Kinetic studies of the reduction of neutrophil cytochrome *b*-558 by dithionite

Irit AVIRAM and Michaela SHARABANI

Department of Biochemistry, George S. Wise Faculty of Life Sciences, University of Tel-Aviv, Ramat-Aviv, Tel-Aviv 69978, Israel

The reduction with dithionite of neutrophil cytochrome *b*-558, implicated in superoxide generation by activated neutrophils, was investigated by a stopped-flow technique in non-ionic-detergent extracts of the membranes and in crude membrane particles. The dependence of the pseudo-first-order rate constants on the concentration of dithionite was consistent with a mechanism of reduction that involves the dithionite anion monomer $\text{SO}_2^{\cdot-}$ as the reactive species. The estimated second-order rate constant was $7.8 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ for Lubrol PX-solubilized cytochrome *b*-558 and $5.1 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the membrane-bound protein. The similarity of the kinetic constants suggests that solubilization did not introduce gross changes in the reactive site. Imidazole and *p*-chloromercuribenzoate, known as inhibitors of NADPH oxidase, did not affect significantly cytochrome *b*-558 reduction rates. The reaction rate of cytochrome *b*-558 with dithionite exhibited a near-zero activation energy. The first-order rate constant for reduction decreased with increasing ionic strength, indicating a positive effective charge on the reacting protein.

INTRODUCTION

Cytochrome *b*-558 has been implicated as an essential component of the plasma-membrane superoxide-generating enzymic complex of activated neutrophils, known as the NADPH oxidase (Segal & Jones, 1979a; Cross *et al.*, 1982b; Segal *et al.*, 1983; Bellavite *et al.*, 1983; Gabig & Lefker, 1984). It has been shown to reside in the plasma membrane and specific-granule membranes of neutrophils (Segal & Jones, 1979b; Borregaard *et al.*, 1983). Evidence for activation-linked translocation of cytochrome *b*-558 from specific granules to the plasma membrane has been presented (Borregaard *et al.*, 1983), but the originally suggested dependence of the activation of NADPH oxidase on this traffic has been questioned (Parkos *et al.*, 1985).

Cytochrome *b*-558 was characterized as a haemoprotein carrying a non-covalently bound haem IX (Pember *et al.*, 1984). The bands of the ferrous form at 558, 528 and 428 nm are typical of a low-spin-haem iron protein. The low reactivity of cytochrome *b*-558 with haem-binding ligands, e.g. cyanide, azide (Pember *et al.*, 1984; Cross *et al.*, 1985) and CO (Cross *et al.*, 1982a; Bellavite *et al.*, 1983), suggests hexa-co-ordination of the metal ion. In spite of the low affinity for CO, ferrocytochrome *b*-558 is oxidized rapidly by molecular O_2 (Cross *et al.*, 1981, 1982a). This property, as well as the low oxidation-reduction potential of -245 mV (Cross *et al.*, 1981; Bellavite *et al.*, 1983), renders cytochrome *b*-558 capable of functioning as a terminal oxidase that reduces O_2 to $\text{O}_2^{\cdot-}$ ions at the expense of electrons delivered from NADPH by a flavoprotein component (Babior & Kipnes, 1977; Cross *et al.*, 1982b; Gabig, 1983).

A definite verification of the function of cytochrome *b*-558 in the superoxide-generating activity of activated neutrophils requires demonstration of its presence in pure preparations of oxidase or reconstitution of oxidase activity from pure components including cytochrome *b*-558. Better characterization of the chemical reactivity of the latter will also contribute to the understanding of

its biological activity. In view of this we have investigated the mechanism of reduction of cytochrome *b*-558 by dithionite, a powerful reducing agent, used routinely in work with electron-transfer proteins (Mayhew & Massey, 1973; Lambeth & Palmer, 1973; Hintz & Peterson, 1980; Davydov *et al.*, 1985). Kinetic studies of haemoproteins indicated that reduction by aqueous solutions of $\text{Na}_2\text{S}_2\text{O}_4$ occurs mainly by a mechanism consistent with participation of the product of dissociation of the $\text{S}_2\text{O}_4^{2-}$ ion, namely with the anion monomer $\text{SO}_2^{\cdot-}$. Similar results were obtained with particulate and solubilized preparations of cytochrome *b*-558. These studies, reported below, were conducted with unpurified extracts or membrane particles, to reflect as closely as possible properties of the haemoprotein *in situ* and to avoid artifacts introduced during the purification.

