Kinetic analysis of metsulphmyoglobin and metmyoglobin reduction by Fe(EDTA)^{2-}

Anthony R. LIM and A. Grant MAUK*

Department of Biochemistry, University of British Columbia, Vancouver, B.C. V6T 1W5, Canada

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Metsulphmyoglobin prepared from horse heart myoglobin was purified by ion-exchange chromatography to yield a product that on reduction with Fe(EDTA)^{2-} has an A_{617}/A_{561} ratio greater than 3.5:1. The kinetics of reduction of this purified metsulphmyoglobin and of native metmyoglobin by Fe(EDTA)^{2-} were studied under various conditions of pH, ionic strength and temperature to compare the relative electron-transfer reactivities of a metallochlorin and a metalloporphyrin in identical protein environments. Although the rate of metsulphmyoglobin reduction is 2–7 times that of metmyoglobin under a variety of conditions, this difference can be more than compensated for by the reported difference in mid-point reduction potential between the two forms of the protein. The electrostatic and activation parameters observed for native metmyoglobin and metsulphmyoglobin are essentially identical, and small differences are found in the pH-dependence of the reduction reaction. These findings lead us to conclude that conversion of the porphyrin prosthetic group into a chlorin has relatively little effect on the electron-transfer reactivity of the central metal atom.

Sulphmyoglobin is a derivative of myoglobin in which the haem group has been chemically modified by reaction of ferrylmyoglobin with a sulphide (Nicholls, 1961; Morell *et al.*, 1967; Berzofsky *et al.*, 1971). The exact nature of the modification is unknown, although radiolabelling experiments have established that one atom of sulphur is incorporated into the prosthetic group in some manner (Berzofsky *et al.*, 1972). Furthermore, the optical properties of sulphmyoglobin are reminiscent of those of chlorins (Berzofsky *et al.*, 1971, 1972), which suggests that one of the pyrrole rings is reduced in the modified haem. This conclusion has been corroborated by comparison of resonance Raman spectra of model chlorin compounds (Andersson *et al.*, 1984a) with those of sulphmyoglobin (Andersson *et al.*, 1984b). As several metalloenzymes are now known to be chlorin-containing proteins (see, e.g., Chang, 1982), sulphmyoglobin and the related derivatives of haemoglobin (Nichol *et al.*, 1968; Carrico *et al.*, 1978), catalase (Nicholls, 1961) and lactoperoxidase (Nakamura *et al.*, 1984) should provide relatively convenient vehicles for evaluating some of the functional options that are available to such proteins.

In the present paper we report a method for purifying metsulphmyoglobin by ion-exchange chromatography and present a kinetic analysis of the reduction of metsulphmyoglobin through use of the mild reducing agent Fe(EDTA)^{2-} as a means of assessing the relative electron-transfer reactivity of metallochlorin- and metalloporphyrin-containing proteins possessing identical protein environments.

Experimental

Horse heart metmyoglobin was obtained from Sigma Chemical Co. (type III, cat. no. M-1882), dissolved in 0.1M-sodium phosphate buffer, pH 8.0, and centrifuged to remove insoluble material. Residual reduced protein was oxidized by addition of a few grains of $\text{K}_3\text{Fe(CN)}_6$, which was subsequently removed by dialysis or repeated ultrafiltration (Amicon YM-5 membrane) and dilution. For experiments with native myoglobin, the fully oxidized protein was purified by the method of Tomoda *et al.* (1981), except that CM-cellulose (Whatman CM-52) was used instead of CM-Sephadex. Metmyoglobin concentration was determined at pH 8.0 on the basis of $\epsilon_{411} = 119000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Antonini & Brunori, 1971).

* To whom correspondence should be addressed.

Bovine liver catalase (Sigma Chemical Co.; cat. no. C-100), H_2O_2 (BDH Chemicals; cat. no. ACS399) and Na_2S (Matheson, Coleman and Bell; cat. no. SX770) were obtained commercially as indicated.

