Effect of Organic Phosphates on Methemoglobin Reduction by Ascorbic Acid

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AKIO TOMODA, SHIGERU MATSUKAWA, MASAZUMI TAKESHITA,* and YOSHIMASA YONEYAMA

From the Department of Biochemistry, Kanazawa University, School of Medicine and *the Department of Medical Technology, Kanazawa University, School of Paramedicine, Kanazawa, Japan 920

The rate of methemoglobin reduction by ascorbic acid was accelerated in the presence of ATP, 2,3-diphosphoglycerate (2,3-DPG), and inositol hexaphosphate (IHP). The acceleration was as much as three times, four times, and ten times in the presence of ATP, 2,3-DPG, and IHP at pH 7.0, respectively.

The changes of the concentrations of methemoglobin and ascorbic acid during the methemoglobin reduction were determined, and the reaction was found to proceed stoichiometrically in the presence of IHP.

The reduction rate of methemoglobin by ascorbic acid was compared at different concentrations of organic phosphates (ATP, 2,3-DPG, and IHP) at various pH values (6.5, 7.0, 7.4). From the changes in the reduction rate under different concentrations of organic phosphates, the dissociation constants of ATP, 2,3-DPG, and IHP to methemoglobin could be determined and were estimated to be $3.3 \times 10^{-4}$ M, $2 \times 10^{-5}$ M, and $8 \times 10^{-6}$ M at pH 7.0, respectively.

On the basis of these results, the acceleration mechanism of methemoglobin reduction by ascorbic acid due to the presence of organic phosphates was described. The physiological role of 2,3-DPG in human red cells was discussed in relation to the reduction of methemoglobin by ascorbic acid.

Although 2,3-diphosphoglycerate and ATP, which are in high concentration and regulate the oxygen affinity of hemoglobin in human red cells, were originally assumed to bind exclusively to deoxyhemoglobin, it is now generally accepted that these organic phosphates bind to methemoglobin as well as oxyhemoglobin (3-8). Furthermore, it has been ascertained by recent reports that the conformational change of the hemoglobin molecule accompanying the transition from the R to the T form occurs due to the binding of inositol hexaphosphate (9, 10), which is a stronger effector than 2,3-diphosphoglycerate and ATP.

On the other hand, it was demonstrated by ESR measurements that in the presence of ATP, high spin methemoglobin was increased (11, 12). Recently transition from the R to the T state of methemoglobin and the increase in high spin methemoglobin were observed in the presence of IHP (13-15).

It is therefore expected that the reduction rate of methemoglobin, i.e., the reactivity of ferric iron in hemoglobin with the reductant, might be affected by the binding of organic phosphates. However, there was little investigation on the effect of organic phosphates on methemoglobin reduction except our previous report that 2,3-DPG accelerated the methemoglobin reduction by ascorbic acid (16). This prompted us to investigate the acceleration mechanism of 2,3-DPG and the effect of organic phosphates such as ATP and IHP.

Since we found that ATP and IHP as well as 2,3-DPG accelerated methemoglobin reduction by ascorbic acid, we studied the effect of these organic phosphates under various conditions and have discussed the acceleration mechanism in this paper. We also considered the physiological significance of 2,3-DPG in methemoglobin reduction by ascorbic acid.

**Experimental Procedures**

*Materials*—Dowex 1-8 and Dowex 50 were purchased from Muromachi (Japan) and Sephadex G-25 from Pharmacia. Bis-Tris and Tris were obtained from Sigma, 2,3-DPG and ATP from Boehringer, and IHP from Sigma. Phosphoglycerate mutase, 3-phosphoglycerate kinase, and glyceraldehyde-3-phosphate dehydrogenase were purchased from Boehringer. Glycolate 2-phosphate was obtained from Sigma and sodium ascorbate from Wako (Japan). 2,3-DPG (cyclohexyl amine salt) was converted into a free acid by treatment with Dowex 50 and neutralized with $1 \text{N NaOH}$. ATP was neutralized with $1 \text{N NaOH}$ and IHP with $1 \text{N HCl}$ before use. The buffer used was bis-Tris (0.05 M, containing 0.1 M NaCl, pH is indicated elsewhere).

