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Formation of sulphmyoglobin during expression of horse heart myoglobin in *Escherichia coli*

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Abstract

Expression of recombinant horse heart myoglobin in *Escherichia coli* has been found to result in the production of both native and variable amounts (~16–17% total) of two sulphmyoglobin isomers. The recombinant sulphmyoglobin produced consists primarily of the A and B isomers as identified by ^{1}H NMR spectroscopy with no evidence for production of the C isomer. Conversion of recombinant sulphmyoglobin to the native protein can be achieved by reconstitution with protohaem IX. The possible relationship of this observation to recombinant expression of other heme proteins is discussed.

Key words: ^{1}H NMR; Sulphmyoglobin; *Escherichia coli*

1. Introduction

In recent years, efficient expression of genes coding for human [1], sperm whale [2], porcine [3], and horse heart [4] myoglobin (Mb) in *Escherichia coli* has been reported. In our work concerning recombinant forms of horse heart Mb, we have frequently observed that the transformed bacterial cultures producing this protein can assume a bright green colour. In addition, the electronic absorption spectra of the wild-type and variant forms of Mb purified from these cultures can exhibit absorption maxima that are not characteristic of the wild-type protein. As amino acid substitutions at the active site of Mb might reasonably be expected to produce changes in the electronic spectrum of the protein, it is important to establish whether new spectroscopic features result from the amino acid substitution itself or from some chemical modification of the protein inherent in the expression system.

The green colour of the cultures and the spectroscopic properties of the purified protein led us to suspect that expression of horse heart Mb in *E. coli* results in sulphmyoglobin (sulphMb) formation, a derivative of Mb that is distinguished by its green colour (e.g. reference [5]). The formation of sulphMb in vitro is achieved by the action of inorganic sulphides on ferryl Mb (Fe(IV) = O) [5].

$$\text{metMb} + H_2O_2 \rightarrow \text{ferrylMb}$$
$$\text{ferrylMb} + H_2S \rightarrow \text{Fe(III)sulphMb}$$

NMR studies have demonstrated that in vitro preparations of sulphMb give rise to a heterogeneneous mixture of at least three major products that cannot be distinguished by electronic spectroscopy but that exhibit characteristic NMR spectra [6–10] particularly in the cyanmet form of the protein. The initial product, S_AMb, is consistent with addition of a sulphur atom as an episulphide across the β–β bond of pyrole II of the protoporphyrin IX group, Fig. 1A. Under acidic conditions this product decays predominantly to S_BMb, the structure of which is uncertain but has been proposed to be as depicted in Fig. 1B [10]. Under basic conditions decay of S_AMb yields mainly S_CMb, the thermodynamically stable product [6,7,10], which has been shown to contain a chlorin prosthetic group in which the vinyl group of pyrole II has formed an exocyclic thiolene ring [8,9], Fig. 1C. In addition, a number of pharmaceutical agents can produce sulphhemoglobin derivatives of undetermined but presumably related structures in vivo [11].

To determine whether or not sulphMb is produced during expression of recombinant horse heart Mb in *E. coli*, we have studied the electronic and NMR spectra of recombinant Mb preparations in which significant quantities of the contaminant are present. We find that sulphMb can, in fact, form during bacterial expression of horse heart Mb, and discuss the possible implications of this and related findings for the bacterial expression of haemproteins in general.

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2. Experimental

2.1 Fermentation

Expression of a synthetic gene for horse heart Mb in *Escherichia coli* has been described [4]. LE392 cells (*supE44, supF58, lac Y1, gal K2, gal T22, mt B1, Trp R55, lambda*$^-$) containing the pGYM plasmid were grown in superbroth (tryptone (10 g/l)(BDH), yeast extract (8 g/l)(BDH), NaCl (5 g/l)(BDH) and ampicillin (100 μg/ml) (Sigma)). Preparative scale cultures (600 ml) were grown (18 h) in 2-liter Erlenmeyer flasks placed in a shaker-incubator (37°C). After harvesting by centrifugation, the cells were resuspended in 20 mM Tris-HCl pH 8.0, and lysozyme (250 mg, Sigma) was added. The cells were shaken at 4°C until the suspension became viscous, and the lysed cells were then frozen until needed. In general, bacteria grown in the flasks produced cultures that varied in colour from bright green to red. For the discussion below, Mb isolated from the green and red coloured cultures is referred to as 'green' and 'red' Mb, respectively.