Reduced sulphmyoglobin was prepared by the method of Berzofsky *et al.* (1971). In our hands, this method yielded material with an A_{617}/A_{561} ratio of 2.8–3.2:1. The reduced sulphmyoglobin was then oxidized by addition of $\text{K}_3\text{Fe}(\text{CN})_6$, and the excess oxidant was removed as described above. This metsulphmyoglobin (approx. 60 mg) was loaded on to a 2.5 cm $\times$ 7 cm column of DEAE-cellulose resin (Whatman DE-52), at 4°C, that was equilibrated with 5 mM-Tris/HCl buffer, pH 8.4 at 25°C. The column was developed, at 4°C, with 2 column volumes of the same buffer at a flow rate of 1.6 ml/min, after which the purified metsulphmyoglobin was eluted with 10 mM-Tris/HCl buffer, pH 8.0 at 25°C. Invariably a dark-coloured material remained bound tightly at the top of this column. The purity of individual fractions was assessed by diluting a small sample of each (50 μl) with ice-cold Tris or phosphate buffer (1.8 ml), reducing the protein with a small volume (0.15 ml) of a concentrated $\text{Fe}(\text{EDTA})^{2-}$ solution, and determining the A_{617}/A_{561} ratio at 15°C after the absorbance at 617 nm had stabilized. The best fractions observed in some preparations had ratios as high as 4.0:1. Fractions with ratios of 3.5:1 and greater were pooled and used in the kinetics experiments. Protein samples treated with reductant for determination of the purity ratio were discarded. Metsulphmyoglobin could be concentrated by ultrafiltration (Amicon YM-5 membrane), but this treatment sometimes resulted in a decrease in the purity ratio observed on subsequent reduction with $\text{Fe}(\text{EDTA})^{2-}$. The concentration of sulphmyoglobin was estimated from $\epsilon_{404} = 117000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Berzofsky *et al.*, 1971).

Electronic spectra were obtained with a Cary 219 spectrophotometer. The reduction kinetics were studied with a computer-interfaced stopped-flow spectrophotometer and data-analysis techniques described previously (Reid & Mauk, 1982). $\text{Fe}(\text{EDTA})^{2-}$ solutions were prepared anaerobically as described by Wherland *et al.* (1975). Reduction of metsulphmyoglobin and native metmyoglobin (2–4 μM before the mixing in the stopped-flow apparatus) was monitored at 404 and 410 nm respectively with reductant in pseudo-first-order excess over protein.

Results

The spectrum of reduced sulphmyoglobin prepared as described above has been presented and discussed previously (Andersson *et al.*, 1984b). The

principal feature of this spectrum is an enhanced A_{617}/A_{561} ratio relative to that described by Berzofsky *et al.* (1971). Reduction of both native metmyoglobin and metsulphmyoglobin with $\text{Fe}(\text{EDTA})^{2-}$ produced first-order plots that were linear for at least 85% of the reaction. The observed rates (k_{obs}) obtained for both proteins were linearly dependent on reductant concentrations, as demonstrated by a representative experiment with metsulphmyoglobin shown in Fig. 1. The second-order rate constant calculated from these data is $1.08 (\text{s.d.} \pm 0.03) \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ [pH 7.0 (phosphate), 10.1, 25°C].

The pH-dependence of this reaction is shown in Fig. 2, along with data for native myoglobin. As $\text{Fe}(\text{EDTA})^{2-}$ does not have an ionizable functional group with a pK_a in this range (Schwarzenbach & Heller, 1951; Belcher *et al.*, 1955), this variation in rate can be interpreted (Reid & Mauk, 1982) in terms of a redox-linked functional group on sulphmyoglobin. With this assumption, we can calculate the rate of reduction of the protonated form of the protein as $1.42 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ and the rate of reduction of the unprotonated form as $4.7 \times 10 \text{ M}^{-1} \cdot \text{s}^{-1}$ [25°C (phosphate), 10.5]. The pK_a for this process is 6.8. The line drawn through the data in Fig. 2(a) represents the non-linear least-squares fit to the observed second-order rate constants that is consistent with these calculated parameters. An identical analysis of native metmyoglobin is shown in Fig. 2(b) for comparison. In this case, $\text{pK}_a = 5.8$, $k_a = 60 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_b = 9 \text{ M}^{-1} \cdot \text{s}^{-1}$.

Fig. 1. Dependence of observed rate of reduction of metsulphmyoglobin by $\text{Fe}(\text{EDTA})^{2-}$ on reductant concentration [pH 7.0 (phosphate), 10.1, 25°C]

The continuous line represents a weighted linear least-squares fit to the data. The error bars for the rate constants are no larger than the dimensions of the symbols and are not shown.