EXPERIMENTAL

Human neutrophils were prepared from fresh buffy coats by dextran sedimentation of erythrocytes and subsequent lysis as described by Curnutte & Babior (1974). Cells suspended in 0.34 M-sucrose containing 1 mM-phenylmethanesulphonyl fluoride were disrupted by sonication. Nuclei and whole cells were sedimented at 250 g for 10 min, and supernatants were centrifuged at 144000 g_{av} in the Ti 60 rotor of the L8 70 Beckman ultracentrifuge for 30 min. Pelleted membranes were resuspended in appropriate buffers and used immediately or stored in 0.34 M-sucrose containing 1 mM-phenylmethanesulphonyl fluoride at -70°C . Before extraction of cytochrome *b*-558, the membranes were washed with Krebs-Ringer phosphate solution (15.7 mM-sodium phosphate buffer, pH 7.4, containing 131 mM-NaCl, 5.2 mM-KCl, 1.3 mM-MgSO₄, 0.9 mM-CaCl₂ and 5 mM-glucose). Extracts of cytochrome *b*-558 were prepared by the addition to the membranes of 1% (w/v) Lubrol PX/0.1 M-NaCl solution buffered at pH 7.5 with either 15 mM-Tris/HCl or 10 mM-Hepes/NaOH buffer. The

extraction was performed at a concentration of 3 mg of the membrane protein/ml. The suspensions were stirred on ice for 30 min and centrifuged at $144000\ g_{av.}$ in the Ti 60 rotor of the centrifuge for 45 min.

$Na_2S_2O_4$ for use in the stopped-flow experiments was freshly dissolved in buffers through which a stream of N_2 was passed for 15 min and kept under N_2 or in air-tight syringes. Its concentration was evaluated on the basis of the dry weight of the reagent. Cytochrome *b*-558 solutions were also deoxygenated by passing N_2 through the solution. In view of the large excess of dithionite used in the stopped-flow experiments strict anaerobiosis was not attempted. The experiments were carried out in the Aminco-Morrow dual-wavelength spectrophotometer at 428 nm–412 nm at constant temperature. The pseudo-first-order rate constants for the reduction were calculated from semi-logarithmic plots of the reaction traces or were evaluated from the $t_{1/2}$ times of the reactions. Each constant was based on at least five determinations. When the effect of ionic strength was investigated, NaCl was added to the dithionite-containing solutions. In experiments with *p*-chloromercuribenzoate and imidazole, the inhibitors were added initially to the Lubrol PX extracts at 1 mM and 0.4 M (pH 7.5) respectively.

Kinetic absorption-difference spectra were calculated from the oscilloscope traces by measuring the total transmittance change at each wavelength. Absorption spectra were recorded on the Cary 118 recording spectrophotometer.

Protein was determined by the procedure of Bradford (1976), with bovine serum albumin as standard.

RESULTS

Solubilization of neutrophil cytochrome *b*-558

Neutrophil membrane pellets were treated with the non-ionic detergent Lubrol PX in 0.1 M-NaCl solution buffered at pH 7.5. Fig. 1 depicts the absorption-difference reduced-minus-oxidized spectrum of the supernatant. The bands at 558, 528 and 428 nm are characteristic of cytochrome *b*-558 and indicate that our solubilization procedure permitted almost complete separation from the main haemoprotein of neutrophils, myeloperoxidase, which remained associated with the membrane pellet. In view of this, most of the kinetic work was carried out on the Lubrol PX extracts.

Stopped-flow measurements of the reaction of cytochrome *b*-558 with dithionite

Lubrol PX extracts were mixed in the stopped-flow apparatus with large excess of dithionite, and the reaction was followed at 428 nm–412 nm, where reduction is accompanied by the largest change in the absorption coefficient (Fig. 1). The oscilloscope trace of the reaction is shown in Fig. 2. The reaction appears monophasic and of first-order with respect to the reacting chromophore. The kinetic difference spectrum constructed from the oscilloscope traces in the Soret-band region (Fig. 3a) clearly exhibits the 428 nm band of reduced cytochrome *b*-558 and indicates that transmittance changes recorded at 428 nm–412 nm indeed reflect its reduction.

