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MEASUREMENT OF MYOGLOBIN DIFFUSIVITY IN THE MYOPLASM OF FROG SKELETAL MUSCLE FIBRES

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SUMMARY

1. Experiments were carried out on intact, single skeletal muscle fibres from frog in order to estimate the apparent diffusion constant of myoglobin (denoted D_{APP}) in the myoplasm of living muscle cells. An optical technique was employed to measure myoglobin concentration along the fibre axis following injection of metmyoglobin (denoted metMb) at a point source. The concentration profiles were fitted by the one-dimensional diffusion equation to give estimates of D_{APP}. The method relied on the fact that myoglobin is normally absent from these frog fibres, thus permitting resolution of the myoglobin-related absorbance above the intrinsic absorbance of the fibre.

2. One complication in the method was that metMb became significantly reduced to oxymyoglobin (denoted MbO_2) during the elapsed time before measurement of the concentration profile. The rate of reduction was evaluated by fitting myoglobin-related absorbance spectra, measured at different times following injection of metMb, with *in vitro* absorbance spectra of metMb and MbO_2. Results from four experiments indicated that reduction could be described by a first-order, irreversible reaction having an average rate constant of 0·0164 min^{-1} (22 °C). The effect of reduction on the fitting of D_{APP} was taken into account.

3. D_{APP} was determined under three fibre conditions: (1) long sarcomere spacing (3·6–3·8 μm) at 16 °C, (2) long sarcomere spacing at 22 °C, and (3) normal sarcomere spacing (2·4–2·7 μm) at 22 °C. The average values for D_{APP} under these conditions were: (1) 0·12 ($n = 5$); (2) 0·17 ($n = 5$); and (3) 0·15 ($n = 7$) $\times 10^{-6}$ cm^2 s^{-1}. The average value at 22 °C, 0·16 $\times 10^{-6}$ cm^2 s^{-1}, is about 4 times smaller than values for myoglobin diffusivity at 20 °C commonly assumed in models of facilitated transport of oxygen by myoglobin.

4. In order to test the possibility that the unexpectedly low value of D_{APP} found in intact fibres might be due to the binding of myoglobin to relatively immobile sites in myoplasm, experiments were carried out in a cut-fibre preparation using a technique described by Maylie, Irving, Sizto & Chandler (1987*b*) for determining the diffusion constants and degree of myoplasmic binding of absorbance dyes. Values for D_{APP} and the factor (denoted $1+\beta$) by which the total myoglobin concentration exceeded the free myoglobin concentration were obtained by fitting the absorbance data by solutions of the one-dimensional diffusion equation. The average value

($n = 4$) for $1+\beta$ ranged from 1·02 to 1·24, depending on the rate assumed for metMb reduction in cut fibres. Even the highest estimate of $1+\beta$ is much lower than the value of 4 required in order to attribute the small value of D_{APP} measured in intact fibres to the presence of bound (and immobile) myoglobin.

5. Experiments were also performed in intact fibres to test whether possible convective movements of myoplasm associated with the mechanical events of muscle contraction might enhance D_{APP}. No significant increase in D_{APP} was observed when fibres were either tetanically stimulated to contract, or passively stretched and shortened, during the period following myoglobin injection and prior to measurement of its concentration profile.

6. A partial theoretical analysis of facilitated oxygen transport indicates that, even with the low value of D_{APP} reported here, under conditions of low free oxygen concentration, myoglobin can still double the transport of oxygen for a given free oxygen gradient.

INTRODUCTION

Myoglobin, like haemoglobin, binds oxygen and undoubtedly serves some function in the oxygen supply of cardiac and red skeletal muscle tissues. One hypothesis is that myoglobin acts to buffer fluctuations in the myoplasmic free oxygen concentration during muscle contraction (Millikan, 1937; Millikan, 1939; see also Tamura, Oshino, Chance & Silver, 1978). Another proposed function is that myoglobin facilitates transport of oxygen through myoplasm by a mechanism involving the diffusion of oxymyoglobin (MbO_2). While it has been demonstrated that myoglobin can facilitate oxygen transport in aqueous solutions (see reviews by Wittenberg, 1970; and Kreuzer, 1970), the degree to which myoglobin might facilitate oxygen transport *in vivo* has eluded firm experimental demonstration (Wittenberg, Wittenberg & Caldwell, 1975; Cole, Wittenberg & Caldwell, 1978; Cole, 1982; Wittenberg & Wittenberg, 1987; see also Jones & Kennedy, 1982; Wittenberg & Wittenberg, 1985).

The physiological importance of facilitated transport of oxygen by myoglobin should depend on a complex interaction between oxygen delivery from capillaries to muscle cells, the myoplasmic oxygen concentration, the rate of oxygen metabolism, and various other parameters including the intracellular diffusion constants of oxygen and myoglobin. Mathematical modelling studies considering various aspects of this problem, including those of Murray (1974), Taylor & Murray (1977), Fletcher (1980), Federspiel (1986), Salathe & Kolka (1986) and Loiselle (1987), have served to theoretically quantify the possible importance of facilitated oxygen transport by myoglobin under assumed, *in vivo* conditions. Of the various assumptions required in these models, one of the most critical and least certain has been the value chosen for myoglobin's diffusion constant in myoplasm (denoted D_{Mgb}). Most modelling studies have used the data of Riveros-Moreno & Wittenberg (1972), who determined D_{Mgb} at 20 °C in solutions varying in myoglobin concentration. The assumption made was that D_{Mgb} in muscle would correspond to that measured in an 18% protein solution. The values chosen ranged from about 0·5 to $0{\cdot}7 \times 10^{-6}$ cm^2 s^{-1} at 20 °C, which are about half of the value measured in dilute solution. Supportive of the range of values typically assumed by modellers at 20 °C was a nuclear magnetic resonance

(NMR) study by Livingston, La Mar & Brown (1983) (see also Jacquez, 1984) purporting to measure the rotational relaxation time of myoglobin in bovine heart muscle. However, generally overlooked have been the measurements by Moll (1968) of myoglobin diffusion between adjacent layers of homogenized muscles, one layer from muscle rich in myoglobin and the other poor in myoglobin. Moll measured a value for D_{Mgb} of $0{\cdot}15 \times 10^{-6}$ cm^2 s^{-1} at 20 °C and a value of $0{\cdot}27 \times 10^{-6}$ cm^2 s^{-1} at 37 °C. These estimates are about a factor of 4 lower than the commonly assumed values.

Clearly there is considerable uncertainty about the value of D_{Mgb} in muscle. The principal goal of this work was to measure myoglobin's diffusion constant in the myoplasm of living muscle, in order that the physiological importance of facilitated transport of oxygen could be more reliably evaluated. The primary method used involved injecting myoglobin into frog skeletal muscle fibres, a fibre type normally devoid of myoglobin, and measuring the longitudinal spread of myoglobin after about an hour. Fitting of the data to the one-dimensional diffusion equation yielded a value for the apparent diffusion constant of myoglobin (denoted D_{APP}). The value measured here for D_{APP} in living frog fibres, $0{\cdot}16 \times 10^{-6}$ cm^2 s^{-1} at 22 °C, is very close to the value determined by Moll (1968) in homogenized mammalian muscle.

The results also indicate that there was no significant enhancement of D_{APP} if a fibre was either tetanically stimulated to contract or passively stretched and shortened. These results argue against the possibility that facilitated transport of oxygen is greatly enhanced by convective transport of MbO_2 during muscle contraction, as suggested by Gayeski & Honig (1983) and Honig, Gayeski, Federspiel, Clark & Clark (1984).

The low value of D_{APP} reported here significantly reduces any assessment of the degree to which myoglobin might facilitate oxygen transport in myoplasm. However, a partial theoretical analysis indicates that, under conditions of low free oxygen concentrations, myoglobin can still double the transport of oxygen for a given free oxygen gradient.

A preliminary account of some of the results has appeared (Pape, 1987).

METHODS

Intact-fibre experiments

The apparatus and basic technique used to measure the apparent diffusion constant of myoglobin in myoplasm was identical to that developed by Baylor, Hollingworth, Hui & Quinta-Ferreira (1986) to measure the apparent diffusion constants of indicator dyes introduced into intact frog skeletal muscle fibres. Single twitch fibres were dissected from hindlimb muscles (semitendinosus or iliofibularis) of English frogs (*Rana temporaria*) and mounted on an optical bench apparatus for measuring intensities of transmitted light (Baylor, Chandler & Marshall, 1982). The chamber contained a normal Ringer bathing solution (in mM: NaCl, 120; KCl, 2·5; $CaCl_2$, 1·8; Na_2PIPES (sodium salt of piperazine-*N*,*N*′-bis(2-ethane-sulphonic acid)), 5) titrated to pH 7·10. The temperature of the bath was measured and maintained at either 16 °C (±1) or 22 °C (±1). Metmyoglobin (metMb) from horse skeletal muscle (Sigma Chemical Co., St Louis, MO, U.S.A.) was pressure-injected into the myoplasm from a micropipette containing a filtered solution (pore size 0·22 μm) of approximately 12 mM-metMb in distilled water. About an hour following the injection, absorbance measurements made at various points along the fibre established how far the myoglobin had diffused away from the injection site. Application of the one-dimensional diffusion equation to the data yielded an estimate of the apparent longitudinal diffusion constant of

myoglobin in myoplasm. An advantage of using frog skeletal muscle is that this fibre type does not normally contain myoglobin and the absorbance due to injected myoglobin is generally well resolved above the relatively small intrinsic absorbance of a single fibre. A possible disadvantage of the method is that myoglobin diffusion in the myoplasm of frog skeletal muscle might be significantly different from that applicable to red muscle. This question is considered in the Discussion.

Optics and data acquisition

The optical arrangement was identical to that described previously (Baylor *et al.* 1986). Briefly, light from a 100 W tungsten–halogen bulb was passed through a field diaphragm and an interference filter with a half-band of 10 nm to select a particular wavelength range, and was focused onto the fibre by means of a long-working-distance objective. Most myoglobin-related measurements were made in the wavelength range 410–420 nm, where myoglobin has an unusually large peak in absorbance (cf. Fig. 1). Masks placed near the field diaphragm limited the illumination of the muscle fibres to circular spots ranging in diameter between 43 and 73 μm. The spot size was chosen to be less than the fibre diameters, which ranged between 53 and 116 μm. The light from the fibre was collected with an identical long-working-distance objective and passed through a Thompson calcite beam-splitting prism which produced two beams of polarized light, one beam polarized in a plane parallel (denoted 0 deg) and the other perpendicular (denoted 90 deg) to the long axis of the fibre. The polarized light beams were projected onto two identical silicon diode photodetectors (model UV-100B, EG&G Inc., Salem, MA, U.S.A.), which gave voltage outputs linearly proportional to transmitted light intensity, thus permitting the measurement of polarized absorbances. The voltage outputs of the photodetectors were simultaneously sampled by a computer-controlled multiplexer and analog-to-digital converter and stored for later analysis.

Metmyoglobin injection

The injection of metMb into fibres was carried out by means of pressure (Picospritzer II, General Valve Corp., Fairfield, NJ, U.S.A.) applied to the back of a moderately blunt micropipette. The micropipettes were made from acid-cleaned microelectrode glass (type TW150F-4, WP Instruments, Inc., New Haven, CT, U.S.A.) pulled on a microelectrode puller (Model 27-9, Narishige Scientific Instrument Lab., Tokyo, Japan) and had resistances of about 2–5 MΩ if filled with a 3 M-KCl solution. Pipette tips were filled by capillary action with a solution of metMb in distilled water.