Fig. 2. Dependence of second-order rate constants (k_{12}) for the reduction of metsulphmyoglobin (a) and native metmyoglobin (b) by Fe(EDTA)^{2-} on pH [10.5 (phosphate), 25°C]

The continuous line is a non-linear least-squares fit to the data calculated as described in the text.

The ionic-strength-dependences of metsulphmyoglobin and native metmyoglobin reduction by Fe(EDTA)^{2-} are shown in Fig. 3. These data may be analysed by fitting them to a form of the Debye-Hückel equation (e.g., Reid & Mauk, 1982). If we assume a radius of 1.84 nm for myoglobin (Mauk & Gray, 1979), the resulting apparent electrostatic charges are 7 (S.D. ± 1) for native myoglobin and 6.2 (S.D. ± 0.8) for metsulphmyoglobin [pH 7.0 (phosphate), 25°C].

The activation parameters for metsulphmyoglobin reduction by Fe(EDTA)^{2-} were determined from an Eyring plot as shown in Fig. 4. For this reaction, $\Delta H^\ddagger = 41.4$ (S.D. ± 0.8) kJ/mol [9.9 (± 0.2) kcal/mol] and $\Delta S^\ddagger = -66$ (S.D. ± 4) $\text{J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ [-16 (± 1) e.u.] [pH 7.0 (phosphate), 10.1]. These values are very similar to those reported previously for the native protein by Cassatt *et al.* (1975) [$\Delta H^\ddagger = 50$ (S.D. ± 4) kJ/mol [12 (± 1) kcal/mol] and $\Delta S^\ddagger = -50$ (S.D. ± 20) $\text{J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ [-13 (± 5) e.u.]].

Fig. 3. Ionic-strength-dependence of second-order rate constants for the reduction of metsulphmyoglobin (a) and native metmyoglobin (b) by Fe(EDTA)^{2-} [pH 7.0 (phosphate), 25°C]

The continuous line is a non-linear least-square fit to the data calculated as described in the text. Error bars are displayed only where they extend beyond the dimensions of the symbol.

Discussion

The method described here for purification of sulphmyoglobin appears to function by allowing removal of native myoglobin and possibly side products arising from the preparation of the modified protein. That such products should be formed in the generation of sulphmyoglobin by the procedure used here is not surprising, because the first step involves treatment of metmyoglobin with 30% H_2O_2 .

Attempts to reduce metsulphmyoglobin with dithionite generated a protein product with a relatively poor purity ratio, in agreement with the observations made by Nicholls (1961). We suspect that peroxide formation arising from use of dithionite results in oxidation of the modified prosthetic group and regeneration of myoglobin. Use of the mild reducing agent Fe(EDTA)^{2-} appears to avoid this problem and allowed us to

Fig. 4. Eyring plot for the reduction of met-sulphmyoglobin by Fe(EDTA)^{2-} [pH 7.0 (phosphate), 10.1, 25°C]. The continuous line is a weighted linear least-squares fit to the data. Error bars are smaller than the dimensions of the symbols.

purify the modified protein in the more stable oxidized state while retaining the ability to assess the quality of the product spectrophotometrically in the reduced state.

The rate of reduction of met-sulphmyoglobin by Fe(EDTA)^{2-} is 2–7 times that observed for reduction of the native protein under any one set of experimental conditions. The results observed here for native myoglobin are consistent with the work of Cassatt *et al.* (1975). One major contribution to the enhanced rates observed for sulphmyoglobin is the relatively high mid-point reduction potential of sulphmyoglobin. The reduction potential of native horse heart myoglobin is approx. (versus normal hydrogen electrode; pH 7, 10.2, 30°C) (Taylor & Morgan, 1942; Behlke & Scheler, 1961), and Nicholls (1961) has estimated the reduction potential of sulphmyoglobin to be 130 mV (versus normal hydrogen electrode; pH 6.5, 10.036, 20°C). Attempts to obtain a more accurate mid-point reduction potential for sulphmyoglobin by spectro-electrochemical techniques in conjunction with the present kinetic studies were unsuccessful, owing to the formation of native myoglobin during the course of the potentiometric titrations. Nevertheless, the reduction potential for sulphmyoglobin reported by Nicholls (1961) and those reported for myoglobin (Taylor & Morgan, 1942; Behlke & Scheler, 1961) can be used with the potentials reported for the Fe(EDTA)^- – Fe(EDTA)^{2-} couple (Schwarzenbach & Heller, 1951; Belcher *et al.*, 1955; Reid, 1984) to correct (Reid, 1984) the second-order rate constants observed here under the most comparable experimental conditions to a thermodynamic driving force of zero. The results of this calculation show that native metmyoglobin reacts about 2.5 times as rapidly as sulphmyoglobin. We conclude, therefore, that the electron-

transfer reactivity of sulphmyoglobin is comparable with or slightly lower than that of native myoglobin.

