

SHORT COMMUNICATION

LACK OF CORRELATION BETWEEN CARDIAC MYOGLOBIN CONCENTRATION AND *IN VITRO* METMYOGLOBIN REDUCTASE ACTIVITY

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Myoglobin (Mb) is an intracellular protein found in red skeletal muscle and heart. It binds oxygen reversibly and enhances oxygen consumption, especially under conditions of low oxygen availability (Bailey *et al.* 1990; Wittenberg and Wittenberg, 1989). A variety of biochemical and biophysical experiments reveals that Mb functions by facilitating the diffusion of oxygen from the extracellular space to the mitochondria (Wittenberg and Wittenberg, 1989). Each molecule of Mb contains a single iron atom which may bind to one molecule of O₂ when the iron is in the ferrous state (i.e. Mb-Fe²⁺). Mb-Fe²⁺ undergoes spontaneous oxidation *in vitro* to the ferric state (Mb-Fe³⁺), known as metmyoglobin (metMb), which cannot bind O₂. Autoxidation occurs for myoglobin isolated from numerous sources with a half-time of the order of hours under conditions approximating *in vivo* pH, ionic strength and temperature (e.g. Kitahara *et al.* 1990; Tajima and Shikama, 1987; Livingstone *et al.* 1986). It is generally accepted that spontaneous autoxidation must occur *in vivo*; however, metmyoglobin accumulation under physiological conditions has never been reported. Since the turnover time for Mb may exceed several months (Hickson and Rosenkoetter, 1981), a mechanism must exist to reconvert metMb to Mb and any failure in this mechanism could impair oxygen delivery to the mitochondria. Reduction of metMb has been observed in isolated, perfused rat and sea raven hearts following treatment with NaNO₂, which oxidizes Mb (Tamura *et al.* 1980; Bailey and Driedzic, 1988). Two fundamentally different enzyme mechanisms have been proposed to account for metMb reduction *in vivo*.

On the basis of studies with isolated enzymes, the redox state of Mb is considered to be maintained by the enzyme metmyoglobin reductase, which has been characterized as NADH-cytochrome *b*₅ oxidoreductase. The postulated mechanism assumes a rapid reduction of the enzyme by NADH, followed by enzymatic reduction of cytochrome *b*₅ Fe³⁺ to cytochrome *b*₅ Fe²⁺. The ferrous cytochrome then nonenzymatically reduces metMb (Livingstone *et al.* 1985). The enzyme has been isolated from mammalian heart (Hagler *et al.* 1979) and fish skeletal muscle (Levy *et al.* 1985) and has a relative

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molecular mass of about 30 000. There is an absolute requirement for NADH as neither NADPH nor FADH₂ is a suitable substrate for the enzyme-catalyzed reduction of Mb-Fe³⁺. *In vitro*, cytochrome *b*₅ and Mb bind to the enzyme in a one-to-one stoichiometry (Livingston *et al.* 1985). Microsomal cytochrome *b*₅ has been identified by immunological procedures in heart (Ito, 1980).

It has not been proved that the enzyme described above as metmyoglobin reductase is responsible for the restoration of Mb-Fe²⁺ *in vivo*. Taylor and Hochstein (1982) argue that other non-specific reductases referred to as 'diaphorases' [NAD(P)H-quinone oxidoreductase] in the earlier literature could be involved. These 'diaphorases' require electron donors such as Methylene Blue and may use either NADH or NADPH under *in vitro* conditions. Support for this enzyme system as the physiologically important one is based on the similar impact of pharmacological inhibitors of metMb reduction on isolated myocytes and on *in vitro* activity of purified enzyme (Taylor and Hochstein, 1982).

In the present paper we report on the relationship between Mb content and the activity of enzymes potentially involved in metMb reduction. The comparative approach was capitalized upon by selecting closely related species that have extremely different levels of cardiac Mb. We assume that the formation of metMb *in vivo*, and consequently the requirement for reduction, is proportional to the total amount of Mb regardless of species. Moreover, if the enzyme termed 'metmyoglobin reductase' or 'diaphorase' were primarily responsible for metMb reduction, there should be a positive relationship between maximal *in vitro* enzyme activity and Mb content.

Sea raven (*Hemitripterus americanus*) and ocean pout (*Macrozoarces americanus*), both teleost fish, were captured by otter trawl in Passamaquoddy Bay off St Andrews, NB. They were transported to Mount Allison University where they were held in recirculating sea water at 10 °C until use. Bullfrogs (*Rana catesbeiana*) and toads (*Bufo marinus*) were obtained from a biological supply house (Boreal). Bullfrogs were kept at 5 °C in a flow-through aquarium until use. Toads were kept at 18 °C until use. Rats (Sprague Dawley, CD strain) were obtained from Charles River Laboratories and kept in individual cages until use with unlimited access to food and water. Rabbits were obtained from local breeders immediately prior to use.