Fibre viability

All-or-none fibre activity, elicited by action potential stimulation triggered by a brief localized shock from a pair of extracellular electrodes, was checked at the beginning of each experiment. While most of the seventeen fibres reported in Table 1 also showed an all-or-none response at the end of the experiments (eleven of the seventeen), one showed a graded response, two were not checked and three gave no response on stimulation. The criteria for keeping an experiment in the latter cases required that the fibre appeared structurally intact and remained immobile during the course of the experiment. The diffusion constants determined for the latter six fibres were not significantly different from those determined in the fibres with an all-or-none response throughout.

Calculation of myoglobin-related absorbance

The method used to obtain the myoglobin-related absorbance in muscle fibres is essentially the same as that described by Baylor *et al.* (1986) for measuring dye-related absorbance signals. The apparent absorbance, A, of the muscle fibre is given by the relationship

$$A = \log_{10}(J/I), \tag{1}$$

where J and I are the intensities of light incident upon and transmitted through the muscle fibre, respectively. In order to obtain the myoglobin-related absorbance, the raw absorbance had to be corrected for the intrinsic absorbance of the muscle fibre. The latter was estimated by extrapolating absorbance measurements, made at a reference wavelength (710 or 720 nm) at which both metMb and oxymyoglobin (MbO_2) do not absorb, to the wavelengths used to measure myoglobin absorbance. Baylor *et al.* (1982, 1986) found that the fibre intrinsic absorbance is described by an empirical relationship of the form

$$A(\lambda) = A(\lambda_{ref})\,(\lambda_{ref}/\lambda)^z. \tag{2}$$

$A(\lambda)$ and $A(\lambda_{ref})$ are absorbances measured at wavelengths λ and λ_{ref}, where λ is the wavelength of interest (e.g. 410 or 420 nm) and λ_{ref} is the reference wavelength. By fitting eqn (2) to absorbance data from uninjected fibres, Baylor *et al.* (1986) determined average values for the exponential index x of 1·3 for 90 deg polarized light and 1·1 for 0 deg polarized light in the wavelength range from 480 to 810 nm. We have used a similar procedure in three fibres for which intrinsic absorbance at 710–720 nm was used to predict intrinsic absorbance at 420 nm and found that values for x of 1·8 for 90 deg and 1·4 for 0 deg polarized light more accurately described the data. For the experiments reported here, the latter values of x were used in eqn (2) in combination with absorbance measurements made at 710–720 nm to provide the major correction for fibre intrinsic absorbance at wavelengths between 400 and 430 nm. The former values for x, 1·3 for 90 deg polarized light and 1·1 for 0 deg polarized light, were used to estimate the fibre intrinsic absorbance in the wavelength range 460–610 nm from the 710–720 nm measurements.

Beer's law and optical path length

Beer's Law, given by the following equation:

$$C = A/(\epsilon l), \tag{3}$$

was used to relate myoglobin concentration (C) to myoglobin absorbance (A), where ϵ is the molar extinction coefficient of myoglobin and l is the optical path length through the muscle fibre. The value of l was estimated from measurements of resting birefringence and the diameter of the fibre as viewed in a plane perpendicular to the light path (Baylor, Chandler & Marshall, 1983).

One complication which arose and had to be dealt with was that metMb became reduced in the myoplasm to MbO_2. In the case where two separate absorbing species (metMb and MbO_2) are present, eqn (3) no longer applies, unless absorbance is measured at an 'isosbestic' wavelength (in which case the extinction coefficients for metMb and MbO_2 are the same). The more general case for relating absorbance to concentration, when both metMb and MbO_2 are present, is given by eqn (8) (p. 260 in Results).

In vitro calibrations of metmyoglobin and oxymyoglobin

Calibrations were carried out to measure the *in vitro* absorbance spectra of metMb and oxymyoglobin (MbO_2) so that the degree to which metMb became reduced to MbO_2 in the myoplasm during the course of an experiment could be determined. All measurements were made with solutions produced from a stock solution having a nominal concentration of 0·5 mM-metMb in 'internal' solution (100 mM-KCl, 10 mM-K_2PIPES, 20 μM-K_2EGTA (potassium salt of ethyleneglycol-bis-*N*,*N*,*N*′,*N*′-tetraacetic acid) at pH 7) designed to mimic the ionic environment of the myoplasm. The myoglobin concentration is nominal, because it refers to the concentration which would have been achieved assuming that the metMb from Sigma Chemical Co. was 100% dehydrated and pure, which apparently is not the case (see below). MbO_2 was formed by the rapid addition of about a fivefold molar excess of a strong reducing agent, sodium dithionite. Absorbance spectra of these metMb and MbO_2 solutions were measured with a UV-visible spectrophotometer (Model 4050, LKB Instruments, Paramus, NJ, U.S.A.) and closely resembled those reported by Yamazaki, Yokota & Shikama (1964). However, a least-squares fit of the absorbances for metMb and MbO_2 measured at 408, 418, 500 and 545 nm in the spectrophotometer with values taken from Yamazaki *et al.* (1964) indicated that our MbO_2 solution was only 91% reduced, with 9% still in the form of metMb. In addition, the extinction constants for MbO_2 reported for the peaks at 418, 544 and 582 nm by Yamazaki *et al.* (1964) indicated that the actual concentration of myoglobin present in our calibrating solutions was lower by 24%, i.e. was 0·38 mM. Sigma Chemical Co. reports that the metMb used in this study (lot No. 75F7035) is 96% pure based on a spectrophotometric assay as well as an elemental analysis (for C, N and Fe). The remaining discrepancy may therefore be due to hydration of the myoglobin.

Figure 1 compares cuvette calibrations of metMb and MbO_2 made on the optical bench using interference filters (symbols) with those made on the spectrophotometer (curves). The values determined on the optical bench between 460 and 590 nm agreed very closely with those measured in the spectrophotometer (Fig. 1*B*). However, there is some discrepancy in the values measured between 400 and 430 nm (Fig. 1*A*). Since knowledge of the shapes and relative amplitudes of the metMb and MbO_2 spectra in this wavelength range was important in the analysis of many experiments to be described, various factors were considered for identifying the source of this discrepancy. This was important, because most of the experiments to be reported were performed

prior to our knowledge that metMb became reduced to MbO_2 in frog fibres and only one wavelength, 410 nm, was used to monitor myoglobin concentration. The spectral feature of particular importance in this analysis is the ratio of the extinction constant of metMb to that of MbO_2 at 410 nm. The experimentally determined values for this ratio were 1·42 in the spectrophotometer and 1·27 on the optical bench. The following analysis explains this discrepancy.

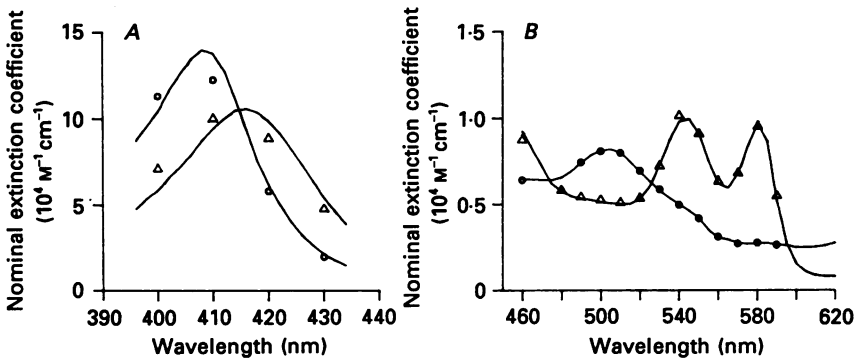

Fig. 1. *In vitro* spectra of metMb (○) and MbO_2 (△) measured on the optical bench and in a spectrophotometer (curves). The ordinates plot nominal extinction coefficients (absorbance divided by the product of path length times nominal myoglobin concentration) *versus* wavelength on the abscissa. *A*, measurements were made in 0·1 cm cuvettes containing nominal myoglobin concentrations of 0·05 and 0·1 mM on the optical bench and in the spectrophotometer, respectively. *B*, measurements were made with a nominal myoglobin concentration of 0·5 mM in either a 0·1 cm cuvette (optical bench) or a 0·05 cm cuvette (spectrophotometer). Scaling factors applied to the spectrophotometer results were 0·974 (metMb) and 0·960 (MbO_2) obtained by least-squares fit of the spectrophotometer data to the optical bench data.

An effective extinction coefficient (ϵ_{eff}) for the optical bench measurements was estimated using the following relationship:

$$\epsilon_{eff} = \frac{\int \epsilon(\lambda)R(\lambda)I(\lambda)T(\lambda)\,d\lambda}{\int R(\lambda)I(\lambda)T(\lambda)\,d\lambda}, \quad (4)$$

where $\epsilon(\lambda)$ is the actual extinction coefficient, $R(\lambda)$ is the responsivity of the photodetector, $I(\lambda)$ is the intensity of the lamp and $T(\lambda)$ is the fraction of light transmitted through the filter over the wavelength range $\lambda \pm d\lambda/2$. This relationship was used to predict the ratio of the effective extinction coefficients of metMb and MbO_2 using the 410 nm (±5 nm) bandwidth filter used on the optical bench. The integrations were evaluated with 2 nm step sizes between 396 and 424 nm. The values used for $\epsilon(\lambda)$ were those measured with the spectrophotometer, $R(\lambda)$ and $I(\lambda)$ were obtained from the manufacturers' specifications for the photodetector and lamp, respectively, and $T(\lambda)$ was measured with the 410 nm filter in the spectrophotometer. The ratio of the extinction coefficients of metMb to MbO_2 predicted with this analysis was 1·28, in essential agreement with the ratio of 1·27 measured on the optical bench. The most important factor in this decrease (1·42 to 1·28) was the rapid drop-off in the absorbance of metMb above 410 nm combined with the fact that the filter allows significant light intensities up to and beyond ±5 nm of the peak transmission wavelength (410 nm).

Given the above agreement, the optical bench calibrations of Fig. 1 were used for analysing experimental results from muscle fibres for the degree of reduction of metMb to MbO_2. It was assumed for this analysis that the MbO_2 data in Fig. 1 corresponded to 91% MbO_2 and 9% metMb, and that the total myoglobin concentration was 0·76 of nominal (see above).

Additional absorbance spectra determined in cuvette showed that metMb, also dissolved in 'internal' solution, was relatively insensitive to possible changes in intracellular pH and calcium

concentrations. For example, absorbances at 410 nm, the primary wavelength used to monitor myoglobin concentration, varied by only 2% between pH 6·60 and 7·60 ([calcium] = 0) and by only 1% between calcium concentrations of 0 and 800 μM (pH = 7·10).