*Preparation of Hemoglobin Solution*—Fresh heparinized blood was centrifuged at 3,000 rpm for 10 min. After removal of serum and buffy coats, red cells were washed three times with isotonic saline (0.9% NaCl solution). Then the red cells were hemolyzed and the stroma were removed by centrifugation at 10,000 rpm for 15 min. The hemoglobin concentration was determined at 540 nm as cyanmet-
hemoglobin using a millimolar extinction coefficient ε_{630} = 11.0 cm^{-1} M^{-1} heme (20).

Preparation of Stripped Methemoglobin—Hemoglobin was converted to the ferric form by addition of a slight excess of potassium ferricyanide. After removal of ferricyanide with Dowex 1-8, methemoglobin was desalted with Sephadex G-25 previously equilibrated with 0.05 M bis-Tris buffer (pH 7.0) containing 0.1 M NaCl and diluted to the appropriate concentration with the same buffer.

Preparation of Carboxypeptidase A-Digested Methemoglobin—Carboxypeptidase A-digested hemoglobin [des(146-His,145-Tyr)] was obtained according to the method by Kilmin and Wootton (21), and converted to stripped methemoglobin as indicated above.

Measurement of Methemoglobin Reduction by Ascorbic Acid—A 1.5-ml standard solution containing 23 μM methemoglobin as hemoglobin tetramer, 0.05 M bis-Tris, and 0.1 M NaCl was used for the assay system. With regard to the measurement of the reduction of carboxypeptidase A-digested methemoglobin by ascorbic acid, 17 μM concentration of this protease-digested methemoglobin was used in place of methemoglobin. The experiments were performed with and without organic phosphates (2,3-DPG, ATP, and IHP). The reaction was started by the addition of final concentration of 6 mM sodium ascorbate and the rate of methemoglobin reduction was measured spectrophotometrically with the Hitachi 124 double beam spectrophotometer by the decrease in absorbance at 630 nm at 25°C.

Determination of Dehydroascorbic Acid—Dehydroascorbic acid was determined by the decrease in absorbance at 630 nm in the presence of 100 μM IHP compared with the control. Such a stimulation of methemoglobin reduction was also observed at pH 6.3 in the presence of the same concentration of IHP at pH 7.0. On the other hand, at pH 7.7, more than 600 μM IHP was necessary for maximal acceleration and the maximal rate was at most three times faster than the control.

RESULTS

Effect of 2,3-DPG, ATP, and IHP on Rate of Methemoglobin Reduction by Ascorbic Acid—Fig. 1 shows the effect of 2,3-DPG, ATP, and IHP on the rate of methemoglobin reduction by ascorbic acid for an initial 8 min, which was measured by the decrease at 630 nm. When 9 mM 2,3-DPG or 3.9 mM ATP was added, 4-fold and 5-fold acceleration of the reaction rate was observed, respectively, while 10-fold acceleration was noticed in the presence of 100 μM IHP compared with the control. (The rate constant was calculated as 9 × 10^8 M^{-1} min^{-1} in the presence of IHP and 8.4 × 10^8 M^{-1} min^{-1} in the absence of IHP from the overall reaction curve.)

Stoichiometrical Studies on Reaction between Ascorbic Acid and Methemoglobin—Ascorbic acid is known to reduce methemoglobin directly (24-26) and the reaction was shown by Gibson to proceed in the second order (25). These suggest that methemoglobin reduction by ascorbic acid proceeds stoichiometrically. Since ascorbic acid is a 2-potential donor, it was expected that 2 M ferric iron of methemoglobin might be reduced by 1 M ascorbic acid and that 1 M dehydroascorbic acid might be produced. Our results in Fig. 2 confirmed the expected stoichiometrical changes which were observed in the presence of IHP.

Effect of IHP, 2,3 DPG, and ATP Concentration on Rate of Methemoglobin Reduction at Different pH Values—In order to examine the mode of molecular interaction between methemoglobin and organic phosphates such as IHP, 2,3-DPG, and ATP, different concentrations of these organic phosphates were mixed with methemoglobin and the rate of methemoglobin reduction by ascorbic acid was compared at various pH values.

