Conversion of oxyhaemoglobin into methaemoglobin by ferricytochrome b_5

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Ferricytochrome b_5 was found to convert oxyhaemoglobin into methaemoglobin under conditions previously found to be optimal for complex-formation between ferricytochrome b_5 and methaemoglobin [Mauk & Mauk (1982) *Biochemistry* **21**, 4730–4734]. As this reaction is completely inhibited by CO, it is proposed that oxyhaemoglobin is oxidized after O_2 dissociation, as has been suggested for the oxidation of oxyhaemoglobin by inorganic complexes. From the present analysis, ferricytochrome b_5 seems unlikely to contribute significantly to methaemoglobin formation *in vivo*. Nevertheless, this observation provides a relatively convenient means of investigating the mechanism by which these two proteins interact.

In the erythrocyte, cytochrome b_5 serves as the principal physiological reductant of methaemoglobin by mediating the transfer of electrons from NADH: methaemoglobin oxidoreductase to methaemoglobin (Hultquist & Passon, 1971; Sannes & Hultquist, 1978). We have previously studied complex-formation between ferricytochrome b_5 and methaemoglobin by electronic difference spectroscopy (Mauk & Mauk, 1982) and by interactive computer graphics (Poulos & Mauk, 1983) to characterize better the mechanism of interaction between these two proteins. During subsequent attempts to obtain difference spectra arising from the interaction of ferricytochrome b_5 and other derivatives of haemoglobin, we made the unexpected observation that mixing ferricytochrome b_5 with oxyhaemoglobin at ratios as low as 1:20 on a haem basis results in the conversion of oxyhaemoglobin into methaemoglobin under certain conditions. The present paper reports our initial characterization of this phenomenon.

Experimental

The tryptic fragment of bovine liver microsomal cytochrome b_5 was purified from fresh ox liver as described previously (Reid & Mauk, 1982). This form of the hepatic protein was used in lieu of erythrocytic cytochrome b_5 owing to the large quantities required and the near-identity of their structures (Slaughter *et al.*, 1982). The erythrocytic cytochrome b_5 is solubilized *in vivo* by catheptic activity (Schafer *et al.*, 1982) and is more closely related in structure to the trypsin-cleaved hepatic protein than to the detergent-solubilized form.

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Human haemoglobin A_0 was purified in the oxygenated form from outdated blood-bank blood by the method of Williams & Tsay (1973). Both proteins were stored in liquid N_2 . Buffers of known ionic strength were prepared from cacodylic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.) as previously described (Mauk & Mauk, 1982) or by adding solid Bistris {2-[bis-(2-hydroxyethyl)-amino]-2-(hydroxymethyl)propane-1,3-diol} (Sigma Chemical Co.) to dilute solutions of HCl (prepared from standardized stock solutions) to obtain the required pH. All protein concentrations are expressed in terms of haem determined on the basis of published absorption coefficients (Ozols & Strittmatter, 1964; Linder *et al.*, 1978).

Difference spectra were obtained with matched tandem quartz cuvettes (M. R. Mauk *et al.*, 1982) and a Cary 219 spectrophotometer that was interfaced to an OLIS (Jefferson, GA, U.S.A.) model 3820 data system through a Varian digital interface port; this system is based on a modified NorthStar Horizon (Z80) microcomputer. The scan rate was computer-controlled to vary inversely with the rate of change of absorbance with wavelength. At each wavelength increment, absorbance was monitored until it had stabilized to $\pm 0.0001 A$ unit. Ten values of the absorbance reading were then acquired at 67 ms intervals and averaged to obtain the absorbance reading used for the wavelength. Data-analysis software permitted a variety of data-manipulation options that included signal-averaging and addition or subtraction of stored spectra to or from each other. Temperature was regulated to $\pm 0.1^\circ C$ with a Lauda model RC3 refrigerated circulating water bath. Temperature

was monitored to $\pm 0.2^\circ\text{C}$ with a Fluke (Everett, WA, U.S.A.) model 2175A digital thermometer fitted with a subminiature copper-constantan thermocouple.

