ACKNOWLEDGMENTS

We thank Drs. G. Hölzemann, H. U. Gremlich, and H.-E. Radunz for interesting and stimulating discussion and for providing substance P.

Registry No. SP, 33507-63-0; POPG, 81490-05-3; POPC, 26853-31-6; POPG-POPC mixture, 118460-95-0.

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Effects of Oxygen on the Metabolism of Nitroxide Spin Labels in Cells†

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Received June 21, 1988; Revised Manuscript Received October 11, 1988

ABSTRACT: The products of the reduction of nitroxides in cells are the corresponding hydroxylamines, which cells can oxidize back to the nitroxides in the presence of oxygen. Both the reduction of nitroxides and the oxidation of hydroxylamines are enzyme-mediated processes. For lipid-soluble nitroxides, the rates of reduction are strongly dependent on the intracellular concentration of oxygen; severely hypoxic cells reduce nitroxides more rapidly than cells supplied with oxygen. In contrast, the rates of oxidation of hydroxylamines increase smoothly with increasing intracellular oxygen concentration up to 150 μM. In order to separate the effects on the rates of metabolism of nitroxides due directly to oxygen from effects due to the redox state of enzymes, we studied the cells under conditions in which each of these variables could be changed independently. Oxygen affects the metabolism of these nitroxides primarily by interacting with cytochrome *c* oxidase to change the redox state of the enzymes in the respiratory chain. Our results are consistent with the conclusions that in these cells reduction of lipophilic nitroxides occurs at the level of ubiquinone in the respiratory chain in mitochondria, and oxidation of the corresponding hydroxylamines occurs at the level of cytochrome *c* oxidase.

Nitroxides, in addition to their role as probes of molecular dynamics of membranes and cells (Berliner, 1976, 1979; Swartz & Swartz, 1983; Morse, 1985), might also serve as effective contrast or imaging agents for NMR (Brasch et al., 1983; Brasch, 1983; Swartz et al., 1986a,b; Swartz, 1988) or ESR (Berliner & Fujii, 1985; Bacic et al., 1988) imaging and in vivo spectroscopy. An attractive feature of this idea is that the reduction of nitroxides, heretofore considered a liability, can be used to advantage, since the reduction of nitroxides and oxidation of hydroxylamines by cells are strongly dependent on the concentration of oxygen (Swartz et al., 1986a,b; Swartz,

† This work was supported by NIH Grants GM-35534 and GM-34250 and used the ESR Center facilities supported by NIH Grant RR-01811. J.F.G. received support from NIH Training Grant CA-09067.

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0006-2960/89/0428-2496$01.50/0

1988). Thus, the metabolism of nitroxides can be used to measure oxygen concentrations and related redox metabolism, with the potential to detect hypoxia in vivo.

Although the metabolism of nitroxides has been studied extensively (Stier & Reitz, 1971; Giotta & Wang, 1972; Stier & Sackmann, 1973; Baldassare et al., 1974; Rosen & Rauckman, 1977; Quintanilha & Packer, 1977; Perkins et al., 1980; Mehlhorn & Packer, 1982; Couet et al., 1984, 1985; Chapman et al., 1985; Chen & McLaughlin, 1985; Swartz et al., 1986a,b; Eriksson et al., 1987; Keana et al., 1987; Chen et al., 1988), there is a paucity of quantitative and mechanistic studies on the effect of oxygen on this metabolism. The rates of reduction of most nitroxides are more rapid in hypoxic cells than in normoxic cells (Swartz et al., 1986a,b). The products of the reduction of nitroxides in cells are the corresponding hydroxylamines (Chen & Swartz, 1988; Chen et al., 1988). The reduction of nitroxides has been suggested to occur at the level of the ubiquinone in the respiratory chain in isolated submitochondrial particles (Quintanilha & Packer, 1977) and rabbit spermatozoa (Chapman et al., 1985). In cultured mammalian cells, it appears that the reduction of lipophilic nitroxides principally occurs at the level of the ubiquinone in the respiratory chain in mitochondria (Chen et al., 1988). In liver microsomes the reduction of nitroxides appears to be mediated by the cytochrome P-450 system (Rosen & Rauckman, 1977).

Reduction of lipophilic nitroxides is reversible in the presence of oxygen (Chen & Swartz, 1988). It has been suggested that mitochondrial cytochrome oxidase is involved in much of the observed oxidation of lipophilic hydroxylamines in mammalian cells because cyanide and azide can inhibit this reaction but antimycin A and myxothiazol do not (Chen & Swartz, 1988). Thus, the presence of oxygen can affect the rates of both the oxidation of hydroxylamines and the reduction of nitroxides.

