farrés, J.[b]; AL354616.1
Streptomyces coelicolor A3	*hmp*A	1197	Farrés, J.[b]; AL158061
Streptomyces coelicolor A3 cosmid J11		1308	AL109949.1
Thermobifida fusca		1281	NZ_AAAQ01000041.1
Vibrio cholerae		1185	AE004358.1
Vibrio parahämolyticus		1185	U09005.1
Xylella fastidiosa 9a5c		1194	AE003859.1NC_002488.1
Yersinia pestis OC92		1191	AJ414154.1

[a]The deposited genome data can be accessed at http://www.ncbi.nih.gov. Hb-encoding genes and putative Hb sequences are marked with an asterisk *.
[b]Personal communication.

ished bacterial genomes using VHb as query sequence, further bacterial Hb proteins can be readily identified (Table 1). We have isolated the Hb coding genes from *Campylobacter jejuni* (CHb) and *Clostridium perfringens* in our laboratory and the latter is being characterized currently [30] (Farrés, J., personal communication). CHb has been analyzed experimentally for its potential use in improving growth under oxygen limitation or protection from nitrosative and oxidative stress [30,31].

The strictly respiratory, denitrifying bacterium *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) synthesizes an alternative form of bacterial globin: a flavoHb termed FHP [13]. The coding sequence for this protein is located on a plasmid [32]. The *fhp* gene was isolated from a megaplasmid library, using FHP-specific antibodies and oligonucleotide probes based on the N-terminal polypeptide sequence of a yeast flavoHb, determined previously by Zhu and Riggs [14]. The *fhp* gene encodes a soluble monomeric polypeptide of 403 amino acids, having a molecular mass of 44 kDa [33].

The flavoHb from *Escherichia coli* (HMP) was isolated in an attempt to identify dihydropteridine reductase activities and to date it is the best-characterized member of the flavoHb protein family [34].

FlavoHbs from *Erwinia chrysanthemi*, *Bacillus subtilis*, *Salmonella enterica* serovar Typhimurium and *Mycobacterium tuberculosis* have been isolated and characterized [35–39]. Other flavoHb proteins have recently been cloned from *Deinococcus radiodurans*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *S. enterica* serovar Typhi (Table 1) [30]. FlavoHbs typically possess a molecular mass of approximately 44 kDa.

Table 2
Binding of oxygen and nitric oxide to various Hb and flavoHb proteins

Ligand pair[a]	Association (mM^{-1} s^{-1})	Dissociation (s^{-1})	Reference
O_2			
HMP	38	0.44	[75]
HMP (Tyr-B10→Phe)	50	34 (3.3)[b]	[74]
VHb	78	5000	[25]
	200	4.2 (1.5)[b]	[64]
FHP	50	0.2	[75]
NO			
HMP (Fe(II))	26	0.0002	[75]
HMP (Fe(II)) (Tyr-B10→Phe)	33 (2.8)[b]	0.0001	[74]
FHP (Fe(II))	10–20		[75]
HMP (Fe(III))	44	4000	[75]
HMP (Fe(III)) (Tyr-B10→Phe)	42 (5.2)[b]		[74]
FHP (Fe(III))	2.4	1200	[75]

[a]Ligand-binding pair, O_2 exclusively binds to the heme in the ferrous form, whereas NO binds to the ferrous (Fe(II)) and the ferric (Fe(III)) heme iron.
[b]Value in brackets represents slow phase in a biphasic process.

A compilation of bacteria carrying Hb and flavoHb genes is shown in Table 1. The genes have either been isolated from these microorganisms or predicted to exist due to high sequence homology to currently known proteins. The alignment of the primary amino acid sequences reveals the occurrence of several conserved residues in both the Hb domain and in the redox-active domain (Fig. 1). The role of these conserved residues is discussed below.

3.2. Intracellular localization of VHb and HMP

Studies on the localization of heterologously expressed VHb in *E. coli* have revealed a distribution of the protein into both the cytoplasmic and periplasmic space, with up to 30% of the active protein being found in the latter [40]. The export of VHb into the periplasm was believed to be due to the presence of a 16-amino-acid-long sequence at the N-terminus of VHb that could direct the protein into the periplasm [40].

Analysis of the amino acid sequence revealed several unusual features not typically found in bacterial signal sequences. Instead of having positively charged residues at its amino terminus, it has a negative charge. The overall hydrophobicity of the central region of this putative signal sequence is significantly lower than that found in typical leader peptides due to the presence of charged residues not cleaved during or after the translocation process (since the region is an intrinsic part of the Hb protein) [40,41]. Fusions of the N-terminal sequence of VHb (16–23 amino acids) to *pho*A allowed the export of alkaline phosphatase into the periplasm of *E. coli* [40]. A similar distribution of VHb was observed in *Vitreoscilla* [40].

Recently, Webster and co-workers [42] analyzed VHb localization using electron microscopy in *Vitreoscilla* and *E. coli*. The results clearly indicate that VHb has mainly a cytoplasmic and not a periplasmic localization in both organisms. The authors suggest that the observed periplasmic localization of VHb might be due to the overexpression and extrusion of VHb and not due to active export [42]. It cannot be ruled out that the osmotic shock procedure used for the periplasmic sample preparation generated these putatively false results.

The N-terminal sequence of HMP is highly similar to that of VHb. HMP has also been found to localize into both the periplasmic and cytoplasmic spaces of *E. coli*, with approximately 30% of the total HMP amount present in the former. The distribution into cytoplasmic and periplasmic spaces was found in an HMP overproducing strain as well as in a strain bearing only a single chromosomal copy of *hmp*. In contrast to VHb, spectral analysis revealed that biochemically active HMP is exclusively found in the cytoplasmic fraction [43].

3.3. Structure of bacterial Hbs and flavoHbs

3.3.1. Globin domain

The three-dimensional (3D) structures of FHP, HMP and VHb have been resolved by X-ray crystallography [44–48]. The Hb domains of FHP, HMP and VHb adopt the classical globin-fold [2]. The heme molecule is embedded in a hydrophobic crevice formed by helices B, C, E, F, G and H. A rigid binding between the polypeptide chain and the porphyrin ring is provided predominantly by hydrophobic side chains pointing towards the proximal site of the heme molecule. No protein–heme interactions are detected at its distal site. A clear difference of these structures, relative to mammalian Hb, is the lack of helix D. The residues connecting helices C and E do not adopt the usual α-helical conformation but rather form a loop. The observed disorder in the CE region in FHP has been interpreted as a potential site of interaction between the globin domain and the FAD module of its reductase domain [45]. By analogy, this would provide a potential contact site for VHb with the NADH metHb reductase. [47,49]. A striking difference between VHb and HMP on