Cut-fibre experiments

The cut-fibre preparation, chamber and optical apparatus are described in Irving, Maylie, Sizto & Chandler (1987). Cut fibres (Hille & Campbell, 1976) from semitendinosus muscles of cold-adapted *Rana temporaria* were used. A stretched muscle bundle was exposed to a Ca^{2+}-free, high-K^+ 'relaxing' solution (in mM: potassium glutamate, 120; PIPES, 5; EGTA, 0·1; $MgSO_4$, 1·0). A short length of a single fibre was isolated and transferred to a Lucite chamber containing 'relaxing' solution. The fibre ends were attached to Lucite clamps mounted on movable pedestals. The fibre was then stretched to sarcomere spacings between 3·1 and 3·5 μm. Vaseline seals were applied, establishing three extracellular compartments, two end pool regions and a central pool. Lucite covers provided additional isolation of the end-pool solutions and significantly reduced possible evaporation by minimizing surface area in the end-pool solutions.

In order to permit access of myoglobin to the myoplasm, the fibre membranes in the end pools were made permeable by a 10 min exposure to a solution containing 0·01% saponin (Sigma Chemical Co.). This treatment was followed by a thorough rinsing with saponin-free relaxing solution. The somewhat arbitrary 10 min exposure time to saponin was 5 times longer than that employed by Maylie, Irving, Sizto & Chandler (1987*b*, *c*) and Maylie, Irving, Sizto, Boyarsky & Chandler (1987*a*) for indicator dyes having molecular weights between 300 and 800 Da. (As reported in Results, the diffusion constant of myoglobin in cut fibres was somewhat higher than obtained with the intact-fibre preparation, indicating that there was no significant resistance to myoglobin entry from the end pools.) The end-pool solutions were then exchanged for internal solution (in mM: caesium glutamate, 45·5; PIPES, 5; Cs-EGTA, 20; $MgSO_4$, 6·8; glucose, 5; Cs_2 ATP, 5·5; caesium creatine phosphate, 20). Because of the long duration desired for these experiments, the relaxing solution in the central pool was exchanged for a depolarizing solution (in mM: K_2SO_4, 94; $CaSO_4$, 7; Na_2HPO_4, 2·15; NaH_2PO_4, 0·85), so that it would not be necessary to pass holding current for a long time. As reported in Results, the use of a depolarizing solution resulted in successful experiments which lasted from 4·5 to 9 h. The temperature in the central pool was monitored and maintained at 17 ± 1 °C.

Myoglobin absorbance was determined using an interference filter with the same specifications as the one used in the intact-fibre experiments (410 nm peak transmission with a 10 nm half-bandwidth). The fibre intrinsic absorbance was estimated from measurements made at 710 nm and extrapolated to 410 nm as described in Irving *et al.* (1987). For the analysis of the experiments, myoglobin absorbances measured at the centre of the fibre were normalized by the myoglobin absorbance of the end-pool solutions measured in cuvette on a spectrophotomer. The latter was corrected to the predicted value of the absorbance which would have been measured at the centre of the fibre if the myoplasm was in chemical equilibrium with the end-pool solution and no binding of metMb occurred. Included in this normalization procedure was the observation that the effective extinction coefficient of the metMb end-pool solution measured with the 410 nm filter on the cut-fibre optical apparatus was 85% of the extinction coefficient measured at 410 nm on the spectrophotometer. (Note that for the apparatus used for the intact-fibre experiments this value was 89%; cf. Fig. 1). The optical path length for the myoplasmic solution was taken as the fibre diameter observed in the microscope in the plane perpendicular to the light path (a potential source of error if the fibre is not cylindrical) times the fraction of fibre volume occupied by myoplasmic water, taken as 0·7 (Baylor *et al.* 1983).

RESULTS

Apparent diffusion constant of myoglobin: intact-fibre results

In both the intact- and cut-fibre experiments, measured myoglobin absorbance profiles were fitted by the method of least squares with solutions of the one-dimensional diffusion equation to yield estimates of D_{APP} and a second parameter, proportional to the total amount of myoglobin present. In addition to diffusion, two

other phenomena which were examined include binding of myoglobin in the myoplasm and reduction of metMb to MbO_2. The formulation presented here assumes a linear relationship between bound (and immobile) myoglobin and free myoglobin, which is given by the following equation:

$$[\mathrm{Mgb}]_B = \beta[\mathrm{Mgb}]_F. \tag{5}$$

$[\mathrm{Mgb}]_B$ and $[\mathrm{Mgb}]_F$ are the bound and free myoglobin concentrations, respectively, and β is a constant. It will be shown later in Results that the measurements of myoglobin diffusion in the intact fibre are consistent with this assumption, and, further, that measurements in the cut-fibre experiments indicate that β is small.

Given the assumption in eqn (5), the one-dimensional diffusion equation then becomes

$$\frac{\partial[\mathrm{Mgb}]_F}{\partial t} = \frac{D_{FREE}}{(1+\beta)} \frac{\partial^2[\mathrm{Mgb}]_F}{\partial x^2}, \tag{6}$$

where t is time, x is the distance along the long axis of the fibre and D_{FREE} is the diffusion constant of free (unbound) myoglobin. Binding would have the effect of slowing the rate at which myoglobin appears to diffuse. The slowed rate is characterized by the apparent diffusion constant, D_{APP}, which is defined here as $D_{FREE}(1+\beta)^{-1}$. Solutions to this problem are described in Crank (1956). The solution applied to the intact fibre (see also Baylor *et al.* 1986), in which myoglobin is assumed to be introduced instantaneously in the middle ($x = 0$) of a long fibre, is given by the following equation:

$$[\mathrm{Mgb}]_T(x,t) = \frac{M}{2(\pi D_{APP} t)^{1/2}} \exp\left(\frac{-x^2}{4D_{APP}t}\right), \tag{7}$$

where t is time from metMb injection, $[\mathrm{Mgb}]_T$ is the sum of both free and bound myoglobin concentrations and M is total amount of myoglobin per cross-sectional area of the fibre injected at $t = 0$.

It will be shown later in Results that metMb is reduced to MbO_2 inside the myoplasm. Because of mass conservation, eqns (5), (6) and (7) still apply with [Mgb] referring to the sum of the concentrations of metMb and MbO_2, if it is assumed that D_{FREE} and β are the same for both metMb and MbO_2. Because of the large size of myoglobin, the factors which affect D_{FREE}, namely the size and shape of the molecule, should be essentially the same for all forms of myoglobin considered.

Resolution of myoglobin absorbance in muscle fibres

In the several minutes following large injections into intact fibres, myoglobin, by virtue of its reddish-brown colour, could be visually observed (at 320× magnification) to diffuse away from the injection site, both radially across the diameter and longitudinally along the axis of the muscle fibre. Radial gradients dissipated relatively quickly due to the short diffusion distance across the diameter of the fibre. The concentration profile of myoglobin along the long axis of the fibre would then be expected to broaden as predicted from the one-dimensional diffusion equation.

Figure 2 provides an example of the raw measurements, the correction for the fibre intrinsic absorbance and the fitting method employed to obtain D_{APP}. The experiment of Fig. 2 was chosen to illustrate the resolution of the myoglobin-related

absorbance signal in a fibre containing a relatively small concentration of myoglobin (cf. columns 7 and 8 in Table 1, Fibre No. 092986.2). All four panels of Fig. 2 plot absorbance (ordinate), determined between 45 and 61 min following the injection of metMb into the myoplasm, *versus* distance from the site of injection (abscissa).

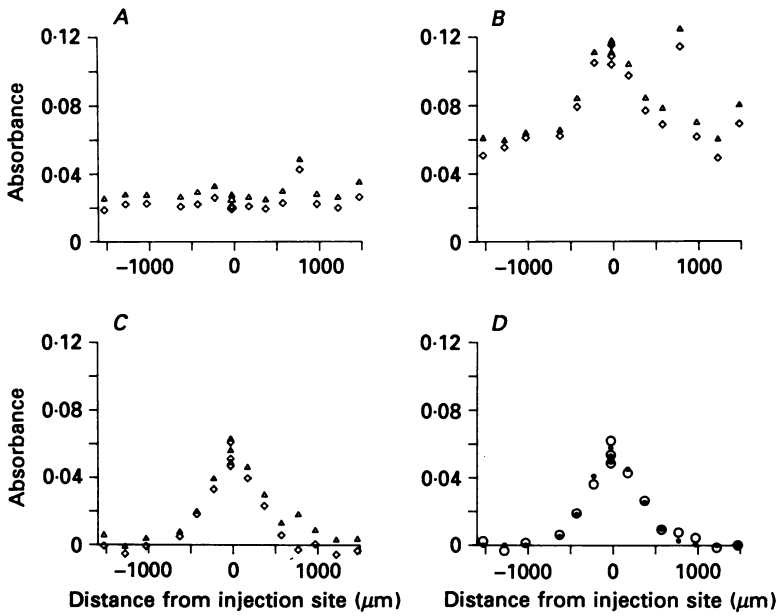

Fig. 2. Sample measurement of myoglobin absorbance profile in an intact fibre and determination of D_{APP}. All four panels plot absorbance on the ordinate *versus* distance from the myoglobin injection site on the abscissa. *A* and *B* show the 90 deg (◇) and 0 deg (△) polarized absorbances measured at 710 and 410 nm, respectively, between 45 and 61 min after myoglobin injection. *C* shows the 90 deg (◇) and 0 deg (△) myoglobin-related absorbances found by subtracting the fibre intrinsic absorbance, estimated from the 710 nm absorbance data of *A*, from the 410 nm absorbance data shown in *B*. *D* plots the average of the 90 and 0 deg myoglobin absorbances (○) and the best-fit solution of this data by eqn (7) of the text (●). (Note that each measured point represents a different time, so that the best-fit solution cannot be represented as a single curve as a function of distance. Note also that the procedure for removing the intrinsic absorbance also corrected for the large deviation seen at 800 μm, which was probably due to extraneous material, e.g. dust or connective tissue, on the fibre. The presence of such material was usually recognized by visual observation in the microscope field, and such regions were generally avoided when making absorbance measurements.) Fibre No., 092986.2; sarcomere spacing, 2·35 μm; temperature, 22 °C.

Figure 2*A* and *B* plots the raw absorbances measured at 710 and 410 nm, respectively. The absorbances measured at 410 nm are larger than at 710 nm, both because the intrinsic absorbance was larger at shorter wavelengths, and because myoglobin had been injected into the myoplasm. Intrinsic absorbances at 410 nm were estimated by extrapolating the 710 nm absorbances of Fig. 2*A* to 410 nm as described in Methods. Figure 2*C* shows the myoglobin-related absorbances at 410 nm, obtained by subtracting the estimated intrinsic absorbances. In Fig. 2*A*, *B* and *C*, absorbances of 90 and 0 deg polarized light are shown as open diamonds and

triangles, respectively. There is no correlation between the small differences of the 90 and 0 deg absorbances and the absolute absorbance in Fig. 2*B*. Thus, within the resolution of the method, myoglobin absorbance in myoplasm had no resolvable 'dichroism' (inequality of 90 and 0 deg absorbances), in this fibre or any of the other fibres in Table 1. The presence of dichroism would have been indicative of myoglobin binding to oriented structures such as the contractile proteins (cf. Baylor *et al.* 1986).

