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Functional consequences of haem orientational disorder in sperm-whale and yellow-fin-tuna myoglobins

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Ligand-binding kinetics of native and reconstituted sperm-whale myoglobin were studied in relation to haem orientational disorder by rapid kinetic methods. In addition, native yellow-fin-tuna myoglobin with significant amount of haem disorder was also used. The O_2 dissociation and association rates were found for the proteins with different degrees of haem disorder, and these results suggest that the isomers are characterized by almost identical kinetic parameters. Rates of CO recombination after photolysis were also identical for the two orientational isomers. The results clearly indicate that the rotation of the haem about the α–γ *meso* axis has little or no effect on the ligand-binding properties of these myoglobins.

INTRODUCTION

Single-crystal X-ray studies have shown that many haem proteins contain their haem prosthetic groups in a fixed orientation [1]. Such studies were, however, unable to detect the presence of a minor component with a 'reversed' haem orientation within the protein pocket. Structural heterogeneity was detected in various native and reconstituted haem proteins by using high-resolution proton n.m.r. [2–5]. This phenomenon is now apparently well established in many O_2-carrying proteins [2–5], including human haemoglobin [6–8].

In particular, the proton-n.m.r. spectrum of sperm-whale myoglobin clearly shows the existence of two, slowly interconverting, forms of the proteins differing only in the 180° rotation of the haem about the α–γ *meso* axis (Fig. 1) [9–11]. The rate of interconversion is sensitive to pH and nature of the bound ligand [12].

The reaction of apoprotein with haem has been reported [10] to yield, initially, a 1:1 mixture of the orientational isomers, which then interconvert to yield a final equilibrium at which 90% of the haem is in the orientation indicated by X-ray crystallographic studies while 10% remain in the 'reversed' orientation. This state of equilibrium is also present in the native myoglobin. The two isomers are indistinguishable from their optical spectra, but can be resolved by n.m.r. methods. Recently, however, circular dichroism in the Soret region has also been shown to distinguish the two isomers [13].

Comparative investigations show the degree and nature of disorientation to be species-dependent. Yellow-fin-tuna myoglobin *in vivo* contains 3:2 mixture of both native and reversed orientations respectively, and upon isolation interconversion between the two forms occurs [14]. The orientation of haem found in *Chironomus thummi thummi* haemoglobin is actually 'reversed' relative to sperm-whale myoglobin [3].

Although the phenomenon of orientational disorder has been extensively studied by n.m.r. apparently little is known about its effect on functional properties of proteins. Many haem proteins contain the same functional unit (protohaem) and yet exhibit remarkably different physiological properties. Thus it is obviously the protein–haem interactions that are responsible for modulating the haem function, and any changes in such interactions may affect the overall function of the haem protein.

The haem orientation results in the haem vinyl groups at positions 2 and 4 being interchanged with the methyl groups at positions 1 and 3 respectively. This may modulate to some small extent the peripheral contacts of these groups with the globin. To ascertain whether such small alterations in haem–protein interactions significantly affect the function of sperm-whale and tuna myoglobins is the aim of the present study.

A short communication [15] on the O_2-binding properties of sperm-whale myoglobin revealed significant differences in the O_2 affinities of the two isomers. The minor isomer was reported to have about 10 times the affinity of the major isomer. Livingston *et al.* [15] measured the O_2 affinities by measuring the half-saturation pressure (p_{50}) from O_2-dissociation curves of sperm-whale myoglobin. Any changes in p_{50} (or O_2 affinity) must result from changes in either the O_2 'on' rates and/or the O_2 'off' rates shown in eqn. (1):

$$\mathrm{Mb} + \mathrm{O_2} \underset{k}{\overset{k'}{\rightleftharpoons}} \mathrm{MbO_2} \qquad (1)$$

where Mb represents myoglobin. We have measured both the 'on' rate (k') by photolytic methods and the 'off' rate (k) by stopped-flow techniques. Our results show virtually no difference in either rate constant, suggesting no difference in O_2 affinity of the two isomers. These results are in agreement with earlier reports showing identical ligand-binding kinetics of native and reconstituted myoglobins [16–21]. Whether a similar situation pertains for the complex, interacting, O_2-binding proteins such as haemoglobin and erythrocrurins remains to be investigated.