2.2. Protein purification

Commercial horse heart Mb (Sigma M1882) was chromatographically purified as described by Tomoda et al. [12]. Wild-type recombinant Mb was purified as previously described [4] except that the crude Mb extract was loaded onto a column of DEAE Sepharose CL6B (Pharmacia) (3 × 15 cm) equilibrated in 20 mM Tris-HCl pH 8.0. Myoglobin does not bind to this resin under these conditions, so fractions eluting directly from the column were loaded onto a column of Chelating Sepharose Fast Flow (Pharmacia) (3 × 10 cm) prepared by sequentially washing with water (300 ml), 50 mM ethylenediaminetetraacetic acid (300 ml), a solution of 35 mM $ZnSO_4$ that contained 25 mM acetic acid (300 ml), and buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 8.0) (300 ml). After binding Mb to this column, it was washed with 20 mM Tris-HCl (0.5 M NaCl, pH 8.0) (200 ml) before developing with 20 mM Tris-HCl (0.5 M NaCl, 50 mM imidazole pH 8.0) (100 ml). The partially purified Mb was then concentrated by centrifugal ultrafiltration (Centriprep-10, Amicon) to a volume of ~ 15 ml and loaded onto a column of Sephadex G-50 (Pharmacia) equilibrated and eluted with 20 mM Tris-HCl containing 1 mM EDTA (pH 8.0) for further purification. Myoglobin fractions ($A_{408}/A_{280} \geq 3.5$) were pooled and exchanged into 20 mM ethanolamine (pH 9.0). Final purification of the Mb was achieved by elution over an HR10/10 Mono-Q anion-exchange column (Pharmacia) that was initially equilibrated with 20 mM ethanolamine pH 9.0 and developed with a linear gradient of NaCl (0–0.06 M). The final product ($A_{408}/A_{280} \geq 3.5$) migrated as a single band by SDS-PAGE. Separation of green and red Mb was not achieved by the FPLC method described above.

MetMb was prepared by addition of four equivalents of potassium ferricyanide, and excess oxidant was removed by anion exchange chromatograpy [13]. ApoMb was prepared as previously described [14] (ε_{280} = 16 $mM^{-1} \cdot cm^{-1}$ [15]). ApoMb in sodium phosphate buffer (pH 7, μ = 0.10 M) was reconstituted by addition of 1.1 equivalents of iron protoporphyrin IX (10 mg/ml in 0.2 M NaOH) (Porphyrin Products, Logan, Utah) followed by elution over a column of Sephadex G-50 (2.5 × 75 cm). Reconstituted protein for NMR analysis was stored at 4°C prior to use to allow for reorientation of the haem group [16,17]. SulphMb was prepared as described previously [18].

2.3. NMR and mass spectrometry

Electrospray mass spectrometry was performed with a triple quadrupole mass spectrometer (API III MS/MS system; Sciex, Thornhill, Ontario, Canada) fitted with a pneumatically assisted electrospray interface [19]. Samples of native and unreconstitued recombinant Mb were prepared in water (50 μl, 1 mg/ml). NMR spectra were recorded at 20°C with a Bruker MSL-200 spectrometer. Protein solutions (~ 2 mM) were exchanged into 50 mM deuterated sodium phosphate buffer pH 7.0 (uncorrected pH meter reading).

3. Results

3.1. Mass spectrometry

The molecular mass determined by electrospray mass spectrometry of apoMb from native Mb was 16951.7 ± 2.0, which compares to a mass of 16950.5 predicted from the amino acid sequence. For the recombinant sample, two principal molecular ions were identified with masses of 16950.5 ± 3.5 and 17083.4 ± 3.1. These values correspond to the mass of the apo-protein and to the mass of the apo-protein plus the N-terminal methionine, respectively. The molecular masses of the red and green Mbs determined in this manner were identical. Mass spectrometry of the prosthetic groups of these derivatives was not attempted.

3.2. Electronic absorption spectra

The electronic spectra of native horse heart Mb in the met-aquo, deoxy and carbonyl forms are shown in Fig. 2A. Examination of the corresponding spectra of purified green and red Mbs (Fig. 2B and C; Table 1) reveals spectra that differ substantially from those of the native protein. These differences are most pronounced for the deoxy and carbonyl derivatives. The Soret absorbancies of the green and red Mbs exhibit maxima intermediate between those expected for native Mb and sulphMb, as anticipated for a mixture of two forms. Similar spectra