Plotted in Fig. 2 *D* are the averages (sum divided by 2) of the 90 and 0 deg myoglobin-related absorbances of Fig. 2*C* (○). These data fall to an average value of zero far from the injection site. A similar finding was observed in approximately half of the fibres. In most of the other fibres, a small offset ranging from −0·005 to +0·002 absorbance units (or −16 to +4% of the peak myoglobin-related absorbance measured at the injection site) was observed. For these fibres, a small offset was subtracted so that the myoglobin-related absorbance far from the injection site was zero. In effect, this procedure used the absorbances in regions of the fibre where negligible myoglobin was present, in order to correct for fibre-to-fibre variations in the functional form chosen to relate the fibre intrinsic absorbance at 410 nm and that at the 710 nm reference wavelength. The overall effectiveness of the above procedure to correct for the fibre intrinsic absorbance is seen by comparing the averaged 90 and 0 deg myoglobin-related absorbance (○) of Fig. 2*D* with the noisier data of Fig. 2*B*.

The absorbance profile of Fig. 2*D* is explained by diffusion of myoglobin away from the injection site and contains information sufficient to estimate D_{APP} for myoglobin. The small filled circles are the least-squares fitted solution of eqn (7) to the muscle data (○), obtained by adjusting the two parameters D_{APP} and M. (Note that the parameter M is independent of the shape of the profile and simply represents a scaling factor proportional to the amplitude of the profile. Conversely, D_{APP} is uniquely determined by the shape of the profile and should be independent of the total amount of myoglobin present in the fibre.) This experiment typifies the generally good agreement observed between measured and fitted absorbance data. The value determined for D_{APP} in this fibre was $0{\cdot}14 \times 10^{-6}$ cm^2 s^{-1}. It may be noted that this value of D_{APP} at 22 °C is closely similar to the value reported by Moll (1968), $0{\cdot}15 \times 10^{-6}$ cm^2 s^{-1} at 20 °C, for myoglobin diffusion in homogenized mammalian muscle.

One potential problem with our determination of D_{APP} in intact fibres is that pressure injection commonly distributed substances on the order of 50 μm or so away from the site of injection. A non-ideal initial distribution would lead to an overestimate of D_{APP} determined from diffusion profiles measured too soon after the injection. The effect of the initial distribution on the determination of D_{APP} was examined in the case of the calcium indicator dye Antipyrylazo III, by fitting D_{APP} under the assumptions that the injected dye was either localized at the injection site or initially spread over a length of fibre ± 50 μm from the site of injection (Baylor *et al.* 1986). The values of D_{APP} determined for these different initial distributions differed by less than 2%. This analysis should apply as well to the results presented here for myoglobin, since the apparent diffusion constants and time periods of measurements of the diffusion profiles were similar for Antipyrylazo III and

myoglobin. Therefore, given an elapsed time of about 40 min before the absorbance measurements were made, the value of D_{APP} determined should be insensitive to the exact initial spread of myoglobin.

Effect of metMb reduction on the determination of D_{APP}

Another problem, not initially recognized in the experiments, was that metMb in myoplasm became significantly reduced to MbO_2 during the elapsed time before the optical measurements. This possible confounding effect on the estimate of D_{APP} was examined in detail in six experiments, using the fact that absorbances measured at 410 and 420 nm fall on opposite sides of an isosbestic wavelength for metMb and MbO_2 (see Fig. 1*A*). In particular, if measurements made at 410 nm yield an underestimate of D_{APP} because of metMb reduction to MbO_2, then measurements at 420 nm should yield an overestimate of D_{APP}. In these experiments the value for D_{APP} determined using the 410 nm signal was on average only 6·5 (±2·1 S.E.M.)% less than that determined with the 420 nm measurements, whereas the true value for D_{APP} should fall in between these estimates. Given the calibration data of Fig. 1*A*, a linear 'isosbestic' combination of the 410 and 420 nm absorbances can be used to estimate the concentration of total myoglobin (metMb plus MbO_2) at each point, so that the fitted parameters would be insensitive to whether the myoglobin is present as metMb or MbO_2. This analysis was done for the same six experiments; the value of D_{APP} based on just the 410 nm absorbances was only 2·2 (±1·9 S.E.M.)% smaller than that of the best estimate of D_{APP} based on the isosbestic combination of absorbances. Correspondingly, M in the same six experiments, when analysed under the assumption that all of the myoglobin was present as metMb, was underestimated by 18 (±6 S.E.M.)%. These results therefore indicate that reduction produced a negligible error (around 2%) in the estimates of D_{APP} made from the 410 nm measurements alone in the other eleven experiments. The major effect of reduction was to produce a significant error in M, which, however, is not the parameter sought.

Determination of D_{APP} under different fibre conditions

D_{APP} was determined by the method of Fig. 2 under three fibre conditions: (1) long sarcomere spacing (3·6–3·8 μm) at 16 °C, (2) long sarcomere spacing at 22 °C, and (3) normal sarcomere spacing (2·4–2·7 μm) at 22 °C. The results for the three fibre conditions are shown in Table 1. The average values for D_{APP} under the three fibre conditions were: (1) 0·12 (±0·03 S.E.M.), (2) 0·17 (±0·02 S.E.M.) and (3) 0·15 (±0·02 S.E.M.) $\times 10^{-6}$ cm^2 s^{-1}. The values of D_{APP} determined at long and at short sarcomere spacing at 22 °C are not significantly different, indicating that sarcomere spacing *per se* does not affect myoglobin's diffusion rate. The average value, $0{\cdot}16 \times 10^{-6}$ cm^2 s^{-1}, at 22 °C compared to that at 16 °C suggests a Q_{10} for diffusion of 1·6. However, the scatter in the data and the small temperature range leave some uncertainty about the Q_{10}. The main point to be emphasized is that the average value at 22 °C is about 4 times smaller than the values commonly assumed in models of facilitated diffusion of oxygen by myoglobin (see Introduction).

If diffusion were the only physical process influencing the longitudinal spread of myoglobin in the muscle fibre and the intracellular environment for diffusion of

TABLE 1. Intact-fibre results: measurement of D_{APP}

(1) Fibre no.	(2) Fibre diameter (μm)	(3) Sarcomere spacing (μm)	(4) Temporal variation (min)	(5) Spatial variation (μm)	(6) No. of points	(7) ⟨[Mgb]⟩ at $x=0$ (μM)	(8) M (μM cm)	(9) D_{APP} $\times 10^6$ (cm^2 s^{-1})
			Long sarcomere spacing at 16 °C					
050886.1	81	3·66	73–85	−1000, +1100	10	862	74·5	0·11
050886.2	72	3·53	51–68	−1000, +1000	11	404	28·3	0·08
061886.1	76	3·64	73–95	−1000, +1000	11	91	8·0	0·15
061886.2	75	3·64	75–110	−1000, +1000	14	94	9·0	0·11
092386.1	78	3·64	65–95	−1600, +1500	18	23	2·0	0·15
Mean ($n = 5$)								0·12
S.E.M.								0·03
			Long sarcomere spacing at 22 °C					
051386.1	95	3·53	32–42	0, +750	5	354	23·5	0·15
062586.1	88	3·53	70–105	−1500, +1200	12	66	6·9	0·16
091186.1	99	3·53	39–55	−1500, 0	7	32	3·9	0·19
091686.1	87	3·68	42–70	−750, +1500	11	17	1·8	0·19
091686.2	90	3·75	42–67	−1300, +1600	14	31	2·3	0·14
Mean ($n = 5$)								0·17
S.E.M.								0·02
			Short sarcomere spacing at 22 °C					
071486.1	105	2·50	65–124	−1400, +1750	14	33	3·4	0·17
092686.1	69	2·50	42–73	−1600, +400	12	144	12·1	0·16
092986.2	107	2·35	45–61	−1500, +1500	16	30	2·2	0·14
100186.1	116	2·50	70–90	−1500, +1250	16	100	9·4	0·14
100286.2	83	2·61	66–99	−1100, +1560	18	40	3·9	0·17
030387.1	53	2.50	74–105	−1100, +1100	12	72	6·7	0·12
042487.2	100	2·73	53–65	−1200, +400	11	112	8·5	0·13
Mean ($n = 7$)								0·15
S.E.M.								0·02

Column 1 gives the fibre reference. Column 2 gives the fibre diameter. Column 3 gives the sarcomere spacing. Column 4 gives the time period during which measurements were made. Column 5 gives the range of distances from the site of injection over which measurements were made. Column 6 gives the number of locations along the fibre where measurements were made. Column 7 gives the average concentration of myoglobin at the injection site during the diffusion run. Columns 8 and 9 give the values for M and D_{APP} obtained from fitting the data to eqn (7). Column 7, 8 and 9 were calculated assuming all myoglobin was present as metMb.

myoglobin did not change with time, it follows that the value of D_{APP} should be independent of myoglobin concentration and the time period during which measurements were made. While the scatter in the data and the small sample sizes preclude any definite conclusions, the data in Table 1 indicate that there were no significant correlations between D_{APP} and ⟨[Mgb]⟩, the average concentration of myoglobin at the injection site (column 7), nor between D_{APP} and the time after injection (column 4) during which absorbance measurements were made. The absence of correlations in these variables supports the assumption made in the analysis that the movement of myoglobin follows a one-dimensional diffusion process

as described in eqns (5) and (6). For example, if significant saturable binding of myoglobin to some component in the myoplasm had occurred, D_{APP} would have been expected to show a positive correlation with $\langle[Mgb]\rangle$.

Reduction of metmyoglobin

While reduction of metMb to MbO_2 was shown to have little effect on the value of D_{APP} determined in the intact-fibre preparation, the rate of reduction has some influence on the estimation of the degree to which myoglobin binds inside fibres, as determined in the cut-fibre preparation (next section). Experiments were therefore performed to determine the rate of reduction in the intact-fibre preparation. It is expected that this rate should be similar to (or possibly overestimate) the rate of reduction in the cut-fibre experiments (see p. 263).

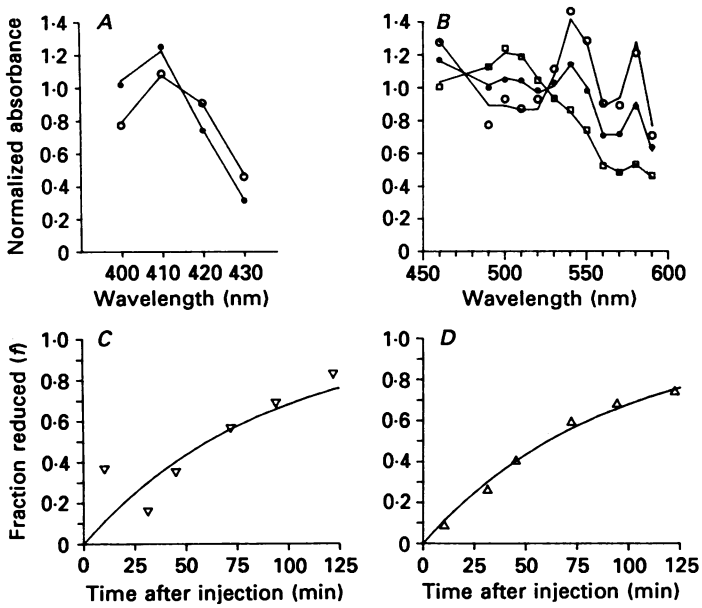

Fig. 3. Reduction of metMb to MbO_2 as a function of time after injection. *A* and *B* show myoglobin absorbance spectra measured between 400 and 430 nm and 460 and 590 nm respectively, at 10 (□, *B* only), 45 (●) and 122 (○) min after injection. The spectra are normalized to the isosbestic points between 410 and 420 nm in *A* and between 520 and 530 nm in *B*. The continuous lines in *A* and *B* show the least-squares best fit of the data by eqn (8) in the text. *C* and *D* plot on the ordinate the fraction of total myoglobin reduced to MbO_2 (symbols), obtained by fitting the muscle data in parts *A* and *B*, respectively, by eqn (8), *versus* time after injection on the abscissa. The continuous curves in both *C* and *D* were obtained by fitting the estimates in *D* by eqn (9), which gave a value for k of 0·012 min^{-1}. Fibre No., 042887.1; sarcomere spacing, 3·8 μm; temperature, 22 °C.

