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A Hemin-Analogous Corrphycene Derivative: Suppression of Heme Oxygenase and Reconstitution with Apomyoglobin

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Summary. A closely hemin-analogous corrphycene derivative was prepared in good overall yield. By spectroscopic measurements it was shown that it complexes with the stress protein heme oxygenase and apomyoglobin in a similar way as hemin. However, due to its molecular structure it is not attacked by heme oxygenase, but is able to block this enzyme to some degree. In addition, the complex with apomyoglobin displays oxygen and carbon monoxide ligation comparable to myoglobin. These properties make this novel corrphycene derivative a candidate to be used as heme oxygenase blocker or otherwise as a blood pigment substitute.

Keywords. Blood substitute; Heme oxygenase; Myoglobin; Corrphycene; Protein.

Introduction

Hemin (**1**) is one of the fundamental pyrrole pigments of life [1]. With respect to pathological problems involving this compound two areas seem to be of peculiar interest. First, ailments resulting from an enhanced degradation of **1** might be alleviated (*e.g.* neonatal jaundice, or even more important, cancer cells could be made more vulnerable to oxidative stress if deprived from antioxidants like bilirubin). Second, the search for artificial blood substitutes [2] would benefit from hemin-analogous compounds, which are not easily metabolized. With respect to the first aspect only a few agents to block the stress protein and key enzyme of heme metabolism, heme oxygenase, have been devised (*e.g.* zinc or tin protoporphyrin [3]). With respect to the second aspect an iron corrphycene [4] and an iron porphycene derivative [5] representing hemin-analogous compounds have been prepared among other non-pigment derived systems. We imagined that for both purposes a hemin-analogous derivative based on the porphyrin isomer corrphycene

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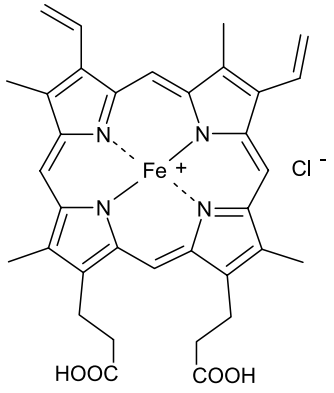
In memoriam of *H. Ruis*



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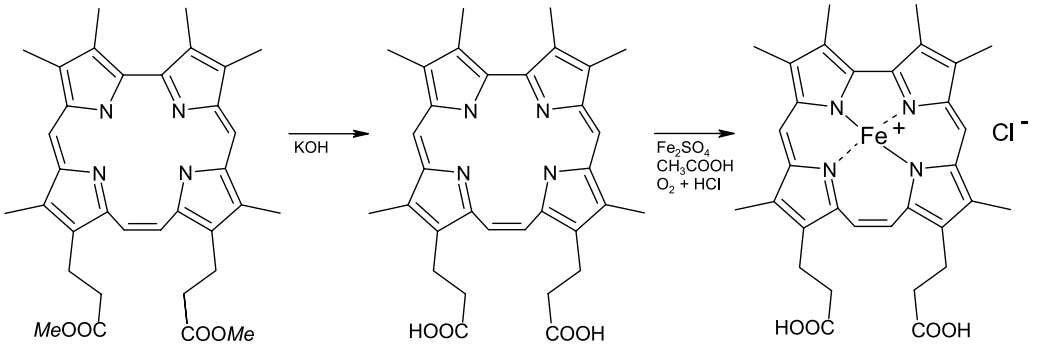
[6] would be a promising target because the missing α-methine bridge might make the pigment invulnerable to metabolic oxidative attack at this position.