MATERIALS AND METHODS

Preparations

Sperm-whale myoglobin (type II) and haemin (type III) were used as obtained from Sigma Chemical Co.

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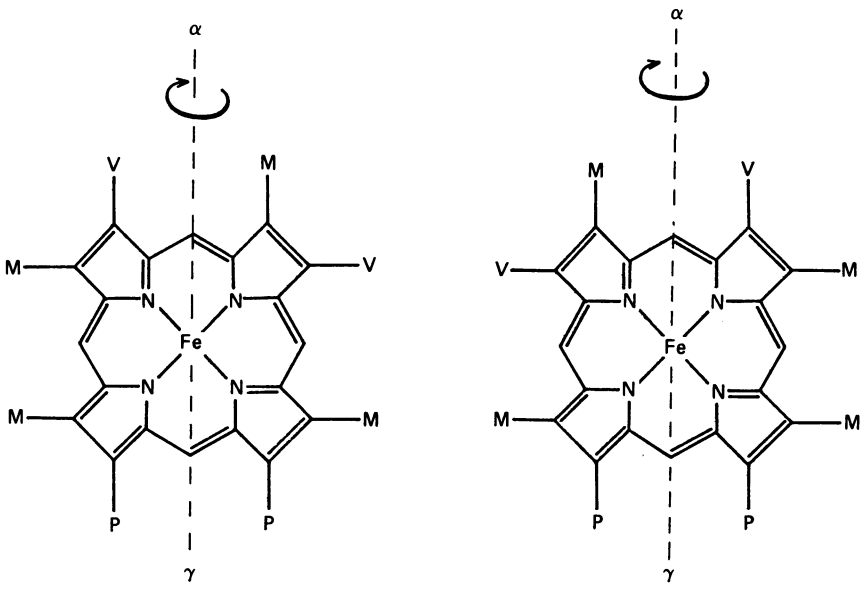

Fig. 1. The two possible haem orientations, viewed from the haem proximal side, as found in sperm-whale and yellow-fin-tuna myoglobins

(A) is the predominant form in the native proteins, and (B) is the disordered form resulting from 180° rotation about the α–γ *meso* axis. M, V and P are respectively methyl, vinyl and propionate groups on the periphery of the haem group.

Sephadex G-25 (fine grade) was purchased from Pharmacia. Deep-frozen yellow-fin tuna (*Thunnus albacares*) was supplied by Dr. J. Bonaventura and Dr. C. Bonaventura (Duke University Marine Laboratories, Beaufort, NC, U.S.A.). Myoglobin was isolated from the muscle tissue by the method of Rice *et al.* [22] and used soon after dialysis against chilled 0.1 M-sodium phosphate buffer, pH 7.4.

The procedure for the preparation of apo-myoglobin and its reconstituted product has already been described [13]. In all the experiments involving reconstituted myoglobin the measurements were carried out within half an hour from the addition of haem to its apoprotein. These measurements were repeated after the samples had been left to equilibrate, for up to a week at pH 7.4 or for 2 days at pH 5.5, at room temperature.

Sperm-whale oxymyoglobin was prepared by the reduction of ferric derivative with a slight excess of $Na_2S_2O_4$ followed by gel filtration on a Sephadex G-25 column (20 cm × 4 cm) equilibrated with 0.1 M-sodium phosphate buffer, pH 7.4, containing 1 mM-EDTA. This procedure was carried out at 4 °C to minimize autoxidation of oxymyoglobin. Only preparations of oxy derivatives with A_{581}/A_{543} ratio greater than unity were used.

Anaerobic $Na_2S_2O_4$ solutions were prepared from degassed buffers under N_2. These solutions were used within a few hours of preparation. CO-saturated solutions were made by equilibrating degassed buffer with CO gas (100 kPa). The concentration of CO in solution was checked by direct titration with a known concentration of dithionite-reduced myoglobin.

Absorption spectra were recorded on a Perkin–Elmer type 575 spectrophotometer.