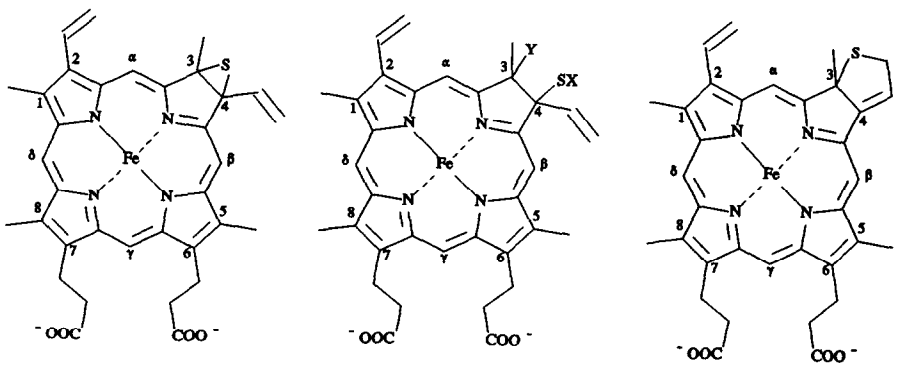

Fig. 1. Proposed structures of (A) S_AMb, (B) S_BMb and (C) S_CMb [6–10,18].

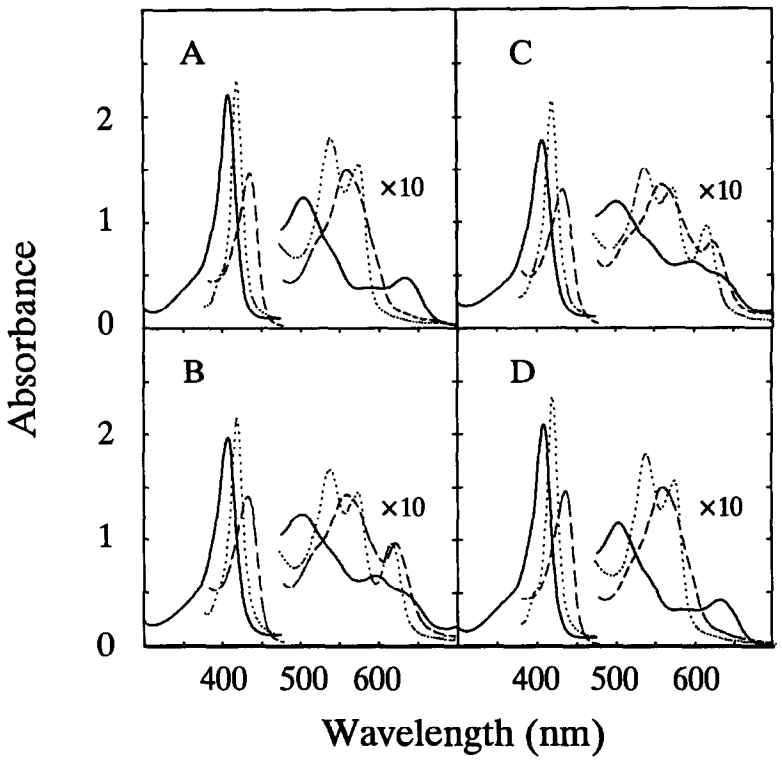

Fig. 2. Electronic spectra (sodium phosphate buffer, pH 6.0, μ = 0.10 M, 25.0°C) of the met (——) deoxy (----) and CO-bound (....) forms of (A) native horse heart Mb, (B) green Mb, (C) red Mb and (D) green Mb reconstituted with fresh iron protoporphyrin IX.

have been reported for samples of hemolysate taken from patients with sulphemoglobinemia [11]. Preparation of apo-Mb from the sample in Fig. 2B followed by reconstitution with fresh hemin, yields the spectrum shown in Fig. 2D, which is identical to that observed for native metMb.

3.3. NMR spectroscopy

The ^{1}H NMR spectrum of the cyanmet form of native Mb is shown in Fig. 3A. The haem resonances a–h have been assigned previously for sperm whale [20] and horse heart [21] Mb. Fig. 3B shows the corresponding spectrum for the cyanmet form of a sulphMb sample prepared in vitro as described above. The sample consists of sulphMb plus some unreacted native Mb (compare Figs. 3A and 3B). The major resonances observed in this spectrum correspond with those previously found for sulphMb [6,10], but some differences are apparent. In previous reports [6,10], three isomeric forms of sulphMb were identified, S_AMb, S_BMb and S_CMb. The relative amounts of these components depend on oxidation state, solution pH, chromatographic methods employed and the age of the sample. We expect that sulphMb formed in vivo will be a similarly heterogeneous mixture, the spectroscopic properties of which are unlikely to duplicate exactly those of pure sulphMb isomers formed under controlled conditions in vitro. For this reason, we have not prepared purified sulphMb isomers. Our intention is simply to establish a correlation between the contaminating resonances in the NMR spectra of the red and green samples with those observed in authentic sulphMb samples.

