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# THERMODYNAMIC STUDY OF METABOLIC REACTIONS IN AN AQUEOUS PROTEIN SOLUTION

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The time dependences of the thermal power of aqueous myoglobin solutions were measured by microcalorimeter at 298.15 K. Exothermic reactions occurred in aqueous myoglobin solutions due to the metabolism of aerobic microbes, and these roughly consisted of four phases. The generation times obtained were about (55±5) min for the logarithmic exothermal reaction phase. The total energies were considerably dependent on the amount of oxygen present, suggesting strongly that the exothermic reaction was caused by aerobic microbes. The apparent thermal metabolic rates were positively dependent on the concentration of myoglobin, probably because of the effects of myoglobin as a food source and/or as a donor of oxygen.

***Keywords:*** *aerobic microbe, metabolism, microcalorimeter, myoglobin, spectroscopy*

## Introduction

In researching the preservation of protein and food, it is very important to observe and prevent putrefaction of the substances. Calorimeters with high stability and sensitivity are suitable for measuring slow thermal reactions such as enzyme activity or solid reactions, because the calorimeter can detect total thermal reactions of samples both thermodynamically and kinetically [1–5]. Although this slow measurement process can totally and sensitively reflect many metabolic reactions, as well as complicated molecular interactions and conformational changes of proteins, it is difficult to use calorimetry to determine conformational changes of molecules. Spectroscopic techniques such as ultra violet absorption (UV) and circular dichroism (CD) are complementary methods to the calorimetric measurements because they can roughly determine the secondary and ternary conformational changes of proteins [6]. In this study, time dependences of thermal power, absorbance, and CD of aqueous myoglobin solutions were observed in various conditions at 298.15 K. Thermodynamic and kinetic analyses of metabolism in aqueous myoglobin solutions were also performed.

## Experimental

### *Materials*

Horse heart myoglobin was purchased from SIGMA and was dissolved with milli Q water just before each calorimetric and spectroscopic measurement. Other additives such as sodium chloride, sodium azide, and hydrochloric acid were special grade reagents purchased from Kishida Chemical.

### *Calorimetric measurements*

A twin microcalorimeter of the heat-conduction type, Thermal Activity Monitor (Thermometric AB, Järfäll, Sweden) was used for the measurements of aqueous myoglobin solutions at 298.15 K with a glass ampoule. The maximum volume of the ampoule is 3.26 $cm^3$. All calorimetric measurements were started as soon as possible after preparing solutions. Concentrations of myoglobin, $c$, were from 0.285 to 2.279 mM, and the volumes of aqueous myoglobin solutions, $V$, were from 0.50 to 2.0 $cm^3$ for calorimetric measurements.

### *Spectroscopic measurements*

Conformational changes of myoglobin were monitored by means of absorption and CD measurements using a Shimadzu UV-visible spectrophotometer UV-1650PC and a Jasco J-720 spectropolarimeter with a quartz cell of 10 and 2 mm path length, respectively. For spectroscopic measurements, fourteen aqueous myoglobin solutions ($c$=0.015 mM) were prepared at certain intervals by diluting an original aqueous myoglobin solution ($c$=2.279 mM) incubated at 298.15 K.

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## Results and discussion

### *Calorimetric measurements of aqueous myoglobin solution*

Figure 1 shows typical thermograms of aqueous myoglobin solutions ($c$=2.279 mM, $V$=1.0 cm$^3$) and water in an atmosphere of air at 298.15 K. Remarkable exothermic reactions were observed in aqueous myoglobin solutions, although no thermal reactions were observed in water. The value of total exothermic energy, $Q$, was about 7.6 J in this reaction. The exothermic reaction could be roughly divided into five periods; (I) first period, no thermal reaction; (II) second period, logarithmic increase in exothermic reaction; (III) third period, complicated exothermic reaction; (IV) fourth period, logarithmic decrease in exothermic reaction; and (V) fifth period, no thermal reaction as in the first period. In most cases, the reaction times of the first and second periods were about 16 and 6 h, respectively, and value of the exothermic energy in the second period was about 1.5% of the total exothermic energy. As shown in Fig. 1, the exothermic reaction was not observed when sodium azide (40 mM) or NaCl (1.7 M) were added, as they had an aseptic effect. These results indicated that the exothermic reactions in aqueous myoglobin solutions were due to metabolic energies produced by microbial growth. Microbial growth normally consists of four phases: a lag phase, a logarithmic growth phase, a stationary phase, and a declining phase [7]. These four phases correspond well with the observed periods of the exothermic reaction, as previously mentioned. The complicated exothermic reaction after the second period probably suggests the existence of some kinds of microbes in the aqueous myoglobin solutions.