The presence of disordered myoglobin was checked by c.d. [13].

Stopped-flow

A Durrum–Gibson [23] stopped-flow apparatus with 2 cm observation chamber and dead time of approx. 3 ms was used. O_2 dissociation was achieved by rapidly mixing oxymyoglobin with $Na_2S_2O_4$ solutions, and the rate constants (k) were determined from the progress curve. Care was taken to exclude contributions from metmyoglobin (often a contaminant of reconstituted oxymyoglobin) by making measurements at a wavelength of 419 nm. In a separate set of control experiments this wavelength was found to be strictly isosbestic for the reduction of metmyoglobin.

O_2 'off' rates were also determined by an O_2-displacement method in which oxymyoglobin is rapidly mixed with CO solutions of various concentrations and the displacement reaction followed by stopped-flow. This is an especially good method since it excludes spectral contributions arising from side reactions involving contaminating ferric protein.

Flash photolysis

The basic design of the apparatus was similar to the stopped-flow with some modifications. The reaction chamber was transparent with a flash system directly above it. Flashes of few milliseconds duration (Metz 45 CT-1) were used for photodissociation of CO. The absorbance changes associated with CO recombination were observed via a monochromator coupled to photomultiplier and storage oscilloscope.

A little dithionite in solution was present to ensure complete formation of carbonmonoxymyoglobin derivative.

Laser photolysis

As the quantum yield for the photodissociation of O_2

from myoglobin is low ($\phi = 0.03$), a procedure developed by Gibson [24] (see ref. [17]) was used in which CO was photodissociated from the carbonmonoxymyoglobin derivative, and O_2, physically dissolved in solution, was allowed to combine with the resulting deoxymyoglobin. The photodissociation was achieved with an Nd-Yag laser delivering pulses of a few nanoseconds duration at 532 nm and with energy of 5 mJ.

Immediately following flash and before replacement begins the following reactions occur:

$$Mb + O_2 \xrightarrow{k'} MbO_2$$

$$Mb + CO \xrightarrow{l'} MbCO$$

However, when the concentration of CO is low relative to that of O_2 then $k'[O_2] \gg l'[CO]$. The combination of O_2 is then the predominant reaction. Over a longer period, following flash, the O_2 is replaced by CO to return the system to the pre-flash condition. In this way series of photolytic events can be recorded and averaged for each sample.

The optical cell, filled with oxymyoglobin solution of known O_2 concentration, was tightly sealed, and additions of anaerobic or CO solutions were made by injection with a hypodermic syringe.

Data collection and analysis

For stopped-flow and flash-photolysis experiments the transmission changes from the photomultiplier were displayed on a storage oscilloscope and also fed into a transient recorder interfaced to a Commodore Pet microcomputer and a digital plotter. The program fitting the data gives plots of transmission changes, absorbance changes (ΔA) and $\log(\Delta A)$ as function of time. Most experiments were under pseudo-first-order conditions with respect to the ligand under study.

In the laser-flash experiments up to 64 flashes, taken at 10 s intervals, were signal-averaged (Datalab 102) and analysed on a BBC microcomputer. Depending on the type of data, the responses can be fitted to a single-exponential or double-exponential decay by a simple non-linear least-squares procedure. In all cases the first few points were ignored because of distortion by the laser flash.

RESULTS

Figs. 2(*a*) and 2(*b*) show the O_2-dissociation kinetics of native and reconstituted sperm-whale myoglobin at 419 nm. The dissociation in both cases is clearly a single-exponential process conforming to first-order kinetics. The value of the 'off' rate (k of Table 1) determined from such traces was found not to vary from one preparation to another. To check for a possible wavelength-dependence the measurements were made at several different wavelengths. It became clear that if small amounts of contaminating metmyoglobin were present the results became complicated and the progress curve biphasic at wavelengths other than 419 nm. In our experience we have found that the reconstituted myoglobin is more susceptible to autoxidation, and very small amounts of contaminating ferric form are often present even in the best preparations. Oxymyoglobins purified on a DEAE-cellulose (DE-32) column to remove metmyoglobin give identical kinetics at all wavelengths. This procedure, however, has the disadvantage of allowing more time (passage though the column) for the orientational isomers to equilibrate.