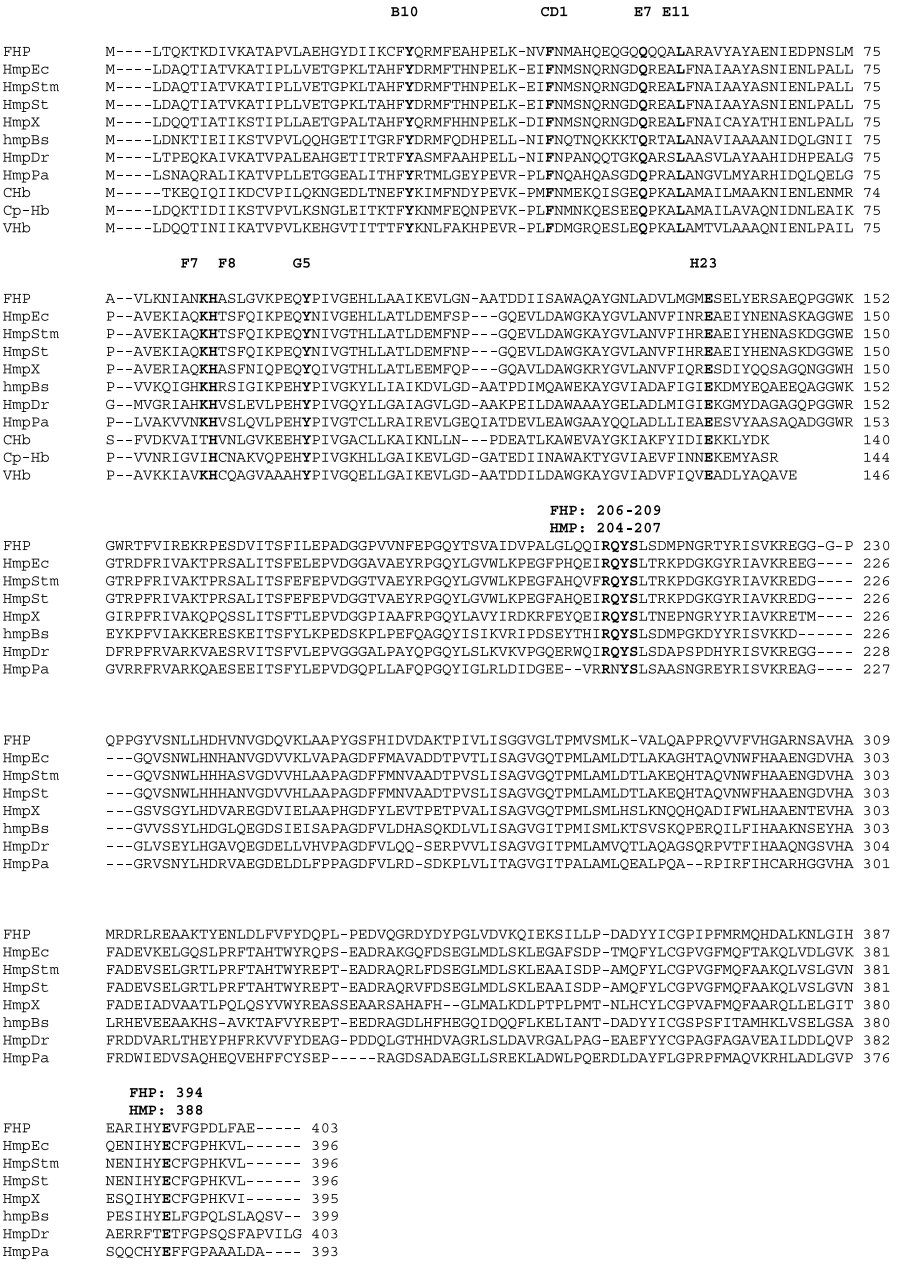

Fig. 1. Multiple amino acid alignment of flavoHbs and Hbs, which have recently been studied. VHb (*Vitreoscilla* [26,27]), HmpEc (*E. coli*, also called HMP [34]), FHP (*R. eutropha* [33]), HmpX (*E. chrysanthemi* [38]), HmpBs (*B. subtilis* [37]), HmpStm (*Salmonella* serovar Typhimurium [35,36]), CHb, HmpPa, HmpDr and HmpSt (*C. jejuni*, *P. aeruginosa*, *D. radiodurans*, *Salmonella* serovar *typhi* [30]) and Cp-Hb (*C. perfringens* (Farrés, J., personal communication)). Annotation of the globin domain is according to the topological positions, in the reductase domain according to the residue number. Important residues are given in bold. His-F8, Tyr-G5 and Glu-H23 form the catalytic triad at the proximal site. Tyr-B10 and Gln-E7 have been suggested to be involved in the stabilization of heme-bound dioxygen. Residues 206–209 (in FHP) and 204–207 (in HMP) are involved in FAD binding. Lys-F7, Glu (FHP: 394, HMP: 388) and Tyr (209, 207) are influencing the redox potential of the prosthetic heme and FAD groups.

one hand and the globin domain of FHP on the other hand is the displacement of helix E in FHP, opening the angle between helix E and F. The heme-binding crevice is therefore enlarged, thus providing more space at the ligand-binding site [45]. A phospholipid has been found to bind inside the hydrophobic crevice, but the function of this ligand is not known [50]. The negative charge of the phosphate group, which is positioned between the heme and the FAD group, is assumed to affect the electrochemical potential of the prosthetic groups and therefore also the electron transfer between the heme and the FAD moiety. Substitution of Ala-E14 for Tyr prevented phospholipid binding, possibly as a result of the Tyr side chain protruding into the lipid-binding site. In contrast, HMP displayed only weak affinity for phospholipids [50]. In HMP, the cavity formed by the CE loop was found to

harbor an anion, such as chloride or phosphate, which might participate in modulating the heme iron ligand-binding properties [48].

A number of residues, which are invariant in all known sequences, are encountered within the globin domain (Fig. 1). Hydrophobic clusters are formed around Phe-CD1 and Trp-H7. These two clusters are essential for folding and stability. The proximal site residues His-F8, Tyr-G5 and Glu-H23 have been proposed to form a hydrogen bond network, which might modulate the redox properties of the heme-iron atom [45]. Indeed, as proposed, the structural rearrangements at the proximal site relative to the classical globin-fold resemble the catalytic triad observed in cytochrome *c* peroxidase and are a strong support for a physiologically different role of flavoHb than pure oxygen storage and delivery [45,51]. Interpreting Raman spectroscopy data, it has been suggested that the imidazole ring of the proximal His-F8 possesses an imidazolate character due to a strong hydrogen bond to Glu-H23. This proximal hydrogen-bonding interaction in HMP provides a strong electronic push for the activation of the heme-bound oxygen [52]. Furthermore, infrared spectra of CO adducts of HMP and also VHb display an absorption maximum around 1960 cm^{-1}, which is similar to data obtained from cytochrome CO adducts and different from CO adducts of myoglobins [53,54].

Interestingly, Tyr-B10 and Gln-E7, previously suggested to be involved in heme ligand stabilization, were found to be shifted away from the heme in unliganded ferric HMP. Instead, the Leu-E11 side chain, protruding into the distal site, prevents interaction of Tyr-B10 with the heme ligand. Previously, the occurrence of two distinct forms of the distal heme pocket has been proposed: an open conformation without hydrogen bonding and a closed structure with strong hydrogen-bonding interactions between ligand and Tyr-B10 [52]. The resolution of the X-ray structure of HMP allowed Ilari et al. [48] to modify the above model and they proposed that a ligand-binding-induced displacement of the Leu-E11 side chain enables approaching and contacting between the heme ligand and the hydroxyl group of Tyr-B10 [48]. A similar conformational change of Leu-E11 was considered necessary for azide and thiocyanate binding to ferric VHb, as such a conformational change was clearly required to make the sixth iron-coordination position accessible for ligand binding [47,55]. Furthermore, Gln-E7 was not found to stabilize iron-bound ligands through hydrogen bonding in VHb [47]. The latter finding has been confirmed by analysis of ligand-binding properties of a VHb mutant where Gln-E7 had been replaced by Leu [56].

Whereas for VHb the occurrence of a dimeric form is well established, Stevanin et al. recently proposed that, due to the observed cooperativity in cyanide binding, HMP might also exist as a dimer in vivo [57]. The quaternary assembly of homodimeric VHb, which has not been observed before within the globin family, is based on a molecular interface defined by helices F and H of both subunits and essentially involves Van der Waals contacts among the two subunits. The two heme-iron atoms approach each other to a distance of 34 Å [47].

3.3.2. Reductase domain

In contrast to the classical globins, flavoHbs contain a C-terminal redox-active domain with binding sites for NAD(P)H and FAD. The reductase domain belongs to the ferredoxin $NADP^+$ reductase-like protein family [58]. The reductase domain can be subdivided into two structurally and functionally independent domains: a FAD-binding domain (residues 153–258 in FHP, 151–251 in HMP) and an NAD(P)H-binding domain (residues 266–403 in FHP, 259–396 in HMP). The former consists of a six-stranded antiparallel β-barrel, whereas the latter is built up of a five-stranded parallel β-sheet, flanked by two N-terminal helices and one C-terminal helix, respectively. The FAD group non-covalently contacts all three domains of FHP. Heme and FAD molecules approach each other to a minimal distance of 6.3 Å (FHP) and 5.9 Å (HMP), respectively, enabling electron transfer from FAD to the heme moiety in a predominantly polar environment, provided by several amino acid side chains [45,48]. The relative spatial orientation of the NAD(P)H-binding domain is changed, i.e. in HMP it is rotated clockwise compared to FHP. This produces a large interdomain cleft in HMP [48]. However, it is known that the relative spatial arrangement of FAD- and NAD(P)H-binding domains within members of the ferredoxin $NADP^+$ reductase-like protein family allows variance without disturbing the function [58].

Several residues are highly conserved in the reductase domain of flavoHbs (Fig. 1). Tyr (FHP: 208, HMP: 206) contacts Lys-F7, Glu (FHP: 394, HMP: 388) and the isoalloxazine ring of FAD; Gln (FHP: 207, HMP: 205) stabilizes a water molecule that bridges FAD and the heme moiety. Arg (FHP: 206, HMP: 204), Tyr (FHP: 208, HMP: 206) and Ser (FHP: 209, HMP: 207) contact the FMN moiety. In the NAD-binding domain, Glu (FHP: 394, HMP: 388) is highly conserved and its side chain contacts Lys-F7, forming a salt bridge. Lys-F7 and Glu (FHP: 394, HMP: 388) are part of a number of polar residues, whose predominantly large side chains originate from all three domains. These residues may influence the electrochemical potential of the prosthetic heme and FAD groups [45], and are considered to be essential for transport of electrons from FAD to the heme iron.