Three experiments, performed with usual amounts of injected myoglobin, showed time-dependent spectral changes between 400 and 430 nm, consistent with some reduction of metMb to MbO_2 during the course of these experiments. In order to confirm the presence of this effect, an experiment was performed in which a relatively

large amount of myoglobin was injected, in order to resolve the more dramatic changes in spectral shape expected to occur between 460 and 590 nm, namely the appearance of the two MbO_2 peaks around 545 and 580 nm (cf. Fig. 1). Figure 3 shows the results from this experiment. In this fibre absorbance spectra were measured at the site of injection at various times ranging from 10 to 122 min following injection. Bracketting measurements between 400 and 430 nm made at the start and finish of each spectral run (each of which lasted about 5 min) were used to estimate the total myoglobin concentration (metMb and MbO_2) and the fraction of total myoglobin reduced to MbO_2 at the beginning and end of the spectral runs. The measured spectra (symbols) in Fig. 3*A* and *B* have also been normalized to the isosbestic points between 410 and 420 nm and 520 and 530 nm respectively, in order to facilitate an examination of the change in spectral shape with time. The spectral changes which occurred in both wavelength ranges are explained by an increase in the fraction of total myoglobin in the reduced state with time (cf. Fig. 1), confirming that reduction does in fact take place in the myoplasm of frog twitch fibres.

In order to determine the rate of reduction, these spectral data were analysed for the degree of reduction and concentration of total myoglobin (metMb plus MbO_2) by a least-squares fit of the data by a linear combination of the metMb and MbO_2 spectra determined in cuvette on the optical bench (see Methods). The fitted spectrum, $a(\lambda)$, is defined as follows:

$$a(\lambda) = Cl[f\epsilon_{oxy}(\lambda) + (1-f)\epsilon_{met}(\lambda)], \tag{8}$$

where C = the concentration of total myoglobin (metMb plus MbO_2), l = the optical path length through the muscle fibre, $\epsilon_{oxy}(\lambda)$ = the effective molar extinction coefficient of MbO_2 at wavelength λ, $\epsilon_{met}(\lambda)$ = the effective molar extinction coefficient of metMb at wavelength λ and $f = [MbO_2]/([MbO_2]+[metMb])$, the fraction of total myoglobin in the reduced state. (Note that essentially all the reduced myoglobin will be present as MbO_2 rather than deoxymyoglobin, since the partial pressure of oxygen was essentially that of the air, i.e. about 150 mmHg.) The fit yielded the degree of reduction (given by f) and the concentration of total myoglobin, C.

Values for C and f were obtained by separately analysing the two wavelength ranges, 400–430 and 460–590 nm. The values for C determined in the wavelength range from 400 to 430 nm averaged 10 ($\pm$9 S.E.M.)% higher than the values determined for the wavelength range from 460 to 590 nm. This difference can be explained by small errors (0·004 absorbance units) in the corrections for fibre intrinsic absorbance between 460 and 590 nm where myoglobin absorbance is much smaller. The continuous lines in Fig. 3*A* and *B* show that the measured absorbance data are well fitted, thus justifying the use of the above analysis for obtaining f and C, with its implied assumption that MetMb and MbO_2 are the only species of myoglobin present.

Figure 3*C* and *D* shows, as a function of time, the values of f (symbols) determined using absorbances measured between 400 and 430 and between 460 and 590 nm, respectively. The continuous line is the same for each panel and shows the linear least-squares fit to the 460–590 nm absorbance data assuming the metMb reduction

is a first-order, irreversible reaction. Since the rate of conversion of MbO_2 back to metMb is very slow (Yamazaki *et al.* 1964 and our observations) and in addition the value of f appears to approach 1·0 in this and other experiments, the assumption of irreversibility is probably valid. The appropriate function with which to fit the data is then

$$f = 1 - e^{-kt}, \tag{9}$$

where k is the rate constant for reduction and t is time.

One purpose of the experiment of Fig. 3 was to confirm whether the spectral changes associated with reduction that occur between 400 and 430 nm correspond to those occurring between 460 and 590 nm, since the three other experiments with smaller concentrations of injected myoglobin allowed resolution of the 400–430 nm signal only. The 400–430 nm data in Fig. 3*C* are, in fact, adequately described by the same function describing the 460–590 nm data in Fig. 3*D*. Equation (9) also gave good fits to the data of the three other experiments (not shown). Data from the four experiments (carried out at 22 ± 1 °C) indicate that metMb reduction does take place and can be described by a first-order, irreversible reaction rate constant averaging 0·0164 (±0·0050 S.E.M.) min^{-1}, which corresponds to a half-time of 42·4 min.

MetMb reduction has been observed in several species, including fish (Al-Shaibani & Price, 1977), pigeon breast muscle (Wittenberg *et al.* 1975), ground beef (Stewart, Hutchins, Zipser & Watts, 1965), bovine heart extract (Hagler, Coppes & Herman, 1979), and rat heart myocytes (Taylor & Hochstein, 1982). The results reported here are the first observations of metMb reduction in a fibre type normally devoid of myoglobin. The first-order rate constant reported here for reduction in frog fibres, 0·0164 min^{-1} at 22 °C, is about 4 times less than the value of 0·070 min^{-1} obtained in rat heart myocytes at 37 °C (calculated from the results of Taylor & Hochstein, 1982, which indicate that 4·3 nmol of metMb $(10^6 \text{ cells})^{-1}$ were reduced to MbO_2 at an initial rate of 0·30 nmol min^{-1} $(10^6 \text{ cells})^{-1}$).

D_{APP} and binding of myoglobin in muscle: cut-fibre results

One explanation for the small value of D_{APP} reported here could be that myoglobin binds to relatively immobile sites accessible to myoplasm. In order to determine whether binding takes place, experiments were carried out in Dr W. K. Chandler's laboratory at Yale University using a technique developed to measure the diffusion constants of indicator dyes in cut fibres and to estimate the degree to which they might bind to intracellular sites (Maylie *et al.* 1987*a*, *b*, *c*). The regions of the fibre exposed to the end-pool solution were made permeable to myoglobin by 'chemical skinning' (see Methods). At the start of the experiment 0·38 mM metMb was added to the end-pool solutions. Myoglobin-related absorbance at 410 nm was monitored at the centre of the fibre as a function of time following this exposure. The rate at which myoglobin reached the centre of the fibre and approached a steady-state concentration should depend on myoglobin's apparent diffusion constant in myoplasm, whereas the final steady-state level approached should depend on the degree to which myoglobin was bound inside the muscle. In particular, in the presence of binding, the concentration of myoglobin in the centre of the fibre would, at steady-state, be larger than in the end pools.

One-dimensional diffusion equation for the cut-fibre preparation

The solution of the one-dimensional diffusion equation (eqn (6), P. 254) applicable to the cut-fibre experiments is described in Crank (1956, p. 45) and Maylie *et al.* (1987*b*), namely,

$$\frac{[\mathrm{Mgb}]_T(0,t)}{(1+\beta)[\mathrm{Mgb}](L)} = 1-\frac{4}{\pi}\sum_{n=0}^{\infty}\frac{(-1)^n}{(2n+1)}\exp\left(\frac{-D_{APP}(2n+1)^2\pi^2 t}{4L^2}\right) \quad (10)$$

where t is time from adding metMb to the end pools and β and D_{APP} have the same definitions given on p. 254). $[\mathrm{Mgb}]_T(0, t)$ is the sum of the concentrations of free plus bound myoglobin at the centre of the fibre, $[\mathrm{Mgb}](L)$ is the concentration of myoglobin in the end pools, and L is the distance from the centre of the fibre ($x = 0$) to the end pools, which is taken to be 550 μm (Maylie *et al.* 1987*b*). The solution given by eqn (10) assumes that the limiting concentration of free myoglobin throughout the fibre approaches $[\mathrm{Mgb}](L)$ at long times. Because of mass conservation, eqn (10) also applies even if metMb becomes reduced to MbO_2, as long as D_{APP} and β are the same for both metMb and MbO_2. In this case [Mgb] refers to the sum of the concentrations of metMb and MbO_2. However, because Mgb-related absorbance measurements were made at 410 nm only, separate solutions for metMb and MbO_2 were needed in order to estimate the effect of metMb reduction in the cut-fibre experiments. These solutions are described in the Appendix.

Determination of β and D_{APP}

Figure 4 illustrates the results and analysis of one cut-fibre experiment. This fibre was exposed to metMb solution for 5 h and 40 min, at which time the end-pool solutions were exchanged for myoglobin-free solution. Myoglobin diffusion out of the fibre was observed thereafter for 3 h and 20 min. The ordinates of Fig. 4 *A*, *B* and *D* plot (as crosses) the measured myoglobin absorbance at the centre of the fibre (denoted $A(0, t)$), normalized to the predicted absorbance of free myoglobin at the fibre centre at infinite time if no reduction of metMb to MbO_2 occurred. This latter absorbance has been denoted $A(L)$, since it was calculated from the measured absorbance of myoglobin in the end-pool solution (see p. 253 in Methods). Included in the calculation is the assumption that myoglobin has equal access to all of the myoplasmic space, taken to be 70 % of the fibre volume. (Note again, because of mass conservation, that even if reduction of metMb takes place, the concentration of free myoglobin (sum of metMb and MbO_2) at infinite time should be the same throughout the fibre, and thus, should be equal to the concentration of metMb in the end pools, denoted $[\mathrm{metMb}](L)$.) The ratio $A(0,t)/A(L)$ for the best-fit solutions (continuous lines in panels *A*, *B* and *D* of Fig. 4) is given by the following equation:

$$\frac{A(0,t)}{A(L)} = \frac{[\mathrm{metMb}]_T(0,t)}{[\mathrm{metMb}](L)} + \frac{\epsilon_{oxy}}{\epsilon_{met}}\frac{[\mathrm{MbO_2}]_T(0,t)}{[\mathrm{metMb}](L)}, \quad (11)$$

where $\epsilon_{oxy}/\epsilon_{met}$ is the ratio of the extinction constants of MbO_2 and metMb determined for the 410 nm interference filter (taken as $1{\cdot}28^{-1}$ or 0·781, Methods). The ratios $[\mathrm{metMb}]_T(0, t)/[\mathrm{metMb}](L)$ and $[\mathrm{MbO_2}]_T(0, t)/[\mathrm{metMb}](L)$ are the solutions of the one-dimensional diffusion equation obtained from equation (A 10) and

the difference of eqn (10) and (A10), respectively. (Note that for a given rate of reduction, the shape of the diffusion profile is uniquely determined by D_{APP}, while the amplitude is determined by $1+\beta$.) In panels *A*, *C* and *D* of Fig. 4 the value for the first-order, irreversible rate constant for reduction was fixed at 0·0164 min^{-1}, the