Dependence of reaction rates on the concentration of dithionite

At the pseudo-first-order conditions of the reaction, with dithionite in large excess, the relationship between

Fig. 1. Absorption-difference dithionite-reduced-minus-oxidized spectrum of Lubrol PX extract of neutrophil membranes

Neutrophil membranes (3 mg of protein/ml) were extracted with 1% Lubrol PX/0.1 M-NaCl in 15 mM-Tris/HCl buffer, pH 7.5, as described in the text.

Fig. 2. Oscilloscope trace of the reaction of Lubrol PX-solubilized cytochrome *b*-558 with dithionite

A solution of 20 mM-dithionite in 0.1 M-Tris/acetate buffer, pH 7.5, was mixed with neutrophil membranes suspended in 1% Lubrol PX/0.1 M-NaCl in 10 mM-Hepes/15 mM-Tris/HCl buffer, pH 7.5. Observation wavelengths, 428 nm–412 nm; transmittance, 5 mV/division; time scale, 20 ms/division; temperature, 23 °C.

the concentration of the reductant and the observed rate constants, $k_{obs.}$, was not linear (Fig. 3b). Straight lines were, however, obtained when $k_{obs.}$ values were plotted against the square root of dithionite concentration (Fig. 3c). This finding is consistent with a reaction mechanism which involves the dithionite anion monomer $SO_2^{\cdot-}$ as the reactive species (Lambeth & Palmer, 1973) according to the following equations:

Fig. 3. Dependence of the pseudo-first-order rate constants for the reduction of cytochrome *b*-558 on the concentration of dithionite

(a) Kinetic difference spectrum obtained from the oscilloscope traces by varying the wavelength of the observation monochromator. The reference monochromator was set at 412 nm. (b) Plot of k_{obs} against dithionite concentration. (c) Plot of k_{obs} against the square root of dithionite concentration. Δ , neutrophil membrane particles suspended in Krebs-Ringer phosphate buffer; $\circ$, same in the presence of 1% Lubrol PX; $\bullet$, Lubrol PX extract. The temperature was 23 °C.

$\text{SO}_2^{\cdot-}$ is formed through dissociation of dithionite, as shown below:

On the basis of these equations, the absolute rate constant for the reduction may be calculated:

$$k = k_{\text{obs.}} / [K(\text{S}_2\text{O}_4^{2-})]^{1/2} \quad (3)$$

This constant is listed in Table 1 in comparison with analogous values reported for other haemoproteins. The same value was obtained when, instead of the extract, crude neutrophil membranes in detergent extraction buffer were mixed with excess dithionite (Fig. 3). When Lubrol PX was omitted, the reaction followed a similar time course, though calculated rates were somewhat lower (Table 1 and Fig. 3). With several preparations of membranes the presence of a small fraction (approx. 15%) of slowly reacting material could be detected. The origin of this finding was not further investigated.

In two experiments cytochrome *b*-558 was extracted from phorbol 12-myristate 13-acetate-activated neutrophils ($1 \mu\text{g}/10^8$ cells, 5 min at 37 °C). Its reaction

Table 1. Rate constants for reaction of $\text{SO}_2^{\cdot-}$ with haemoproteins

	k ($\text{M}^{-1} \cdot \text{s}^{-1}$)
Cytochrome <i>b</i> -558 (solubilized)	7.8×10^6 *
Cytochrome <i>b</i> -558 (membrane-bound)	5.1×10^6 *
Cytochrome <i>c</i>	3.9×10^7 †
Horse metmyoglobin	2.7×10^6 †
Cytochrome <i>b</i> ₅ (membrane-bound)	3.4×10^6 ‡
Cytochrome <i>P</i> -450 _{cam}	6.4×10^4 §

* k was obtained from the slope of the plot in Fig. 3(c) and the reported value of $K = 1.45 \times 10^{-9}$ M at 10.1 (Chien & Dickinson, 1978).