Fig. 3A shows the stimulative effect of IHP which was the most pronounced of the organic phosphates examined. The effect reached a maximum at concentrations above 50 μM versus 24 μM methemoglobin at pH 7.0 and the maximal acceleration was more than 10 times compared with the control. Such a stimulation of methemoglobin reduction was also observed at pH 6.3 in the presence of the same concentration of IHP at pH 7.0. On the other hand, at pH 7.7, more than 600 μM IHP was necessary for maximal acceleration and the maximal rate was at most three times faster than the control.

Fig. 3B shows the effect of 2,3-DPG which was also studied at various concentrations and pH values. At pH 7.0, the acceleration of the reduction rate by ascorbic acid in the presence of 2,3-DPG reached a maximum at concentrations above 6 mM versus 24 μM methemoglobin with a maximal acceleration of four times compared with the control. At pH 6.3, maximal acceleration was observed above 4 mM 2,3-DPG and the rate was six times larger than the control. At pH 7.7, there was no acceleration of methemoglobin reduction even at the high concentration of 2,3-DPG.

The rate of methemoglobin reduction was also accelerated by the addition of ATP and reached a maximum at a concentration above 1 mM at pH 7.0 and 500 μM at pH 6.3 (Fig. 3C). The maximum acceleration was three times and five times at pH 7.0 and 6.3, respectively. Such an acceleration was not observed in the presence of high concentrations of ATP at pH 7.7.

Fig. 2. Stoichiometrical studies on the reaction between ascorbic acid and methemoglobin. A 1.5-ml solution of methemoglobin (64 μM) in 0.05 M bis-Tris buffer (pH 7.0) containing 0.1 M NaCl was mixed in the presence of IHP (100 μM). The reaction was started by the addition of ascorbic acid (6 mM) and the absorption spectrum was measured at 630 nm for 37 min for analysis of methemoglobin at 25°C. In parallel with this experiment, 9 ml of methemoglobin solution containing the same constituents were mixed and the reaction was started simultaneously by the addition of ascorbic acid (6 mM). At intervals 1.5-ml aliquots were taken out for analysis and deproteinized with 5% metaphosphoric acid. The supernatant was used for measuring dehydroascorbic acid. X, 2X dehydroascorbic acid; O, amount of ferric iron reduced.
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Fig. 3. Effect of IHP, 2,3-DPG, and ATP on the rate of methemoglobin reduction by ascorbic acid at different pH. Experimental conditions and procedure are essentially the same as those described under Fig. 1. Experiments were carried out at various pH values (pH 6.3, 7.0, 7.7) and at various concentrations of IHP, 2,3-DPG, and ATP. A, effect of IHP. The numbers in parentheses show the concentration of IHP at pH 7.7. B, effect of 2,3-DPG. C, effect of ATP.

Thus the acceleration of methemoglobin reduction by ascorbic acid may be due to the binding of these organic phosphates and this is largely influenced by the concentration of hydrogen ion.

Effect of NaCl on Methemoglobin Reduction by Ascorbic Acid in Presence and Absence of IHP — Since the binding of organic phosphates with deoxy- and oxyhemoglobin is known to be influenced by high concentrations of neutral salts (27), it is probable that binding of organic phosphate to methemoglobin is also influenced by these salts. Hence we studied the effect of NaCl concentrations on methemoglobin reduction by ascorbic acid in the presence and absence of IHP at various pH values (6.3, 7.0, 7.7) as shown in Fig. 4. At lower concentrations of NaCl, the rate of methemoglobin reduction was markedly accelerated in the presence of 100 μM IHP, although the pH dependence was pronounced. At 10 mM NaCl, the reduction rate was accelerated 15 times, 11 times, and 7.5 times faster than the control at pH 6.3, 7.0, and 7.7. However, the stimulative effect of IHP was almost lost by high concentrations of NaCl (450 mM) at each pH. In the absence of IHP, there was little acceleration or difference in the reduction rate regardless of the difference in pH.

This inhibitory effect of NaCl on the acceleration by IHP are interpreted as competition of chloride ion with IHP for binding sites of methemoglobin, as is well known with deoxy- and oxyhemoglobin (28). These results further support the view that the binding of organic phosphates such as IHP to methemoglobin is essential to the acceleration of methemoglobin reduction by ascorbic acid.

