JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Temperature and pH Dependence of the Autoxidation Rate of Bovine, Ovine, Porcine, and Cervine Oxymyoglobin Isolated from Three Different Muscles—*Longissimus dorsi*, *Gluteus medius*, and *Biceps femoris*

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This study examined the temperature and pH dependence of the in vitro autoxidation rate of bovine, ovine, porcine, and cervine oxymyoglobin that had been isolated and purified from three muscles of different oxidative stability—*Longissimus dorsi*, *Gluteus medius*, and *Biceps femoris.* To avoid obtaining unreliable estimates of autoxidation rate as has occurred in many previous studies, in this study, precautions were taken to eliminate the effects of freezing, chemical reduction with hydrosulfite, and contaminating metal ions on the reaction rate. When these precautions were taken, the rate constants for the different myoglobins studied were similar to each other but were 2−7-fold lower, and the Ea (activation energy) was 20−100% higher than that reported in most previous studies. The type of muscle the myoglobin was isolated from had no effect on the reaction rate or the Ea; however, the species did have a significant effect ($p < 0.05$) with porcine myoglobin having a 10% lower reaction rate and a 20% lower Ea than myoglobin from the other species. Increasing the reaction pH from 5.50 to 6.50 produced an exponential increase in reaction rate but only a small curvilinear change in Ea that had a maximum at pH 6.00.

KEYWORDS: Myoglobin; autoxidation; ovine; bovine; porcine; cervine; temperature dependence; muscle type; pH

INTRODUCTION

Research over the last 20 years has shown that there is up to a 12-fold difference in the rate at which myoglobin oxidizes in postmortem mammalian muscles depending on the species and muscle type (*1*−*3*). One explanation for this difference is that the myoglobin from different muscles and different species oxidizes at different rates. For example, research on myoglobin from different marine animals has shown that there are differences in oxidation rate of the myoglobins from different species (*4*). Similarly, more recent research on myoglobin from different bovine muscles also indicates that there are differences in oxidation rate of myoglobins from the different muscle types (*5*).

These differences in oxidation rate of myoglobin from different muscles and different species are not surprising since it is well-established that there are structural and chemical differences between myoglobin from different sources (*4*, *6*−*9*). For example, differences in crystallographic, immunological, and electrophoretic behavior among various myoglobins from different mammalian species have been known for many years (*10*, *11*). Moreover, differences in amino acid composition of myoglobins from different species has also been well-established (*12*−*15*). In particular, it has been demonstrated that various tuna myoglobins, in contrast to mammalian myoglobins, contain free sulfhydryl groups that could potentially influence the myoglobin oxidation rate (*16*, *17*).

Although there are a number of differences between different myoglobins, most of the properties of myoglobin are conserved. For instance, early X-ray analyses on myoglobin from different species indicated that tertiary structures for different myoglobins are essentially the same (*10*, *18*). Also, the functional properties of tuna myoglobins, including a high affinity for oxygen, are similar to each other and to that of mammalian myoglobins (*19*−*21*).

There are other possible reasons for the different rates at which myoglobin oxidizes in postmortem mammalian muscles that are related to intrinsic properties of the muscles. For example, different concentrations of endogenous antioxidant (ascorbate and carnosine (*22*), uric acid (*23*), carotenoids, and tocopherols (*24*)) and pro-oxidant compounds (copper and iron (*25*)) in muscle will affect myoglobin oxidation rate. Similarly, differences in the activity of reducing enzymes in muscle (such

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10.1021/jf0112769 CCC: $22.00
Published on Web 03/27/2002

as superoxide dismutase and glutathione peroxidase), which can reduce oxidized myoglobin, will also affect the rate at which myoglobin oxidizes in postmortem muscle (*26*).

Nevertheless, it is unclear whether the structural differences between myoglobins are reflected in different autoxidation properties. One of the problems of trying to relate historic in vitro autoxidation rates of different myoglobins to the autoxidation in muscle is that results obtained in earlier studies need to be interpreted cautiously (*4*, *7*, *27*). The reason for this is that numerous factors that were not considered in earlier myoglobin studies have since been found to have a substantial effect on the rate of myoglobin autoxidation. The most important of these factors includes the use of sodium hydrosulfite to reduce myoglobin that has been oxidized during purification (*8*), freezing myoglobin or the muscle myoglobin is extracted from (*8*, *28*), and contamination of myoglobin preparations with metal ions (*29*, *30*). Hence, to obtain reliable estimates of myoglobin oxidation rates, it is important that these effects are eliminated.