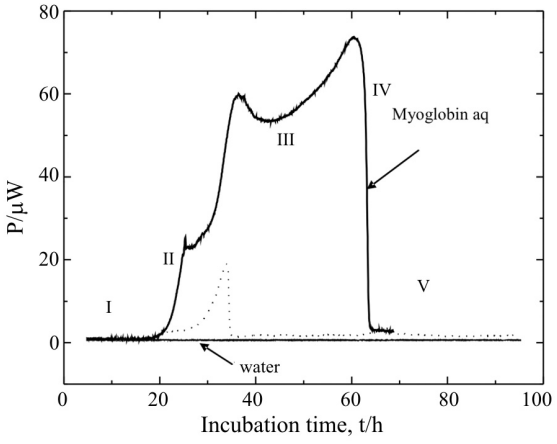


**Fig. 1** Typical thermograms of aqueous myoglobin solution and water at 298.15 K. The thick solid line and dotted line are those of aqueous myoglobin solutions ($c$=2.279 mM, $V$=1.0 cm$^3$) in an atmosphere of air and nitrogen gas, respectively. Other solid lines show the thermograms of water and aqueous myoglobin solution with sodium azide and NaCl

### *Spectroscopic measurements of aqueous myoglobin solutions*

In order to clarify the possible contribution of structural changes of myoglobin to the exothermic reaction process observed by calorimetric measurements, UV and CD spectra of aqueous myoglobin solutions were recorded. Figure 2 shows UV and CD spectra of aqueous myoglobin solutions incubated from 0 to 94 h. As shown in Fig. 2a, an obvious decrease and increase in the absorbance could be seen at around 420 and 280 nm, which could reflect respectively the states of the heme-iron and of aromatic amino acids. These changes in absorbance might be caused by remarkable structural changes of myoglobin and isolation of heme-iron from myoglobin due to metabolisms of microbes. As shown in figure 2b, the shapes of CD spectra were almost unchanged, although intensities of the CD spectra were considerably decreased. These re-