The objectives of this study are to determine the dependence of intracellular oxygen on the rates of reduction of nitroxides and oxidation of hydroxylamines and to elucidate the mechanisms by which oxygen affects these rates. We approached these objectives by (1) measuring rates of reduction of nitroxides and oxidation of hydroxylamines as a function of the concentration of intracellular oxygen; (2) using metabolic inhibitors, uncouplers, and substrates to change selectively the redox state of the enzymes in the mitochondrial electron transport chain while maintaining a constant intracellular oxygen concentration; and (3) varying oxygen concentrations under conditions in which the redox states of the enzymes were kept constant.

Our results, obtained with TB cells, a mammalian cell line, indicate that the metabolism of nitroxides is strongly affected by the local concentration of oxygen, that reduction of lipophilic nitroxides occurs at the level of ubiquinone in the respiratory chain in mitochondria, and that the redox state of the enzymes in the respiratory chain, rather than intracellular oxygen concentration per se, is primarily responsible for affecting the rate of metabolism of lipophilic nitroxides in these cells.

Materials and Methods

Cells. Mouse thymus–bone marrow (TB) cells were obtained from Professor P. Wong of the University of Illinois, cloned from a single cell, and prepared as described previously (Swartz et al., 1986a,b).

Chemicals. Nitroxides were purchased from Molecular Probes (Junction City, OR) and used without further purification. 4-Oxo-2,2,6,6-tetramethylpiperidine-d_{16},1-^{15}N-1-oxyl ([^{15}N]PDT)[1] was purchased from MSD Isotopes (St. Louis, MO). Inorganic chemicals were of analytical grade. SKF-525A was a gift from Smith Kline & French laboratories (Philadelphia, PA). Rotenone, antimycin A, propyl gallate, CCCP,[1] and Dextran were purchased from Sigma Chemical Co. (St. Louis, MO). Malonate was purchased from Eastman Kodak Co. (Rochester, NY). Myxothiazol was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Labeling. Cells were washed with serum-free McCoy's medium before labeling. Cells (10^8/mL corresponding to approximately 4.5 mg of protein/mL) were labeled with lipid-soluble nitroxides as follows. An amount of nitroxide in ethanol that provided a final nitroxide/lipid ratio of 0.011 (Nettleton et al., 1988) was pipetted into a 6 × 50 mm glass culture tube and dried to make a uniform film of nitroxide on the side of the tube. The cell suspension (100 μL) was then added, vortexed intermittently for 0.5–2 min, and removed for ESR studies. These procedures as well as the subsequent manipulations used for the ESR measurements did not alter cell viability, as measured by exclusion of 0.4% Trypan Blue, and labeled cells were capable of replicating normally. As shown recently (Nettleton et al., 1988), this procedure labels all of the membranes of the cell.

ESR. All spectra were taken on a Varian E-109E ESR spectrometer. To prevent cells from settling out of the sensitive volume of the cavity, the cavity (Varian TE_{102}) was mounted so the sample was horizontal. The cell suspension contained 10% Dextran to retard settling of the cells. Samples were drawn into a gas-permeable Teflon tube (Zeus Industrial Products, Inc., Raritan, NJ) with an inside diameter of 0.813 mm and a wall thickness of 0.038 ± 0.014 mm. This tube was folded in half and inserted into a quartz ESR tube open at each end. Samples were kept at 37 °C by using a Varian gas flow temperature controller. The time-dependent changes in the concentration of nitroxides were monitored by setting the magnetic field of the spectrometer on the peak of the midfield line of the ESR spectrum of the nitroxide and turning the field sweep to zero. A modulation amplitude of 5 G was used to broaden the peak and minimize the effect of any drift of the magnetic field or line-width changes during the course of the measurements. The data were digitized on a Houston HI-PAD digitizing board, and the rates of reduction were determined from the slopes of the line of concentration vs time for the first 3–5 min. We determined the rates of reduction from the initial slopes, before significant amounts of hydroxylamines were produced, in order to minimize contributions to the rates due to oxidation of the hydroxylamines, but the observed rates of reduction of nitroxides in the presence of oxygen could have been slightly less than the actual values because of oxidation of hydroxylamines. ESR spectra were collected, stored, and manipulated with an IBM PC computer with an IBM DAC data acquisition card (Morse, 1987).