Results and Discussion

Aspects of Preparation

The corrphycene-analogous hemin derivative, which has been described already [4], suffered from the fact that the two carboxyethyl groups directly attached to the macrocyclic ring system at the positions of the propionic acids of **1** could not be hydrolysed to the corresponding acids (only this substitutent would make it a true hemin analogue) without decomposition of the macrocycle. Thus, this derivative could not suit its purpose. To prepare an analogue closer in substitution to hemin, *i.e.* the corrphycene derivative **2** (as a minor change only the hemin vinyl groups were replaced by methyl), we saponified the diester **3** prepared recently [7] (it should be mentioned that the route to **3** described in this reference proved still to be superior to the other methods reported to prepare the corrphycene skeleton [6, 8]). The resulting diacid **4** was completely stable under acidic and basic conditions. The diacid **4** was then complexed with iron(II) and subsequently air oxidized to eventually provide the hemin-analogous corrphycene derivative **2** in 64% overall yield starting from **3** (Scheme 1). This corrphycene derivative **2** proved to be stable and similar in its properties to hemin (**1**). Interestingly enough, **2** did not suffer from the precipitation tendency of **1** [9] upon dissolving in water and accordingly gave stable aqueous solutions. The absorption spectra of **1** and **2** dissolved in phosphate-buffer are displayed in Fig. 1. Nevertheless, as in the case of **1** the rather broad and red shifted *Soret* band of **2** (from $\lambda = 390$ to 402 nm) points to an association phenomenon [9].

The magnetic moment of **2** was measured by means of the *Evans* NMR method [10] in comparison with that of **1**. Whereas we found for **1** dissolved in *DMSO* and *DMF* $\mu_B = 5.76$ and $5.69\,JT^{-1}$ well in accord with literature data [11] on this high spin (5/2) complex, these values were found to amount to $\mu_B = 3.03$ and $3.22\,JT^{-1}$ for **2**. This result agreed with a recent study of corrphycene complexes [12]. The



3 **4** **2**

Scheme 1

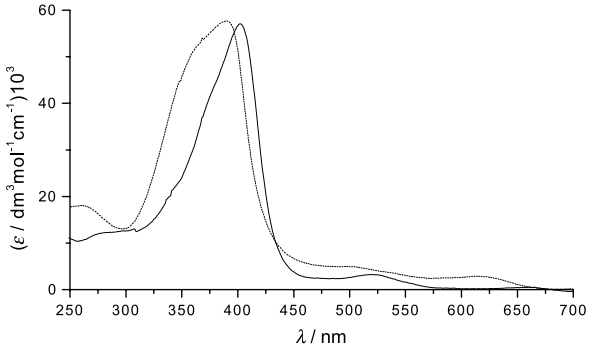


Fig. 1. UV-Vis spectra of **1** (· · ·) and **2** (—) in 50 m*M* phosphate buffer ($pH = 7.0$), $c = 5\,\mu M$, $t = 20°C$

authors of this study interpreted such lower values as originating from a mixture between $5/2$ and $3/2$ spin states.

*Interactions of **2** with Heme Oxygenase*

Heme oxygenase (EC 1.14.99.3, also known as heat shock protein-32) is the ubiquitous key enzyme in the metabolism of heme [13]. Complex formation of **2** was compared to the one of **1** under the conditions published for the latter in Ref. [14]. As shown in Fig. 2 the hemin analogous corrphycene derivative **2** behaved similarly upon complexation with heme oxygenase resulting in a characteristic *Soret* absorption at $\lambda = 412$ nm as compared to **1**, which absorbs at $\lambda = 405$ nm. Accordingly, **2** formed a complex with heme oxygenase in a comparable way as has been observed for **1**. However, upon a competition experiment it could be shown that **2** becomes gradually expelled from the complex with heme oxygenase by addition of an excess of **1**.

As demonstrated with the assay of Fig. 3, the corrphycene **2** was not attacked by heme oxygenase in comparison with **1** as described for the latter in Ref. [15]. The absorption spectrum of the heme oxygenase complex with **1** changed

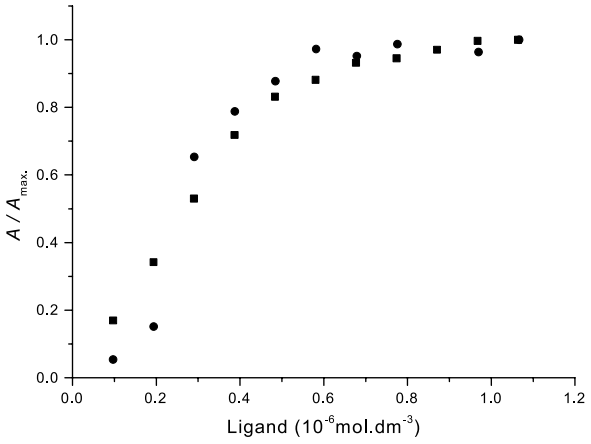


Fig. 2. Complexation of **1** (filled squares) and **2** (filled circles) with heme oxygenase (1 μM heme oxygenase in 30 mM phosphate buffer ($pH = 7.4$), containing "solution A" was titrated with the respective ligand, $t = 22°C$)