Dissociation Constants of 2,3-DPG, ATP, and IHP to Methemoglobin — The binding of organic phosphates to methemoglobin seems to be closely connected with the acceleration of the methemoglobin reduction shown in Fig. 3, A to C, so it is possible to estimate the dissociation constants of organic phosphates such as 2,3-DPG, ATP, and IHP to methemoglobin from these results.

The values listed in Table I are the dissociation constants of 2,3-DPG, ATP, and IHP which were obtained from the kinetic treatment of the results shown in Fig. 3, A to C, and compared with the values in the literature. The detailed method is mentioned under "Appendix."

Reduction of Carboxypeptidase A-digested Methemoglobin by Ascorbic Acid — Table II shows the rate of the ascorbic acid reduction by carboxypeptidase A-digested methemoglobin in the presence and absence of IHP at pH 7.0. The reduction rate was not altered regardless of the presence and absence of IHP.

DISCUSSION

According to Perutz et al. (9), the conformational change of methemoglobin due to the binding of organic phosphates includes a transition from the R to the T state. This suggests that the T state of methemoglobin is favored for the reaction with ascorbic acid than the R state. The carboxypeptidase A-digested methemoglobin is considered to take an R form even in the presence of organic phosphates (13) and it is anticipated that the reduction rate will not be affected by organic phosphates. The results shown in Table II support the possibility that the conformational change is involved in the acceleration of methemoglobin reduction.

On the other hand, it is known that methemoglobin has a
high spin state at acidic and neutral pH (aquomethemoglobin), and low spin at alkaline pH (hydroxymethemoglobin). Rein et al. showed by FSR measurement that ATP changes the spin state of ferric iron to high spin (12). Recently Perutz et al. reported that the change of the quaternary structure of methemoglobin due to the binding of IHP accompanies the shift of spin state to high spin, which was ascertained by the measurement of paramagnetic susceptibility and ESR (13). Furthermore, Hensley et al. (14) indicated that the conformational change of methemoglobin is largely independent of spin state. Olson (15) showed that the conformational changes of methemoglobin occur partially after these changes in spin state.

We investigated to see if the high spin state of methemoglobin favors ascorbic acid attack. As shown in Table III, there was no difference in the reduction rate for the region between pH 6.3 and 9.0 in the absence of organic phosphates. As the conformation of methemoglobin is predominantly the R form in these pH regions (29), this result suggests that only the change of the spin state to high spin is not sufficient for the acceleration of methemoglobin reduction by ascorbic acid.

Judging from these results, it might be possible that for the acceleration of methemoglobin reduction, both the change of conformation of methemoglobin from the R to the T state and the change to high spin are indispensable. However, detailed elucidation of the acceleration of methemoglobin reduction necessitates further analysis of the quaternary structure of methemoglobin and the state of heme iron.

It is possible to estimate the dissociation constants of 2,3-DPG, ATP, and IHP from the reduction rate if kinetic treatment is applied as mentioned under "Appendix." The dissociation constants obtained are summarized in Table I in comparison with those appearing in the literature, although the precise conditions were not necessarily the same.

The dissociation constant of IHP to methemoglobin obtained from the present results is $2.9 \times 10^{-6} M$, $8 \times 10^{-5} M$, and $2 \times 10^{-3} M$ at pH 6.3, 7.0, and 7.7, respectively. The values at pH 6.3 and 7.0 are consistent with the value at pH 6.65 reported by Perutz et al. (9), and the value at pH 7.7 is roughly one order larger than their estimation at pH 7.45 (13).

With regard to the dissociation constant of 2,3-DPG to methemoglobin, Chanutin and Hermann estimated it to be $1.1 \times 10^{-3} M$ at pH 6.5 (2). Recently, Perutz et al. estimated it to be $0.2$ to $2.0 \times 10^{-3} M$ at pH 6.6 (9). The values obtained from our results are $4.5 \times 10^{-4} M$ and $2 \times 10^{-3} M$ at pH 6.3 and 7.0, respectively, and these roughly correspond to the values by these authors. As there was little acceleration of methemoglobin reduction by ascorbic acid at pH 7.7, the estimation of the dissociation constant of 2,3-DPG at this pH was difficult, and the value might be far larger than those at lower pH.