Kinetics measurements were made with the spectrophotometer and data system just described. Absorbance/time data points obtained at fixed wavelength and digitized by the computer were used to determine initial rates by the method of Boeker (1982). As adapted in the present work, this method involved collecting data for the first 25% of the reaction, plotting the slope between successive points ($d[\text{methaemoglobin}]/dt$) versus the concentration of product formed ($[\text{methaemoglobin}]$), fitting the data by a linear least-squares analysis and extrapolating to zero $[\text{methaemoglobin}]$ to determine the initial rate. For each rate measurement, control measurements were made of the rate of autoxidation of oxyhaemoglobin to permit correction of the absorbance readings for this process. In addition, absorbance data were corrected for the contribution of complex-formation between ferricytochrome b_5 and methaemoglobin under those conditions for which it has been possible to determine association constants (Mauk

& Mauk, 1982). The magnitude of the correction for autoxidation varied inversely with the rate of reaction and resulted in a decrease in k_{obs} , ranging from 2 to 27%. The correction for the absorbance change arising from complex-formation between cytochrome b_5 and methaemoglobin increased k_{obs} by 0–2%.

Results

The difference spectra observed at various time intervals after mixing cytochrome b_5 with oxyhaemoglobin at equal concentrations of haem [pH 6.2 (cacodylate buffer), 1.0×10^{-5} , 25°C] are shown in Fig. 1(a). The conditions used in this experiment are those that we have previously found to be near-optimal for complex-formation between cytochrome b_5 and methaemoglobin (Mauk & Mauk, 1982). The spectra in Fig. 1(a) are indicative of methaemoglobin formation from oxyhaemoglobin in the reaction cuvette and can be simulated by appropriate combinations of spectra of oxyhaemoglobin and methaemoglobin. There is no evidence for formation of detectable amounts of reduced cytochrome b_5 in this process. Ferro-

Fig. 1. (a) Difference spectra observed at different time intervals after mixing ferricytochrome b_5 and oxyhaemoglobin, and (b) difference spectrum obtained after mixing ferricytochrome b_5 and carbonmonoxyhaemoglobin in an analogous manner. (a) Ferricytochrome b_5 ($100 \mu\text{M}$) was placed into one side of a tandem cuvette, and an identical mass ($\pm 0.1 \text{ mg}$) of oxyhaemoglobin ($100 \mu\text{M}$) was placed into the other [both solutions were pH 6.2 (cacodylate buffer), 1.0×10^{-5} , 25°C]. This cuvette was placed in the sample beam of the spectrophotometer, an identical cuvette prepared in the same manner was placed in the reference beam, and the baseline was recorded. The proteins in the two sides of the sample cuvette were then mixed and the spectrum was recorded after 2 min (A), 12 min (B), 22 min (C), 32 min (D) and 50 min (E). (b) In this case, identical masses of carbonmonoxyhaemoglobin ($195 \mu\text{M}$) and ferricytochrome b_5 ($200 \mu\text{M}$) were used [pH 6.2 (cacodylate buffer), 1.0×10^{-5} , 25°C]. The spectrum shown resulted after mixing the proteins present in the two compartments of the cuvette in the sample beam. No change in this spectrum was observed up to 45 min after mixing. The matched tandem cells had a pathlength of 0.874 cm .

Fig. 2. Initial-rate analysis of methaemoglobin formation resulting from mixing oxyhaemoglobin and cytochrome b_5 . ●, pH 6.2 (Bistris buffer), 10.005, 25°C. Initial protein concentrations were 8.90 μM -oxyhaemoglobin and 174.1 μM -ferricytochrome b_5 . The continuous line is an unweighted linear least-squares fit to the data after deletion of the first four points. ○, pH 6.2 (Bistris buffer), 10.1, 25°C. Initial protein concentrations were 8.60 μM -oxyhaemoglobin and 176.1 μM -ferricytochrome b_5 . The broken line is an unweighted linear least-squares fit to the data. All data were corrected for absorbance changes attributable to autoxidation of oxyhaemoglobin. The 10.005 data were also corrected for the complex-formation between ferricytochrome b_5 and methaemoglobin.

Fig. 3. pH-dependence of the rate of methaemoglobin formation as a function of cytochrome b_5 concentration. Conditions: Bistris buffer, 10.005 and 25°C; Δ, pH 5.8; ●, pH 6.2; ○, pH 6.4; ■, pH 6.7; □, pH 7.0. A weighted linear least-squares fit was applied to the data at pH 6.7 and 7.0. The lines shown for data at pH 5.8, 6.2 and 6.4 have no numerical significance. All data were corrected for autoxidation of oxyhaemoglobin. Data at pH 5.8, 6.2 and 6.4 were also corrected for complex-formation between ferricytochrome b_5 and methaemoglobin. Error bars are displayed only where they are larger than the dimensions of the symbols.