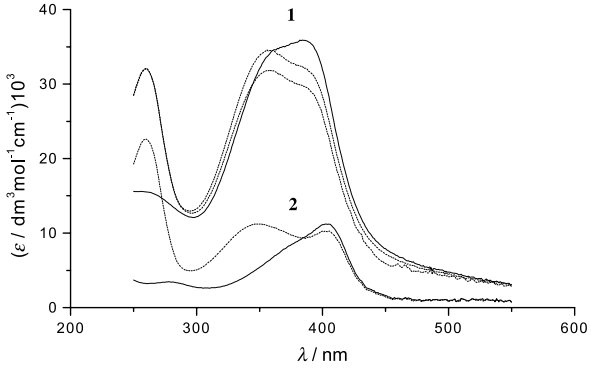


Fig. 3. Reaction of **1** and **2** (15 μM) with heme oxygenase and *NADPH* ($t = 22°C$); a solution of 0.34 nM heme oxygenase in 0.2 cm^3 of 100 mM phosphate buffer ($pH = 7.4$) (—), immediately after addition of 15 μM *NADPH* (· · ·), and after 1.5 h (· · ·)

characteristically by addition of *NADPH* and in the course of time the *Soret* absorption of this system faded. A corresponding change was observed when the complex of heme oxygenase and **2** was treated with *NADPH*, however, this spectrum did not change with time. Accordingly, **2** was not cleaved by heme oxygenase and because the complexation behavior of **1** and **2** are similar, **2** constitutes a candidate of a heme oxygenase blocker.

*Reconstitution of **2** with Apomyoglobin*

Apomyoglobin serves as a working horse in studies concerning analogies of hemin binding to a protein [16]. Upon reconstitution of **2** with apomyoglobin the 1:1 complex could be isolated and proved to be similarly stable as myoglobin. The absorption spectra and CD curves of reconstituted myoglobin and the apomyoglobin complex of **2** are displayed in Fig. 4. These data are quite similar and

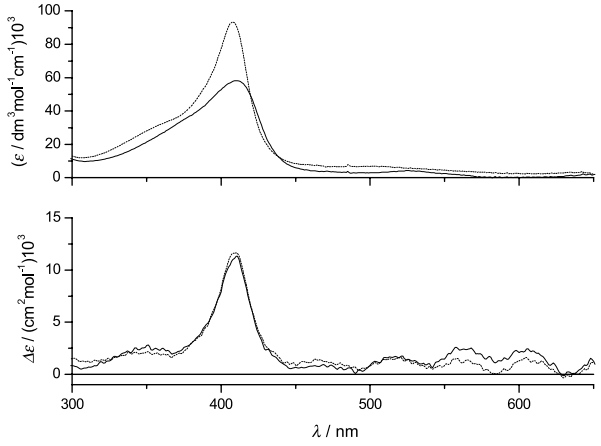


Fig. 4. UV-Vis spectra and CD of myoglobin (···) and the apomyoglobin complex of **2** (—) in 50 m*M* phosphate buffer ($pH = 7.0$), $c = 6\,\mu M$, $t = 22°C$

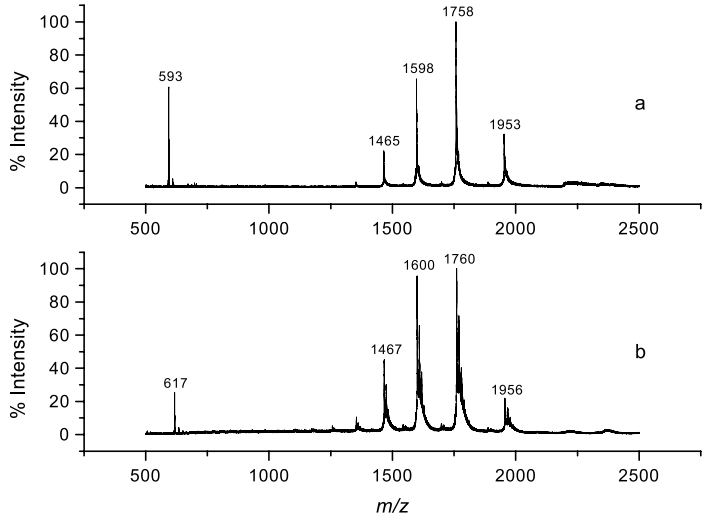


Fig. 5. Electrospray-ionization mass spectra of the apomyoglobin complex with **2** (a) and myoglobin (b)

accordingly emphasize the structural similarities of the two macrocyclic iron(III) apomyoglobin complexes. As in the case of the heme oxygenase complex the *Soret* band of **2** became red shifted from $\lambda = 402$ nm by 7 nm to $\lambda = 409$ nm.