† Obtained by Lambeth & Palmer (1973).

‡ Calculated from the data of Davydov *et al.* (1985) by using $K = 1.45 \times 10^{-9}$ M.

§ Reported by Hintz & Peterson (1980) at 12.

Fig. 4. Arrhenius plot of the reduction of cytochrome *b*-558 by dithionite

$k_{\text{obs.}}$ values were measured at 5 mM-dithionite in Lubrol PX extracts of neutrophil membranes. Dithionite was in 50 mM-potassium phosphate buffer, pH 7.5.

with dithionite was unaffected by the previous stimulation (results not shown).

Effects of *p*-chloromercuribenzoate and imidazole on the reaction rates

p-Chloromercuribenzoate (Bellavite *et al.*, 1983; Cross *et al.*, 1985) and imidazole (Iizuka *et al.*, 1985) have been described as inhibitors of the NADPH oxidase activity. This inhibition was attributed to interaction with the cytochrome *b*-558 moiety of the enzyme. The effects of these compounds on the reduction rates were tested in stopped-flow experiments. At 0.5 mM-*p*-chloromercuribenzoate and 0.2 M-imidazole $k_{\text{obs.}}$ values determined at 5 mM-dithionite at 24 °C were $21.2 \pm 0.7 \text{ s}^{-1}$ and $18.2 \pm 0.5 \text{ s}^{-1}$ respectively. $k_{\text{obs.}}$ for untreated solubilized cytochrome *b*-558 was $25.1 \pm 0.8 \text{ s}^{-1}$.

Dependence of the reaction rates on temperature

Stopped-flow experiments were performed at different temperatures and constant 5 mM-dithionite concentration. An Arrhenius plot of the $k_{\text{obs.}}$ values is shown in Fig. 4. The apparent energy of activation was $1.85 \text{ kJ} \cdot \text{mol}^{-1}$.

Ionic-strength-dependence of the reaction of cytochrome *b*-558 with dithionite

The effect of ionic strength was studied by manipulation of the NaCl content in the dithionite solution. The results indicated decrease of the observed pseudo-first-order rate constants with ionic strength (Fig. 5a). This could simply reflect diminished concentration of the monomer SO_3^{2-} , due to the reported suppression of dithionite dissociation at high ionic strength (Lambeth & Palmer, 1973; Chien & Dickinson, 1978). This possibility was checked by calculating the true rate constant, k , at each ionic strength introducing values of K estimated by Chien & Dickinson (1978). The corrected plot shown in Fig. 5(b) retains its negative slope with respect to I , suggesting interaction of a positively charged site on the molecule of cytochrome *b*-558 with the negatively charged reductant.

DISCUSSION

The reduction of neutrophil cytochrome *b*-558 by dithionite occurs by a mechanism consistent with the participation of SO_3^{2-} as the reducing species (Fig. 3). Similarity of the kinetic constants obtained in particulate and detergent-solubilized preparations suggests similar conformation of the reacting site. Moreover, since Lubrol PX extracts do not contain myeloperoxidase (Fig. 1), it may be also concluded that its presence does not affect reactivity of cytochrome *b*-558 and that no interaction between these two haemoproteins takes place during the reaction with dithionite. The slight increase in the rates upon solubilization (Fig. 3) may reflect increased exposure to the reductant. The high rates of the reaction of cytochrome *b*-558 as compared with other haemoproteins (Table 1) reflect good accessibility to the hydrophilic and charged reagent.

Fig. 5. Dependence of reduction rates on ionic strength of the medium

(a) $k_{\text{obs.}}$ values determined at 10 mM-dithionite. (b) Second-order rate constants were calculated according to eqn. (3) by using values of K reported by Chien & Dickinson (1978).

Both *p*-chloromercuribenzoate and imidazole were described as inhibitors of the NADPH oxidase. The effect of the former on the oxidation–reduction potential (Bellavite *et al.*, 1983) and of the latter on the low-temperature absorption spectrum of cytochrome *b*-558 (Iizuka *et al.*, 1985) implicated cytochrome *b*-558 in this inhibition. The finding that both compounds had only a minor effect on the kinetics of reduction therefore seems surprising, since it implies that reduction of cytochrome *b*-558 by SO_2^{2-} is insensitive to oxidation–reduction potential and to the origin of the iron ligand at the sixth co-ordination position of the metal.