Therefore, the objective of this study was to determine the in vitro autoxidation rate of myoglobin isolated from three muscles of different oxidative stability: high—*Longissimus dorsi* (LD); intermediate—*Gluteus medius* (GM); and low—*Biceps femoris* (BF); and from four different species (ovine, porcine, bovine, and cervine). To ensure that reliable estimates of myoglobin autoxidation rate were obtained, precautions were taken as follows: (i) the muscles used for myoglobin extraction and the purified myoglobin were not frozen, (ii) an extraction and purification technique was used that prevented autoxidation of myoglobin during purification and the need for chemical reduction with hydrosulfite (*25*); the interference of contaminating metal ions and autoxidation products was eliminated by using a chelating agent (DPTA [diethylenetriaminepentaacetic acid]) and catalase, respectively (*30*).

MATERIALS AND METHODS

Materials. All chemicals used in this study were of the highest grade available commercially and were used as received without further purification. All solutions were prepared with deionized and glass-distilled water. Catalase from bovine liver (Sigma) was obtained commercially as a crystalline suspension and was resuspended in buffer immediately prior to analysis. The muscles used in this study were obtained from commercially produced and slaughtered animals of similar physiological maturity (the approximate age of the four species was as follows: porcine, 6 months; bovine, 14 months; ovine, 6 months; and cervine, 12 months). The muscles were obtained 18 h postmortem after they had cooled to 5 °C and were stored at 0 °C in oxygen-permeable poly(vinyl chloride) bags until the myoglobin was extracted and purified (within 24 h).

Myoglobin Extraction and Purification. Immediately prior to extraction, the surface of each muscle was trimmed to remove any surface muscle that could contain oxidized myoglobin, and 600 g of the trimmed muscle was used to extract and purify the myoglobin (*25*). This method produces high purity (>96%) reduced myoglobin (>98% oxy form) within 48 h. If necessary, the myoglobin was concentrated to 2.0 mg/mL (by ultrafiltration under an oxygen atmosphere [400 kPa] using a low-binding, 10 000 MW cutoff filter [Millipore]). It was then filter-sterilized and stored in a sealed glass tube at 0 °C in the absence of light and used within 1 week of preparation. The concentration and filtration methods had no adverse effect on the myoglobin oxidation rate (*25*).

Myoglobin Autoxidation. The autoxidation rate of myoglobin purified from muscles from each of the four species and three muscle types was measured at three pH levels (pH 5.50, 6.00, and 6.50) and at three temperatures (20, 30, and 40 °C). The pH conditions used in this study are similar to those found in postmortem muscle—the temperature conditions used are much higher than refrigerated temperatures; however, lower temperatures could not be used because even at 10 °C myoglobin oxidation rate was so slow that even after 72 h meaningful kinetic parameters could not be calculated at high pH. The autoxidation rate was determined at the three different temperatures so that the activation energy (Ea) could be calculated for each of the 12 myoglobin types at the three different pH levels.

Myoglobin oxidation rate was determined at a myoglobin concentration of 1.0 mg/mL in 10 mM phosphate buffer containing 5 μM DTPA and 0.1 μM catalase at predetermined pH levels (5.50, 6.00, and 6.50) and temperatures (20, 30, and 40 °C). The DTPA and catalase were added to eliminate the possible effect of contaminating metal ions and byproducts of oxidation on the autoxidation rate (*30*). Prior to the start of the reaction, the purified myoglobin (2.0 mg/mL) and 20 mM phosphate buffer (pH 5.50, 6.00, or 6.50) containing 10 μM DTPA and 0.2 μM catalase were separately incubated at the required temperature (either 20, 30, or 40 °C) in 1.0 cm path length plastic cuvettes (Banksia Scientific). To initiate the reaction, 1.5 mL of phosphate buffer (containing DTPA and catalase) and 1.5 mL of purified MbO were mixed in the cuvettes.

The autoxidation rate of myoglobin was monitored by measuring the decrease in absorbance of reduced myoglobin (at 572 nm corrected for turbidity measured at 730 nm) (*25*). Absorbance was measured at 15 min intervals using a CARY UV−Vis spectrophotometer equipped with a 12 cell, temperature-controlled sample holder (Varian Pty Ltd., Australia).