The electrospray ionization mass spectrum of the complex of apomyoglobin with **2** also clearly demonstrated the bound state between the pigment and the globin. As illustrated with Fig. 5 the result compares nicely with the corresponding myoglobin experiment (*cf.* Ref. [17]) – the two pigments **1** and **2** are characterized by m/z values of 617 and 593, and the whole complex by the ionization cascade with the M^{10+} peak at $m/z = 1760$ and 1758 corroborating the pigment–globin binding ($\Delta M_{\mathbf{1-2}} = 24$, thus $\Delta M^{10+}{}_{\mathbf{1-2}} = 2$).

The complex between apomyoglobin and **2** could be further characterized by its ^1H NMR spectrum in comparison with the one of myoglobin [18] as illustrated

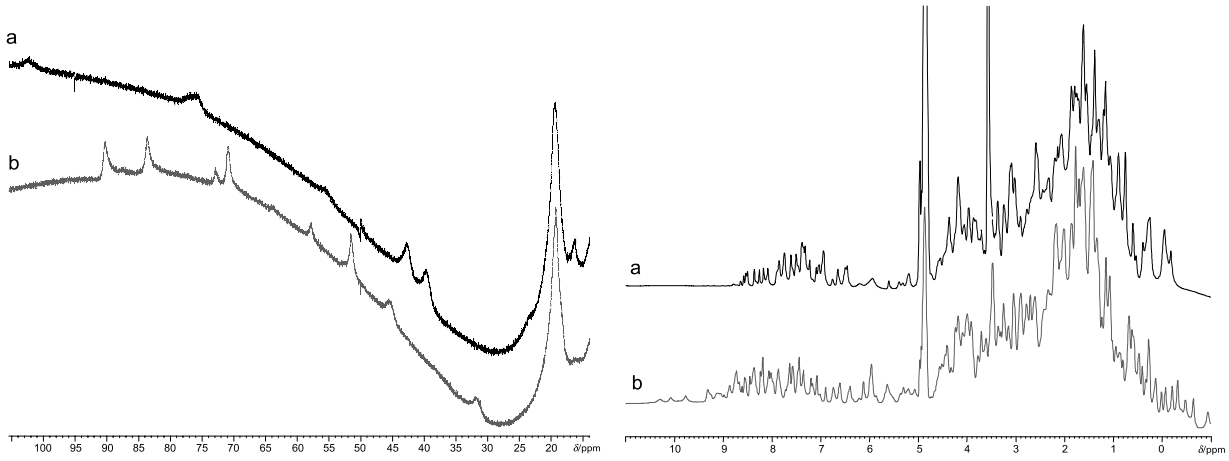


Fig. 6. ^{1}H NMR spectra of the complex between apomyoglobin and **2** (a) and of myoglobin (b), $c = 100\,\mu M$ in D_2O, $t = 30°C$

with Fig. 6. The paramagnetically shifted proton signals of the protons attached to the macrocycle between $\delta = 30$–100 ppm are clearly discernible – the protein proton signals observed between $\delta = 0$ and 10 ppm are similar, but in certain regions clearly distinct from each other. These observations indicate that the corrphycene **2** is similarly bound to the binding pocket of apomyoglobin as compared to hemin (**1**).

With respect to the binding of **2** to apomyoglobin we found that the association constant is comparable to the one of hemin [19], *i.e.* in the order of 10^{12}–$10^{15}\,M^{-1}$. The phenomenon is illustrated by the similar plots of the data of **1** and **2** in Fig. 7. However, in a competition experiment **2** became slowly expelled from its complex with apomyoglobin upon addition of **1** indicating a slightly smaller association constant.

