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Determination of myoglobin based on its enzymatic activity by stopped-flow spectrophotometry

Qi Zheng*, Zhihong Liu, Ruxiu Cai

Department of Chemistry, Wuhan University, Wuhan 430072, PR China

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Abstract

A new method has been developed for the determination of myoglobin (Mb) based on its enzymatic activity for the oxidation of *o*-phenylenediamine (OPDA) with hydrogen peroxide. Stopped-flow spectrophotometry was used to study the kinetic behavior of the oxidation reaction. The catalytic activity of Mb was compared to other three kinds of catalyst. The time dependent absorbance of the reaction product, 2,3-diamimophenazine (DAPN), at a wavelength of 426 nm was recorded. The initial reaction rate obtained at 40 °C was found to be proportional to the concentration of Mb in the range of 1.0×10^{-6} to 4.0×10^{-9} mol L^{-1}. The detection limit of Mb was found to be 9.93×10^{-10} mol L^{-1}. The relative standard deviations were within 5% for the determination of different concentrations of Mb. Excess of bovine serum albumin (BSA), Ca(II), Mg(II), Cu(II), glucose, caffeine, lactose and uric acid did not interfere.

Keywords: Stopped-flow spectrophotometry; Myoglobin; Enzymatic activity

1. Introduction

Myoglobin (Mb) is a small single heme protein present in high concentration in both cardiac and skeletal muscle, where it acts as a transportation vehicle for oxygen. Even a small degree of muscle cell damage will result in a substantial and rapid increase in the concentration of Mb in plasma. It has been found that Mb is released into the circulatory system after cardiac muscle damage due to myocardial infarction. Mb is the commercially available marker that most effectively fits the role of an early marker for AMI [1–3]. Since it plays such an important role in life, Mb has always been of interest from many aspects. The sophisticated oxygen delivery function, oxygen binding mechanism and the structure of Mb have been investigated [4–8]. The determination of Mb is also a quite significant work, and some methods have been proposed for the purpose [9–11]. Among these reported methods, ELISA is the most widely used. However, it requires multiple steps and is time-consuming. O'Regan et al. developed an amperometric immunosensor for the rapid detection of Mb in whole blood [9]. Hashimoto et al. have detected heme proteins by chemiluminescence [12]. Liang et al. detected Mb with high-performance size-exclusion chromatography [13]. To our knowledge, no stopped-flow spectrophotometric method has been developed to determine Mb thus far. Moreover, the determination of Mb based on its enzymatic activity, as is described in the present work, is a novel idea.

The search for satisfying substitutes for HRP is one of the most interesting trends in enzymatic analysis [14–19]. As is well know, the active center of the naturally occurring peroxidase HRP is a Fe(II) contained heme. Therefore, some heme derivations and synthetic metalloporphyrins have been applied in simulating HRP in some peroxidatic reactions. However, because these substitutes lack a three-dimensional structure, the peroxidatic activity of such mimetic enzymes is not very satisfactory, and is seriously inhibited by competing substrates. Fortunately, as we know, the Mb molecule contains a polypeptide subunit and a single heme as the active center. And what is more attractive, it has the naturally occurring quaternary structure of proteins. Heme we expected Mb would be a competent mimetic enzyme of HRP.

* Corresponding author. Tel.: +86 278 7684184; fax: +86 278 7647617.
E-mail address: zq_1101@sina.com (Q. Zheng).