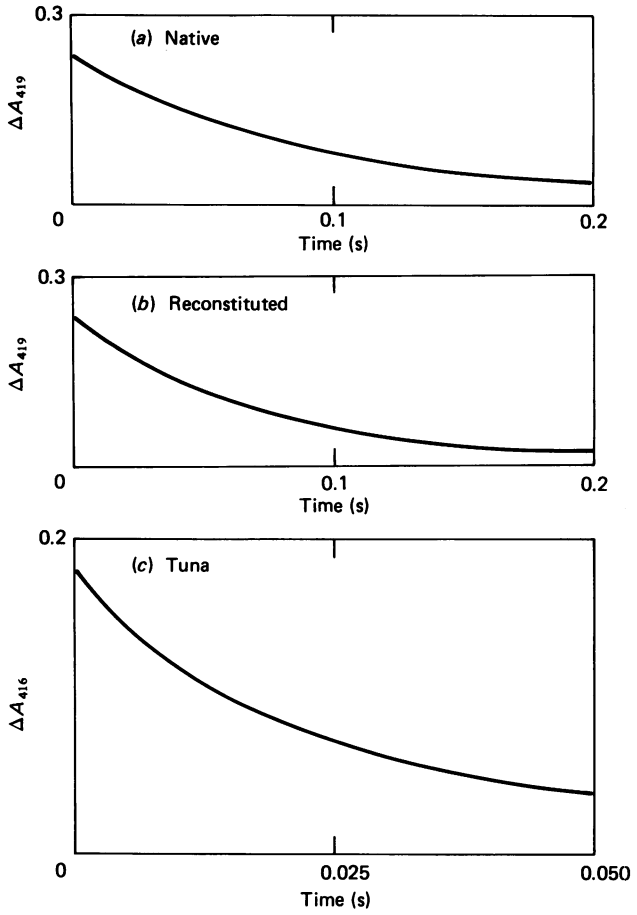

Fig. 2. O_2-dissociation kinetics of (*a*) native and (*b*) reconstituted sperm-whale myoglobin and (*c*) native yellow-fin-tuna myoglobin in 0.1 M-phosphate buffer, pH 7.4, at 20 °C

Determinations were made at 419 nm for sperm-whale myoglobin and at 416 nm for tuna myoglobin. Concentrations after mixing were 5 μM-protein and 15 mM-dithionite.

Fig. 2(*c*) shows a typical progress curve for O_2 dissociation from tuna myoglobin. The dissociation, although faster, is similar in nature to that from sperm-whale myoglobin.

The results of experiments in which CO was used to displace O_2 from oxymyoglobin are shown in Fig. 3. The displacement was also a single-exponential process at all CO concentrations, and showed no wavelength-dependence. The results were analysed according to eqn. (2) [17,25]:

$$\frac{1}{R} = \frac{1}{k} + \frac{k'[O_2]}{kl'[CO]} \qquad (2)$$

where R is the first-order rate constant for approach to equilibrium (i.e. O_2 displacement), k is the O_2 'off' rate constant and l' and k' are the combination rate constants for the CO and O_2 respectively. A plot of $1/R$ versus $[O_2]/[CO]$ gives an intercept of $1/k$. The intercept was

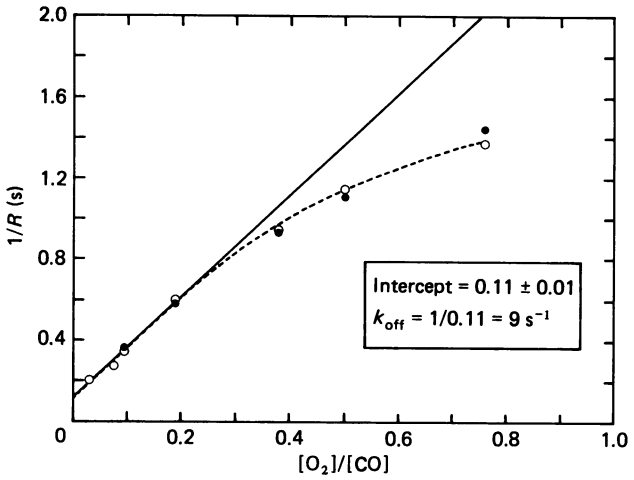

Fig. 3. Plot of the reciprocal rate of O_2 displacement (R) by CO for native (●) and reconstituted (○) sperm-whale myoglobin in 0.1 M-phosphate buffer, pH 7.4, at 20 °C