cytochrome b_5 should be readily detected owing to the large change in absorbance expected at 555 nm ($\Delta\epsilon = 17500 \text{ M}^{-1} \cdot \text{cm}^{-1}$). Separation of the reaction mixture by ion-exchange chromatography confirmed the presence of ferricytochrome b_5 , methaemoglobin and oxyhaemoglobin. After 20–24 h, conversion of oxyhaemoglobin into methaemoglobin was complete. The formation of methaemoglobin was observable even at low concentrations of cytochrome b_5 (e.g. oxyhaemoglobin/cytochrome b_5 ratios 20:1 on a haem basis). Significantly, repeating this experiment with carbonmonoxyhaemoglobin in place of oxyhaemoglobin produced a stable weak difference spectrum (Fig. 1b). No change in this difference spectrum was detected up to 45 min after mixing.

Analyses of the time courses for the reaction of cytochrome b_5 and oxyhaemoglobin conducted at both 577 nm and 630 nm were consistent with at least four rate processes. Control experiments conducted in the absence of cytochrome b_5 confirmed that the two slower processes involved autoxidation of oxyhaemoglobin and haemichrome formation. The two faster processes occurred only in the presence of cytochrome b_5 and were

therefore the reactions of interest. All subsequent kinetic experiments were carried out at 577 nm to maximize absorbance changes. Bistris buffers were used instead of cacodylate buffers to avoid complications that might arise from possible residual iron contamination in the recrystallized cacodylic acid.

Subsequent kinetic experiments involved extraction of the initial rate from results collected during only the first 25% of the total expected absorbance change (as determined by addition of ferricyanide), since haemichrome formation was not detectable during this time period. The absorbance/time data were also corrected for autoxidation of oxyhaemoglobin and for complex-formation between ferricytochrome b_5 and methaemoglobin as described above (see the Experimental section).

Examples of data used in the initial-rate analyses for experiments conducted at two widely different ionic strengths and constant pH are shown in Fig. 2. From these results it is apparent that at low ionic strength there are two components, whereas at higher ionic strength only one is observable. The magnitude of the faster component was found to be greater at low pH and low ionic strength. As this phase of the reaction was too rapid to be analysed accurately by these techniques, the data were

Fig. 4. Ionic-strength-dependence of the rate of methaemoglobin formation as a function of cytochrome b_5 concentration

Conditions: pH 6.2 (Bistris buffer) and 25°C; ●, I 0.005; ○, I 0.01; ■, I 0.05; □, I 0.1. A weighted linear least-squares fit was applied for I 0.05 and I 0.1. The lines drawn through the data for I 0.005 and I 0.01 have no numerical significance. All data were corrected for autoxidation of oxyhaemoglobin. Data at I 0.005 and I 0.01 were also corrected for complex-formation between ferricytochrome b_5 and methaemoglobin. Error bars are displayed only where they are larger than the dimensions of the symbols.

inspected visually and those data points affected by the faster component were deleted in all subsequent analyses.

The variation of the slower cytochrome b_5 -dependent component with pH at constant ionic strength is shown in Fig. 3. The uncertainties in the values of k_{obs} determined from the linear least-squares analyses (as illustrated in Fig. 2) are indicated by the error bars where they extend beyond the dimensions of the symbols that represent data points. At higher pH, the rate of reaction is relatively low and is linearly dependent on cytochrome b_5 concentration. As the pH is lowered, the rate of reaction increases and ceases to vary in a linear fashion with cytochrome b_5 concentration. A similar dependence of this phase on ionic strength is shown in Fig. 4. At higher ionic strength, the reaction is relatively slow and demonstrates a linear dependence on cytochrome b_5 concentration. At lower ionic strength, the rate increases, and the rate-dependence on cytochrome b_5 concentration becomes non-linear.

Discussion

The results of the present series of experiments demonstrate that, under appropriate conditions of pH and ionic strength, ferricytochrome b_5 can

oxidize oxyhaemoglobin to methaemoglobin. This observation provides a relatively simple means for studying the interaction of these two proteins under aerobic conditions and in the absence of enzymes or other proteins. By analogy of the present reaction to that of ferricyanide (Antonini *et al.*, 1965) and $\text{Fe}(\text{EDTA})^-$ (Yamada *et al.*, 1978) with oxyhaemoglobin, the following minimal mechanism is proposed to account for the results presented above:

where cyt represents cytochrome and Hb represents haemoglobin. In this scheme, the electron-transfer reaction actually involves ferricytochrome b_5 and deoxyhaemoglobin. This hypothesis is consistent with the complete inhibition of reaction by CO, which has a much greater affinity for haemoglobin than does O_2 . Carbonmonoxyhaemoglobin, of course, is oxidized by ferricyanide, but at a much lower rate than oxyhaemoglobin (see, e.g., Antonini *et al.*, 1964; Lanir *et al.*, 1982). For this reason, we cannot eliminate the possibility that ferricytochrome b_5 oxidizes carbonmonoxyhaemoglobin at an undetectably low rate. The ferrocyanide b_5 produced in this process is rapidly autoxidized, as described by Berman *et al.* (1976). Clearly, reactions (1) and (2) are oversimplifications, insofar as deoxygenation and oxidation of haemoglobin are most probably multi-step phenomena. For the present analysis, however, a more detailed mechanism would be inappropriate.

As the reduction potential of cytochrome b_5 [$E_{\text{m},7}^0$ 5 mV versus normal hydrogen electrode at I 0.1 (phosphate buffer) at 25°C (Reid *et al.*, 1982)] is significantly lower than that of haemoglobin (methaemoglobin $\rightleftharpoons$ deoxyhaemoglobin) [$E_{\text{m},7}^0$ 150 mV versus normal hydrogen electrode at I 0.1 (phosphate buffer) at 25°C (Antonini *et al.*, 1964)], it is surprising that haemoglobin oxidation is observed at relatively low ratios of ferricytochrome b_5 to oxyhaemoglobin. The requirement for ligand dissociation before electron transfer explains how this reaction occurs at equimolar concentrations of the two proteins, because, in fact, under these conditions ferricytochrome b_5 is present at great excess over deoxyhaemoglobin. From the O_2 -binding data of Imai & Yonetani (1975) and the Adair equation (Adair, 1925), it can be shown that a 9 μM solution of haemoglobin equilibrated with air (at atmospheric pressure) contains deoxyhaemoglobin at a concentration of 13–16 nM

(pH 6.5–7, 25°C). Thus an equimolar aerobic mixture of cytochrome b_5 and haemoglobin contains the cytochrome at 600-fold excess over deoxyhaemoglobin, so our kinetic measurements can be regarded as employing pseudo-first-order conditions. We further note that the immediate autoxidation of ferrocycytochrome b_5 maintains the oxidant concentration constant throughout the course of the reaction and accounts for our failure to observe ferrocycytochrome b_5 formation.

From this argument, it should be possible to estimate the second-order rate constants for cytochrome b_5 oxidation and reduction of haemoglobin on the basis of our data. As discussed by Lanir *et al.* (1982), a plot of $\log[\text{oxyhaemoglobin}]$ versus time should be linear with a slope described by eqn. (3):

$$\text{Slope} = 2.303 \frac{k_{+1}k_{+2}[\text{ferricytochrome } b_5]}{k_{-1}[\text{O}_2] + k_{+2}[\text{ferricytochrome } b_5]} \quad (3)$$

For our experiment at pH 7 and 1.005×10^{-3} M (25°C), $[\text{ferricytochrome } b_5] = 22.6 \mu\text{M}$ and $[\text{O}_2] = 250 \mu\text{M}$ (Wilhelm *et al.*, 1977). The rate constants for the O_2 association (k_{-1}) and dissociation (k_{+1}) reactions with R-state haemoglobin may be approximated at $5.5 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ and 22 s^{-1} respectively (Olsen, 1981). With these assumptions, our data produce a value for k_{+2} of $3 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$. The equilibrium constant, K_{eq} , for eqn. (2) can be calculated from the reduction potential of haemoglobin (150 mV; Antonini *et al.*, 1964), which appears to be relatively insensitive to ionic strength at this pH, and from the reduction potential of cytochrome b_5 (–35 mV at pH 7, 1.005×10^{-3} M and 25°C) estimated from its ionic-strength-dependence (Reid *et al.*, 1982). The resulting K_{eq} value (1.35×10^3) can be used with the value calculated above for k_{+2} to deduce the rate of methaemoglobin reduction by ferrocycytochrome b_5 (k_{-2}). The resulting value is $4 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. The only other value reported for this rate constant was derived by Kuma (1981) from a steady-state analysis of a reconstituted methaemoglobin reductase system that used erythrocytic cytochrome b_5 . The rate constant he reported, $2.7 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ (pH 7.0), is significantly lower than ours, as would be expected (Sannes & Hultquist, 1978) from the much greater ionic strength (0.22 M) and lower temperature (20°C) used in his experiments.