Measurement of Intracellular Oxygen Concentration. We have demonstrated that the intracellular oxygen concentration ($[O_2]_{int}$) is equivalent to the extracellular concentration ($[O_2]_{ext}$) in control samples and in the presence of inhibitors of respiration, but that the situation changes when cellular respiration is stimulated by CCCP; in this case gradients of up to 50 μM can develop between $[O_2]_{ext}$ and $[O_2]_{int}$. Under these conditions, measurement of $[O_2]_{ext}$ no longer gives an accurate representation of the actual oxygen tension inside the cell, and thus it becomes necessary to measure $[O_2]_{int}$ rather than $[O_2]_{ext}$ in order to describe accurately the relationship

[1] Abbreviations: [^{15}N]PDT, 4-oxo-2,2,6,6-tetramethylpiperidine-d_{16},1-^{15}N-1-oxyl; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Cat_1, 4-(trimethylammonio)-2,2,6,6-tetramethylpiperidine-1-oxyl.

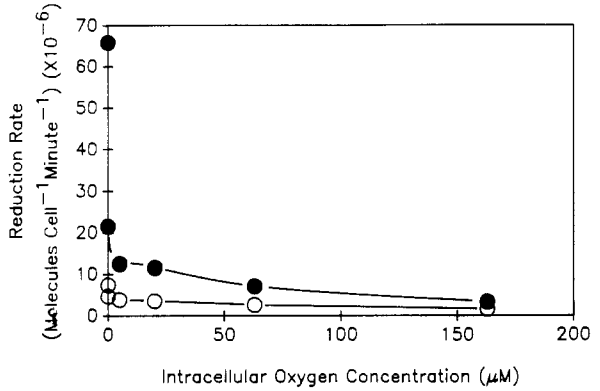

FIGURE 1: Effect of intracellular oxygen concentration on the initial rates of reduction of doxylstearates. The filled circles are for 5-doxylstearate, and the open ones are for 10-doxylstearate. The bulk concentration of the doxylstearates is 0.05 mM. Experimental conditions and methods are described under Materials and Methods.

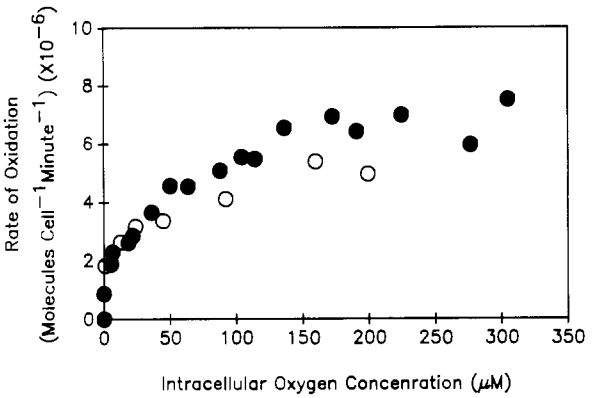

FIGURE 2: Effect of intracellular oxygen concentration on the rate of oxidation of the hydroxylamines of 5- and 10-doxylstearates. The filled circles are for 5-doxylstearate, and the open ones are for 10-doxylstearate. The hydroxylamines were produced by anaerobic cellular reduction of the corresponding doxylstearates. The bulk concentration of the hydroxylamines is 0.05 mM. Oxidation was measured after reintroducing oxygen into the chamber. Experimental conditions and methods are otherwise described under Materials and Methods.

between $[O_2]$, redox potential, and the reduction rate of nitroxides. The method of measuring $[O_2]_{int}$ is described in greater detail elsewhere (Swartz & Pals, 1988) and involves the use of a membrane-permeable nitroxide, [^{15}N]PDT, whose line width is very sensitive to oxygen concentration, in conjunction with potassium ferricyanide, a charged, membrane-impermeant paramagnetic broadening agent. When [^{15}N]PDT is added to a cell suspension, the nitroxide distributes throughout the intracellular and extracellular space. Ferricyanide, on the other hand, remains exclusively in the extracellular space and broadens away the signal from the extracellular [^{15}N]PDT. The remaining signal arises only from the intracellular nitroxide.

For calibration purposes, cellular respiration was inhibited with 5 mM NaCN so that $[O_2]_{int}$ and $[O_2]_{ext}$ were equivalent and could be related to the oxygen tension of the perfusing gas. The changes of line width (ΔW) were plotted as a function of $[O_2]_{int}$, and the resulting curve was fit to a second-order regression equation. This calibration curve then was used to convert values of ΔW measured in respiring cells into appropriate values for $[O_2]_{int}$.