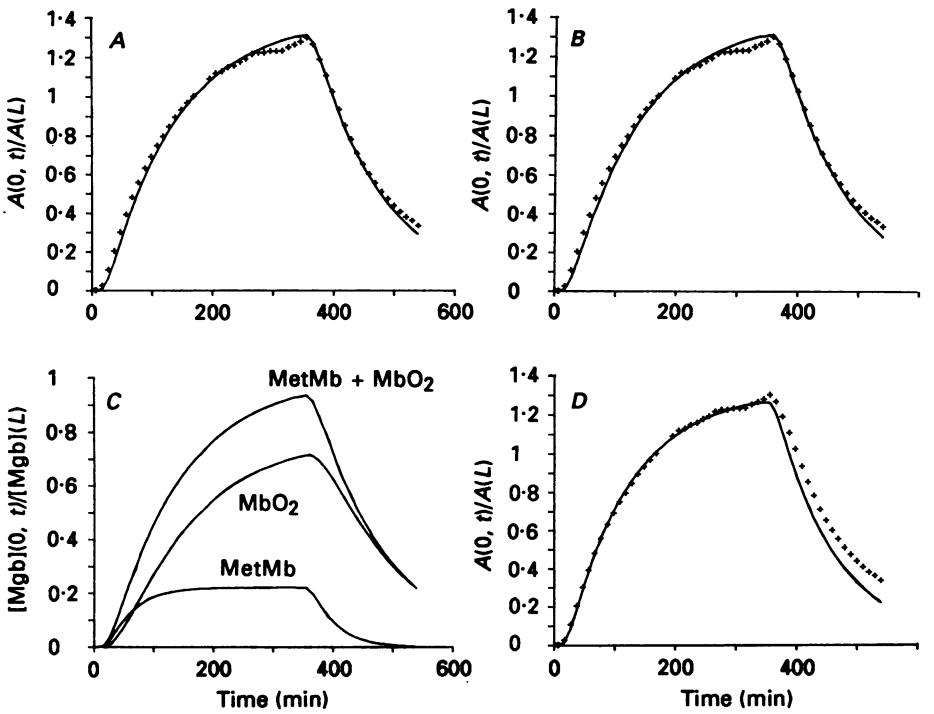

Fig. 4. Results and analysis in a cut-fibre experiment. Panels *A*, *B* and *D* plot the myoglobin-related absorbance (at 410 nm) measured at the centre of a cut fibre (×) as a function of time following addition of 0·38 mM-metMb to the end pools. Absorbances are normalized to the absorbance of the end pools as described in Methods. The end pools were exchanged for myoglobin-free solution at 340 min. *A*, the best-fit values for D_{APP} and $1+\beta$ assuming $k = 0{\cdot}0164$ min^{-1} were $0{\cdot}174 \times 10^{-6}$ cm^2 s^{-1} and 1·69, respectively. *B*, the best-fit values for D_{APP} and $1+\beta$ with no reduction of metMb assumed ($k = 0$) were $0{\cdot}183 \times 10^{-6}$ cm^2 s^{-1} and 1·39, respectively. All the data were used in the fits of panels *A* and *B*, while only the data up to 340 min were fitted in panel *D*. *D*, the best-fit values for D_{APP} and $1+\beta$ assuming $k = 0{\cdot}0164$ min^{-1} were $0{\cdot}198 \times 10^{-6}$ cm^2 s^{-1} and 1·58, respectively. Panel *C* shows the normalized free concentrations of metMb and MbO_2 and the sum of metMb and MbO_2 at the centre of the fibre if $k = 0{\cdot}164$ min^{-1} and $D_{APP} = 0{\cdot}174 \times 10^{-6}$ cm^2 s^{-1} (see text for details). Fibre No., 112186.1; temperature, 17 °C.

average value determined in the intact-fibre preparation (see above), whereas in panel *B*, k was fixed at zero. (Note that if $k = 0$, the last term in eqn (11) equals zero and the normalized absorbance approaches $1+\beta$ at infinite time.)

The solutions in Fig. 4 *A* and *B*, obtained by fitting all of the data points, indicate that the data are fitted equally well when k is fixed at 0·0164 min^{-1} or 0. However, with $k = 0{\cdot}0164$ min^{-1} the values for $1+\beta$ are 22% higher, and for D_{APP} 5% lower, than the values determined with k set at zero. The explanation for the slightly lower

value of D_{APP} is illustrated by panel C, which shows the normalized solutions for free metMb and MbO_2 that gave the best-fit solution shown in panel A. Also shown in panel C is the sum of the metMb and MbO_2 solutions, which would have approached 1 if the metMb concentration in the end pools had been maintained for infinitely long time. While reduction does not affect the time course of total myoglobin at the centre of the fibre, the rise in $[MbO_2]$ has a slower time course than that of [metMb]. The net effect when combined with eqn (11) is to require a lower value of D_{APP} in the fit in order to explain the observed absorbance measurements at 410 nm.

In panel D only the data up to 340 min, when the end pools were exchanged for myoglobin-free solutions, were used in obtaining the best fit. In comparison with panel A, a better fit was achieved for the rising phase of the data, but at the expense of the falling phase being less well fitted. There could be several explanations for this, including the possibility that towards the end of this very long experiment (lasting 9 h) myoglobin diffusion was in fact a little slower than at the onset. Nevertheless, the good fits of the data in panels A and B indicate that binding (if it occurs) is essentially reversible, since the one-dimensional diffusion equation adequately describes the diffusion of myoglobin into as well as out of the fibre.

Table 2 summarizes the results of this analysis from four cut-fibre experiments. The average value for D_{APP} (columns 3 and 6) was about 40% higher than the value determined in intact fibres. A similar difference was found previously for the diffusion constants of several other substances (all indicator dyes of lower molecular weight) determined in both the intact- and cut-fibre preparations (Baylor *et al.* 1986; Maylie *et al.* 1987*b*, *c*). This difference suggests that, in general, diffusion is faster (or binding is less) in cut fibres compared with intact fibres. The value measured in intact fibres is likely to more closely reflect the physiologically relevant value of D_{APP} for myoglobin.

The values for $1+\beta$ determined when k was set to 0·0164 min^{-1} averaged 22% higher than the values determined when k was set equal to zero (see column 10 of Table 2). This is expected, since the extinction coefficient of MbO_2 at 410 nm is less than that of metMb; thus observed values of $A(0, t)$ require larger concentrations of total Mgb to be present if reduction occurs. The reason the average value of 22% was not closer to the upper limit of 28%, set by the ratio $\epsilon_{met}/\epsilon_{oxy}$ (= 1·28, see Methods), is because the solution to the one-dimensional diffusion equation predicts that there will be some metMb present at the centre of the fibre when steady state is reached at long times (Fig. 4*C* and column 9 of Table 2). This is a consequence of the assumption that only metMb was present in the end pools and not MbO_2. This assumption is justified because the volumes of the end pools were large compared to the volume of the fibre, and the end pools were periodically mixed and occasionally flushed with fresh metMb solution.

The actual value of k applicable to the cut fibres probably falls between 0 and 0·0164 min^{-1} for the following reasons: (1) the rate of reduction was estimated in intact fibres at 22 °C, while the cut-fibre experiments were carried out at 17 °C, and (2) the diffusional loss of substrates, co-factors and/or enzymes from cut fibres would tend to lower k. As a result, the best estimate of $1+\beta$ should probably fall between the value 1·02 obtained when k was set equal to zero and the value 1·24 obtained when k was set equal to 0·0164 min^{-1}.

As reported in the previous section, the average value for D_{APP} measured in intact frog fibres at 22 °C agrees with that reported by Moll (1968) for myoglobin diffusion in homogenized mammalian muscle, but is about 4 times smaller than the values commonly assumed at 20 °C. The average values for $1+\beta$ (1·02 when $k = 0$ and 1·24

TABLE 2. Cut-fibre results

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
		$k = 0$			$k = 0{\cdot}0164$ min^{-1}				
Fibre no.	Maximum time (min)	D_{APP} (cm^2 s^{-1}) $\times 10^6$	$1+\beta$	D_{FREE} (cm^2 s^{-1}) $\times 10^6$	D_{APP} (cm^2 s^{-1}) $\times 10^6$	$1+\beta$	D_{FREE} (cm^2 s^{-1}) $\times 10^6$	metMb at $x = 0$, $t = \infty$ (%)	(7) ÷ (4)
111886.1	404	0·129	0·82	0·105	0·123	1·01	0·124	14·9	1·24
111986.1	268	0·183	1·00	0·183	0·173	1·22	0·210	22·2	1·22
112086.2	395	0·214	0·87	0·186	0·200	1·05	0·210	25·7	1·21
112186.1	539	0·183	1·39	0·255	0·174	1·69	0·293	22·3	1·21
Mean	—	0·177	1·02	0·182	0·168	1·24	0·210	21·3	1·22
S.E.M.	—	0·031	0·22	0·053	0·028	0·27	0·060	—	—

Column 1 gives the fibre reference. Column 2 gives the duration of the experiment following exposure to metMb in the end pools. Column 3 and 4 give parameters associated with fitting the one-dimensional diffusion equation to the experimental points and column 5 gives the product of columns 4 and 5. Columns 6, 7 and 8 correspond to columns 3, 4 and 5 except that the rate of reduction was fixed at 0·0164 min^{-1} instead of zero. Column 9 gives the percentage of the total myoglobin present as metMb at the centre of the fibre at infinite time predicted when $k = 0{\cdot}0164$ min^{-1}. Column 10 gives the ratio of column 7 to column 4. Temperature for all experiments was 17 ± 1 °C.

when $k = 0{\cdot}0164$ min^{-1}) given in columns 4 and 7 of Table 2 are much smaller than the value of 4 required to explain the 4-fold discrepancy. Therefore, the important additional conclusion obtained from the cut-fibre experiments is that the low values of D_{APP} found in both intact fibres and cut fibres cannot be attributed to binding of myoglobin in frog myoplasm. Other possible explanations for the low diffusivity will be considered in the Discussion.

Consideration of the role of convection in facilitated transport

Convection, if it occurs, is generally much more effective than diffusion in achieving mass transfer. An additional mechanism by which myoglobin might facilitate oxygen transport is via convective transport of MbO_2. This mechanism might be important if there were a stirring of myoplasm resulting from the mechanical events associated with contraction, as suggested by Honig and co-workers (Gayeski & Honig, 1983; Honig *et al.* 1984).

In order to investigate this possibility, experiments measuring D_{APP} were made in intact fibres by the usual method except that the fibres, at a sarcomere spacing of 3·0 μm, were either electrically stimulated or passively stretched and shortened during the time intervening between the injection of myoglobin and the absorbance measurements. In one type of experiment a brief tetanus was periodically imposed by a series of ten stimulated action potentials spaced 20 ms apart. This stimulation was repeated every 15 s, for a total of 15 min in one fibre and 24 min in another fibre,

and measurements of myoglobin absorbance profiles were made 33–51 and 39–49 min, respectively, after injection. The values for D_{APP} determined for these two fibres, 0·150 and $0{\cdot}175 \times 10^{-6}$ cm^2 s^{-1}, were not significantly different from those determined in the absence of stimulation (column 9 of Table 1) at the same temperature (22 °C). These results suggest that the mechanical events of a normal contraction do not lead to a significant increase in D_{APP}, at least for the type of stimulation employed here.