Fish were stunned by a blow to the head and the heart quickly excised. Blood was flushed from the heart with Ringer's solution. Frogs and toads were double-pithed, the hearts quickly excised and flushed with amphibian Ringer. Mammals were anaesthetized with sodium pentobarbitol, the hearts were excised and flushed by Langendorff-type perfusion with Krebs-Henseleit bicarbonate buffer. Ventricles from all hearts were weighed and homogenized (10% w/v homogenate) in ice-cold phosphate buffer (10 mmol l⁻¹ total phosphate:1 mmol l⁻¹ EDTA, pH 7.0). This crude homogenate was separated into two parts. One part was then further diluted 10-fold, with 10 mmol l⁻¹ phosphate buffer, for use in metmyoglobin reductase assays, and the other part was centrifuged at 10 000 g for 15 min. The supernatant was used for Mb and diaphorase assays.

Mb content was assayed with a Gilford spectrophotometer. A wavelength scan from 500 to 610 nm was carried out comparing the supernatant to phosphate buffer. The peak absorbance above background light scatter at 583 nm was determined. Mb concentration

was calculated using 12.8 as the millimolar extinction coefficient (Hardman *et al.* 1966) and is expressed as nmol g^{-1} wet mass. Lack of a characteristic absorption peak for metMb around 500 nm indicates that the Mb in the extract was reduced.

Metmyoglobin reductase assays were carried out under the following conditions: 0.15 mmol l^{-1} metMb (from horse heart, Sigma), 0.1 mmol l^{-1} NADH (Sigma), 0.3 mmol l^{-1} $\text{K}_4\text{Fe}(\text{CN})_6$ in 2 ml of phosphate buffer (30 mmol l^{-1} total phosphate:1 mmol l^{-1} EDTA) pH 7.0 at 20 °C (from Hagler *et al.* 1979). The reaction mixture was allowed to temperature-equilibrate in a water-jacketed cuvette holder, then 50 μl of dilute homogenate was added and the resulting reduction in metMb was measured spectrophotometrically at 583 nm. Preliminary experiments involving repeated wavelength scans showed that the metMb was being reduced. In other preliminary experiments, no activity was observed with NADPH. Similar optimal conditions were identified for purified beef heart and tuna muscle enzyme (Levy *et al.* 1985; Hagler *et al.* 1979). This suggests that the assay employed is relatively specific for metmyoglobin reductase; however, the possibility that there is an alternative enzyme with similar substrate preferences and pH profile cannot be ruled out. Previous studies have shown that non-specific NADH oxidase activity is minimal in heart homogenates (Driedzic *et al.* 1987). Therefore, differences in depletion of one of the cosubstrates across species was not a confounding factor.

Diaphorase assay conditions were as follows: 0.15 mmol l^{-1} metMb, 0.1 mmol l^{-1} NADPH in 2 ml of phosphate buffer (30 mmol l^{-1} total phosphate:1 mmol l^{-1} EDTA) pH 7.0 at 20 °C (modified from Taylor and Hochstein, 1978). The assay mixture was allowed to temperature-equilibrate, 2.5 μmol of Methylene Blue was added and non-enzymatic reduction of metMb was recorded for 5 min. 100 μl of supernatant was then added and metMb reduction was assayed at 583 nm.

Enzyme activities were calculated from the change in absorbance using a millimolar extinction coefficient of 12.8 (Hardman *et al.* 1966). Activities are expressed as $\mu\text{mol min}^{-1} \text{g}^{-1}$ wet mass. Comparisons between mean maximal enzyme activities were carried out with the Student's *t*-test. Correlations between Mb content and maximal enzyme activities were determined by linear regression analysis. *P* values of less than 0.05 were considered significant and *P* values of less than 0.01 were considered highly significant.

Fig. 1A shows Mb concentration in various species. Sea raven have the greatest amount (195 nmol g^{-1}), then rat (158 nmol g^{-1}), bullfrog (93 nmol g^{-1}), rabbit (53 nmol g^{-1}) and finally ocean pout and toad, which do not have detectable levels of cardiac Mb. Fig. 1B shows the maximal *in vitro* activity of metmyoglobin reductase. Toads display the greatest amount of activity and then ocean pout. There is no significant difference in activity between sea raven and bullfrog but these both display greater activity than either rat or rabbit. The two mammalian species are not significantly different in metmyoglobin reductase activity. There is no correlation between maximal metmyoglobin reductase activity and Mb concentration. Fig. 1C shows the pattern of diaphorase activity across the species utilized. In this case the greatest activity was found in rabbit heart. There was no significant difference between rat, bullfrog and toad heart diaphorase activity when compared pairwise. Sea raven and ocean pout displayed the