The first-order rate constant (k, h^{-1}) was determined using the Varian Kinetics Application software (version 3.1), which allowed interactive selection of the data range. To ensure reliable calculations of the rate constant, the initial (A_0) and the final absorbance (A_α) values that were calculated using this technique were compared to the literature values for myoglobin at 1.0 mg/mL ($A_0 = 0.60$; $A_\alpha = 0.18$ (*31*)).

Calculation of Ea. The Ea of myoglobin was determined at pH 5.50, 6.00, and 6.50 over a range of three temperatures (i.e., 20, 30, and 40 °C) using the Arrhenius equation (*32*)

$$\log(k_2/k_1) = \mathrm{Ea}/(2.303 \times R) \times ((T_2 - T_1)/(T_1 \times T_2))$$

where k_1 = rate constant value of myoglobin at temperature 1 (k, h^{-1}); k_2 = rate constant value of myoglobin at temperature 2 (k, h^{-1}); Ea = activation energy; R = gas constant; T_1 = temperature 1 (K); T_2 = temperature 2 (K).

Experimental Design. The design used for the experiment was a complete 4 × 3 factorial with four species (bovine, porcine, ovine, and cervine) and three muscle types (LD, GM, and BF). The effect of pH (5.50, 6.00, and 6.50) and temperature (20, 30, and 40 °C) was determined by using a split-plot for each muscle (*33*). Because the number of samples to assay in the experiment was greater than the number of cells in the spectrophotometer, a randomized incomplete block design was used, which also took into consideration possible variations in the two light sources in the spectrophotometer (*33*). The experiment was carried out in duplicate.

Data Analysis. Data were analyzed by analysis of variance (ANOVA) using the generalized linear model procedure (SAS [SAS Institute Inc., 1994]). When ANOVA showed significant treatment effects ($p < 0.05$), mean separations were carried out using the least significant difference test of SAS (*34*).

RESULTS AND DISCUSSION

Autoxidation Rate of Myoglobin. ANOVA of the data showed that there was no effect ($p > 0.05$) of muscle type on the autoxidation rate of myoglobin for any of the four species studied (**Table 1**). These results are in contrast with those previously obtained using a ^{1}H NMR, which indicated that there was a difference in autoxidation rate of myoglobin isolated from two different bovine muscles (*5*). No previous studies other than this have shown any effect of muscle type on myoglobin autoxidation rate.

Analysis of the results also showed that there was an effect of species on the myoglobin autoxidation rate (**Table 1**). Porcine myoglobin had a significantly lower autoxidation rate than

Table 1. Autoxidation Rate Constants (k, h^{-1}) of Myoglobin from Porcine, Bovine, Ovine, and Cervine; LD, GM, and BF muscles Averaged over pH and Temperature[a,b]

	rate constant values (k, h^{-1})			
	muscle type			
species	LD	GM	BF	mean
porcine	0.155^{ax}	0.150^{ax}	0.161^{ax}	0.155^{a}
bovine	0.180^{bx}	0.178^{bx}	0.179^{bx}	0.179^{b}
ovine	0.179^{bx}	0.181^{bx}	0.168^{bx}	0.176^{b}
cervine	0.170^{bx}	0.170^{bx}	0.168^{bx}	0.169^{b}
mean	0.171^{x}	0.170^{x}	0.169^{x}	0.170

[a] a and b in the same column with different superscripts are significantly different ($p < 0.05$). [b] x, y, and z in the same row with different superscripts are significantly different ($p < 0.05$).

myoglobin from any of the other species ($p < 0.01$). Although an effect of species on myoglobin autoxidation rate has not been shown previously for mammalian myoglobin, differences have been shown to occur with myoglobin from different marine animals (*4*).

There was a consistent effect of pH on the myoglobin autoxidation rate for the different species and temperatures (Figure 1). The values for the rate constant, averaged over species and temperature, at pH 5.50, 6.00, and 6.50 were 0.288, 0.156, and 0.062, respectively. Hence, for all species, the autoxidation rate of myoglobin was very pH-dependent and decreased by approximately 50% for each 0.5 unit increase in pH. This effect of pH is very similar to that reported by previous researchers over a similar pH range (*35*−*37*).