In the latter two experiments, the fibres, although held isometrically at the tendon ends at a sarcomere spacing of 3·0 μm, undoubtedly underwent some shortening during each tetanus. In order to investigate whether length changes *per se* might result in convective motion and a faster D_{APP} for myoglobin, two experiments were performed in which fibres were passively stretched and shortened for a 23–25 min time period intervening between myoglobin injection and the absorbance measurements. These continuous length changes were produced at one tendon end of the fibre at a frequency of 2–3 Hz, with the resulting changes in sarcomere spacing measured at the myoglobin injection site ranging from 2·7 to 3·3 μm, i.e. a $\pm 10\%$ change in length. Absorbance measurements for the determination of D_{APP} were made 40–55 min after myoglobin injection at a sarcomere spacing of 3·0 μm, following the cessation of passive manipulation. In these experiments the values for D_{APP}, 0·133 and $0{\cdot}177 \times 10^{-6}$ cm^2 s^{-1} (22 °C), were also not significantly different from those summarized in Table 1. The results from both types of experiments involving mechanical movements during the time of myoglobin diffusion support the tentative conclusion that muscle contraction does not lead to convective motions of myoplasm that significantly increase the apparent diffusion constant of myoglobin.

DISCUSSION

The experiments presented here indicate that myoglobin diffuses in myoplasm much more slowly than is commonly assumed. Two basic questions are raised by this finding. Why is the value of D_{APP} so low? What is the physiological significance of this finding?

Possible explanations for the low value of D_{APP}

The value reported here for the apparent diffusion constant of myoglobin in myoplasm at 22 °C is roughly 7 times less than reported in dilute aqueous solution at 22 °C, $1{\cdot}13 \times 10^{-6}$ cm^2 s^{-1} (Polson, 1937) and $1{\cdot}06 \times 10^{-6}$ cm^2 s^{-1} (Riveros-Moreno & Wittenberg, 1972). If $1+\beta$ for myoglobin is 1·24, the upper limit suggested by the cut-fibre results (column 7 of Table 2), then D_{FREE} (cf. eqn (6)) for myoglobin in frog skeletal muscle is about 6 times less than it is in dilute solution. The results of Kushmerick & Podolsky (1969) and Maylie *et al.* (1987*a*, *b*, *c*), however, indicate that the free diffusion constants in muscle of several substances ranging in molecular weight between 200 and 800 Da are roughly half the values either measured or predicted by the Stokes–Einstein equation in dilute aqueous solution. This suggests that an upper limit for the effective viscosity of myoplasm (including tortuosity factors) is 2 times the viscosity of water (Kushmerick & Podolsky, 1969). Therefore, the value for D_{FREE} of myoglobin in myoplasm reported here is about 3 times less than expected for diffusion in a solution with the effective viscosity of myoplasm.

The possibility may be raised that, because the myofilaments have a net negative charge (Naylor, Bartels, Bridgman & Elliott, 1985), the effective myoplasmic solution space accessible to myoglobin may be less than the 70% of fibre volume assumed in the method for estimating $1+\beta$. If myoglobin had had access to less than 70% of fibre volume, then the value estimated for $1+\beta$, and thus D_{FREE}, would have been too low. However, since the isoelectric point for myoglobin is 6·8–7·0 (Wyman, 1958; Lehninger, 1975), the net charge on myoglobin at the pH expected for myoplasm in these experiments (6·8–7·0) should be between +1 and −1. Thus possible effects on β related to electrostatic repulsion or attraction are probably negligible. Additionally, the estimates of D_{FREE} for three Ca^{2+} indicator dyes (Arsenazo III, molecular weight 776, net charge −5 at pH 7; Antipyrylazo III, molecular weight 746, net charge −3 at pH 7; and tetramethylmurexide, molecular weight 344, net charge −1 at pH 7) in cut fibres (Maylie *et al.* 1987*a*, *b*, *c*), which depend directly on the assumption that the percentage of accessible fibre volume is 70%, are in agreement with the diffusion constants estimated in skinned fibres for uncharged substances of similar molecular weight (Kushmerick & Podolsky, 1969). Since the negative charges of the indicator dyes do not appear to have affected their access to the myoplasmic solution space, then electrostatic effects for myoglobin should also be negligible.

In agreement with there being a low myoglobin diffusivity in myoplasm are the apparent diffusion constants of several other large molecular weight substances reported from other laboratories (Table 3). While the possibility of myoplasmic binding of these other substances was not ruled out, the results suggest that larger molecular weight substances diffuse more slowly in myoplasm, by at least a factor of 3, than is predicted by the combined viscosity and tortuosity effects applicable to smaller molecular weight substances. It should be noted, however, that the Stokes–Einstein prediction of diffusion constants is derived from an analysis of the drag experienced by a sphere moving in a solution of infinite extent (see e.g. Bird, Stewart & Lightfoot, 1960). A larger drag, and hence smaller diffusion constant, is predicted in the case of a sphere moving near an immobile structure. For molecules the size of myoglobin, molecular diameter of 3·5 nm (assuming a molecular weight of 18000 Da and a protein density of 1·38 g cm^{-3}), there are likely to be viscous drag effects not applicable to molecules whose size is small with respect to the dimensions of the spaces in which diffusion takes place. For example, the diameter of the thick filament, not including the cross-bridge array, is about 15 nm, and including the cross-bridge array is about 30 nm (Cantino & Squire, 1986), whereas the centre-to-centre distance between thick filaments is about 35–40 nm (see e.g. Gauthier, 1983). Thus, the disproportionately large reduction in myoplasmic diffusion constants of myoglobin and other large molecular weight substances may be a direct consequence of the relatively larger effects of viscous drag imposed by the internal structures of muscle.

An additional effect applicable to the longitudinal diffusion of larger molecules may be that passage is limited at the Z-line. One argument against rate limitation at the Z-line is the similarity of the values of D_{APP} for myoglobin determined under short and long sarcomere spacing (Table 1). Since the longitudinal spacing between Z-lines (and possibly the Z-lattice structure) is different under these conditions, a significant difference in D_{APP} in the two conditions might have been expected if

passage through the Z-line were the rate-limiting step. Another argument against limitation at the Z-line is that values determined for the radial diffusion constants of aequorin and parvalbumin (Table 3) and other large molecular weight substances (Maughan & Wegner, 1987) are also low. The agreement between the value of D_{APP} of myoglobin reported here and in homogenized muscle (Moll, 1968) also argues

TABLE 3. Measurements of apparent diffusion constants for other large substances in muscle

(1) Substance	(2) Mol. wt	(3) Direction	(4) Preparation	(5) Diffusion constant ($cm^2\ s^{-1}$) $\times 10^6$
Aequorin	20000	Radial	Skinned barnacle at 20 °C (Ashley, Moisescu & Rose, 1974)	0·1–0·15
Aequorin	20000	Axial	Intact frog at 14 °C (Blinks, Rudel & Taylor, 1978)	0·05
Parvalbumin (and other proteins)	10000 50000–150000	Radial	Skinned frog at 20 °C (Maughan & Wegner, 1987)	All about 10 times less than aqueous solution

Column 1 gives the substance whose diffusion constant was measured. Column 2 gives the molecular weight of the substance in daltons. Column 3 gives the diffusion direction referred to the fibre axis. Column 4 gives the preparation and reference. Column 5 gives the apparent diffusion constant measured.

against the possibility that myoglobin movement was rate limited by passage through the Z-line, since the Z-line lattice would have been disrupted in the homogenized muscle. While none of these arguments is especially convincing by itself, together they suggest that limited passage through the Z-line is not the explanation for the small value of D_{APP} reported here.

Relationship to myoglobin diffusion in red muscle

These considerations in turn suggest that the longitudinal diffusion constant measured for myoglobin in frog fibres should provide an upper limit to the more physiologically relevant radial diffusion constant in mammalian red muscle. In particular, the presence of mitochondria and sarcoplasmic reticulum in red skeletal and cardiac muscles probably present significant barriers to radial diffusion. Mitochondria and sarcoplasmic reticulum encircle the I bands of myofibrils of red skeletal muscle (see e.g. Eisenberg, 1983), indicating that about 40% of the surface of myofibrils is impermeant to myoglobin. In cardiac muscle the density of mitochondria is greater than in skeletal muscle, with the myofibrils typically surrounded by mitochondria along the entire sarcomere length (Griepp, 1983). In addition, we have estimated, by calculating average path lengths around thick filaments, that tortuosity factors reduce radial diffusion through myofilaments of the A-band by a factor of 1·5 compared with longitudinal diffusion (assuming the diameter of thick filaments is half the centre-to-centre spacing between thick filaments; see two paragraphs above). These factors suggest that the value for D_{APP} reported here for the longitudinal diffusion of myoglobin in frog fibres could overestimate the more physiologically relevant radial diffusion constant in red

skeletal and cardiac muscles by a factor of 2 or more. Moreover, our measurement of D_{APP} is in close agreement with that of Moll (1968) on homogenized mammalian muscle, who also argued that his value, $0{\cdot}15 \times 10^{-6}$ cm^2 s^{-1} (20 °C), was likely to be an upper limit to that applicable to intact red muscle.

Physiological significance of the results

The primary objective of these experiments was to obtain a more reliable estimate of D_{APP} of myoglobin in myoplasm for incorporation into mathematical models of oxygen transport that aim to quantify myoglobin's role in supporting the oxygen supply to the mitochondria. The relevant value for D_{APP} suggested by our experiments is at least 4 times smaller than commonly assumed in these models and possibly 8 times smaller. Moreover, experiments carried out on mechanically stimulated fibres (p. 265) suggest there is no enhancement of D_{APP} associated with contractile activity of skeletal muscle.