Lys-F7 is conserved in VHb, but is replaced by Thr and Ile in the Hb from *C. jejuni* and *C. perfringens*, respectively. The absence of the charged residue in the Hb from *C. jejuni* and *C. perfringens* might therefore prevent electron transfer from an external reductase, such as the metHb reductase present in *Vitreoscilla*, to the heme moiety as proposed for VHb [30].

Overall reaction:

$$NO + O_2 + NAD(P)H \longrightarrow NO_3^- + NAD(P)^+$$

Reaction proceeding at heme:

$$Fe^{+2}\text{-}O\text{-}O + N{=}O \longrightarrow [Fe^{+3}\text{-}OONO^-] \longrightarrow Fe^{+3} + NO_3^-$$

Fig. 2. The reaction mechanism of the HMP-catalyzed NOD reaction as outlined by Gardner et al. [167]. The reaction has been suggested to proceed via a heme-bound peroxynitrite [52].

3.4. Biochemical properties of Hbs and flavoHbs

Hbs are generally associated with oxygen binding and transport. Indeed, bacterial Hbs and flavoHbs are able to reversibly bind molecular oxygen. However, their structural properties (i.e. the occurrence of an intramolecular redox domain in flavoHb and the presence of the catalytic triad at the distal heme site in both Hb and flavoHb) may bring into question the role of bacterial Hb and flavoHb in simple oxygen storage and delivery.

3.4.1. Ligand binding

Bacterial Hb and flavoHb bind various heme ligands, such as O_2, CO, NO and CN^-. The observed absorption spectra are characteristic of Hb proteins [8,59–63]. Whereas oxygen and CO bind only to the ferrous heme, NO binds to ferrous and ferric heme, although the affinity for NO is strongly dependent on the oxidation state of the heme. Upon aerobic incubation of ferric HMP with NAD(P)H it is reduced to the oxygenated ferrous state. This oxy complex is not stable and, upon exhaustion of NADH with residual oxygen, decays into a form in which heme and flavin are oxidized. The reversibility of oxygen binding and the rapid reassociation kinetics after photodissociation confirm the globin-like features of HMP [62]. Addition of dithionite and nitrite to the ferric protein results in the formation of a nitrosyl complex [62,63].

3.4.2. Reaction kinetics of Hb and flavoHb

An overview of ligand binding kinetics and affinities of Hb and flavoHb is given in Table 2. For VHb two different values for oxygen release have been published. Orii and Webster reported a very fast release of oxygen from VHb ($k_{off} = 5600\ s^{-1}$), a value which is several folds higher than the rate of any other known Hb [25]. Recent data indicate a more moderate rate of oxygen release from VHb (biphasic release with $k_{off} = 4.2\ s^{-1}$ (fast phase) and 0.15 s^{-1} (slow phase)) [64]. This biphasic behavior of oxygen release from VHb has been associated with conformational changes occurring in response to the breakage of hydrogen bonds between heme-bound oxygen and Tyr-B10. The k_{off} values for VHb reported by Giangiacomo et al. [64] are in good agreement with the data obtained in our laboratory (Farrés, J., personal communication).

Replacement of the distal Gln-E7 residue by His had deleterious effects on oxygen-binding properties of VHb [56]. Binding of O_2 to the mutated protein is impaired (absence of a stable oxy form), suggesting that the distal His is coordinating the heme iron and transforming it to a hexacoordinated state. Interestingly, a change of Gln-E7 to Leu had little effect on oxygen-binding characteristics, indicating that the distal glutamine does not stabilize the bound oxygen with a hydrogen bond, a finding supported by the structural arrangement of the heme pocket [47,56].

3.4.3. Catalytic activities of flavoHbs and Hbs

HMP can mediate electron transfer either from NADH (via FAD) to a heme-bound ligand such as oxygen (or NO) or, in the absence of a ligand, from NADH (via FAD) to an external electron acceptor [65]. In the presence of oxygen and NADH, HMP has NADH oxidase activity, which leads to the rapid consumption of NADH and to the generation of superoxide and H_2O_2, both in vitro and in vivo [66,67]. Upon exhaustion of oxygen, the oxygenated species disappears to generate the deoxy-ferrous heme, whereupon the flavin becomes reduced [68]. Various electron acceptors are reduced by HMP in vitro, including dihydropteridine, ferrisiderophores, ferric citrate, cytochrome *c*, and Fe(III)-hydroxamate [34,65,69,70]. However, it is unknown if these reactions are of any biological significance in vivo [34,70]. Despite the lack of a reductase domain, purified, aerobic preparations of VHb generate hydrogen peroxide in the presence of NADH and O_2 in a 1:1 stoichiometry [71].

3.4.4. Aerobic detoxification of NO by flavoHb

Gardner et al. [72] identified an inducible, O_2-dependent, cyanide-sensitive, protective enzymatic activity that is able to shield the NO-sensitive aconitase in *E. coli*. Exposure of aerobically growing cells to NO elevated this activity. Gardner et al. [72] and Hausladen et al. [73] attributed the observed NO detoxification to HMP expression, which was shown to play a central role in the inducible response to nitrosative stress. The authors stated that NO detoxification consumes O_2 and NADH and converts NO and O_2 in equistoichiometric amounts to nitrate, and designated the activity as nitric oxide dioxygenase (NOD) (Fig. 2).

HMP-mediated NO degradation proceeds at a rate of 94 s^{-1} (20°C) and 240 s^{-1} (37°C) at an oxygen concentration of 200 μM and an NO concentration of 1 μM [74,75]. It has been suggested that NOD activity is hampered when the ratio of NO/O_2 exceeds 1:100 [74]. However, no significant reduction of NO-degrading activity was observed for HMP at NO/O_2 ratios of 1:20 and 1:10 [31]. Mills et al. [67] considered an O_2 concentration of 35–100 μM to be required for maximal oxygenase activity in vivo. Below an O_2 concentration of 30 μM, the NOD activity of HMP was ineffective [67]. Substitution of Tyr-B10, a residue proposed to stabilize the Fe^{2+}-O_2 complex, by Phe, Glu or His diminished the NOD activity of HMP approximately 15–35-fold.

Therefore, Hausladen et al. [76] suggested an alternative enzymatic reaction for HMP with NO: a denitrosylase activity (O_2 nitroxylase). They pointed out that due to the higher affinity of ferrous HMP for NO, relative to O_2, and the inability to bind O_2 in the ferric form, HMP would preferentially bind NO at biologically relevant O_2 concentrations [74,76]. During steady state turnover, HMP was found in the ferric state. The formation of a heme-bound nitroxyl equivalent and its subsequent oxidation is a possible novel enzymatic function of HMP. Despite the suggested NO inhibition of HMP activity [67,74], the denitrosylase reaction proceeds at a rate (at 40 μM NO and 10 μM O_2) which is only approximately 50% slower than the NO turnover at high O_2 concentrations of HMP.

The NOD activity of purified FHP was determined to be 7.4 s^{-1} (20°C) and 120 s^{-1} (37°C), showing that the NOD activity of FHP is more temperature dependent than that of HMP [75]. Furthermore, aerobic NO consumption in vitro (at 10 μM NO, room temperature) with a concomitant formation of nitrate was recently reported for the flavoHbs from *P. aeruginosa* (26 s^{-1}), *K. pneumoniae* (95 s^{-1}), *Salmonella typhi* (76 s^{-1}) and *B. subtilis* (68 s^{-1}). NO turnover of HmpBs was shown to be concentration dependent (10 μM NO: 68 s^{-1}; 20 μM NO: 128 s^{-1}) [31]. Interestingly, the NO degradation by *D. radiodurans* flavoHb was not significantly different from non-enzymatic NO decay [31].

NO-degrading activity of the single domain proteins VHb and CHb was also proposed to account for the observed protective effects of these proteins against nitrosative stress [31,77]. However, in vitro NO-degrading activity of cell extracts containing either of these proteins was not significantly different from control extracts, suggesting that a membrane-linked reductase could be involved in NO detoxification in vivo [31]. The influence of the reductase domain on NO-degrading activity has therefore been analyzed more thoroughly. The truncated hemoglobin domain of FHP (FHPg) (3.4 s^{-1}) displayed a four-fold lower NO turnover rate than the native FHP (15 s^{-1}) [31]. In contrast, engineered VHb protein, carrying an intramolecular reductase domain originating from FHP, increased the NO turnover rate by a factor of four, thus highlighting the importance of the reductase domain for the NO-degrading activity [31].