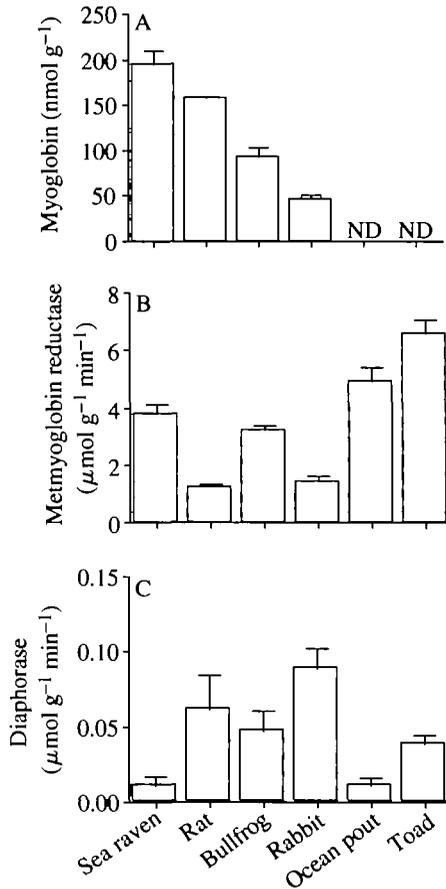


Fig. 1. (A) Heart Mb content in various species. All values are means + S.E.M. ND indicates that Mb was undetectable by the assay used. $N=8$ for sea raven, 6 for rat, bullfrog, ocean pout and toad and 5 for rabbit. (B) Maximal *in vitro* metmyoglobin reductase activities in various species. All values are means \pm S.E.M. and the N values are the same as above. (C) Maximal *in vitro* diaphorase activities in various species. All values are means + S.E.M. and the N values are the same as for the top panel.

least amount of activity. Diaphorase activity was at least 20-fold lower than the metmyoglobin reductase activity within species.

Mb content in vertebrate hearts is extremely species-specific and ranges from undetectable levels to values approaching 500 nmol g^{-1} (Sidell *et al.* 1987). Hearts with the lowest level of Mb appear white following blood washout and hearts with high levels are deep red following blood washout. Mb content is not related to phyletic position as there are some fish species with Mb content higher than those of some mammals and some mammals with extremely low levels. Rather, Mb content appears to be related to oxygen demand and delivery characteristics so that hearts facing potentially low extracellular oxygen levels because of environment (fish in hypoxic water) or behaviour (diving mammals) appear to have elevated levels of Mb.

Maximal activities of the metmyoglobin reductase system of non-mammalian species always exceeded those of the mammalian species. We felt it was necessary to run assays for rat and rabbit hearts at lower than physiological temperature for a direct comparison with the ectothermic species involved in the study. Increasing the reaction temperature to 37 °C had only a small impact on the rate of either metmyoglobin reductase or diaphorase ($Q_{10} \approx 1.2$, data not shown) from rat or rabbit hearts. Therefore, even after adjustment of the assay temperature to physiological levels, the maximal activity of metmyoglobin reductase was lower in mammals than in ectotherms. In contrast, diaphorase activities were always greater in the mammalian species.

We hypothesized that the greater the Mb content of hearts the greater the level of metmyoglobin reductase, but there was no correlation between Mb content and metmyoglobin reductase activity. Indeed, hearts with undetectable levels of Mb displayed the greatest amount of enzyme activity. This does not mean that the metmyoglobin reductase enzyme does not function in the reduction of oxidized Mb but simply that the enzyme did not co-evolve with Mb and is not dedicated to metMb reduction. It is quite possible that the primary function of this enzyme lies elsewhere, for example in the regeneration of NAD^+ from NADH or the reduction of microsomal cytochromes.

A similar situation exists for the other metMb reducing enzyme found in hearts, the diaphorase enzyme. There is no correlation between cardiac Mb concentration and diaphorase activity. This enzyme displays considerably less maximal activity than the metmyoglobin reductase enzyme. In each species examined the metmyoglobin reductase activity ranged from at least 20-fold greater (rabbit) to 400-fold greater (sea raven) than the diaphorase activity.

Reduction of Mb in intact cells has been observed following treatment with NaNO_2 to oxidize Mb and subsequent washout of NaNO_2 . For instance, metMb reduction, monitored by reflectance spectroscopy in perfused rat hearts, was of the order of $30 \text{ nmol g}^{-1} \text{ min}^{-1}$ at 25–27 °C (Tamura *et al.* 1980). This technique, however, only measures what is occurring on a very small section of the epicardium. Taylor and Hochstein (1982) observed metMb reduction at a rate of $10 \text{ nmol g}^{-1} \text{ min}^{-1}$ in isolated rat heart cells at 37 °C. In the present study, rat heart homogenates show maximal *in vitro* activities of metMb reduction of the order of $1.0 \mu\text{mol g}^{-1} \text{ min}^{-1}$ with metmyoglobin reductase (the ferrocyanide-activated enzyme) and $60 \text{ nmol g}^{-1} \text{ min}^{-1}$ for diaphorase (the Methylene-Blue-activated enzyme) at 20 °C. Either of the enzymes measured in the current study has adequate catalytic potential to keep pace with the *in vivo* rates of metMb reduction.

In conclusion, this study has demonstrated a lack of correlation between either metmyoglobin reductase or diaphorase activities and cardiac Mb content. Paradoxically, those hearts that have the least amount of Mb display the greatest *in vitro* level of metmyoglobin reductase. The current data also reveal that in a wide range of species metmyoglobin reductase activity greatly exceeds diaphorase activity. Measurements of rates of metMb formation *in vivo* are required to assess whether diaphorase has the catalytic potential to maintain the Mb redox state.

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