A lower value of D_{APP} necessarily reduces predictions concerning the degree to which myoglobin facilitates oxygen transport in myoplasm. Nevertheless, the following analysis estimating the relative contribution of facilitated flux to total oxygen flux indicates that myoglobin might still significantly facilitate oxygen transport when the oxygen concentration is low. The question of how a lower value for D_{APP} changes predictions concerning the facilitation of oxygen transport can be addressed with an application of Fick's law. The total oxygen flux due to diffusion at any point, including both the flux of physically dissolved (or free oxygen) and facilitated flux, is, when normalized to the free oxygen flux, given by the following expression:

$$\frac{\text{total oxygen flux}}{\text{free oxygen flux}} = \frac{D_{O_2}\nabla[O_2] + D_{APP}\nabla[MbO_2]}{D_{O_2}\nabla[O_2]}, \tag{12}$$

where D_{O_2} and D_{APP} are the apparent diffusion constants of oxygen and myoglobin respectively, and $\nabla[O_2]$ and $\nabla[MbO_2]$ are the local gradient vectors of the concentrations of oxygen and MbO_2, respectively. Assuming instantaneous equilibrium between oxygen and myoglobin, one can readily obtain the following analytical expression (see Appendix, eqns (A 11)–(A 15)), which gives an upper limit for this ratio under steady-state conditions:

$$\frac{\text{total oxygen flux}}{\text{free oxygen flux}} = \frac{D_{O_2}K_d + D_{APP}[Mb]_T(1-y)^2}{D_{O_2}K_d}. \tag{13}$$

$[Mb]_T$ is the total myoglobin concentration, including both MbO_2 and deoxymyoglobin (Mb), y is defined as the fraction of total myoglobin present as MbO_2 ($[MbO_2]/([Mb]+[MbO_2])$), and K_d is the dissociation constant for the oxygen–myoglobin binding reaction. Since the kinetics of the oxygen–myoglobin binding reaction are very fast compared with the time constant for diffusion on the length scale of a fibre radius, this upper limit should actually be very close to the true value of this ratio at most locations in a cell under steady-state conditions. The factor by which myoglobin facilitates the oxygen flux (eqn (13)) is plotted on the ordinate in Fig. 5 as a function of both y and the partial pressure of oxygen on the abscissa. In this calculation the value assumed for the dissociation constant for oxygen

binding to myoglobin was 4·6 μM at 37 °C (Antonini & Brunori, 1965; a K_d = 4·6 μM corresponds to a partial pressure of oxygen for half-saturation of myoglobin of about 3·4 Torr at 37 °C), the total myoglobin concentration was 0·5 mM, and the diffusion constant of oxygen was $1{\cdot}3 \times 10^{-5}$ cm^2 s^{-1} (see Federspiel, 1986). (Note that both

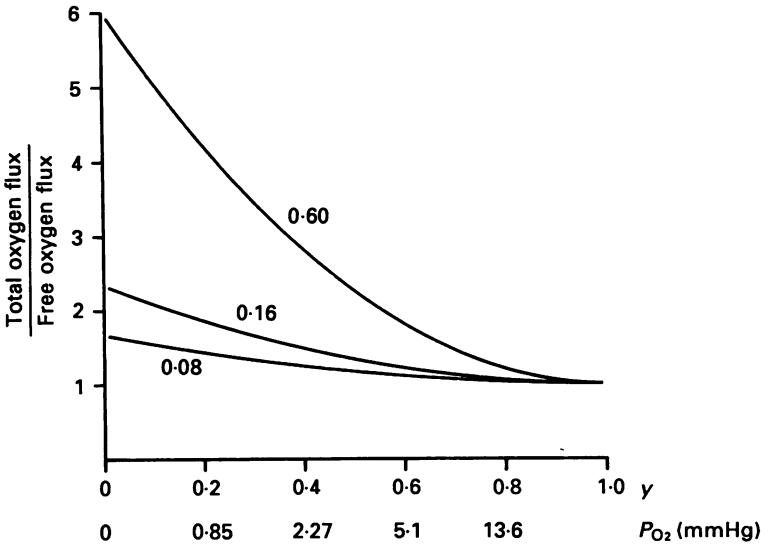

Fig. 5. Effect of myoglobin's apparent diffusion constant (D_{APP}) on facilitated transport of oxygen by myoglobin. The results of eqn (13) in the text are plotted as a function of the degree of myoglobin saturation, y, and the partial pressure of oxygen, P_{O_2} (in units of mmHg). Values assumed for D_{APP} (in units of 10^{-6} cm^2 s^{-1}) are indicated next to the curves. See text for values assumed for the other parameters of eqn (13).

the latter value and D_{APP} for myoglobin are for 20 °C, but the ratio of the diffusion constants should be the same at 37 °C, since the Q_{10} values are very similar (from Grote & Thews, 1962, and Moll, 1968). Also note that the diffusion constant assumed for oxygen is one-half the value applicable to free solution.) The upper curve in Fig. 5 was calculated assuming a diffusion constant for myoglobin of $0{\cdot}6 \times 10^{-6}$ cm^2 s^{-1}, which is in the range of values commonly assumed for myoglobin's diffusion constant by mathematical modellers (see p. 248). The middle curve was calculated using $0{\cdot}16 \times 10^{-6}$ cm^2 s^{-1}, the value reported here for D_{APP} in the longitudinal direction in myoplasm of intact frog fibres. The lower curve was calculated using $0{\cdot}08 \times 10^{-6}$ cm^2 s^{-1}, an estimate which may more closely approximate the physiologically relevant value for D_{APP} in the radial direction of red skeletal and cardiac muscles (see preceding section). As expected, the two lower values for D_{APP} predict a much less prominent role of myoglobin in facilitating oxygen transport. Nevertheless, as the oxygen concentration approaches zero, when facilitated transport would be most useful, the contribution of MbO_2 diffusion approximately doubles the rate of total oxygen transport for a given oxygen gradient. Therefore, facilitated transport of oxygen by MbO_2 diffusion could still play an important role in oxygen delivery in circumstances of limited oxygenation.

In conclusion, the measured value of $0{\cdot}16 \times 10^{-6}$ cm^2 s^{-1} at 22 °C reported here

should probably be considered an upper limit for the radial diffusion constant of myoglobin *in vivo*. This value extrapolates to $0{\cdot}27 \times 10^{-6}$ cm^2 s^{-1} at 37 °C if one uses a value of 1·4 for Q_{10} based on the measurements by Moll (1968) of myoglobin diffusivity in homogenized muscle at 20 and 37 °C. These values should be of utility in the more detailed mathematical models designed to evaluate the degree to which myoglobin facilitates oxygen transport *in vivo*. Several questions remain incompletely answered, however, including exactly why the measured value of the longitudinal diffusion constant for myoglobin in frog skeletal muscle was as low as it was and how accurately does this value approximate the physiologically relevant radial diffusion constant of myoglobin in red skeletal and cardiac muscles.

APPENDIX

The purpose of this Appendix is to derive mathematical equations used in the body of the paper.

Solution of the one-dimensional diffusion equation for the cut-fibre experiment

In order to analyse the cut-fibre results under the assumption that reduction of metMb to MbO_2 takes place, the following solution to the one-dimensional diffusion equation was found. The formulation of the problem is based on the differential equation

$$\frac{\partial[\text{metMb}]_F}{\partial t} = \frac{D_{FREE}}{(1+\beta)}\frac{\partial^2[\text{metMb}]_F}{\partial x^2} - k[\text{metMb}]_F, \tag{A 1}$$

where $[\text{metMb}]_F$ is the concentration of free (unbound) metMb, k is the first-order irreversible rate constant for reduction, x is the distance from the centre of the fibre, and t, β and D_{FREE} are defined on p. 262. The appropriate boundary conditions are

$$\frac{\partial[\text{metMb}]_F}{\partial x} = 0 \quad \text{at } x = 0 \tag{A 2}$$

and

$$[\text{metMb}]_F(L) = \text{a constant} \quad \text{at } x = L \text{ for } t > 0, \tag{A 3}$$

where L is the diffusion distance from the end pools to the centre of the fibre. The initial condition is

$$[\text{metMb}]_F = 0 \quad \text{for all } x \text{ when t} = 0. \tag{A 4}$$

Making the following substitutions:

$$w = \frac{[\text{metMb}]_F(x, t=\infty) - [\text{metMb}]_F(x,t)}{[\text{metMb}]_F(L)}, \quad z = \frac{x}{L} \quad \tau = \frac{t D_{APP}}{L^2} \quad \text{and} \quad \rho^2 = \frac{kL^2}{D_{APP}}$$

into eqns (A 1) to (A 4) leads to the following formulation in dimensionless form:

$$\frac{\partial w}{\partial \tau} = \frac{\partial^2 w}{\partial z^2} - \rho^2 w, \tag{A 5}$$

where D_{APP} has been substituted for $D_{FREE}\,(1+\beta)^{-1}$ (see p. 254). The boundary conditions become

$$\frac{\partial w}{\partial z} = 0 \quad \text{at } z = 0 \tag{A 6}$$

and $$w = 0 \quad \text{at } z = 1 \text{ for } t > 0, \tag{A 7}$$

whereas the initial condition becomes

$$w(z, \tau = 0) = \frac{[\text{metMb}](z, \tau = \infty)}{[\text{metMb}]_F(L)}. \tag{A 8}$$

The initial condition was chosen so that $w(z, \tau) \to 0$ as $\tau \to \infty$. This initial condition was obtained by solving the steady-state problem associated with eqns (A 1)–(A 4), and is given by

$$w(z, 0) = \frac{e^{\rho z} + e^{-\rho z}}{e^{\rho} + e^{-\rho}}. \tag{A 9}$$

A solution to the problem, as formulated here, can readily be obtained using the technique of separation of variables. The solution at $x = 0$, in terms of the initially defined variables, is given by

$$\frac{[\text{metMb}]_T(0, t)}{(1+\beta)[\text{metMb}]_F(L)} = \frac{2}{e^{\rho} + e^{-\rho}} - 4\pi \sum_{n=0}^{\infty} \frac{(2n+1)(-1)^n}{4\rho^2 + \pi^2(2n+1)^2} \exp\left(\frac{-D_{APP}(4\rho^2 + \pi^2(2n+1)^2 t)}{4L^2}\right), \tag{A 10}$$

where $[\text{metMb}]_T\,(1+\beta)^{-1}$ was substituted for $[\text{metMb}]_F$ (see p. 254). $[\text{metMb}]_T$ is the sum of the free plus bound metMb concentrations. (Note that this equation reduces to eqn (10) in the text if k and thus ρ equal 0.) The corresponding solution for MbO_2 is the difference between eqn (10) and eqn (A 10).

Derivation of eqn (13) *in Discussion*

Under the assumption that oxygen and myoglobin are in instantaneous equilibrium at any point, eqn (12) on p. 269 can be rewritten as

$$\frac{\text{total oxygen flux}}{\text{free oxygen flux}} = \frac{D_{O_2}(d[O_2]/d[MbO_2])\nabla[MbO_2] + D_{APP}\nabla[MbO_2]}{D_{O_2}(d[O_2]/d[MbO_2])\nabla[MbO_2]}$$

or

$$\frac{\text{total oxygen flux}}{\text{free oxygen flux}} = \frac{D_{O_2}(d[O_2]/d[MbO_2]) + D_{APP}}{D_{O_2}(d[O_2]/d[MbO_2])}. \tag{A 11}$$

d is the derivative operator and ∇ is the vector gradient operator. Since first-order reversible kinetics describe the oxygen–myoglobin binding reaction, the equilibrium assumption provides the following relationship:

$$[O_2] = \frac{K_d[MbO_2]}{[Mb]_T - [MbO_2]}, \tag{A 12}$$

so that $$\frac{d[O_2]}{d[MbO_2]} = \frac{K_d[Mb]_T}{[Mb]^2}, \tag{A 13}$$

where [Mb] has been substituted for $[Mb]_T - [MbO_2]$. (Note that $[Mb]_T$ is constant in

both time and space.) Substituting eqn (A 13) into eqn (A 11) yields the following equation:

$$\frac{\text{total oxygen flux}}{\text{free oxygen flux}} = \frac{D_{O_2}K_d[Mb]_T + D_{APP}[Mb]^2}{D_{O_2}K_d[Mb]_T}. \quad (A\ 14)$$

Defining y as the fraction of total myoglobin present as MbO_2,

$$y = \frac{[MbO_2]}{[Mb]+[MbO_2]},$$

allows combination with eqn (A 14) to yield

$$\frac{\text{total oxygen flux}}{\text{free oxygen flux}} = \frac{D_{O_2}K_d + D_{APP}[Mb]_T(1-y)^2}{D_{O_2}K_d}. \quad (A\ 15)$$

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