The intercept shown in the box was obtained by linear-regression fit to the first six points.

obtained by linear regression to the first six points in Fig. 3. A k value of 9 s^{-1} was obtained, and this is in reasonable agreement with the value obtained by the dithionite stopped-flow method (Table 1).

The non-linearity observed at low CO concentrations has been observed before for myoglobins [17]. The reason for this is not clear, but it may be due to breakdown in assumptions made in deriving eqn. (2) and also possibly due to existence of intermediates in the displacement reaction [17].

The rate of O_2 combination to deoxymyoglobin following laser photolysis of the carbonmonoxymyoglobin derivative in the presence of O_2 are also identical, within experimental error, for the native and reconstituted sperm-whale myoglobin (Fig. 4 and Table 1). The slow relaxation, seen more clearly in Fig. 4(*c*) for tuna myoglobin, was found from a separate set of experiments to be due to slow replacement of O_2 by CO in the dark shortly after O_2 combination has taken place. It must also be mentioned that leaving the reconstituted myoglobin to equilibrate (for a week at pH 7.4 or for 2 days at pH 5.5) at room temperature had almost no effect on the k and k' values.

The results of flash photolysis to determine CO-combination rate constants are shown in Fig. 5. The recombination of CO is also monophasic and identical for both native and reconstituted myoglobin. A little 'tail' seen at the start of progress curves is due to interference of the photolytic flash with the observation beam.

DISCUSSION

Table 1 summarizes the kinetic parameters obtained and also shows the percentage of disordered form present in each of the samples as reported by LaMar *et al.* [10,14]. These percentages are also consistent with c.d. measurements [13].

The overall O_2 affinity of the myoglobin is determined by the O_2 'off' and 'on' rates. If, as reported [15], the O_2