3.4.5. *Anaerobic conversion of NO by HMP*

Under anaerobic conditions, the reaction of NO with HMP leads to the generation of nitrous oxide (N_2O) with the concomitant consumption of NAD(P)H. NO binds to the active site of HMP and is reduced to the nitroxyl anion, which then reacts via a dimeric intermediate, forming N_2O. This reaction proceeds at a rate of 0.14 s^{-1} (1 μM NO, 37°C), which is several magnitudes lower than the rate under aerobic conditions [78]. Recent results have shown a novel enzymatic activity in *E. coli* that efficiently removes NO under anoxic conditions and which was identified as a flavorubredoxin reductase [79–81]. The enzyme has been shown to have an NO turnover rate of 14.9 ± 6.7 s^{-1}, which is independent of the NO concentration between 1 and 10 μM NO [81].

4. Regulation and function of bacterial flavoHbs and Hbs

4.1. *VHb*

4.1.1. *Regulation of* vhb *expression*

The oxygen-dependent promoter of *vhb* (P_{vhb}) is induced under oxygen-limited conditions in *Vitreoscilla* [23]. P_{vhb} has been characterized in *E. coli*, and has been shown to be functional in various heterologous hosts, such as *Pseudomonas*, *Azotobacter*, *Rhizobium etli*, *Streptomyces* sp., *Serratia marcescens* and *Burkholderia* sp. [82–88]. P_{vhb} is maximally induced under microaerobic conditions in both *Vitreoscilla* and *E. coli*, when the dissolved oxygen level is less than 2% of air saturation [82,89]. Activity of P_{vhb} in *E. coli* is positively modulated by CRP and FNR. Expression from P_{vhb} is substantially reduced in strains that are unable to synthesize CRP or cAMP. Supplementation of cAMP to a culture of a *cya* mutant strain increased the expression level 10-fold [82]. In contrast, a *cya* deletion had no effect on Φ(*hmp-lac*Z) expression [90]. A CRP-binding site has been predicted to be centered at −97 [82]. Despite the influence of catabolite repression, expression of reporter enzymes from P_{vhb} was not different in glucose- or glycerol-containing media under carbon-limited conditions [91]. In an *fnr* mutant *E. coli* strain, the expression from P_{vhb} was reduced two-fold under microaerobic conditions (*fnr* mutant: 2.5 μg CAT mg^{-1} protein, fnr^+: 5 μg CAT mg^{-1} protein) [92]. The location of the FNR-binding site within the promoter structure has been disputed. Tsai et al. [92] located it at a position typical for activation (around −41.5), whereas Joshi and Dikshit [93] located it around −25.5. The latter site shares only limited homology to the known *E. coli* (TTGATxxaxATCAAt) and non-*E. coli* (TTGATxxxxATCAAg) FNR consensus sequences [94]. The DNA-binding motifs of CRP (TGT-

GAxxxxxxTCACAxTT) and FNR are nearly identical, differing in only one base pair. It has been shown that switching of the non-congruent base pair in the half-site motifs of target promoters can result in their in vivo regulation by the corresponding non-cognate regulator [95]. Thus, it would seem possible that *E. coli* FNR and CRP are able to bind to the identical site in P_{vhb}. Furthermore, repression of P_{vhb} can be achieved by the addition of a complex nitrogen source to the medium (e.g. yeast extract). This represents a third level of regulation of P_{vhb}, whose mechanism has not yet been studied in detail [91].

In bioreactor cultivations, under conditions of maximal induction, the level of recombinant protein expressed from P_{vhb} accounted for up to 10% of total cellular protein [91]. High expression levels, as well as the manifold regulatory mechanisms, have rendered P_{vhb} extremely interesting for applications in biotechnological production processes, since the induction of promoter activity is readily achieved by lowering dissolved oxygen concentrations in bioreactors. Thus, expensive chemical inducers, such as IPTG, can be omitted.

4.1.2. Biochemical function of VHb

Upon expression of VHb in *E. coli* under oxygen-limited conditions, recombinant cells grew to higher final cell densities relative to plasmid-bearing controls [89]. Further experiments showed that the effect of VHb was not restricted to growth improvements, but also resulted in enhanced protein synthesis [96]. Tsai et al. [97] showed that, in *E. coli*, beneficial effects of VHb correlated with VHb expression levels up to a saturation level (3.4 μmol VHb g^{-1} cell), above which no further improvements could be observed. Khosla et al. [96] proposed that VHb expression could facilitate oxygen diffusion and improve aerobic metabolism in *E. coli*.

The cellular energetic parameters of VHb-expressing cells have been analyzed in various *E. coli* mutants lacking one or both of the terminal oxidases. Under aerobic conditions, no differences were seen between VHb^+ and control strains independent of the genetic background. However, under oxygen-limited conditions, the presence of VHb enhanced the growth rate and final cell density in a *cyd* mutant relative to the corresponding control strain [98]. Based on these observations, Kallio et al. [98] formulated a hypothesis, stating that the expression of VHb increases the effective intracellular oxygen concentration under microaerobic conditions. Such an increase would shift the relative activity of the terminal oxidases. The energetically more favorable cytochrome bo_3, which possesses higher proton translocation activity, would predominantly be used in VHb-expressing microaerobic *E. coli* cells [98–100].

This hypothesis has been studied in detail and it was found that expression of VHb increased cytochrome bo_3 content five-fold and cytochrome *bd* content 1.5-fold relative to Hb-free controls. No differences in the level of cytochrome *bd* were observed in a *cyo* mutant strain upon expression of VHb; conversely, VHb expression in a cyo^+ *cyd* mutant strain led to a two-fold increase in the amount of cytochrome bo_3. Furthermore, it was concluded that the specific activity of cytochrome bo_3 was enhanced by VHb [97]. Inspection of interactions between VHb and the various subunits of cytochrome bo_3 and *bd*, using a yeast two-hybrid system, has verified the observation that VHb mainly interacts with cytochrome bo_3. VHb was found to interact specifically with subunit I of the cytochrome bo_3 complexes from *Vitreoscilla*, *E. coli* and *P. aeruginosa* [101].

As a result of enhanced respiratory activity, VHb-expressing cells are able to generate a 50% higher proton flux per reduced oxygen molecule (relative to a VHb-negative control) [97,98]. The higher proton flux led to a 30% higher ATP synthase activity and a 65% higher net ATP turnover, thus supporting the model of Kallio et al. [97,98,102]. Dikshit et al. [103] showed that an *E. coli* mutant lacking both terminal oxidases could grow on the aerobic substrates succinate and lactate when the cells were engineered to express VHb. These results suggest that VHb itself might harbor a terminal oxidase function. However, this potential function of VHb has not been investigated further and we have not been able to verify these results using a different *E. coli* strain lacking both terminal oxidases (Kallio, P., personal communication).

Due to the observed interplay of VHb with terminal oxidase, VHb might affect the electron flux through the respiratory chain in *E. coli*, thus changing the $NAD(P)^+$/NAD(P)H ratio and ultimately the flux through the primary carbon metabolism. Fluorescence measurements indicated that, near anoxic conditions, the steady state level of cellular NAD(P)H was 1.8-fold lower in a VHb-expressing *E. coli* strain relative to control cells. Importantly, no significant differences in the total $NAD(P)^+$/NAD(P)H pool were measured, indicating that the observed reduction of the NAD(P)H level was due to a shift in the ratio of the redox couple. The responses to rapidly changing oxygen tension were slower, suggesting that VHb buffers the intracellular redox state by sustaining normal respiration during the absence of aeration [104].

Furthermore, VHb-positive cells contained increased amounts of tRNA's and active 70S ribosome complexes under microaerobic conditions in *E. coli*. This was also accompanied by a corresponding increase of a marker enzyme activity [105]. This could be due to an increase in the amount of available GTP, required for the binding of tRNA to the small ribosomal subunit during the first step of translation initiation. A previous review [106] summarizes the metabolic pathway of inosine-5′-monophosphate (IMP), which is a common precursor for both AMP and GMP. A high ATP concentration favors GMP synthesis and inhibits the synthesis of IMP from GMP in *E. coli*.

Previous energetic experiments revealed that VHb-ex-

pressing *E. coli* cells have higher ATP levels [98,107], which should also lead ultimately to elevated GTP levels in VHb-expressing strains. VHb-expressing strains have higher ATP levels, based on one-dimensional ^{31}P nuclear magnetic resonance measurements, which measure total NTP levels. A generalized assumption has been made that the increased NTP peak of VHb-expressing cells is due to the enhanced production of ATP [98,107]. However, it cannot be ruled out that the production of either CTP, TTP or GTP has also increased. It could be possible that the relative ratio between ATP and GTP, shown to be approximately 4:1 both in exponentially growing cells and in stationary phase cultures, has remained the same [108,109]. VHb-expressing cells may also have higher GTP levels and faster GTP turnover rates, and for that reason ribosomes are able to utilize more GTP for protein synthesis. GTP is also needed for positioning of the large 50S subunit with the initiation complex in prokaryotes. Thus, elevated GTP levels may also improve the formation of 70S complexes in VHb-expressing *E. coli* relative to controls. A putative explanation for the increased translational activity and the 3.2-fold enhancement of number of active 70S ribosomes at the end of the 30-h bioreactor cultivation, is that VHb-expressing strains have more ATP and more GTP than the VHb-negative controls.