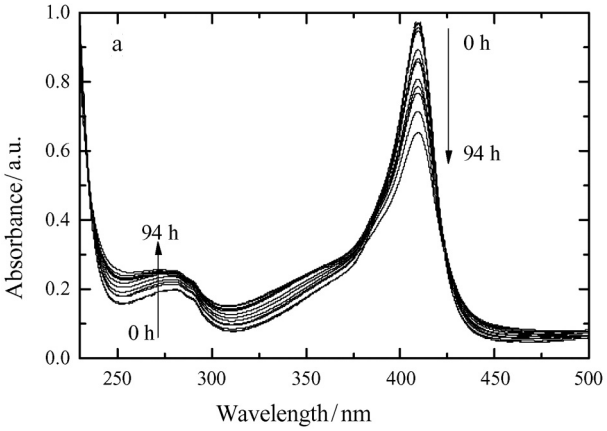


**Fig. 2a** Time dependences of UV spectra of aqueous myoglobin solutions at 298.15 K

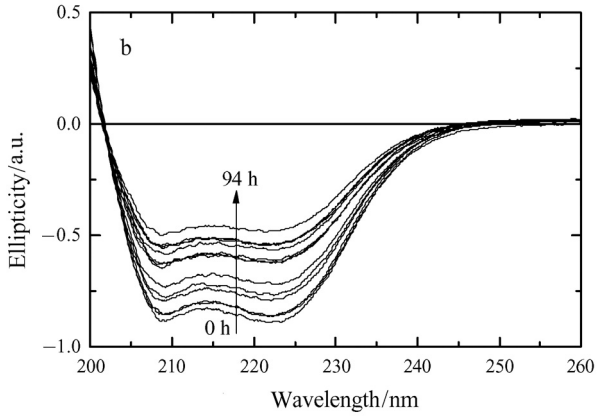


**Fig. 2b** Time dependences of CD spectra of aqueous myoglobin solutions at 298.15 K

sults suggest that the secondary structure of undigested myoglobin by microbes was not changed at all, and the structure of digested myoglobin was completely and vitally influenced by the cutting of peptide bonds, resulting in a decreased concentration of myoglobin. Figure 2c shows time dependences of conformational changes obtained from the spectroscopic measurements. The time dependences of both spectroscopic measurements were very similar to each other. Both spectroscopic results showed no conformational changes of myoglobin occurred until about twenty hours of incubation. These results were consistent with the calorimetric results.

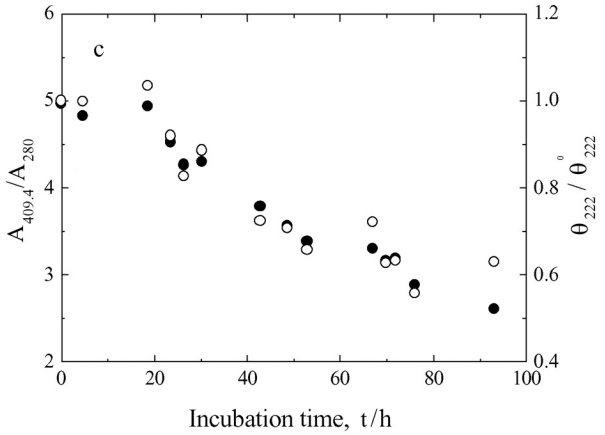


**Fig. 2c** Time dependences of fractions of spectroscopic intensities, $A_{409.4}/A_{280}$ (closed circle) and $\theta_{222}$ normalized by $\theta^{o}_{222}$ at 0 min (open circle)

*Volume of solution - effects of oxygen*

In order to clarify the effects of oxygen on the exothermic reaction, the thermal power of aqueous myoglobin solution ($c$=2.279 mM, $V$=1.0 cm$^3$) was measured in a glass ampoule with an atmosphere of nitrogen gas at 298.15 K (dotted line shown in Fig. 1). In an atmosphere of nitrogen gas, an exothermic reaction could be seen only during the second period, as a logarithmic increase in the exothermic reaction period. The total exothermic energy in the nitrogen atmosphere was about 0.280 J, which corresponded to about 3.6% of that in an air atmosphere. The percentage of the exothermic energy was close to the expected percentage, 1.2%, that was calculated from the solubility of oxygen in water, 0.0057 mL/mL [8]. It is suggested that oxygen was a very important and crucial factor for the exothermic reaction and that the measured reaction was aerobic.

The thermal reactions of aqueous myoglobin solutions were measured as the amount of air in the glass ampoule varied in response to changing the volume of the solution and the results were listed in Table 1. The apparent thermal metabolic rates ($\nu_{app}$), which can be obtained by dividing the total reaction energy by the thermal reaction time, were all about (0.10±0.01) J h$^{-1}$ under this condition ($c$=2.279 mM, $V$=0.2–2.0 cm$^3$). Figure 3 shows the relationship between the total reaction energy and the volume of solution. The total exothermic energies were proportionally decreased with the increase in solution volume in the range of 0.5–2.0 cm$^3$. The $X$ axis intercept, which could be obtained by a linear least squares