The pH-dependence observed for the reduction of native metmyoglobin by Fe(EDTA)^{2-} (Fig. 2) is presumably attributable to thermodynamic factors or kinetic factors or both. The thermodynamic contribution to this effect may be assessed because the variation of the reduction potential of horse heart myoglobin over the pH range studied here is known (Taylor & Morgan, 1942; Behlke & Scheler, 1961), and measurements by Reid (1984) have determined the reduction potential of the Fe(EDTA)^- – Fe(EDTA)^{2-} couple over the same range of pH. Although the effect of pH on both equilibria is small, we have used these values to correct the second-order rate constants observed at various values of pH to a thermodynamic driving force of zero (Reid, 1984). This correction decreases but does not eliminate the dependence of these rate constants on pH, and suggests that both kinetic and thermodynamic factors contribute to the observed dependence. It seems likely that, if a similar analysis were possible for the data obtained for sulphmyoglobin, it would yield qualitatively similar results. In this regard, we note that the $\text{p}K_a$ for the water molecule bound to the iron atom of met-sulphmyoglobin is significantly lower than that of native myoglobin (Berzofsky *et al.*, 1971). This should result in a relatively greater dependence of the reduction potential of sulphmyoglobin on pH over the range of interest here. Such behaviour may help to explain the difference in $\text{p}K_a$ values observed for the rate of reduction of native myoglobin and sulphmyoglobin by Fe(EDTA)^{2-} (Fig. 2).

The electrostatic charges for native myoglobin and sulphmyoglobin estimated from the ionic-strength-dependences are within error of each other. We note, however, that the calculated charge for the native protein, +7, compares with a value of +4.5 estimated naively from the protein sequence (Wherland & Gray, 1977) and with a value of +0.2 (pH 6.6) calculated from titration data (McLennan, 1984, and references cited therein). In general, protein charges estimated from data of the type reported here have been found to correlate reasonably well with those estimated from protein sequences (Feinberg & Ryan, 1981). This agreement is surprising, because many buried functional groups in proteins cannot be expected to contribute significantly to the surface electrostatic charge that would be expected to influence rates of reaction with charged oxidation–reduction reagents. This discrepancy is clearly apparent in the case of myoglobin, as indicated by the charge determined from titration data described above and from the demonstration that

the isoelectric pH of horse heart myoglobin is near neutrality (Radola, 1973). The present electrostatic charges estimated for native myoglobin and sulphmyoglobin may be aberrantly high as the result of inadequacies in the Debye-Hückel analysis. Such deficiencies would include the failure of the known ellipsoidal shape of myoglobin to conform to the spherical shape assumed by this analytical approach. Alternatively, a single, positively charged, functional group strategically located at the site of electron transfer between Fe(EDTA)²⁻ and myoglobin could produce such a result, as we have previously found for cytochrome *b*₅ (Reid *et al.*, 1984). This latter possibility must await crystallographic analysis of horse heart myoglobin for meaningful evaluation. Regardless of the origin of the discrepancy between the expected and the apparent electrostatic charge, however, it is sufficient at present to observe that sulphmyoglobin and native myoglobin demonstrate equivalent electrostatic properties in their reactions with Fe(EDTA)²⁻.

In conclusion, we have described a method for purification of metsulphmyoglobin and have compared the kinetics of its reduction by Fe(EDTA)²⁻ with those of native myoglobin. Although subtle differences were observed in the kinetic behaviour of these proteins, the conversion of the porphyrin prosthetic group into a chlorin appears to generate relatively little change in the inherent electron-transfer reactivity of the protein in the uptake of a single electron. As naturally occurring metallo-chlorin-containing proteins generally catalyse reactions that involve multi-electron-transfer processes, the likelihood of some significant functional role for the prosthetic group in such reactions has been suggested (Chang, 1982). The present results certainly do not rule out this possibility.

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