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## The effect of ligand on the kinetics of the reaction of the sulfhydryl groups of human hemoglobin with p-mercuribenzoate

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*Summary.* The rate of reaction of PMB with the masked sulfhydryls of human hemoglobin derivatives correlates well with the extent of dissociation for various ligands. It is suggested that the $\alpha_1\beta_2$ dimer of hemoglobin participates in the slow step of the reaction with PMB.

In previous studies we have used the rate of reaction of the masked sulfhydryls with p-mercuribenzoate (PMB) as a probe of the extent of dissociation and/or conformation change of the protein under various conditions[1-3]. We have now extended these studies to include the effect of ligand on the kinetics of the reaction.

*Materials and methods.* Human hemoglobin was prepared by the toluene method[4]. After passage through an anion exchange resin the solution was dialyzed against phosphate buffer, pH 7. Methemoglobin was prepared by addition of $K_3Fe(CN)_6$ to oxyhemoglobin[5]. Fluoro- and azidomethemoglobin were prepared by addition of the sodium salt of ligand to methemoglobin[6]. A final concentration of 0.3 M NaF was necessary to convert about 95% of the methemoglobin into fluoromethemoglobin. Since this change in ionic strength would affect the hemoglobin dissociation, an equivalent concentration of NaF was added to the azidomethemoglobin for comparison. That the fluoride anion did not replace the azide ion was confirmed by comparing the visible absorption spectrum of the azidomethemoglobin with and without 0.3 M NaF. The spectra were identical. Experiments involving deoxyhemoglobin were carried out using a Thunberg type cell (Bolab, Inc., Derry, N.H.) which allowed addition of PMB under anaerobic conditions. The concentration of oxyhemoglobin was calculated from $E = 14.6 \times 10^3\ M^{-1}\ cm^{-1}$ (heme) at 575 nm and the concentration of methemoglobin from $E = 4.1 \times 10^3\ M^{-1}\ cm^{-1}$ (heme) at 630 nm. PMB was obtained from Calbiochem, La Jolla, California, and used without further purification. The concentration of PMB was calculated from $E = 1.69 \times 10^4\ cm^{-1}\ M^{-1}$ at 232 nm. The reaction rate of the masked -SH groups with PMB was measured spectroscopically at 25°±0.2 by a method described previously[1]. All spectrophotometric measurements were carried out with the Cary 14 spectrophotometer.

*Results.* The kinetic data were treated according to the method of Yuthavong and Ruenwongsa[7] instead of the pseudo 1st order plot previously used[1]. This method which uses only the initial rate was especially suitable for the reaction with methemoglobin which occasionally showed turbidity after 35 min reaction with PMB. Our results confirmed the rate equation found by Yuthavong and Ruenwongsa:

$$\text{Rate} = k_{obs.}\,[Hb]^{1/2}[PMB]$$

where [Hb] is the total hemoglobin concentration in heme and [PMB] is the concentration of PMB remaining after reaction of the $\beta$-93 sulfhydryls. The observed changes in rate with change in ligand are reported in the table.

*Discussion.* On the assumption that dissociation of the hemoglobin tetramer necessarily precedes the reaction of the masked sulfhydryls with PMB it is worthwhile to compare the observed rate of reaction with the extent of dissociation. The unreactivity of deoxyhemoglobin is understandable since the extent of dissociation is known to be significantly less than that of oxyhemoglobin[8]. It is noteworthy that $\alpha$- and $\beta$-chains completely reacted with PMB exist largely as the tetramer below pH 7 in the deoxy-state[9]. The reactivity of azidomethemoglobin is consistent with the extent of its dissociation which at pH 6 is slightly less than that of methemoglobin[10] and approximately equivalent to that of oxyhemoglobin[8].

The apparently greater reactivity of fluoromethemoglobin is probably due to the presence of 0.3 M NaF which would promote further dissociation since its extent of dissociation is intrinsically somewhat less than that of the other methemoglobin derivatives, although the tertiary and quaternary structures of met- and fluorohemoglobin are identical[10,11]. This was demonstrated by the roughly equivalent rates of the fluoro- and azidoderivatives when 0.3 M NaF was added to the latter. The faster rate for methemoglobin in which the ligand is water is consistent with its greater dissociation as measured by several methods[10,12].