Oxygen and carbon monoxide binding of the complex of corrphycene **2** and apomyoglobin was found to be comparable to that of myoglobin. As shown in

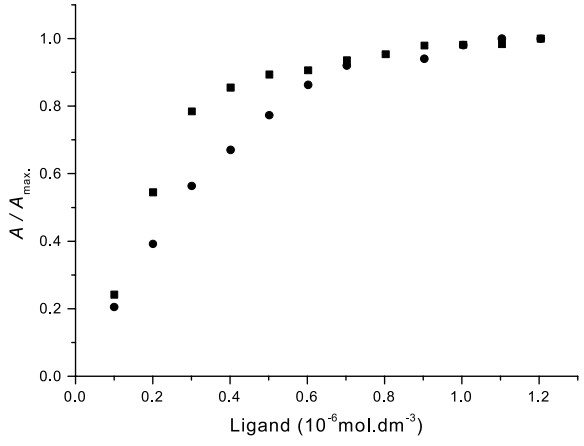


Fig. 7. Complexation of **1** (filled squares) and **2** (filled circles) with apomyoglobin (1 μM apomyoglobin in 30 mM phosphate buffer ($pH = 7.4$), containing "solution A" was titrated with the respective ligand, $t = 22°C$)

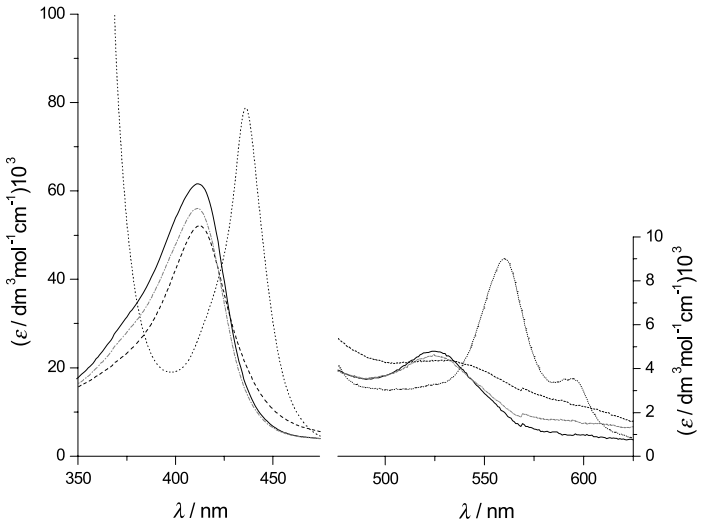


Fig. 8. Reactions of the apomyoglobin complex of **2**, ($c = 6\,\mu M$, 50 mM phosphate buffer ($pH = 7.0$), $t = 20°C$; (—)) with sodium dithionite (· · ·), then molecular oxygen (- - -), and eventually carbon monoxide (- . -)

Fig. 8 the complex became easily reduced to its Fe(II) state by means of dithionite as indicated by the strong bathochromic shift of the *Soret* band ($\Delta\lambda = 24$ nm; *cf.* myoglobin $\Delta\lambda = 25$ nm [20]). Bubbling with molecular oxygen quantitatively reverted the Fe(II) complex back to the Fe(III) state. An oxygen molecule became ligated to the pigment as indicated by a slight bathochromic and hypochromic shift (413 nm) of the *Soret* band as compared to the begin (412 nm) of the experiment. Upon bubbling of this oxygen complex with carbon monoxide the latter expelled oxygen and the characteristic hyperchromic and hypsochromic shift of the *Soret* band (412 nm) upon carbon monoxide ligation was observed. These spectroscopic changes were complemented by the even more characteristic changes in the Q-band region: $\lambda = 524 \rightarrow 560 \rightarrow 530 \rightarrow 524$ nm (Fig. 8).

According to the results described above, the complex between apomyoglobin and corrphycene **2** is comparable in its structural details and ligand binding properties to myoglobin. Given the retarded metabolism of this pigment (heme oxygenase does not attack the macrocyclic corrphycene ring as shown above) might be used as a blood pigment substitute.

In conclusion, we have prepared a close heme-analogous corrphycene **2**, which behaves in the protein binding aspects comparable to hemin, is not cleaved by heme oxygenase – even acting as a blocking agent – and effectively binds in its protein complexed state ligands like molecular oxygen or carbon monoxide. It might thus serve as a heme oxygenase blocker on the one hand, or a blood pigment substitute on the other hand.