Lately it has been revealed that VHb protects cells from nitrosative stress [31,77]. NO-sensitive, cellular respiration of *E. coli* cells was protected by the presence of VHb up to an NO concentration of 10 μM, whereas at 20 μM a more than 50% reduced respiration rate was reported [77]. In contrast, the respiration of an HMP-expressing strain was insensitive to NO up to a concentration of 60 μM [110]. However, NO detoxification activity of VHb in vitro has been judged negligible [31]. Despite the protective effect of VHb against nitrosative stress in vivo, we could not detect any induction of P_{vhb} under such conditions, thus one may question if VHb would protect its native host against toxic nitrogen compounds (Frey, A.D., personal communication). However, activation of P_{vhb} by FNR upon shift to oxygen limitation might protect the host from reactive nitrogen species produced during nitrate/nitrite respiration.

4.2. E. coli *flavoHb HMP*

4.2.1. *Regulation of hmp expression*

To elucidate possible roles of *E. coli* HMP protein, Poole and co-workers [90] have studied *hmp* regulation under various growth conditions. Anaerobic expression is enhanced in an *fnr* mutant [90]. HMP expression was stimulated directly by the presence of nitrate (only anaerobically) and nitrite. Nitric oxide was found to function as an inducer under aerobic conditions. The effects of these nitrogen compounds were mediated independently of NarLP and SoxRS regulatory systems [90]. Furthermore, induction of HMP expression was observed in cells entering the stationary growth phase. This induction is mediated by the stationary phase-specific sigma subunit (σ^S) of RNA polymerase [111].

FNR functions as a repressor under anaerobic conditions. An FNR-binding site occurs at positions −2 to +11 in the promoter region (P_{hmp}), and binding of FNR to this DNA sequence was demonstrated using gel retardation and DNase I protection assays [90,112]. A mode of P_{hmp} induction under anoxic conditions involving FNR has been recently outlined [112]. Based on the observation that NO is able to reversibly inactivate FNR by nitrosylation, the NO response of a Φ(*hmp-lac*Z) fusion was analyzed under anoxic conditions. Upon exposure to NO, Φ(*hmp-lac*Z) was derepressed due to inactivation of FNR. In the *fnr* mutant no effect of NO on the derepressed level of Φ(*hmp-lac*Z) activity was observed [112]. Affinity of FNR to P_{hmp} could be similarly reduced upon incubation of FNR in presence of O_2 and NO. The reversible inactivation of FNR via nitrosylation by NO represents an alternative pathway for regulation of FNR activity [112]. As outlined above, the anaerobic NO detoxifying activity of HMP is only minor and more potent NO detoxifying activities exist in *E. coli* under anoxic conditions.

Induction of *hmp* expression by NO was shown to be dependent on MetR, which is the global regulator of the methionine biosynthetic pathway in *E. coli*. Two MetR-binding sites are located in the intergenic region between *hmp* and *gly*A, which are divergently transcribed [113]. Homocysteine is a co-regulator for MetR and mutations in the methionine pathway (*met*L), the function of which is also needed for homocysteine synthesis, made these mutants sensitive to various NO compounds. Upon feeding of homocysteine to the growing cultures, resistance against nitrosative stress was restored [114]. MetR has also been shown to be necessary for induction of Φ(*hmp-lac*Z) by SNP (sodium nitroprusside) and GSNO (S-nitrosoglutathione), but only the downstream MetR-binding site is essential for induction of *hmp* expression [113]. Induction occurs upon depletion of homocysteine, which allows MetR to bind to the promoter region. Inactivation of homocysteine may occur via a nitrosation reaction [113].

Hmp regulation is also achieved by the addition of an iron chelator, which induced expression 40-fold under anaerobic and five-fold under aerobic conditions [90]. The observed stimulation might result from complexing iron and a subsequent inactivation of the [Fe–S] cluster of FNR, leading to the derepression of promoter activity. However, no Fur-binding site is present in the promoter sequence of *hmp*, which is in marked contrast to the situation in *Salmonella* serovar Typhimurium outlined below.

Membrillo-Hernández et al. [115] also reported the induction of a Φ(*hmp-lac*Z) in the presence of superoxide-producing paraquat. The induction of *sod*A-*lac*Z followed a subtler pattern, as did the Φ(*hmp-lac*Z), the expression of which was induced only by paraquat concentrations

above 200 μM. The independence of HMP upregulation by SoxRS was shown using *E. coli* mutants of either of these two transcriptional regulators. Anjum et al. [116] showed a 28-fold induction of a Φ(*hmp*-*lac*Z) fusion after a prolonged incubation in the presence of paraquat, a treatment that induced HMP expression to spectrally detectable levels.

4.2.2. Function of HMP protein

The observation that HMP is an NADH oxidase, and is thereby able to generate superoxide radicals and H_2O_2, was used to formulate the hypothesis that HMP might be involved in the oxidative stress response in *E. coli* [66,67]. Interestingly, HMP not only generates ROS, but is also needed for full expression of *sod*A and *sox*S. Furthermore, an *hmp* mutant was markedly more sensitive towards paraquat, particularly at lower paraquat concentrations, as shown by a viability assay [117]. Expression of HMP in *E. coli* protected cells against paraquat-induced oxidative stress, as judged from the superior growth behavior relative to a control [31]. However, it might be argued that the role of HMP under oxidative stress might be dose dependent, since the overexpression of HMP led to the production of ROS. Poole and Hughes [118] suggested that HMP might act as an amplifier of superoxide stress, leading to the full expression of SoxRS stress response.

Various classes of proteins, such as [Fe–S], Cu/Fe or heme-containing proteins, are considered to be targets of RNS. Aconitase, a protein containing a radical-sensitive, labile [Fe–S] center, is a prime target for inactivation by RNS. The protective activity of HMP was demonstrated in vivo as well as in vitro [72]. Terminal oxidases contain both heme and Cu and are main targets of NO inhibition. Stevanin et al. [57] reported a protective effect of HMP on the activity of both terminal oxidases in mutant strains lacking either of the terminal oxidases. Respiration of an *hmp* mutant was extremely sensitive to NO and half-maximal inhibition was achieved at an NO concentration of 0.8 μM. However, the degree of inhibition increased at low oxygen concentrations.

4.3. Regulation and function of S. enterica serovar Typhimurium flavoHb (HmpStm)

The flavoHb of *Salmonella* serovar Typhimurium (HmpStm), which shares 94% identity with *E. coli* HMP, was shown to provide resistance against various NO donors and acidified nitrite both aerobically and anaerobically [35,36]. The wild-type and an isogenic *hmp* mutant showed no differences in the minimal inhibitory concentrations of paraquat and H_2O_2. Also, the synergistic action of NO- and O_2^--forming peroxynitrite did not result in differences in the viability, thus implying that HmpStm is exclusively protecting from RNS [35]. Analysis of a Φ(*hmp*Stm-*lac*Z) fusion revealed that the expression in response to NO is independent of SoxS, OxyR and FNR under aerobic conditions. However, in a *fur* mutant complete derepression of Φ(*hmp*Stm-*lac*Z) was observed that could not be augmented by an NO bolus or by addition of an iron chelator. A putative Fur-binding box is present in the promoter region of the *hmp*Stm gene. Additional genes known to be repressed by Fur were induced by the addition of NO, thus showing that NO is indeed able to inactivate Fur. This model of Fur inactivation by NO has been demonstrated for the Fur protein of *E. coli* [119]. Induction of Φ(*hmp*Stm-*lac*Z) by the iron chelator was similar in wild-type and *hmp*Stm mutant strains, with both being insensitive to low iron levels, indicating that *hmp*Stm expression is exclusively regulated via Fur [36].

Stevanin et al. [110] recently reported some evidence that HmpStm expression protects cells in vivo, using human macrophages. A strain with a deleted *hmp*Stm gene was found to be more sensitive to macrophage-mediated killing than the wild-type strain. However, for both strains the number of viable cells was already reduced by approximately 90% 30 min after internalization of the bacteria by non-activated human macrophages and remained at this level.