The prominent resonance in the spectrum of the sulphMb sample observed at 44.3 (i), 38.3 (j), 29.5 (k), 25.6 (l), 24.3 (m), 20.0 (n), 9.7 (o) and 9.3 (p) ppm can also be identified in the green (44.1, 38.1, 29.3, 25.5, 24.1, 9.5 and 9.1 ppm) and red (44.4, 38.2, 29.5, 25.6 and 24.3 ppm) samples, Fig. 3C and D. Peaks o and p are not significant contaminants in the red sample. By comparison with published resonance assignments for the cyanmet forms of each of the three isomeric forms, S_AMb, S_BMb and S_CMb, we see that only S_AMb (peaks i, l, m, and n) and S_BMb (peaks j and k) are present in our samples, with no contamination from S_CMb. Nevertheless, the presence other presumed forms of sulphMb [10] are evident from the presence of the resonances labelled 'x' in Fig. 3 that are not attributable to either S_AMb, S_BMb, S_CMb or native Mb. Examination of the relative intensities of the methyl resonances for S_AMb (i), S_BMb (j) and native Mb (l) in Fig. 3C and D reveals that the *total* amount of these two sulphMb forms is approximately the same in both the green (~ 16%) and red (~ 17%) samples. However, the relative amounts of each of the isomers differ. For the green sample the main contaminant is S_AMb (13% S_AMb, 3% S_BMb), while for the red sample the main contaminant is S_BMb (5% S_AMb, 12%

S_BMb). Reconstitution of the green sample from Fig. 3C yields the spectrum shown in Fig. 3E, which is identical to that of native Mb (Fig. 3A).

4. Discussion

From the results outlined above, it is clear that the expression system previously described [4] for efficient production of horse heart Mb can produce substantial quantities of sulphMb derivatives. The molecular masses for the green and red apo-Mb derivatives obtained from electrospray mass spectrometry measurements, combined with the conversion of the green form to native metMb following reconstitution with protohaem IX, provide compelling evidence for the integrity of the apoprotein and chemical modification of the prosthetic group as expected for sulphMb. We also note that S_AMb, S_BMb and S_CMb are known to be unstable to decay to native deoxyMb following deoxygenation with dithionite [10]. Presumably, this treatment would provide an alternative method for removal of sulphmyoglobin contamination from our samples, though it is not clear whether the unidentified sulphMb products responsible for the resonances labelled 'x' in Fig. 3 would be similarly eliminated.

The presence of S_AMb and S_BMb with no evidence for the formation of S_CMb is consistent with work in vitro [10] showing that the most effective routes for the formation of S_CMb are through incubation of sulphMb with CO (22°C, 3 days) or cyanide (4°C, 2 months). As our protein was never exposed to these conditions, formation of S_CMb is not expected. Formation of small amounts (3–10%) of S_CMb upon incubation of metaquo-sulphMb at 22°C for 8 hours was also reported [10], however, we see no evidence for formation of S_CMb by this route. Interestingly, the *total* amount of sulphMb in the green and the red samples is the approximately the same (~ 16% and ~ 17% respectively), while the relative amounts of S_AMb and S_BMb isomers differ. Presumably, this finding results from the observation that S_BMb forms from the initial product, S_AMb [10], so that the difference between the red and green cultures lies not in the total amount of S_AMb formed initially, but in the relative rates of conversion of S_AMb to S_BMb. As the conversion of S_AMb to S_BMb is dependent on pH, oxidation state, ligation state, temperature and time [10], small changes in any of these variables under the growth conditions employed could account for the varying amounts of S_BMb observed.

Table 1
Absorption maxima (nm) for native Mb, sulphMb (SMb), green Mb (GMb), red Mb (RMb) and reconstituted green Mb (GMb_{rec})

Protein	Soret	Visible
Native Mb		
Fe(III)	408	502, 630
Fe(II)	435	560
Fe(II)CO	424	540, 579
SMb		
Fe(III)	404	593
Fe(II)	421	508, 545, 570, 617
Fe(II)CO	422	613
GMb		
Fe(III)	407.5	503, 596
Fe(II)	431	556, 619.5
Fe(II)CO	422	541, 557, 620.5
RMb		
Fe(III)	407	503, 596
Fe(II)	431	559, 622
Fe(II)CO	422	540.5, 576.5, 619
GMb_{recon}		
Fe(III)	408	503, 631
Fe(II)	434.5	559.5
Fe(II)CO	424	539.5, 578.5