There was a large but inconsistent effect of temperature on the autoxidation rate of the different myoglobins (Figure 1). With the bovine, ovine, and cervine myoglobin, the autoxidation rate increased by approximately 5-fold for every 10 °C increase in temperature. The Q10 values averaged over pH for these three different myoglobins were similar and ranged from 4.67 to 4.89. In contrast, with porcine myoglobin, the effect of temperature was much smaller—the Q10 value was 20% lower at 3.77.

This effect of temperature on the autoxidation rate of bovine, ovine, and cervine myoglobin is similar to that previously reported for bovine myoglobin (*9*). However, the smaller effect of temperature on the autoxidation rate of porcine myoglobin has not been reported previously.

The myoglobin autoxidation rate was also affected by an interaction between species, pH, and temperature (**Figure 1**). This interaction was such that the autoxidation rate of porcine myoglobin increased less with increasing temperature at pH 5.50 and 6.00 (but not at pH 6.50) than myoglobin from the other three species (**Figure 1**). The differential effect of pH on the temperature dependence of the autoxidation rate for the myoglobin from different species is most likely related to the differential ionization of three protonatable groups involved in autoxidation. It has been theorized that the effect of pH on myoglobin autoxidation rate, and subsequently the interaction between pH and reaction temperature, can best be explained by an "acid/base-catalyzed three states model"(*35*). This model involves the catalytic role of the hydrogen ion on the distal histidine and two dissociatable side groups, most likely carboxyl groups, in the heme pocket.

Ea. Both species and pH had a significant effect on the myoglobin Ea ($p < 0.01$); however, there was also a significant ($p < 0.01$) interaction between species and pH (**Table 2**). The species effect occurred because porcine myoglobin had an Ea (averaged over pH) approximately 20% lower than that obtained

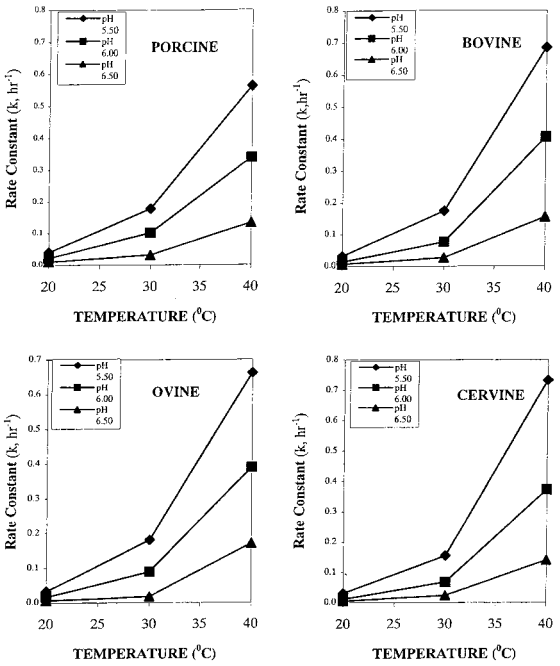

Figure 1. Effect of pH and temperature on autoxidation rate (k, hr^{-1}) of porcine, bovine, ovine, and cervine myoglobin (averaged over muscle type).

Table 2. Ea of Porcine, Bovine, Ovine, and Cervine Myoglobin at pH 5.50, 6.00, and 6.50 Averaged over Muscle Type[a,b]

	Ea			
	pH			
species	5.50	6.00	6.50	mean
porcine	100.6^{ax}	104.3^{ay}	97.7^{ax}	100.9^{a}
bovine	116.0^{bx}	126.9^{cy}	117.9^{bx}	120.3^{b}
ovine	114.5^{bx}	120.0^{by}	124.8^{cz}	119.9^{b}
cervine	119.4^{bx}	124.8^{bcy}	118.5^{bx}	120.9^{b}
mean	112.6^{x}	119.0^{z}	114.8^{y}	115.5

[a] a and b in the same column with different superscripts are significantly different ($p < 0.05$). [b] x, y, and z in the same row with different superscripts are significantly different ($p < 0.05$).

with myoglobin from the other three species (100 cf. 120). Moreover, when averaged over species, pH had a curvilinear effect on Ea with a maximum at pH 6.00. The species−pH interaction resulted from the fact that the Ea of the ovine myoglobin increased approximately linearly with increasing pH, in contrast to the curvilinear increase in Ea exhibited by myoglobin from the other three species.