4.4. Regulation and function of R. eutropha flavoHb (FHP)

FHP expression is 20-fold upregulated under oxygen-limited conditions in the denitrifying bacterium *R. eutropha* [13]. Two potential binding motifs for NarL (centered around −108) and FNR (centered around −86) in the upstream non-coding region of *fhp* have been identified. The regulation of FHP by these two global regulatory molecules suggests a role of FHP in the anaerobic metabolism of *R. eutropha*. Isogenic *fhp* mutants show no significant differences in aerobic or anaerobic growth. However, the mutant strain did not accumulate nitrous oxide during denitrification with nitrite as electron acceptor [33]. Recently, the NOD activity of FHP has been demonstrated, though being five- to 10-fold lower than the NO turnover rate of flavoHb from pathogenic bacteria [31,75]. Furthermore, expression of FHP in *E. coli* cells enhanced resistance against nitrosative stress [31]. These results suggest that FHP may also protect *R. eutropha* from NO and related molecules during denitrification process, which are unavoidable side products. The intracellular NO concentration during denitrification, however, is tightly controlled by NO reductases [120]. Thus, it is not clear if the NO detoxifying activity of FHP is of any effect under denitrifying conditions.

4.5. B. subtilis flavoHb (HmpBs)

Φ(*hmp*-*lac*Z) is induced during nitrate respiration by a shift to anaerobic conditions, but not during fermentative growth in *B. subtilis* [37]. Absence of *hmp*Bs expression

during anaerobic growth and expression during nitrate respiration has recently been verified using two-dimensional gel electrophoresis under strictly defined growth conditions [121]. FNR acts as an inducer on *hmp*Bs and is in turn dependent on ResD-ResE, which controls aerobic and anaerobic respiration in *B. subtilis*. In Δ*res*DE cells, induction of *hmp*Bs was 10-fold lower relative to wild-type cells upon a shift to oxygen limitation, and was abolished by deletion of *fnr* and *nar*G. Addition of nitrite, but not nitrate, restored induction of Φ(*hmp-lac*Z) [37]. The conclusion is that upon a shift to anaerobiosis nitrate respiration occurs, which is dependent on FNR and *nar*G. Deletion analysis of regulatory sequences and DNase I footprinting experiments indicated that the binding site for ResD is located between −67 and −49 in the promoter region [122]. Besides controlling anaerobic induction of *hmp*, ResDE-dependent genes were shown to respond to RNS, albeit to different extents. Deletion of either *Res*D or *Res*E reduced SNP-induced expression, although a basal expression level was observed in both mutant strains. *Hmp* expression is induced 20-fold upon exposure to SNP (aerobic), and almost 100-fold under anaerobic conditions in the presence of SNP (200-fold in the presence of NO) [123], thus indicating that HmpBs also serves to protect from RNS, which was demonstrated recently [31]. Surprisingly, NO-degrading activity was found to be dependent on NO concentration under conditions during which other proteins did not show any response to elevated amounts of NO [31].

4.6. FlavoHbs from various bacterial species

Little is known concerning the flavoHbs from *M. tuberculosis* (HmpMt) and *P. aeruginosa* (HmpPa), two pathogens which cause severe infections and whose treatment is impaired due to the occurrence of antibiotic resistance. *Hmp*Mt seems to be mainly induced upon entry into the stationary phase (five-fold) if the growth is oxygen limited. Furthermore, a short shift to anaerobic conditions was able to induce expression [39]. Using microarray technology, a comparison of 4-day and 100-day cultures revealed a 1.8-fold higher expression level of *hmp*Mt at the latter timepoint [124]. RNS only slightly induced expression of *hmp*Mt (1.5-fold), and ROS decreased expression by 50%. Exposure to ROS or RNS had no effect on cell viability. Besides one flavoHb, *M. tuberculosis* possesses two additional globin proteins, which belong to the group of trHb (HbO and HbN) [19,125].

An interesting observation was recently reported by Firoved and Deretic [126]. Comparison of a mucoid wild-type to a mucoidy suppressor strain revealed a strong 61-fold induction of *HmpPa* expression in the suppressor strain during the exponential growth phase under aerobic conditions. Furthermore, other genes encoding NO reductase were also strongly induced (20- to 50-fold). It has been suggested that the expression of NO detoxifying genes is upregulated to compensate for the reduced protection normally provided by the mucoid phenotype. Previously, we have shown that HmpPa is able to detoxify NO and to protect a heterologous host against nitrosative stress [31].

Early support for the hypothesis that flavoHb proteins function as integral components of stress response was reported by Favey et al. [38]. They showed that upon inactivation of the flavoHb gene (*hmp*X) in the plant pathogen *E. chrysanthemi*, the bacterium became unable to infect plant species. This behavior could be attributed to the reduction in the synthesis of pectate lyases (PL), which are pathogenicity determinants of *E. chrysanthemi* under conditions of low oxygen tension. The synthesis of PL is abolished in *hmp*X mutant cells upon infection of Saintpaulia plants. The regulation of *hmp*X expression was also shown to be dependent on plant host factors, as demonstrated by the upregulation of a Φ(*hmp*X-*gus*) by cocultivation of *E. chrysanthemi* in the presence of a tobacco suspension culture. Microaerobic conditions per se, as encountered upon infection, were not sufficient to start transcription of a Φ(*hmp*X-*gus*) [38]. The involvement of NO compounds in the plant defense has been shown [127], and therefore it is very likely that *hmp*X expression contributes to the resistance against RNS radicals.

5. Hbs and flavoHbs in biotechnology

Oxygen is the electron acceptor of choice in allowing the cell to generate energy in the most efficient way. During respiratory growth on oxygen, energy is produced by coupling substrate oxidation to the reduction of oxygen. Substrates, such as glucose, pyruvate, lactate and succinate, are completely oxidized via the tricarboxylic acid (TCA) cycle with concomitant transfer of electrons to the cellular quinone pool [128]. The electrons are transferred further to one of the two terminal oxidases. Cytochrome bo_3 is the terminal oxidase of choice at atmospheric oxygen levels ($K_m = 0.35$ μM), whereas cytochrome *bd* is active during microaerobic growth ($K_m = 3$–5 nM) [99,100,129].

In the absence of oxygen, but in the presence of an appropriate alternative electron acceptor, *E. coli* cells can produce a set of alternative respiratory oxidoreductases, which catalyze the transfer of electrons to fumarate, nitrate, dimethyl sulfoxide or trimethylamine-*N*-oxide [130,131]. In the complete absence of oxygen and alternative electron acceptors, the cellular metabolism of *E. coli* switches to fermentative pathways [132]. These catabolic pathways involve redox-balanced dismutation of the carbon source, substrate level phosphorylation and the formation of fermentation products such as acetate, formate (H_2 and CO_2), ethanol, lactate and succinate [132].

The switch between different metabolic modes is accompanied by fairly dramatic changes at the transcriptional level. Two global regulators ensure the expression of the appropriate enzymatic configuration: FNR and ArcAB,

which both can function synergistically and antagonistically on the expression of certain genes. Furthermore, their regulatory function is assisted by other regulatory proteins, such as NarL/P [128]. These regulatory mechanisms ensure that the energetically most favorable metabolic pathway is used. Thus, oxygen is used in preference to nitrate, nitrate to fumarate, and fumarate to the endogenously generated electron acceptors, which are produced in the fermentative pathways [133].

The commercial production of a variety of desirable metabolites and important pharmaceuticals employs the overexpression capacity of oxygen-requiring bacteria, fungi and mammalian cells. Oxygen has very low solubility in water and various microorganisms and cultured cell types have high nutritional demand for oxygen, especially during large-scale and high-cell-density production processes. The high demand for oxygen can be partially satisfied by improving process parameters and bioreactor configurations, e.g. improved mixing rates, high-efficiency dispersion systems and modifications of the medium [134].

5.1. Use of VHb to improve cell growth and productivity

Metabolic engineering approaches seek genetic strategies, for example to alleviate adverse effects of oxygen limitation on microorganisms, higher cells and plants [135]. The approach is based on previous observations that *Vitreoscilla* expresses Hb under oxygen deprivation. The *vhb* gene was successfully transferred to *E. coli* and upon expression of VHb, growth and protein production of *E. coli* was enhanced under microaerobic conditions [89,96]. The positive effects of VHb expression, promoting either the efficiency of oxygen-limited growth and/or production of primary and secondary metabolites in numerous microorganisms, are well documented (Table 3). This technology seems to be especially beneficial for mycelium-forming microorganisms, such as the antibiotic-producing *Streptomyces* strains, *Acremonium crysogenum* and *Saccharopolyspora erythraea*. It is known that oxygen transfer to the mycelial pellet is limited and is especially critical when such microorganisms are grown in highly viscous industrial media. In such situations, VHb expression seems to enhance antibiotic production. The VHb expression technology has been successfully tested in collaboration with the biotechnological industry, using an industrial *S. erythraea* strain producing erythromycin. The *vhb* gene was stably integrated into the genome of *S. erythraea* using *Streptomyces* phage ΦC31 attachment site [136]. VHb expression significantly increased erythromycin production, resulting in an enhanced space–time yield of approximately 100% (1.1 g erythromycin l^{-1} day^{-1}) relative to the original strain. The final erythromycin titer was 7.4 g l^{-1} after 9 days of cultivation for the engineered strain (control 4.0 g l^{-1}) [137]. Thus, these results show that this technology may generally be applied to improve the metabolism of industrially important antibiotic-producing strains and of other filamentous microorganisms.