The apparent energy of activation $\Delta E_{\text{obs.}}$ of $1.85 \text{ kJ} \cdot \text{mol}^{-1}$ (Fig. 4) for the reaction of cytochrome *b*-558 with the dithionite monomer deserves special comment. This value includes contribution of the dependence of dithionite monomerization on temperature. When this effect of temperature is taken into account according to the equation $\Delta E_{\text{obs.}} = \Delta E + \frac{1}{2}\Delta H$ (Chien & Dickinson, 1978) and by using $5.09 \text{ kJ} \cdot \text{mol}^{-1}$ as ΔH for dithionite monomerization, ΔE of $-0.69 \text{ kJ} \cdot \text{mol}^{-1}$ is obtained. Small or slightly negative energies of activation are not inconsistent with outer-sphere electron-transfer processes (Marcus & Sutin, 1975, 1985). Interestingly, ΔE for the reduction of cytochrome *P*-450 LM2 by SO_2^{2-} calculated from the reported $\Delta E_{\text{obs.}}$ of $2.4 \text{ kJ} \cdot \text{mol}^{-1}$ (Davydov *et al.*, 1985) is also zero.

The importance of electrostatic interactions in electron-transfer reactions is well documented. The simplest equation relating kinetic constants to ionic strength is the limiting form of the Brönsted–Debye–Hückel equation (Frost & Pearson, 1961):

$$\log k = \log k_0 + 2AZ_1Z_2I^{1/2} \quad (4)$$

where the value of A is 0.509 in aqueous solutions at 25°C , Z_1 and Z_2 are the effective charges on the oxidation–reduction partners and k_0 is the second-order rate constant at zero ionic strength. This equation predicts a linear dependence of $\log k$ on the square root of ionic strength, and is valid for low ionic strength and for relatively small radii of the reacting molecules.

For proteins interacting with small molecules investigation of the dependence of the reaction rates on ionic strength permitted evaluation of the effective charge on the macromolecule. Studies carried out on cytochrome *c* at very low ionic strength demonstrated that the effective charge obtained reflected the net charge of the protein at the pH of the experiment as calculated on the basis of its amino acid composition (Koppenol *et al.*, 1976; Goldkorn & Schejter, 1979). In other cases (Cusanovich, 1978) smaller charges than expected from the composition were found and were interpreted as an indication for the magnitude of charge at the specific electron-transfer site. In the case of cytochrome *b*-558 the negative slope of the plot shown in Fig. 4 implies a positive charge on the haemoprotein. Since our experiments were performed in a detergent solution at relatively high ionic strength and since the dimensions of the cytochrome *b*-558 molecule are unknown, the assumptions necessary for quantitative evaluation of the effective charge may not be applicable. The positive sign of the charge may indicate the charge at the site where electrons are transferred from the negatively charged SO_2^{2-} to the protein or alternatively reflect the overall positive charge on the latter. The possibility that the cytochrome *b*-558 molecule at pH 7.5 is positive is borne out by the finding that it is retained

on cation-exchange columns at this pH (Harper *et al.*, 1984).

Our studies were performed with unpurified preparations of cytochrome *b*-558. This approach is justified by the similarity of the kinetic constants determined in particulate and solubilized preparations (Fig. 3). Moreover, by using the crude extracts, artifacts introduced during the purification procedure may be omitted. Thus three different methods for the purification of cytochrome *b*-558 have recently been published (Harper *et al.*, 1984; Pember *et al.*, 1984; Lutter *et al.*, 1985). Products of these purification methods, though apparently homogeneous with respect to contaminating proteins, differ markedly in their molecular masses, redox properties and composition. Thus, in view of lack of a definite structural characterization of cytochrome *b*-558, the use of unpurified preparations seems preferable.

This work was supported by The Council of Tobacco Research U.S.A., Grant 1651.