Measurement of Oxygen Consumption Rates. Oxygen consumption rates were obtained by measuring $[O_2]_{ext}$ in a closed system over time and finding the slope of the resulting linear plot. Extracellular oxygen was measured with Cat_1,[1] a charged, membrane-impermeable nitroxide. Cat_1 was used without added broadening agent, and a parameter (ΔC) determined from the spectral superhyperfine structure was used to measure $[O_2]_{ext}$ (Morse & Swartz, 1985; Swartz & Pals, 1988). Oxygen consumption rates were measured by using 10^6 TB cells suspended in the medium/Dextran solution described above and 1.5×10^{-4} M Cat_1. Samples were drawn into glass capillary tubes which were then sealed at both ends and placed in quartz ESR sample tubes. Spectra were recorded at 1-min intervals, ΔC was measured for each spectrum and converted into an extracellular oxygen concentration, and the slope of $[O_2]_{ext}$ vs time was obtained to determine the oxygen consumption rate.

RESULTS

Effects of Oxygen on the Reduction of Nitroxides. Figure 1 shows that the initial rate of reduction of 5-doxylstearate, whose doxyl moiety is close to the membrane surface (Jost et al., 1971), is affected by $[O_2]_{int}$. At low levels of $[O_2]_{int}$, the curve is steep. At high $[O_2]_{int}$, the rate gradually decreases with increasing $[O_2]_{int}$. The rate of reduction of 10-doxylstearate, whose doxyl moiety is deep in the membrane (Jost et al., 1971), shows a similar dependence on intracellular oxygen, but the values are always smaller than those of 5-doxylstearate at any given $[O_2]_{int}$, and especially when $[O_2]_{int}$ is below 1 μM. An apparent K_m for oxygen can be calculated from these data by a double-reciprocal plot: it is less than 1 μM.

Table I: Effects of Inhibitors and Other Agents on Oxygen Consumption Rates[a]

inhibitors and other agents	oxygen consumption rates	inhibitors and other agents	oxygen consumption rates
control	23.7 ± 1.2 (12)	myxothiazol	7.0 ± 0.6 (6)
rotenone	7.6 ± 0.6 (4)	cyanide	0.8 ± 0.2 (5)
malonate	24.2 ± 0.6 (4)	CCCP	30.3 ± 2.0 (6)
rotenone + malonate	9.2 ± 1.1 (5)	succinate	26.4 ± 1.5 (7)
		propyl gallate	22.7 ± 3.0 (6)
antimycin	8.6 ± 1.4 (7)	SKF-525A	25.6 ± 1.0 (7)

[a] The unit for the rate is 10^{-10} μmol cell^{-1} min^{-1}. The concentration was 0.2 mM for rotenone; 5 mM for malonate and cyanide; 0.05 mM for antimycin, myxthiazol, SKF-525A, and propyl gallate; and 85 mM for succinate. The results are given as means ± SEM (number of indepedent measurements in parentheses).

Effects of Oxygen on the Oxidation of Hydroxylamines. The rates of oxidation of hydroxylamines (which were produced by prior cellular reduction of 5- and 10-doxylstearates; Chen & Swartz, 1988) are shown in Figure 2 as a function of $[O_2]_{int}$. There is little difference in the rates of oxidation of the hydroxylamines of 5- and 10-doxylstearates. The apparent K_m for oxygen, calculated from a double-reciprocal plot of the data in Figure 2, is about 30 μM.

Effects of Inhibitors and Uncouplers on the Rate of Oxygen Consumption. The inhibitors, uncouplers, and substrates used in this study act at specific sites in the mitochondrial electron transport chain or in the cytochrome P-450 complex and change the redox state of the related enzymes. It was essential to demonstrate that the various inhibitors and uncouplers used were effective at the cellular level, since the experiments described in this paper all involved the use of whole cells rather than isolated mitochondria or microsomes. Table I shows that, at the cellular level, inhibitors of the mitochondrial electron transport chain (NaCN, antimycin A, rotenone, myxothiazol) were all effective in reducing the rate of oxygen consumption whereas the rates measured in the presence of inhibitors of the cytochrome P-450 complex (SKF-525A and propyl gallate)