2,4-Dinitrotoluene (2,4-DNT) is a serious contaminant at munitions facilities. The pathway of 2,4-DNT degradation by *Burkholderia* sp. strain DNT has been analyzed by biochemical and genetic means. Oxygen was shown to be required for three steps in the oxidative degradation pathway [138]. Thus, VHb technology has been considered useful to improve the bioremediation capacity of *Burkhol-*

Table 3
Effects of VHb expression on growth and productivity

Organism	Effects	Reference
Acremonium chrysogenum	3.2-fold increase in cephalosporin C production	[159]
Bacillus subtilis	30% increase in neutral protease, 7–15% increase in α-amylase activity and 1.5-fold increase in protein secretion	[160]
Burkholderia sp.	15% increase in biomass and two-fold increase in the rate of DNT degradation	[88,139]
Chinese hamster ovary cells	40–100% increase in tissue plasminogen activator production	[143]
Corynebacterium glutamicum	30% increase in L-lysine titer and 24% increase in L-lysine yield	[161]
Escherichia coli	30% increase in total cell protein, 80% increase in CAT activity, 40% increase in β-galactosidase activity	[89]
Escherichia coli	61% increase in β-lactamase activity	[105]
Escherichia coli	3.3-fold increase in α-amylase activity	[162]
Escherichia coli	1.8-fold increase in ferritin production	[163]
Nicotiana tabacum	50% faster germination, enhanced growth, 80–100% more dry weight, 30–40% more chlorophyll, 34% more nicotine	[164]
Nicotiana tabacum, suspension culture	10–50% enhanced growth, shortened lag phase	[142]
Pseudomonas aeruginosa	11% increase in viable cell number, increased oxygen uptake	[165]
Rhizobium etli	68% higher nitrogenase activity in nodules of bean plants and 53% higher total nitrogen content	[87]
Saccharomyces cerevisiae	three-fold increase in final cell density	[102]
Saccharopolyspora erythraea	70% increase in final erythromycin titer	[136,137]
Serratia marcescens	enhancement of acetoin and 2,3-butanediol production, increase in cell size	[85,86]
Streptomyces coelicolor	10-fold increase in actinorhodin production	[84]
Streptomyces lividans	50% increase in final cell density	[84]
Streptomyces rimosus	2.2-fold increase in oxytetracycline production	[144]
Xanthomonas maltophila	15% increase in viable cell number, enhanced degradation of benzoic acid	[166]

deria sp. strain DNT and indeed expression of VHb improved the rate of 2,4-DNT degradation [88]. The enhancement of the degradation capacity was shown to be dependent on culture conditions. Feeding of a co-substrate increased the total amount of 2,4-DNT removal. Generally, VHb-expressing strains reached higher final cell numbers at 200 ppm 2,4-DNT, whereas at lower concentrations no differences were seen relative to *Burkholderia* sp. strain DNT. Furthermore, the former displayed significantly improved bioremediation characters [139]. However, it cannot be concluded if the observed effects are due to an enhanced biodegradative activity or are the result of the higher cell numbers due to the improved growth properties of VHb-expressing cells.

The beneficial effects of VHb-expression technology were shown not to be restricted to bacterial organisms. The use of plants and plant cells for the production of natural or recombinant compounds has recently gained momentum [140]. Oxygen supply to plant cells is known to affect both cell growth and the production of metabolites [141]. Therefore, Farrés and Kallio [142] have expressed VHb suspension cultured tobacco cells. VHb expression resulted in a shortened lag phase and 10–50% increased final cell densities in batch cultures relative to control cultures. No differences were seen in sucrose consumption or ethanol production [142]. Furthermore, in a mammalian system, tissue plasminogen activator production has been increased 40–100% in VHb-expressing Chinese hamster ovary cells relative to controls [143].

The above-cited results show that heterologous VHb expression is able to alleviate oxygen limitation in a wide range of distinct organisms. Thus, the inverse metabolic engineering approach, i.e. the genetic transfer of useful phenotypes to heterologous organisms, represents a fast and effective means to alleviate the adverse effects of oxygen limitation [144].

5.2. *Novel globin proteins for improved performance in heterologous hosts*

It has been hypothesized that the biochemical properties of VHb are not optimized for foreign host cells [145]. Therefore, screening of novel globin proteins and generation of VHb mutants has been performed to adapt the VHb technology for certain biotechnological applications.

5.2.1. *VHb mutants and single chain variants of VHb*

Andersson et al. [145] have used error-prone PCR (polymerase chain reaction) to generate a number of randomly mutated *vhb* genes. VHb mutants were screened for improved growth properties under microaerobic conditions in *E. coli*. Several clones were selected and analyzed to elucidate the physiological effects of novel VHb proteins. The expression of four VHb mutants, carried by pVM20, pVM50, pVM104 and pVM134, was able to enhance microaerobic growth of *E. coli* approximately 22% (mutations: Glu-A17 and Glu-H23 to Gly), 155% (His-C1 to Arg and Gln-E20 to Arg), 50% (Ala-E10 to Gly) and 90% (Ile-B5 to Thr), respectively, with a concomitant decrease of acetate excretion into the culture medium. The effects of the amino acid exchanges on the structure have not been analyzed in detail. Glu-H23 is part of a H-bonding network at the proximal site of the heme thought to provide an electronic push and to be partially responsible for the activation of O_2. The absence of this residue might lower the auto-oxidation rate of the heme and therefore favor reversible O_2 binding. Due to the cloning strategy used in their construction, the mutated VHb proteins also contained an extension of eight residues (MTMITPSF) at their N-termini. The experiments indicated that the positive effects elicited by mutant VHb expression from pVM20 and pVM50 were linked to the N-terminal peptide tail. Removal of the N-terminal sequence from pVM20 and pVM50 reduced cell growth approximately 23% and 53%, respectively, relative to native VHb controls [145].

If criteria other than final OD_{600} are applied (e.g. μ_{max}), other VHb mutants outperform native VHb. Of the mutants reaching higher final cell densities, only pVM50 exhibited a higher μ_{max} (50%) than the strain expressing native VHb. The strain carrying a Lys-F2 to Asn mutation grew fastest and had a two-fold higher μ_{max}, and a His-BC1 to Arg substitution enhanced μ_{max} by 50%. Lys-F2 has been predicted to be part of the intramolecular hydrophobic zipper observed between helix F and H. These structures also build the contact region for interaction between the two VHb subunits. Thus, the quaternary architecture might be altered by the Lys-F2 to Asn substitution, e.g. changing the O_2-binding kinetics.

The glucose consumption rate and biomass yield on glucose ($Y_{x/glc}$) for mutants versus native VHb protein has yet to be analyzed. However, experiments have revealed that clear differences in $Y_{x/glc}$ exist among strains expressing various Hbs [30,146]. Our data (Kallio, P., unpublished results) show that growth of cells carrying pVM50 is already glucose-limited after 10 h of growth, in contrast to pVM20 and native VHb-expressing cells, leading to a double limitation (O_2 and carbon source). This clearly indicates that growth protocols, established for VHb-expressing cells, are not ideally suited to assess the growth-promoting potential of cells expressing VHb mutants.

Since VHb exists as a homodimer, it was determined if a single chain variant containing two *vhb* genes separated by a short linker (termed double VHb) could outperform growth properties of a strain expressing native VHb. Expression of double VHb, which was judged to be biochemically active, increased final cell density about 19% relative to the native VHb-expressing *E. coli* cells. Based on a computer model, the authors proposed that formation of oligomeric structures composed of several double VHb might occur [147]. However, it has yet to be analyzed if such structures exist in vivo.

5.2.2. Screening of flavoHb and Hb proteins and construction of chimeric proteins

Due to the high structural similarity between VHb and FHPg, several chimeric protein fusions between VHb and the reductase constituents of FHP were constructed. Truncated forms of FHP were also generated [148]. Upon expression of these constructs in microaerobically grown *E. coli* cells, increased final cell densities were observed after 30-h fed-batch cultivations, relative to control strains. Expression of the native FHP or the chimeric fusion between VHb and the reductase domain of FHP (VHb-Red) resulted in an approximately 50% and 75% higher final cell density, respectively, relative to the VHb-expressing strain. Furthermore, all Hb- or flavoHb-expressing strains accumulated less acetate, indicating a less anaerobic metabolism [148].