The observed rate expression provides evidence as to the identity of the reactive unit of hemoglobin participating in the slow step of the reaction. It has been shown that reaction of the $\beta$-93 sulfhydryls with PMB in the presence

Rate of reaction of masked sulphydryl groups of human hemoglobin derivatives with PMB in phosphate, pH 6.9, temperature 25 °C, NaCl = 0.1 M*

| Derivative | $k_{obs.}$ ($M^{-1/2}\ min^{-1}$) |
|---|---|
| Deoxy- | No reaction |
| Oxy- | 0.56 |
| AzidoMet | 0.51 |
| AzidoMet + 0.3 M NaF | 0.83 |
| FluoroMet | 0.78 |
| Met- | 1.27 |

* Hb concentration = $0.5\text{–}1.6 \times 10^{-5}$ M (Tetramer), PMB concentration = $1.1 \times 10^{-4}$ M.

of 0.1 M NaCl is sufficient to promote dissociation of the hemoglobin tetramer into dimers[13,14]. Rosenmeyer and Huehns have suggested that of the 2 types of dimer possible, $\alpha_1\beta_1$ and $\alpha_1\beta_2$ that dissociation to the $\alpha_1\beta_1$ dimer is more likely since reaction of the $\beta$-93 sulfhydryl which lies near the $\alpha_1\beta_2$ interface should lead to formation of the $\alpha_1\beta_1$ dimer. However, it is in the $\alpha_1\beta_2$ dimer that the masked sulfhydryls $\alpha$-104 and $\beta$-112 are exposed to further reactions with PMB. The rate expression for the reaction of hemoglobin with PMB involves a term with $[Hb]^{1/2}$. It can be shown that this requires participation of the dimer in the slow step. Participation of monomers would require a term involving $[Hb]^{1/4}$. Thus the $\alpha_1\beta_2$ dimer is probably the reactive dimer.


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## Quenching of intrinsic fluorescence accompanies the activation of prococoonase[1]


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*Summary.* The intrinsic fluorescence of prococoonase from *Bombyx mori* is largely quenched upon its activation. The rates of fluorescence quenching and enzyme activation are equal, indicating that both reflect the same process.


Cocoonase is a proteolytic enzyme used by some silkworms to digest the protein sericin of the cocoons, hence facilitating their escape[2-4]. It is synthesized in the galeae first as prococoonase, a zymogen which can be activated by bovine trypsin, proteolytic enzymes in the moulting fluid and cocoonase itself, in the course of which a portion of the polypeptide chain is cleaved from prococoonase[4-6]. In this report it is shown that activation of prococoonase from *Bombyx mori* is accompanied by a large quenching of intrinsic fluorescence.

*Materials and methods.* Prococoonase was prepared by a method adapted from Berger et al.[4]. Galeae from 60 *B. mori* moths on the last day before emergence from cocoons where collected in 0.1 M potassium phosphate buffer pH 6.6 containing 10 mM sodium cyanide (0.8 ml). After grinding in an all-glass homogenizer, the mixture was centrifuged at 12,000 × g for 10 min. The supernatant was made 90% saturated by addition of solid ammonium sulphate. Most of the proenzyme remains in the supernatant which, after dialysis and concentration, was passed through a column of Sephadex G-75 (1 × 50 cm) and eluted with the same buffer. Only a single peak is obtained from the column, and it was shown to be homogeneous on dodecylsulphate-polyacrylamide gel electrophoresis.

The proenzyme (1.5 mg/ml, 0.04 ml) was activated by addition of trypsin solution (0.2 mg/ml in 0.1 M Tris-HCl pH 8.0, 40 mM KCl, 10 mM KCN, 0.16 ml). Enzyme activity was measured by adding the activated mixture (0.02 ml) to a solution of 0.1 M Tris-HCl pH 8.0, 40 mM KCl, 10 mM KCN, 0.5-2 mM benzoylarginine ethyl ester (BAEE) (0.98 ml). Hydrolysis was followed by spectrophotometry at 258 nm (Beckman DU monochromator and

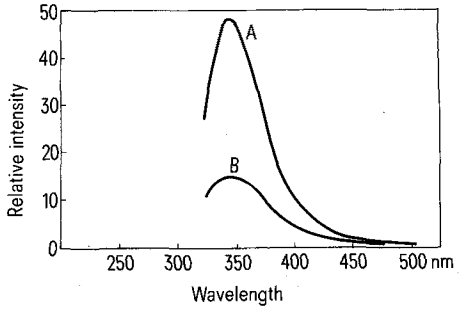


Fig. 1. Fluorescence spectra of (A) prococoonase and (B) the mixture of cocoonase and the activation peptide. The proenzyme was activated as indicated in 'Materials and methods'.

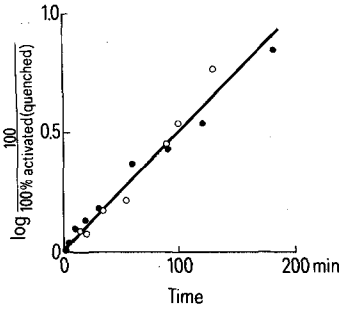


Fig. 2. The plots of log 100/(100-% activated enzyme) against time (○) and of log 100/(100-% maximum quenching) against time (●).