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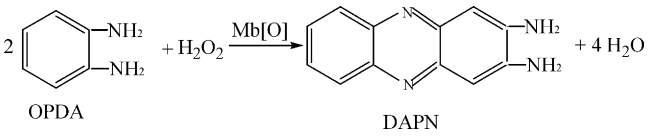
It was certified in the present work that Mb did show quite high peroxidatic activity when it was used to catalyze the oxidation of OPDA by H_2O_2, which generated 2,3-diaminophenazine (DAPN), a yellow product with high absorptivity. The reaction is as follows:



OPDA DAPN

Therefore, a new spectrophotometric method for the determination of Mb based on its catalytic activity was set up. The quantitative determination was built on kinetic analysis by means of stopped-flow, which has been widely recognized as a powerful technique for kinetic studies [20]. The catalytic reaction was studied by measuring the time-dependent absorbance of DAPN, the initial reaction rate was obtained and found to be proportional to the concentration of Mb within a concentration range of 1.0×10^{-6} to 4.0×10^{-9} mol L^{-1}. The method is simple, sensitive and reliable for the determination of Mb.

2. Experimental

2.1. Apparatus

The SFA-12 HI-TECH scientific kinetic stopped-flow accessory (HI-TECH, Japan) was fitted to the UVIKON-941 spectrophotometer (Kontron Instrument, Germany). A circulation water-bath was used to control the temperature in the stopped-flow module and the cell compartment. A microcomputer was applied to control the operation of the instrument and to collect and process the data.

2.2. Reagents

All solutions were prepared using deionized water. Unless indicated otherwise, all dilutions were made in 0.2 mol L^{-1} Na_2HPO_4–citric acid (pH 5.6). Mb solutions were prepared by dissolving a certain amount of Mb (Sigma Chemical Co.) in deionized water and stored below 4 °C. *o*-Phenylenediamine (Shanghai Chemical Agent, Shanghai, China) was sublimed before use; a 0.1 mol L^{-1} *o*-phenylenediamine stock solution was prepared by dissolving sublimed *o*-phenylenediamine in the buffer solution. The working solution was prepared daily by diluting the stock solution with buffer to the desired volume. Hydrogen peroxide solutions were prepared by appropriate dilution of the 30% solution with water (standardized by titration with $KMnO_4$).

2.3. Procedures

Each color comparison tube was filled with 0.40 mL of 1.00×10^{-2} mol L^{-1} H_2O_2, 0.50 mL of 1.00×10^{-2} mol L^{-1}, a proper amount of Mb, and then diluted with pH 5.6 Na_2HPO_4–citric acid buffer solution to 5 mL. The oxidation reaction was monitored by the light absorption at 426 nm.

3. Results and discussion

3.1. UV–vis spectra of the system

Since DAPN was the oxidized product of OPDA with Mb catalyzing, the absorption of the product was likely to be utilized to quantitatively determine the concentration of Mb. The UV–vis spectra of H_2O_2, Mb, OPDA and DAPN were recorded and shown in Fig. 1. It can be seen that DAPN had a strong absorption band that peaked at 426 nm, while none of the other three reagents absorbed at this wavelength. It indicated that the measurement was free of interference from other chemicals in this system. For the purpose of kinetic analysis, the time dependent absorbance of DAPN at 426 nm was recorded during the reaction process. As is shown in Fig. 2, within the first 10 min of the reaction, the absorbance

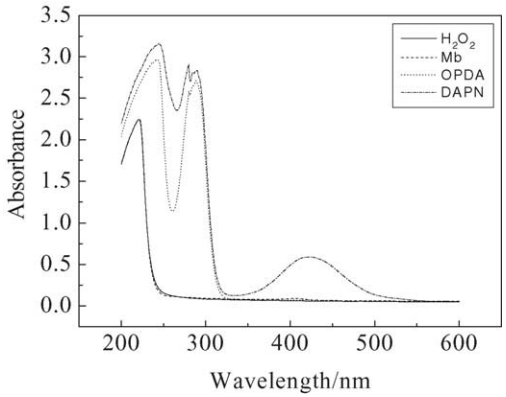


Fig. 1. UV–vis spectra of H_2O_2, Mb, OPDA, DAPN in 0.2 mol L^{-1} Na_2HPO_4–citric acid buffer (pH 5.6). [Mb] = 1.0×10^{-7} mol L^{-1}, [OPDA] = 1.0×10^{-4} mol L^{-1}, [H_2O_2] = 1.0×10^{-3} mol L^{-1}, [DAPN] = 1.0×10^{-4} mol L^{-1}.