**Table 1** The total exothermic energies ($Q$), thermal reaction time ($t_r$), and apparent thermal metabolic rate ($\nu_{app}$) in aqueous myoglobin solutions at 298.15 K

| Experiment | $c$/mM | $V$/cm$^3$ | [HCl]/mM | Atmosphere | $Q$/J | $t_r$/h | $\nu_{app}$/mJ h$^{-1}$ |
|---|---|---|---|---|---|---|---|
| Air | 2.279 | 1.00 | 0.00 | Air | 7.64 | 69 | 111 |
| | 2.279 | 1.00 | 0.00 | $N_2$ | 0.28 | 20 | 14 |
| Volume | 2.279 | 0.50 | 0.00 | Air | 9.08 | 91 | 100 |
| | 2.279 | 1.50 | 0.00 | Air | 5.98 | 52 | 115 |
| | 2.279 | 2.00 | 0.00 | Air | 4.53 | 45 | 101 |
| | 2.79 | 2.00 | 0.00 | Air | 4.53 | 45 | 101 |
| Concentration | 1.140 | 1.00 | 0.00 | Air | 7.79 | 93 | 84 |
| | 0.570 | 1.00 | 0.00 | Air | 7.63 | 141 | 54 |
| | 0.285 | 1.00 | 0.00 | Air | 7.97 | 674 | 12 |
| HCl | 2.279 | 1.00 | 0.01 | Air | 7.61 | 79 | 96 |
| | 2.279 | 1.00 | 0.10 | Air | 7.67 | 47 | 163 |
| | 2.279 | 1.00 | 1.00 | Air | 7.54 | 44 | 171 |
| | 2.279 | 1.00 | 3.16 | Air | 7.68 | 121 | 63 |
| | 2.279 | 1.00 | 10.0 | Air | 0 | NO | NO |

NO – not observed

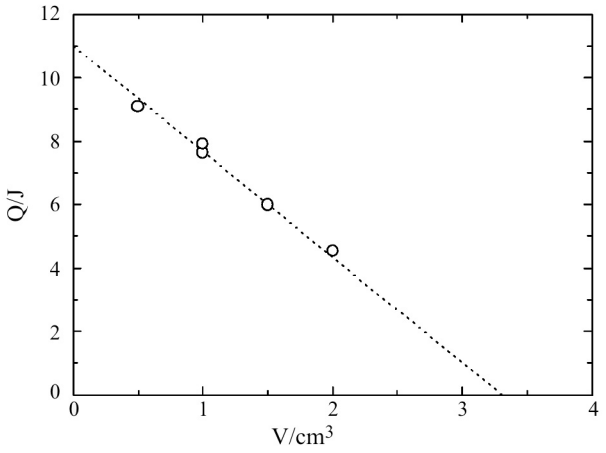


**Fig. 3** Dependence of total metabolic energy, $Q$, on the volume of aqueous myoglobin solutions, $V$, in a glass ampoule at 298.15K

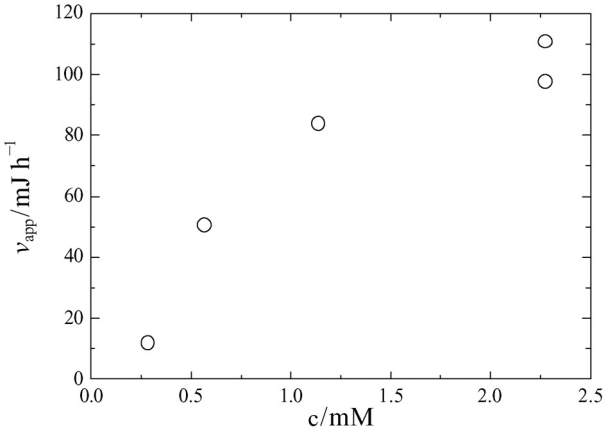


**Fig. 4** Relationship between apparent thermal metabolic rate, $\nu_{app}$, and the concentration of myoglobin, $c$, in water and atmosphere of air at 298.15K

method, was (3.4±0.1) cm$^3$, which was close to the total volume of the glass ampoule, 3.26 cm$^3$. These results strongly suggest that the exothermic reaction of aqueous myoglobin solution was significantly dependent on the amount of oxygen.