REFERENCES

- Babior, B. M. & Kipnes, R. S. (1977) *Blood* **50**, 517–524
- Bellavite, P., Cross, A. R., Serra, M. C., Davoli, A., Jones, O. T. G. & Rossi, F. (1983) *Biochim. Biophys. Acta* **746**, 40–46
- Borregaard, N., Heiple, J. M., Simons, E. R. & Clark, R. A. (1983) *J. Cell Biol.* **97**, 52–61
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Chien, J. C. & Dickinson, L. C. (1978) *J. Biol. Chem.* **253**, 6965–6972
- Cross, A. R., Jones, O. T. G., Harper, A. M. & Segal, A. W. (1981) *Biochem. J.* **194**, 599–606
- Cross, A. R., Higson, F. K., Jones, O. T. G., Harper, A. M. & Segal, A. W. (1982a) *Biochem. J.* **204**, 479–485
- Cross, A. R., Jones, O. T. G., Garcia, R. & Segal, A. W. (1982b) *Biochem. J.* **208**, 759–763
- Cross, A. R., Parkinson, J. F. & Jones, O. T. G. (1985) *Biochem. J.* **226**, 881–884
- Curnutte, J. T. & Babior, B. M. (1974) *J. Clin. Invest.* **83**, 1662–1672
- Cusanovich, M. A. (1978) *Bioinorg. Chem.* **4**, 117–145
- Davydov, D. R., Karyakin, A. V., Binar, B., Kurganov, B. I. & Archakov, A. I. (1985) *Eur. J. Biochem.* **150**, 155–159
- Frost, A. A. & Pearson, R. G. (1961) *Kinetics and Mechanism*, 2nd edn., pp. 150–155, John Wiley and Sons, New York
- Gabig, T. G. (1983) *J. Biol. Chem.* **258**, 6352–6356
- Gabig, T. G. & Lefker, B. A. (1984) *J. Clin. Invest.* **73**, 701–705
- Goldkorn, T. & Schejter, A. (1979) *J. Biol. Chem.* **254**, 12562–12566
- Harper, A. M., Dunne, M. J. & Segal, A. W. (1984) *Biochem. J.* **219**, 519–527
- Hintz, M. J. & Peterson, J. A. (1980) *J. Biol. Chem.* **255**, 7217–7325
- Iizuka, T., Kanegasaki, S., Makina, R., Tanaka, T. & Ishimura, Y. (1985) *Biochem. Biophys. Res. Commun.* **130**, 621–626
- Koppenol, W. H., van Buuren, K. J. H., Butler, J. & Braams, R. (1976) *Biochim. Biophys. Acta* **449**, 157–168
- Lambeth, D. O. & Palmer, G. (1973) *J. Biol. Chem.* **248**, 6095–6103
- Lutter, R., van Schaik, M. L. J., van Zwieten, R., Wever, R., Roos, D. & Hamers, M. N. (1985) *J. Biol. Chem.* **260**, 2237–2244
- Marcus, R. A. & Sutin, N. (1975) *Inorg. Chem.* **14**, 213–316
- Marcus, R. A. & Sutin, N. (1985) *Biochim. Biophys. Acta* **811**, 265–322

- Mayhew, S. G. & Massey, V. (1973) *Biochim. Biophys. Acta* **315**, 181–190
- Parkos, C. A., Cochrane, C. G., Schmitt, M. & Jesaitis, A. J. (1985) *J. Biol. Chem.* **260**, 6541–6547
- Pember, S. O., Heyl, B. L., Kinkade, J. M., Jr. & Lambeth, J. D. (1984) *J. Biol. Chem.* **259**, 10590–10595
- Segal, A. W. & Jones, O. T. G. (1979a) *Biochem. Biophys. Res. Commun.* **88**, 130–134
- Segal, A. W. & Jones, O. T. G. (1979b) *Biochem. J.* **182**, 185–188
- Segal, A. W., Cross, A. R., Garcia, R. C., Borregaard, N., Valerius, N. H., Soothill, J. F. & Jones, O. T. G. (1983) *N. Engl. J. Med.* **308**, 245–251

Received 9 December 1985/4 February 1986; accepted 1 April 1986