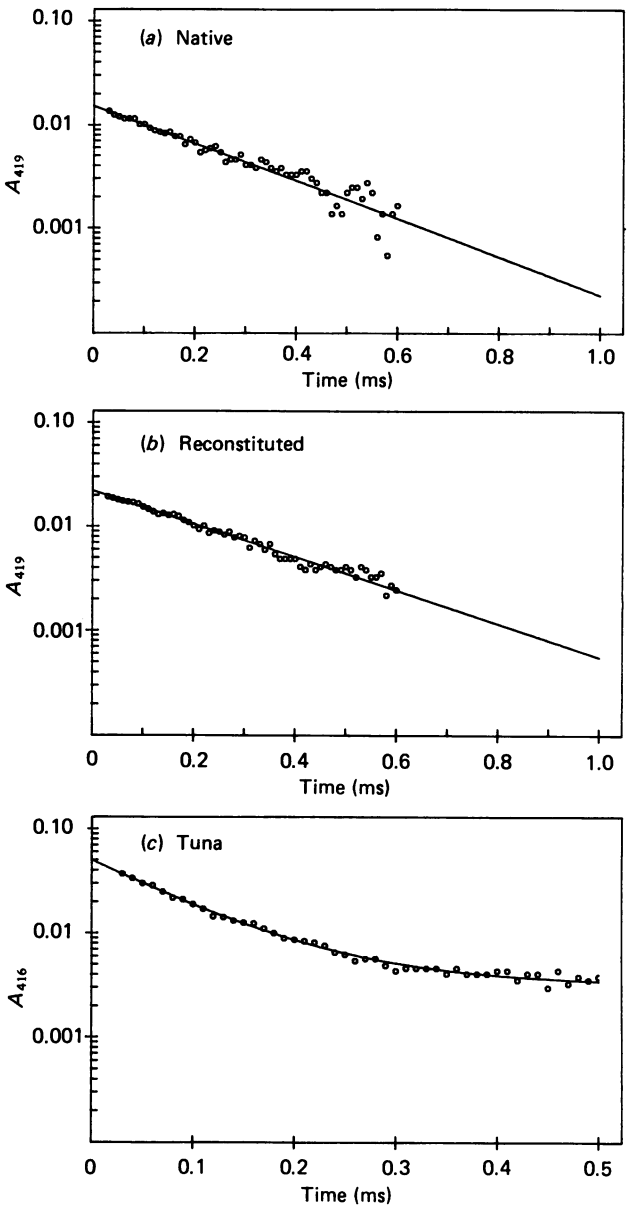

Fig. 4. O_2-combination kinetics of (*a*) native and (*b*) reconstituted sperm-whale myoglobin and (*c*) tuna myoglobin in 0.1 M-phosphate buffer, pH 7.4, at 20 °C

Concentrations were 5 μM-protein, 220 μM-O_2 and 60 μM-CO.

affinities of the isomers differ by a factor of 10-fold one should observe significant differences in their rates of O_2 combination and/or dissociation. Higher affinity would be reflected in low 'off' and/or high 'on' rates. Generally, however, changes in affinity for small gaseous ligands are reflected mainly in the 'off' rates, whereas those of bulky ligands such as isocyanides are reflected in the 'on' rates, which may depend on steric interactions in the vicinity of the haem group.

The O_2-dissociation or -association progress curves of a mixture of isomers characterized by kinetic parameters differing by a factor of 10-fold (or even less) should contain both a fast and a slow phase. Furthermore, since

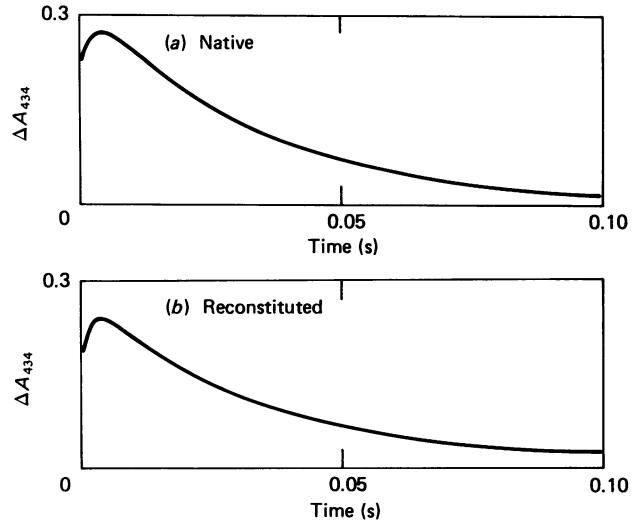

Fig. 5. CO-recombination kinetics of (*a*) native and (*b*) reconstituted sperm-whale myoglobin in 0.1 M-phosphate buffer, pH 7.4, at 20 °C

The wavelength was 434 nm and concentrations were 7 μM-protein and 70 μM-CO.

the two isomers have identical optical spectra [13], the absorbance changes associated with the two phases must reflect the proportions of the isomers present.

Our results clearly show the 'off' and 'on' rates to be identical for native myoglobin and a 1:1 mixture (reconstituted myoglobin) of isomers. No slow phase reflecting higher affinity was observed in either the dithionite or the displacement method. The 'off' rate for the 1:1 mixture was slightly higher than for the native form as measured by the dithionite method. The difference, however, is small and could only reflect an approx. 10% lower affinity of the 1:1 mixture. In addition, the dissociation of O_2 from tuna myoglobin containing a significant amount of disordered form (3:2) further shows the two isomers to have identical 'off' rates (36 s^{-1}).

The O_2- and CO-association kinetics similarly shows no fast phase reflecting higher affinity of these ligands for the disordered form.