After finishing the exothermic reaction of the aqueous myoglobin solution, the same sample solution was measured again in an atmosphere of fresh air. Interestingly, only the third period (complicated exothermic reaction) could be observed, without the first and second periods. These results indicate that the microbes would not be active under the anaerobic conditions within the glass ampoule due to their metabolic reaction, and that refreshing the air could cause the metabolism of the microbes to resume.

*Concentration of myoglobin*

In order to clarify the effects of myoglobin on the exothermic reaction, thermal powers of aqueous myoglobin solution were measured at various concentrations of myoglobin from 0.285 to 2.279 mM. These measurements were performed for constant volume solutions ($V$=1.0 cm$^3$, air), to accommodate the effect of oxygen, as previously disclosed. Table 1 shows the results of these calorimetric measurements. The thermal reaction time was significantly influenced by the concentration of myoglobin, although the total reaction energies were almost constant, about 7.6 J. The reaction time for 0.285 mM myoglobin was about 675h, which was about 10 times as long as the reaction time for 2.28 mM myoglobin. The $\nu_{app}$ were 111 mJ h$^{-1}$ (2.279 mM), 84 mJ h$^{-1}$ (1.140 mM), 54 mJ h$^{-1}$ (0.570 mM), 12 mJ h$^{-1}$ (0.285 mM) within an error of 5%, respectively. Figure 4 shows the dependence of $\nu_{app}$ on the concentration of myoglobin. The $\nu_{app}$ increased and converged to a certain value with the increase in concentration of myoglobin. Such a dependence on myoglobin concentration probably indicates that myoglobin is a medium for microbes, and that myoglobin contributes to the increase in the amount of dissolved oxygen of the aqueous solution by an enzymatic function involving the heme-iron of myoglobin. These results suggest that multiplication of microbes and putrefaction of protein solutions can be suppressed by keeping the concentration of protein low.

*Concentration of hydrochloric acid*

Time dependences of thermal powers of aqueous myoglobin solutions ($c$=2.279 mM, $V$=1.0 cm$^3$, air) were measured with various concentrations of HCl added, from 0 to 10 mM, and the results are shown in Fig. 5 and in Table 1. The exothermic reactions were clearly observed in all myoglobin+HCl aqueous solutions except for 10 mM HCl. All of the total exother-

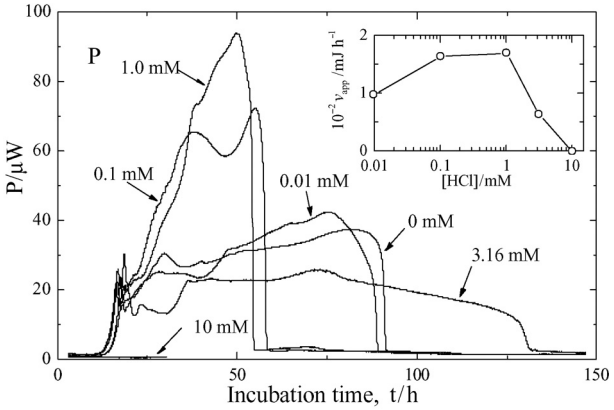


**Fig. 5** Typical curves of aqueous myoglobin solutions ($c$=2.279 mM, $V$=1.0 cm$^3$, air) with various concentration of HCl at 298.15 K. The inset in the figure shows dependence of the apparent thermal metabolic rate, $\nu_{app}$, on the concentration of HCl

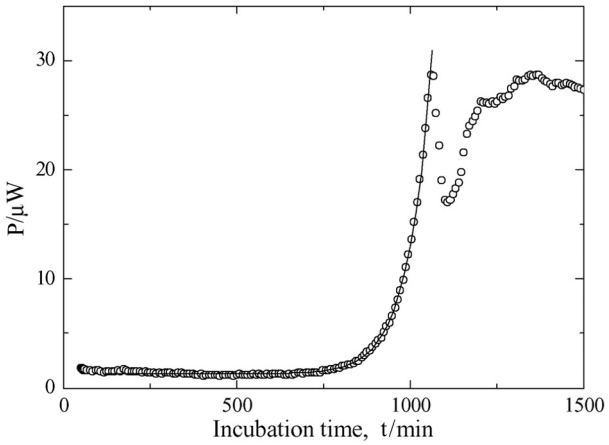


**Fig. 6** An example of fitting for logarithmic growth phase in aqueous myoglobin solution ($c$=2.279 mM, $V$=1.0 cm$^3$, air) at 298.15 K