While researchers agree that the autoxidation of myoglobin is pH-dependent, the effect of pH on the Ea of myoglobin is not so clear. For example, earlier studies found (*39*, *40*) that the Ea for the autoxidation of myoglobin increased approximately 4-fold as the pH decreased from 6.44 to 5.35. However, later studies showed different effects. For instance, one subsequent study found that the Ea of myoglobin was relatively constant in the pH range of 5.0−10.0 (*38*) while another study showed that the Ea was relatively constant in the pH range of 5−7 but there was considerable variation in the Ea outside this pH range (*8*). This large variation in the effect of pH on Ea is most likely related to the method of myoglobin preparation and the procedure used for measuring the autoxidation rate, as is discussed in the following section.

Comparison with Previous Studies. The rate constants and activation energies for bovine myoglobin at pH 6.00 and 30 °C

Table 3. Comparison of Results from the Present Study with Literature Values for Rate Constants (k) and Ea for the Autoxidation of Oxymyoglobin from Several Different Bovine Muscles and Marine Animals

source of oxymyoglobin		oxymyoglobin		conditions			
species	muscle	k (h^{-1})	E_a (kj/mol)	temp (°C)	pH	[buffer] (M)	authors
bovine	BF/GM	0.210	99.2	30	5.70	0.6	Snyder and Ayres, 1961
	GM	**0.077**	**116.5**	**30**	**6.00**	**0.1**	**present study**
	BF	**0.079**	**117.9**	**30**	**6.00**	**0.1**	**present study**
	PM[a]	0.294	100.1	30	6.00	0.1	Renerre et al., 1992
	PM	0.096	96.0	31	6.00	0.1	Foucat et al., 1994
	heart	0.288		30	7.30	0.6	Anderson et al., 1988
	heart	0.004		30	7.30	0.05	Gunther et al., 1999
	heart	0.058		30	6.00	0.05	Gunther et al., 1999
	LD	0.294	100.1	30	6.00	0.1	Renerre et al., 1992
	LD	0.072	104.0	31	6.00	0.1	Foucat et al., 1994
	LD	**0.078**	**118.0**	**30**	**6.00**	**0.1**	**present study**
		0.515	58.2	30	5.90	0.1	Brown and Dolev, 1963a
		0.093	75.4	30	5.70	0.6	Brown and Dolev, 1963a
		0.330		30	5.70	0.6	Snyder and Skrdlant, 1966
sperm whale		0.080	100.5	22	5.78	0.4	Brown and Mebine, 1969
		0.180	136.5	40	7.00	0.1	Livingston et al., 1986
yellow fin tuna		0.180	100.5	22	5.90	0.4	Brown and Mebine, 1969
		0.500	104.7	40	7.00	0.1	Livingston et al., 1986
tuna		0.148	83.3	30	5.90	0.1	Brown and Dolev, 1963a
		0.175	77.9	30	5.90	0.2	Brown and Dolev, 1963a
		0.152	91.7	30	5.90	0.6	Brown and Dolev, 1963a
pacific green sea turtle		0.160	111.0	40	7.00	0.1	Livingston et al., 1986

[a] PM = psoas major.

for the bovine myoglobin from the three different muscles in this study are shown in **Table 3**. For comparison, the table also includes the values from earlier studies (under the same conditions where possible) that have used myoglobin from different bovine muscles and also values for myoglobin from marine animals where species effects on myoglobin oxidation rate have been observed.

The results in **Table 3** highlight the large range in values reported for the rate constant of bovine myoglobin. Many of the earlier rate constants reported for bovine myoglobin are 2−7-fold higher than those reported in the present study. There are, in effect, two categories of results. In the first category are the earlier studies (*2*, *6*−*8*, *29*, *35*, *39*−*41*) that used myoglobin that had been either chemically reduced with hydrosulfite, previously frozen, or used without removing or eliminating the effect of contaminating metal ions. In the second category are the more recent studies (*5*, *30*, *37*, and the present study) that attempted to eliminate some or all of the detrimental factors affecting myoglobin autoxidation that had been ignored in the studies in the first category. The values for the rate constants reported in studies in the first category are 3−7 times higher than those reported in studies in the second category.