The non-linear dependence of the rate of methaemoglobin formation on cytochrome b_5 concentration observed at low pH and low ionic strength (Figs. 3 and 4) can result from a variety of mechanisms or combinations of mechanisms (Yoneda & Holwerda, 1978; A. G. Mauk *et al.*,

1982). The simplest and most likely explanation, however, is that these conditions permit kinetically detectable complex-formation between ferricytochrome b_5 and deoxyhaemoglobin (formation of precursor complex) before electron transfer. This interpretation seems reasonable, because the conditions under which this non-linearity is seen correspond exactly to the conditions that we have previously found to promote complex-formation between ferricytochrome b_5 and methaemoglobin. Similarly, the origin of the rapid phase seen at low ionic strength and low pH (Fig. 2) may involve complex-formation between ferricytochrome b_5 and oxyhaemoglobin that produces an absorbance change similar to that seen for complex-formation between ferricytochrome b_5 and methaemoglobin at the wavelength monitored. This possibility requires further investigation.

Finally, the inevitable question concerning the contribution of this process to the overall oxidation–reduction behaviour of haemoglobin in erythrocytes under physiological conditions must be addressed. From the combined dependences of this reaction on pH and ionic strength, there can be little doubt that at pH 7.4 and an ionic strength of 0.14 this reaction is effectively undetectable. Nevertheless, the usefulness of this reaction in mechanistic studies of the interaction of these structurally well-characterized proteins has been clearly established.

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References

- Adair, G. S. (1925) *Proc. R. Soc. London Ser. A* **109**, 292–300
- Antonini, E., Wyman, J., Brunori, M., Taylor, J. F., Rossi-Fanelli, A. & Caputo, A. (1964) *J. Biol. Chem.* **239**, 907–912
- Antonini, E., Brunori, M. & Wyman, J. (1965) *Biochemistry* **4**, 545–551
- Berman, M. C., Adnams, C. M., Ivanetich, K. M. & Kench, J. E. (1976) *Biochem. J.* **157**, 237–246
- Boeker, E. A. (1982) *Biochem. J.* **203**, 117–123
- Hultquist, D. E. & Passon, P. G. (1971) *Nature (London)* **229**, 252–254
- Imai, K. & Yonetani, T. (1975) *J. Biol. Chem.* **250**, 2227–2231
- Kuma, F. (1981) *J. Biol. Chem.* **256**, 5518–5523
- Lanir, A., Caughey, W. S. & Charache, S. (1982) *Eur. J. Biochem.* **128**, 521–525
- Linder, R. E., Records, R., Barth, G., Bunnberg, E., Djerassi, C., Hedlund, B. E., Rosenberg, A., Benson, E. S., Seamans, L. & Moscovitz, A. (1978) *Anal. Biochem.* **90**, 474–480

- Mauk, A. G., Bordignon, E. & Gray, H. B. (1982) *J. Am. Chem. Soc.* **104**, 7654-7657
- Mauk, M. R. & Mauk, A. G. (1982) *Biochemistry* **21**, 4730-4734
- Mauk, M. R., Reid, L. S. & Mauk, A. G. (1982) *Biochemistry* **21**, 1843-1846
- Olsen, J. S. (1981) *Methods Enzymol.* **76**, 631-651
- Ozols, J. & Strittmatter, P. (1964) *J. Biol. Chem.* **239**, 1018-1023
- Poulos, T. L. & Mauk, A. G. (1983) *J. Biol. Chem.* **258**, 7369-7373
- Reid, L. S. & Mauk, A. G. (1982) *J. Am. Chem. Soc.* **104**, 841-845
- Reid, L. S., Taniguchi, V. T., Gray, H. B. & Mauk, A. G. (1982) *J. Am. Chem. Soc.* **104**, 7516-7519
- Sannes, L. G. & Hultquist, D. E. (1978) *Biochim. Biophys. Acta* **544**, 547-554
- Schafer, D. A., Hogikyan, J. V. & Hultquist, D. E. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 506
- Slaughter, S. R., Williams, C. H., Jr. & Hultquist, D. E. (1982) *Biochim. Biophys. Acta* **705**, 228-237
- Wilhelm, E., Battino, R. & Wilcock, R. J. (1977) *Chem. Rev.* **77**, 219-262
- Williams, R. C. & Tsay, K.-Y. (1973) *Anal. Biochem.* **54**, 137-145
- Yamada, T., Marini, C. P. & Cassatt, J. C. (1978) *Biochemistry* **17**, 231-236
- Yoneda, G. S. & Holwerda, R. (1978) *Bioinorg. Chem.* **8**, 139-159