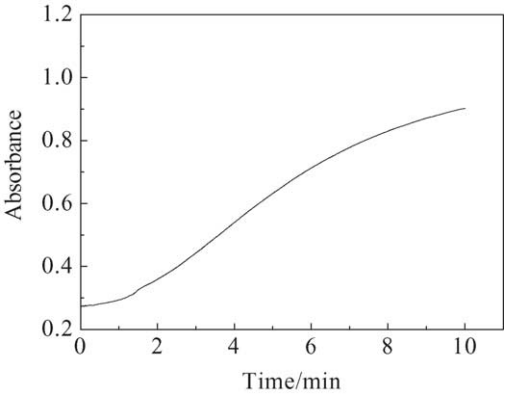


Fig. 2. Single-wavelength stopped-flow traces obtained in 10 min. [Mb] = 1.0×10^{-7} mol L^{-1}, [OPDA] = 1.0×10^{-3} mol L^{-1}, [H_2O_2] = 1.0×10^{-3} mol L^{-1}.

Table 1
Catalytic characteristics of Mb and Hb, βCD–hemin, hemin

Catalyst	K_m (10^{-3} mol L^{-1})	V_{max} (min^{-1})	[E] (10^{-6} mol L^{-1})	K_{cat} (10^6 L mol^{-1} min^{-1})
Hb	8.75	1.60	0.250	6.40
Mb	20.9	0.86	0.20	4.26
βCD–hemin	47.2	0.643	1.20	0.536
Hemin	55.9	0.493	1.20	0.411

of DAPN continuously increased as oxidization proceeded. But there had an obvious lag phase at the very beginning, which, in our opinion, was considered as the typical induced period of enzymatic reactions. That is to say, Mb in this reaction system clearly exhibited enzymatic characteristics.

3.2. Comparison of the catalytic of Mb and Hb, βCD—hemin, hemin

The enzymatic activity of Mb was compared with that of Hb, βCD—hemin and hemin for the catalysis of the reaction between 1,2-diaminobenzene and hydrogen peroxide. The Michaelis–Menten constant K_m and the maximum rate V_{max} were obtained from an Eadic–Hofatee graph (v versus v/[substrate]), and the transformation constant K_{cat} was calculated from the equation $V_{max} = K_{cat}\,[E]_0$, in which $[E]_0$ is the initial concentration of the catalyst. The K_m, V_m and K_{cat} values for Mb, Hb, βCD—hemin and hemin are listed in Table 1. The order of catalytic activities of the catalysts, Hb > Mb > βCD—hemin > hemin, was obtained using these parameters. Since the active center of all four catalysts is just the same iron-porphyrin, the differences in catalytic activity are mainly determined by molecular conformation. Hb has the natural quaternary structure, which may explain why the activity of Hb is higher than that of Mb. However, as a natural protein with smaller molecular size, Mb would be less likely than Hb to block the antigen–antibody immunoreaction because of the spatial effect. As for βCD—hemin, it just simulates the three-dimensional structure of naturally occurring proteins through the cavum of βCD, so its catalytic active is less than that of Hb and Mb but still better than hemin, its prosthetic group model.

3.3. Optimization of experimental variables

Different kinds of buffer solutions with varying pH values were tested for the reaction. The absorbance of DAPN was plotted versus pH for the different buffers (Fig. 3). The maximum absorption occurred in Na_2HPO_4–citric acid buffer within a pH range 5.0–5.6. Therefore, a Na_2HPO_4–citric acid buffer with pH 5.0 was chosen for subsequent experiments.