mic energies were about 7.6 J, which was close to the value in water. As shown in Fig. 5 and in Table 1, however, the thermal reaction time was significantly influenced by the concentration of HCl. The obtained vapp are plotted against the concentration of HCl in the inset of Fig. 5. Although the vapp in 0.01 mM of HCl was about 96 mJ h$^{-1}$, which was close to that in water, the vapp in 0.1–1.0 mM of HCl were about 167 mJ h$^{-1}$, which is about 1.7 times the value in water. This result may indicate the optimum pH for these microbes. The vapp in 3.16 mM HCl was remarkably decreased compared to lower concentrations of HCl, probably because concentrations of HCl higher than 1 mM might result in a pH that is inappropriate for the metabolism system of the microbes. These results suggest that protein solutions can be easily rotted by multiplication of microbes at their optimum pH, and that concentrations of HCl greater than 10 mM can depress the microbe multiplication.

*Analysis of the logarithmic growth phase*

The logarithmic growth phase, which could be seen at the second period of the thermal reaction, can reflect the nature of the microbes, because their growth is not influenced by degradation and volume of the medium in this phase [7]. In order to determine the nature of the microbe in the aqueous myoglobin solution, the thermograms were fitted for the logarithmic growth phase by a nonlinear least squares method using Eq (1).

$$P = A_0 + A_1 2^{\frac{t - t_{lag}}{t_0}} \quad (1)$$

where $P$ is the thermal power (μW), $A_0$ is the value of base line (μW), $A_1$ is the proportionality constant reflecting the energy of metabolic reaction per one microbe (μW), $t$ is the incubation time (min), $t_0$ is the generation time (min), and $t_{lag}$ is the lag phase time (min). The $(t–t_{lag})/t_0$ is the generation number. Figure 6 shows an example of fitting for the logarithmic growth phase in aqueous myoglobin solution ($c$=2.279 mM, $V$=1.0 cm$^3$, air) at 298.15 K. The fact that the thermal power could be fitted by Eq (1) from 120 to 1050 min suggests that this period was a logarithmic growth phase of one kind of microbe. The obtained generation times and lag phase times in most aqueous myoglobin solutions were about (55±5) and (400±100) min, respectively, except for a few cases such as 10 mM HCl aq and low concentration of myoglobin. These results suggest that about 14 cell divisions might occur in the logarithmic growth phase, resulting in the logarithmic exothermic reaction. These divisions could indicate the final number of microbes in the logarithmic growth phase was about 16000 times the number in the initial state. It might be possible to measure the putrefaction of solutions quantitatively by using this analysis.

## Conclusions

In this study, the exothermic reactions that occur in aqueous myoglobin solutions were caused by the metabolism of aerobic microbes. The process of microbe proliferation was accompanied by digestion of myoglobin, as observed by calorimetric and spectroscopic measurements. After a non-thermal reaction corresponding to the lag phase, a logarithmic exothermal reaction corresponding to the logarithmic growth phase was observed. The obtained generation times in this phase were about (55±5) min at 298.15 K, except in a few conditions that were inappropriate for microbial growth. The total exothermic energies were clearly proportional to the amount of oxygen present in the solutions, strongly suggesting that the metabolism reaction was considerably dependent on oxygen. The apparent thermal metabolic rates were positively dependent on the concentration of myoglobin, probably because of the effect of myoglobin as a microbial food and/or as a donor of oxygen. The metabolism reactions were more active from 0.1 to 1.0 mM of HCl, reflecting the optimum pH for the microbes, but they were considerably inactivated at over 10 mM HCl. A precision calorimeter is thus an effective method for observing the putrefaction of protein solutions both quantitatively and kinetically.

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