The source of the sulphur required for formation of sulphMb may be related to the source of the so-called 'inorganic' or 'labile' sulphur implicated in formation of iron-sulphur clusters (e.g. ferredoxins, HiPIPs, nitrogenase). Although little information is available concerning mobilization of this sulphur for formation of clusters, it is known that cysteine is the probable source in *E. coli.* [23]. Significantly, recombinant expression of iron-sulphur proteins requires no additional sulphur supplements to the growth media, so we, therefore, expect that sufficient concentrations of free sulphide exist under our growth conditions to account satisfactorily for the formation of sulphMb. The origin of the oxidising equivalents necessary for formation of the ferrylMb intermediate is also of concern. Efficient sulphMb preparation in vitro requires formation of ferrylMb with hydrogen peroxide, although simple exposure of oxyhemoglobin to H_2S gas is sufficient to produce the characteristic green colour of sulphMb [24]. In the presence of excess oxygen, oxyMb autooxidises to form metMb plus superoxide anion radical [25]. Trace amounts of superoxide thus formed could decay according to the following schemes:

$$O_2^{\bullet -} + O_2^{\bullet -} + H_2O \rightarrow H_2O_2 + O_2 + 2OH^-$$
$$O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

As the Mb produced during bacterial expression is a mixture of oxyMb and metMb, this mechanism could account for the presence of transient amounts of H_2O_2 in vitro and the subsequent formation of sulphMb.

The observations reported here do not necessarily imply that formation of recombinant sulphMb is a characteristic of other reported Mb expression systems because critical, poorly defined differences in bacterial strains, media, and precise growth conditions undoubtedly influence the formation of this derivative. Nevertheless, Olson and co-workers have noted the need to remove small amounts of a green component from some recombinant sperm whale Mb preparations [26], and

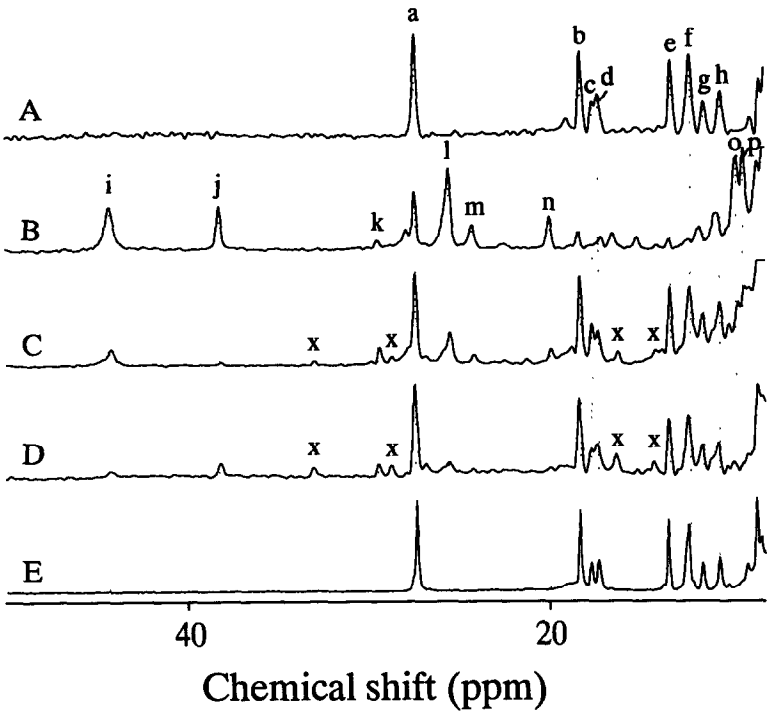

Fig. 3. ^{1}H NMR spectra (200 MHz) of horse heart cyanmetMb derivatives (50 mM sodium phosphate buffer, pH* 7.0, 20°C). (A) native Mb, (B) sulphMb prepared in vitro, (C) green Mb, (D) red Mb and (E) green Mb reconstituted with fresh iron protoporphyrin IX. Cyanmet derivatives were obtained by addition of five equivalents of KCN to ferriMb.

preparation of recombinant human Mb is reported [1] to require reconstitution with fresh hemin, which could account for the absence of spectroscopic anomalies with this system. An additional consideration in the production of recombinant heme proteins is indicated by a recent NMR study of recombinant human Hb expressed in *E. coli* [27] demonstrating that heme inserted into recombinant hemoglobin may not always achieve the correct orientation. Finally, the aberrant spectroscopic forms of recombinant cytochrome *c* peroxidase previously observed [28] may be related in part to the low level of heme incorporation into the recombinant peroxidase by *E. coli* and our observation is that this recombinant holoprotein exhibits an electronic absorption spectrum markedly different from that of the native protein (J.C. Ferrer and A.G. Mauk, unpublished).

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