Temperature is a very important factor that cannot be neglected in kinetic analysis. The effect of temperature on the absorption was studied. As is demonstrated in Fig. 4, the absorption increased before the temperature reached 40 °C. The decrease in the absorption at temperatures higher than 40 °C might have resulted from the loss of the catalytic activity of

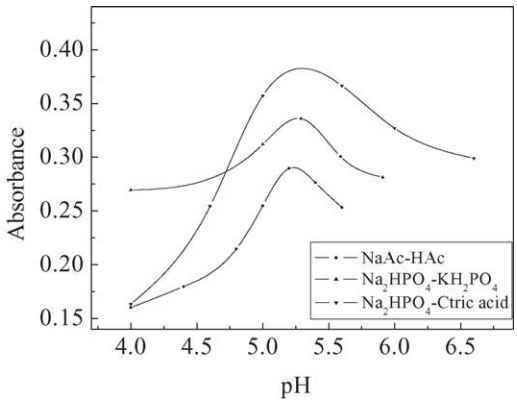


Fig. 3. Effect of pH and buffer system. [Mb] = 1.0×10^{-7} mol L^{-1}, [OPDA] = 1.0×10^{-4} mol L^{-1}, [H_2O_2] = 1.0×10^{-3} mol L^{-1}.

Mb. All absorptions for analysis were measured at a constant temperature of 40 °C.

3.4. Analytical characteristics and application

Under the optimized conditions, the initial reaction rate was found to be proportional to the Mb concentration in a wide range, based on which the kinetic method for determining Mb was built. The results are listed in Table 2.

The interference of other species in the determination of 2 $\times 10^{-7}$ mol L^{-1} Mb was investigated. The results are listed in Table 3. The values represent the tolerable amount (in molar ratio) of foreign species relative to Mb, which resulted in a relative error less than 5%. Excess of BSA, Ca(II), Mg(II),

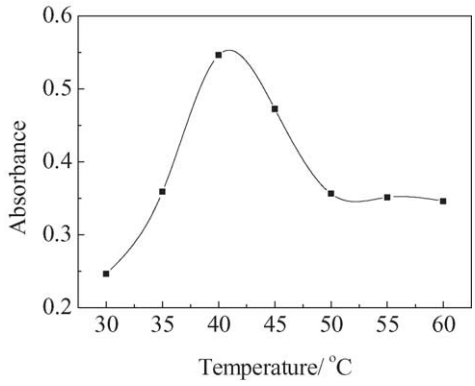


Fig. 4. Effect of reaction temperature. [Mb] = 1.0×10^{-7} mol L^{-1}, [OPDA] = 1.0×10^{-3} mol L^{-1}, [H_2O_2] = 1.0×10^{-3} mol L^{-1}.

Table 2
Calibration curves for the determination of Mb ($n = 7$)

Liner range of Mb (mol L^{-1})	Liner equation	r^2	[OPNA] (mol L^{-1}) ($\times 10^{-3}$)	[H_2O_2] (mol L^{-1}) ($\times 10^{-4}$)	Time (min)
4.0×10^{-9} to 1.0×10^{-7}	$r = 0.054 + 4.26 \times 10^7$ [Mb]	0.9987	1.0	8	5
1.0×10^{-7} to 1.0×10^{-6}	$r = 0.069 + 1.28 \times 10^6$ [Mb]	0.9992	1.0	8	3.5

Table 3
The influence of other species

Species	Tolerance of foreign species to Mb (molar ratio)
Uric acid	2000
Ca(II)	2000
Mg(II)	2000
Cu(II)	100
BSA	1500
Glucose	250
Lactose	200
Caffeine	100
VB_6	50
Zn(II)	40

Cu(II), glucose, caffeine, lactose and uric acid present in the system did not interfere. Zn(II) and VB_6, interfered slightly. Fe(II) is the active metal ion of the heme and hence interferes with the determination.

The precision of the method was also determined at different concentrations of Mb. The relative standard deviations were 4.03, 2.24 and 3.52% for the determination of 4.0×10^{-8}, 1.0×10^{-7} and 1.0×10^{-6} mol L^{-1} myoglobin, respectively.

Acknowledgements

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