The binding of ATP to methemoglobin was studied in detail by Jänich et al. (6) and they estimated the dissociation constant of ATP to methemoglobin to be about $10^{-4} M$ at pH 6.2. Our result at pH 6.3 ($1 \times 10^{-4} M$) corresponds to their estimation, and the value at pH 7.0 was $3.3 \times 10^{-4} M$. Thus the dissociation constant of ATP to methemoglobin at these pH values are smaller than those of 2,3-DPG, however, much larger than those of IHP. Considering our results, it is likely that the dissociation constants of these organic phosphates to methemoglobin are of nearly the same order as those to oxyhemoglobin, and one order larger than those to deoxyhemoglobin.

When equimolar (23 $\mu M$), double (46 $\mu M$), or 10-fold (230 $\mu M$) 2,3-DPG was present against methemoglobin (23 $\mu M$), the rate of reduction of methemoglobin by ascorbic acid was expected to be accelerated by 2.3, 4.5, and 21%, respectively, compared with the rate of methemoglobin reduction in the absence of 2,3-DPG (these values were calculated by the extrapolation of the linear curve in Fig. 3). Taking into account that physiological concentration of 2,3-DPG is nearly equivalent to that of hemoglobin in red cells and that dissociation constant of 2,3-DPG to methemoglobin is nearly the same order as that of 2,3-DPG to oxyhemoglobin, 2.3% acceleration may be anticipated in red cells compared with the case without 2,3-DPG.

Therefore the actual contribution of 2,3-DPG to methemoglobin reduction by ascorbic acid might not be large in human red cells. Nevertheless, present results suggest that 2,3-DPG...
affects the rate of methemoglobin reduction in the red cells when ascorbic acid is applied to the patients suffering from hereditary methemoglobinemia, whose red cells contain high 2,3-DPG (30). Recently an increase in 2,3-DPG has been shown when ascorbic acid is added to ACD blood (31), and this may serve for the maintenance of the integrity of red cells during preservation by accompanying the acceleration of methemoglobin reduction by ascorbic acid.

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APPENDIX

The methemoglobin reduction by ascorbic acid in the presence of 2,3 DPG may be formulated

\[ \text{MetHb} + \text{DPG} \overset{k_1}{\rightarrow} \text{Hb(reduced)} + \text{DPG} \]

where \( k_1 \) and \( k_2 \) are the rate constants of methemoglobin and its complex with DPG and \( K = k_2/k_1 \) association constant of DPG binding to methemoglobin.

The rate constant \( k_1 \) follows first order kinetics and can be determined by measuring the rate of methemoglobin reduction in the absence of DPG.

Thus, the apparent rate of methemoglobin reduction by ascorbic acid \( V \) is described as

\[ V = -\frac{d[\text{MetHb}]}{dt} = k_1[\text{MetHb}] + k_2[\text{MetHb}-\text{DPG}] \]

where \( f_1 \) and \( f_2 \) are the free and DPG-bound methemoglobin fraction in total hemoglobin used (\( \text{MetHb} \)).

Since the association constant \( K \) can be described as \( K = k_2/k_1 \) \( [\text{MetHb}] [\text{DPG}] \) and \( \text{MetHb} + \text{MetHb-EPG} \) equals \( [\text{MetHb} f_1 \) and \( f_2 \) corresponds to \( 1/(1 + K[\text{DPG}]) \), \( K[\text{DPG}]/(1 + K[\text{DPG}]) \), respectively.

Thus, \( V = -d[\text{MetHb}] / dt = [\text{MetHb}] k_1/(1 + K[\text{DPG}]) + k_2 K[\text{DPG}]/(1 + K[\text{DPG}]) \) and the apparent rate constant \( k_{\text{app}} = V/[\text{MetHb}] = k_1 + (K[\text{DPG}]k_2)/[1 + K[\text{DPG}]] \).

As a result the following equation is obtained, i.e.

\[ \frac{1}{k_{\text{app}} - k_1} = \frac{1}{k_2 - k_1} + \frac{1}{k_2 - k_1} K[\text{DPG}] \]

If the reciprocal of \( k_{\text{app}} - k_1 \) is plotted versus the reciprocal of DPG concentration, the equilibrium association constant \( K \) may be obtained from the intercept of a linear curve as shown in Fig. 5, and dissociation constant can be obtained by the reciprocal of the association constant.

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