The higher myoglobin oxidation rates in the earlier studies are consistent with subsequent research in this area. For example, Brown and Dolev (*7*) showed that freezing myoglobin increases its autoxidation rate, as was subsequently shown, because of freeze denaturation of the myoglobin (*42*). Similarly, it has been shown that reduction of myoglobin with hydrosulfite increases the autoxidation rate of myoglobin because of the interaction between myoglobin and the products from the reaction between hydrosulfite and myoglobin (*43*). More recently, it has been shown that trace levels of copper and iron increase the myoglobin autoxidation rate by catalyzing the reaction (*37*).

Importantly, the rate constants obtained with bovine myoglobin in this study agree well with those reported in the more recent studies that have not used chemically reduced oxymyoglobin (*5*, *37*).

The results of the present study agree well (approximately 25% lower) with those obtained previously using bovine myoglobin extracted from cardiac muscle (*37*). This good agreement is due to the fact that the procedures used for determining the rate constant were the same in both studies. Importantly, this is the only other study reported in the literature that has used nonchemically reduced myoglobin and also removed the interfering effect of contaminating copper and iron ions by chelating them with DPTA.

The results from the present study also agree well with the results previously obtained using myoglobin extracted from bovine LD and PM (*Psoas major*) muscle (*5*). Interestingly though, while the rate constant obtained in this study with myoglobin from the LD muscle is virtually identical to that obtained with myoglobin obtained from all bovine muscles in this study, the value obtained by these researchers for myoglobin from the PM muscle was approximately 20% higher. The most likely reason for the higher value obtained with myoglobin for the PM muscle was that no precautions were taken in that study to prevent the effect of contaminating metal ions on the autoxidation rate. Failure by these researchers to take this precaution could explain the differences they observed in the rate constants between myoglobin from the different muscle types. This is because there can be a 20−50% variation in the rate constant of myoglobin isolated from different muscles (even from the same muscle type) if the effect of contaminating metal ions is not removed by chelating with DPTA (*30*).

The other point highlighted in **Table 3** is the difference in myoglobin oxidation rate between different marine animals within a given study. These studies have shown that there are several orders of magnitude difference in autoxidation rate of myoglobin isolated from different marine species (*4*, *8*). However, it is not clear if the same differences in autoxidation rate between species would have been observed if the researchers had used conditions similar to those in the present study that eliminated artifactual results.

The values for Ea in **Table 3** show that there is a large variation (approximate 2-fold) in the values previously reported for the Ea of bovine myoglobin. This result is particularly surprising since although it is well-established that the method for isolating and purifying myoglobin and determining autoxidation rate can affect the rate constant, it would be expected that the effect of temperature on reaction rate would be fairly similar across different studies. As this is not the case, the results in **Table 3** indicate that there is an interaction between the myoglobin preparation technique and temperature, which affects the autoxidation rate of myoglobin. This interaction will in turn influence the Ea of the reaction since Ea is a measure of the temperature dependence of the reaction.

Although, in general, the more recent studies show more consistent values for Ea than the earlier studies, the values obtained in the present study are still 25−40% higher than those previously reported, even those reported in recent studies. The reason for this is unclear. However, the present study is the only study reported in the literature that has determined the Ea of myoglobin using techniques that completely prevents artifactual results. As this is the case, the Ea values for myoglobin reported in this study can be regarded as the most reliable in vitro values reported to date. This ascertain is further supported by the fact that the Ea values for the four different myoglobins used in this study were very reproducible since the standard error for the Ea for each myoglobin type was low (ca. 1.0). The low standard error indicates that within a species there was very little variation in the Ea for different myoglobin preparations used in this study. It has been previously shown that the procedures used in this study for preparing myoglobin and measuring autoxidation rate produce more reliable and reproducible estimates of the rate constant, which presumably also results in more reliable and reproducible estimates of the Ea (*30*).

CONCLUSION

This study has shown that muscle type and species have little effect on myoglobin autoxidation rate and Ea. The study has also shown that to obtain consistent and reliable estimates of the autoxidation rate of myoglobin it is essential to use procedures that avoid the use of sodium hydrosulfite and freezing during myoglobin isolation and purification and to eliminate the effect of contaminating copper and iron ions during analysis. Unless such procedures are used to prevent artifactual results, the rate constant for the reaction will not only be overestimated by 2−7-fold but also the temperature and pH dependence of the reaction will also be incorrectly characterized.

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Received for review September 5, 2001. Revised manuscript received December 24, 2001. Accepted January 14, 2002.

JF0112769