Thus our studies on sperm-whale and yellow-fin-tuna myoglobins indicate haem orientation to have no significant effect on the O_2-binding properties of these proteins. Our results are in agreement with several authors [16–21] who have shown that myoglobins reconstituted with protohaem have ligand-binding properties identical with those of the native forms. Although these authors have not reported precise times between reconstitution and ligand-binding measurements, their samples are likely to contain appreciable amounts of haem disorder (unknown at that time), since the rate of interconversion between the two isomers is extremely slow under physiological conditions [10,26].

Our results conflict, however, with those of Livingston *et al.* [15], who report a 10-fold difference between the O_2 affinities of the two isomers as measured by O_2 titrations made in a spectrophotometric/polarographic device. It is possible that the discrepancies between the findings of these authors and ourselves derive from the unrecognized oxidation of a fraction of haem during the course of their spectroscopic titrations. The oxidation, being irreversible, would tend to give high apparent binding constants for O_2. As mentioned above, freshly reconstituted myoglobin is more susceptible to autoxidation than is the native protein. In addition, we note that the O_2 'off' rates measured by Livingston *et al.* [15] are considerably higher than those that we observe. However, as discussed above, the measurements of O_2 'off' rates are also strongly perturbed by the presence of metmyoglobin, and thus at high dithionite concentration, as is generally employed in such measurements, it is possible to monitor both reduction of the ferric form as well as deoxygenation, depending on wavelength.

It may also be noted that, although native tuna and sperm-whale myoglobins have different degrees of haem disorder present, their O_2 affinities are, however, similar. *Chironomus thummi thummi* haemoglobin, which has 'reversed' haem [3] relative to sperm-whale myoglobin, also has almost identical O_2 affinity [27]. Thus in those myoglobins it appears that haem orientation is of little importance in regulating O_2 affinity. However, since absolute O_2 affinity is a function of many specific haem–protein interactions, which can vary significantly from source to source, the species-independent affinity may result from a number of opposing influences of which one may be haem rotation.

Perhaps it is not surprising that the two isomers have identical O_2 affinity, since rotation of haem merely interchanges peripheral methyl and vinyl groups. These groups, being close to each other on the periphery of the haem group, are unlikely when interchanged to cause significant changes in haem–protein interactions regula-

Table 1. Rate constants for the reactions of sperm-whale (native and reconstituted) and tuna myoglobins with O_2 and CO ligands in 0.1 M-phoshate buffer, pH 7.4, at 20 °C

k is the dissociation rate constant for O_2 and *k'* and *l'* are the association rate constants for O_2 and CO respectively. Values for percentage haem disorder are taken from LaMar *et al.* [10,14] and are consistent with our c.d. measurements.

Haem disorder (%)	Myoglobin	k (s^{-1}) Dithionite method	k (s^{-1}) Displacement method	$10^{-7} \times k'$ ($M^{-1} \cdot s^{-1}$)	$10^{-5} \times l'$ ($M^{-1} \cdot s^{-1}$)
10	Native	10	9	2	4.3
50	Reconstituted	12	9	1.8	4.2
40	Tuna	36	–	4.5	–

ting O_2 affinity. Indeed, the role of these groups has been assessed in reconstitution experiments with haems modified at the 2,4-positions [16–20] and also 'bald' (lacking vinyl and methyl groups) and 'stripped' (lacking all peripheral groups except propionate groups) haems [21] have been used. Such studies have confirmed that complete removal of vinyl and methyl groups, which interchange upon rotation, may affect the O_2 affinity of some proteins by a maximum of 2–3-fold only. This may, however, be explained by the electronic effects of completely removing groups from the haem.

Thus the physicochemical properties of myoglobins appear to be unaffected by haem disorder, and the question remains whether it is of any importance in other haemoproteins. It is possible that haem disorder, although unimportant in simple haemoproteins (myoglobins), actually plays a more significant role in complex proteins (haemoglobins). Thus haem disorder could exert its influences on properties that are absent from the myoglobin molecules. Such properties include the Bohr effect, co-operative ligand binding and allosteric interactions etc. In this context it is noteworthy that Gersonde *et al.* [28] have recently found the Bohr effect in *Chironomus* monomeric haemoglobin to be controlled by haem orientational disorder.

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