To analyze the primary carbon metabolism of the strains expressing VHb, FHP, VHb-Red and FHPg, ^{13}C-labeling experiments were conducted. The active pathway configuration is imprinted into the proteinogenic amino acids, and analysis of the labeling pattern using nuclear magnetic resonance spectroscopy subsequently allows calculation of the flux distribution in the primary carbon metabolism. [149]. Cells were fractionally labeled with [^{13}C]glucose during the late-exponential phase, a timepoint at which growth of control culture ceases rapidly whereas Hb- or flavoHb-expressing *E. coli* cells continue to grow. Interestingly, the carbon routing in microaerobically grown VHb- or VHb-Red-expressing cells and control cells was strikingly similar and closely resembled the labeling pattern of wild-type *E. coli* cells grown under anaerobic conditions, i.e. the TCA cycle was interrupted and found to be replenished by the anaplerotic reaction. Furthermore, 70% of acetyl–CoA was synthesized via pyruvate–formate lyase (PFL) [146,150]. Additionally, dilution of the intracellular acetate pool occurred, which is indicative for re-uptake of the excreted acetate (10% of total amount of acetate). This effect was even stronger if cells were grown in the presence of excess glucose (30–40% of intracellular acetate) (Frey, A., personal communication). In contrast, expression of FHP or FHPg shifted the metabolism to a more aerobic regime, with a functional TCA cycle and a low or absent PFL activity. PFL has a highly oxygen-sensitive radical in its active site and is therefore readily inactivated by the presence of minute amounts of oxygen. Despite the identical carbon flux distribution of VHb- and VHb-Red-expressing cells and control strains, VHb/VHb-Red-expressing cells still exhibited better growth and energetic characteristics, i.e. cells grew faster, displayed a lower production of fermentative byproducts and a higher oxygen consumption rate, and had an overall increased $Y_{x/glc}$ [146].

Bollinger et al. [30] have screened various microbial genomes for the existence of uncharacterized and unidentified Hb and flavoHb genes, which possibly could be used in biotechnological production processes to improve cell growth and metabolic properties under oxygen-limited conditions. Growth behavior and byproduct formation of *E. coli* cells expressing various hemoproteins were analyzed in microaerobic fed-batch cultivations. *E. coli* cells expressing flavoHbs from *P. aeruginosa*, *S. typhi* and *D. radiodurans* grew to similar final cell densities, as did the strain expressing VHb (2.98 g l^{-1} cell dry weight). Although the clones expressing flavoHbs from *E. coli*, *B. subtilis* and *K. pneumoniae* or the Hb from *C. jejuni* outperformed the plasmid-bearing control strain (2.06 g l^{-1} cell dry weight), these clones did not reach the final cell densities measured for VHb-expressing variants. Additionally, increased $Y_{x/glc}$ was determined for all recombinant strains, and an approximately two-fold $Y_{x/glc}$ enhancement was obtained with *D. radiodurans* flavoHb-expressing *E. coli* relative to the *E. coli* control carrying the parental plasmid. These results imply that Hb- and flavoHb-expressing strains are able to utilize the carbon substrate more efficiently [30].

Saccharomyces cerevisiae flavoHb (K_d = 0.03 μM [75]) or horse heart myoglobin (K_d = 0.79 μM [151]) did not improve growth of *E. coli* during microaerobic growth relative to plasmid-bearing control strains. In contrast, the K_d for VHb has been reported to be 70 μM [25]. However, as mentioned before, a second set of kinetic data for VHb exists, which results in a K_d of 0.02 μM [64], which is similar to the value reported for the flavoHb of *S. cerevisiae*. These results suggest that the kinetic properties for oxygen binding and release of the Hb proteins are not suitable to support microaerobic metabolism of *E. coli* as proposed previously [152].

6. Conclusions

Bacterial Hbs and flavoHbs are surprisingly versatile proteins serving several biological functions, including protection of the host cell from nitrosative and oxidative stress and delivering oxygen to respiring cells.

A growing body of evidence has accumulated in the last few years, demonstrating a role for flavoHbs in the response to the threat posed by nitrosative stress. This general role of flavoHb in NO defense is of great interest for clinical microbiology. Pathogenic microorganisms, such as *M. tuberculosis* and *Salmonella* serovar Typhimurium, might profit from this resistance mechanism upon infection of a potential host. Bearing in mind the steadily increasing number of antibiotic-resistant pathogens, flavoHbs might represent a novel drug target for therapy.

The expression of many flavoHb genes is induced by environmental stress conditions such as oxygen deprivation or oxidative and nitrosative stress. Several regulatory proteins have been implicated in the control of gene expression, such as FNR, ResD/E, Fur, NarL/P, MetR and stationary phase responsive σ^S [36,37,39,90]. In general, induction of flavoHb gene expression is independent of

the oxidative stress responsive SoxRS and OxyR transcription factors [36,37,39,90].

Oxidative and nitrosative stress are common phenomena, caused by exposure of cells to ROS and RNS, both of which can damage biomolecules such as proteins, nucleic acids and cellular membranes. Under normal growth conditions, a balance exists between the amount of radicals produced and the cellular defense mechanisms [153]. Defense mechanisms against oxidative and nitrosative stress are generally under the control of SoxRS and OxyR, which exist in a variety of bacteria (for review, see [154]). Exposure to a boost of radicals results in the rapid induction of adaptive defense enzymes independent of, or under the control of, SoxRS and OxyR. Various gene products (e.g. *ahpC*, *metL*, *noxR1*, *noxR3*, *sodA*, *katG* and *gor*) have been shown to contribute to this defense, protecting against either or both kinds of these bacteriocidal products [155].

Besides RNS and ROS, which both serve cytotoxic and immunoregulatory functions, the arsenal of antimicrobial agents also comprises for example myeloperoxidase, elastase, cathepsin G and phospholipase A2. All of these compounds contribute to the host defense. Knockout studies in mice showed that, dependent on the pathogen, different compounds are essential for the host defense. For example, production of ROS by phagocyte oxidase is essential, whereas RNS produced by inducible nitric oxide synthase are only contributory to the defense against *Salmonella* serovar Typhimurium [156]. Thus, it can be anticipated that the role of flavoHb in the bacteria's defense mechanisms is dependent on its host, and its contribution to overall defense varies among different organisms.

In contrast to flavoHbs, Hb proteins may serve different functions and their exact roles are still poorly defined. VHb has been widely used to improve growth of a variety of microorganisms under oxygen-limited growth conditions. The observed beneficial effects of VHb expression on metabolism have been proposed to be linked to increased respiratory activity, i.e. higher oxygen uptake, higher specific activities of terminal oxidases, specific interaction of VHb with cytochrome bo_3 and, therefore, an overall improved cellular energetic charge.

However, recently two independent studies showed that VHb is also able to protect cells against RNS and shields the terminal oxidases from inactivation by NO [31,77]. In the experiments done so far, it has not been possible to determine the true mechanism of action of VHb or other bacterial Hb proteins when expressed in either native or heterologous hosts. It has been suggested that VHb might exist in two alternative forms: as a homodimer or as a heterodimer with a partner reductase [77]. However, the presence of RNS under standard cultivation conditions in *E. coli* is still a matter of debate, in contrast to denitrifying bacteria, where the production of these reactive molecules is well documented. The steady state NO concentrations vary between 1 and 65 nM, depending on species and growth conditions [120]. In a medium containing nitrite, the production of NO at acidic pH might occur via protonation of nitrite and subsequent dismutation into several species of nitrogen oxides, including NO. Under oxygen-limited conditions, notable amounts of organic acids are produced and excreted into the media, lowering the pH, and formation of RNS might occur under these conditions. Furthermore, NO production can occur endogenously using nitrite as an electron acceptor [157]. In our experiments, we have observed a low expression of *E. coli hmp* in the late-exponential phase of growth and also upon entering the stationary phase under oxygen-limited conditions (Frey, A. D., personal communication). Induction of *hmp* might be an indication of the presence of RNS. Thus, expression of heterologous flavoHbs and Hbs might improve protection of organisms against RNS and therefore favor growth of globin-expressing cells. Interestingly, prevention of inhibition of cytochrome activity or increasing the oxygen supply would result in identical effects, previously postulated to be due to a higher intracellular oxygen level.

Protein engineering approaches, either of rational or random origin or based on evolutionary selection, will help to improve the properties of globin proteins used in biotechnological production processes. However, one of the key limitations in the generation of improved VHb proteins is the lack of an efficient screening system. Screening of mutant strains in a defined experimental setup might result in the selection of phenotypically undesired mutant strains, since additional parameters might limit these mutants in outperforming the native protein under the standard conditions. It is quite obvious that previously applied methods may have resulted in the selection of a physiologically unfavorable group of mutants. Therefore, the exact mechanisms for improving growth and productivity have to be clarified first. This knowledge will be required for the setup of a screening system allowing rapid and clear identification of novel proteins with superior characters.

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