Experimental

^{1}H NMR spectra were recorded by means of a Bruker Avance 500 MHz instrument. UV-spectra were measured on a Hewlett Packard HP 8453 and a Varian CARY 100 BIO instrument with a *Peltier* cell

thermostat allowing to set temperatures within ± 0.01°C. CD spectra were obtained on a Jasco J-180 instrument at $t = 20$°C. Magnetic moments were measured by means of the *Evans* ^{1}H NMR (Bruker Avance 200 MHz instrument) method [10] at $t = 25$°C. The TOF ESI spectra were obtained on a (TOF) ESI mass spectrometer (Perkin Elmer) with a nano-electrospray interface in the positive ion mode with the spray tip potential set at 1700 V and the nozzle potential at 80 V, $t = 20$°C, $c = 5\,\mu M$ in H_2O; *cf.* Ref. [17].

Stock solutions of hemin (bovine hemin chlorid; Sigma) and corrphycene were prepared by dissolving a small amount of hemin (7.8 mg) or corrphycene (7.5 mg) in 1 cm^3 of 0.1 *M* NaOH. The concentration of the apomyoglobin (from equine skeletal muscle; Sigma) solution was determined by spectroscopy using $\varepsilon_{280} = 14.272\,\text{m}M^{-1}\,\text{cm}^{-1}$ in H_2O.

The spectrophotometric titration of 1 μM heme oxygenase (*HSP*32; 100 μg solution in 50 m*M* ammonium bicarbonate, $pH = 8.3$; Sigma) with hemin and corrphycene was carried out by stepwise addition of 0.1 μM hemin or corrphycene which was dissolved in 30 m*M* sodium phosphate buffer ($pH = 7.4$) containing "solution A" to both cuvettes. The increments of absorbance as the difference at $\lambda = 405$ for hemin and 412 nm for corrphycene were plotted against the molar ratios of the hemin or corrphycene added and the free heme oxygenase, which was supposed to be present in the sample cuvette (Fig. 2). The mixture designated as "solution A" was a mixture of 1 m*M EDTA*, 0.1% Triton X-100 and 0.1% sodium cholate [14].

Standard reaction mixtures for the assay of heme bound to heme oxygenase in the reaction with *NADPH* (β-nicotinamide-adenine-dinucleotide phosphate, reduced, tetrasodium salt; Fluka) contained in a final volume of 200 mm^3: 15 μM hemin or corrphycene, 0.34 n*M* heme oxygenase, 100 m*M* sodium phosphate buffer ($pH = 7.4$), and 15 μM *NADPH*.

Reconstitution of apomyoglobin with hemin or corrphycene (Figs. 4, 7, 8) was carried out at $pH = 7.0$ in 50 m*M* sodium phosphate buffer at 0°C. A stoichometric amount of hemin or corrphycene was mixed with apomyoglobin to yield the reconstituted holomyoglobin.

For the NMR sample 100 μM apomyoglobin complex of **2** was diluted in 375 mm^3 of D_2O 99.9% (Eurisotop) and 125 mm^3 of 200 m*M* sodium phosphate buffer ($pH = 6$); lyophilised and then diluted with 500 mm^3 of D_2O 100% (Eurisotop). Equilibration took place under Ar for 18 h. The myoglobin sample was treated in the same way except the process of equilibration because natural myoglobin instead of the reconstituted one was used (Fig. 6).

The spectrophotometric titration of 1 μM apomyoglobin with hemin and corrphycene was carried out by stepwise addition of 0.1 μM **1** or **2** which was dissolved in 30 m*M* sodium phosphate buffer ($pH = 7.4$) containing "solution A" to both cuvettes. The increments of absorbance as the difference at $\lambda = 410$ for hemin and 412 nm for corrphycene were plotted against the molar ratios of **1** or **2** added and the free apomyoglobin which was supposed to be present in the sample cuvette (Fig. 7).

The corrphycene-apomyoglobin complex (6 μM, 50 m*M* sodium phosphate buffer ($pH = 7.0$), $t = 20$°C) was reduced with appropriate amounts of solid sodium dithionite to yield the ferrous complex. By bubbling the solution with molecular O_2 and CO the oxy and carbon monoxide forms of myoglobin reconstituted with **2** were obtained (Fig. 8).

Acknowledgments

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