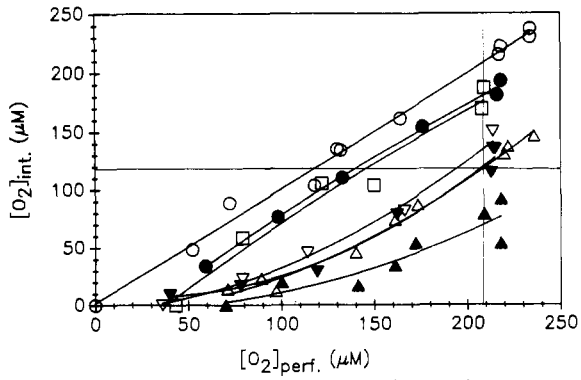

FIGURE 3: Intracellular oxygen concentration vs the oxygen concentration of the perfusing gas for TB cells under the various conditions used in this study. The cells were treated with NaCN (○), antimycin A (●), rotenone and malonate (□), propyl gallate (▽), SKF-525A (▼), or CCCP (▲) before the measurements. The measurements from the control sample (△) also are shown in this figure. $[O_2]_{int}$ is highest in cells whose respiration is most inhibited (NaCN) and lowest in cells whose respiration has been stimulated by CCCP. The vertical line at $[O_2]_{perf}$ = 209 μM illustrates the variation in $[O_2]_{int}$ when all samples are perfused with air. The horizontal line indicates a constant $[O_2]_{int}$. This graph was used to find the values of $[O_2]_{perf}$ needed to maintain samples at an intracellular oxygen concentration of 118 μM.

showed little variation from the control value. The protonophore CCCP stimulated oxygen consumption by approximately 30%.

Effects of Inhibitors and Uncouplers on Intracellular Oxygen Concentrations. When oxygen consumption rates are affected by the addition of inhibitors, $[O_2]_{int}$ can be altered as well. This is illustrated in Figure 3, in which $[O_2]_{int}$ is plotted as a function of the oxygen concentration of the perfusing gas in the presence of various inhibitors. $[O_2]_{int}$ is highest when cellular respiration is strongly inhibited (NaCN) and lowest when respiration is stimulated by the addition of an uncoupler (CCCP). The vertical line in Figure 3 indicates the situation in which all samples are perfused with air (where $[O_2]_{perf}$ is 209 μM). There is clearly a large difference in the values of $[O_2]_{int}$ in the presence of different inhibitors. The horizontal line, in contrast, indicates a constant $[O_2]_{int}$ of 118 μM (which is equivalent to $[O_2]_{int}$ when a control sample is perfused with air), obtained by changing $[O_2]_{perf}$ to compensate for the different respiration rates of the cells in the presence of inhibitors or the uncoupler.

Effects of Inhibitors and Uncouplers on Rates of Reduction. Table II shows that when $[O_2]_{int}$ is 118 μM, cyanide, antimycin A, and myxothiazol (inhibitors of cytochrome *c* oxidase and reductase) dramatically increase the rates of reduction of nitroxides in the presence of oxygen. At the same $[O_2]_{int}$, CCCP, which uncouples the respiratory chain, decreases the rate of reduction whereas an excess of succinate, which is the substrate of succinate dehydrogenase, increases the rate of reduction. Rotenone and malonate together significantly inhibit the initial rates of reduction in the presence of oxygen. Cyanide, antimycin A, or myxothiazol decreases the dependence of the rate of reduction on $[O_2]_{int}$; e.g., the ratio of the rates of reduction in the presence and absence of oxygen is 2.9 with addition of cyanide, compared to 27.5 for the control sample. This effect results in a different equilibrium value of nitroxide in the presence of oxygen; e.g., the equilibrium value with antimycin A is 14% vs 83% in the control (Figure 4).

Inhibitors of cytochrome P-450 and its reductase, SKF-525A, and propyl gallate have little effect on the rate of reduction of nitroxides by cells either with or without oxygen (Table II).

Table II: Effects of Inhibitors and Other Agents on the Rates of Reduction of 5-Doxylstearate[a]

inhibitors and other agents	rates of reduction	
	$[O_2]_{int}$ = 118 μM	N_2
control	0.32 ± 0.02 (20)	8.8 ± 0.8 (10)
rotenone	0.27 ± 0.02 (4)	1.8 ± 0.3 (4)
malonate	0.34 ± 0.05 (4)	8.3 ± 0.8 (4)
rotenone + malonate	0.23[b] ± 0.02 (20)	1.9 ± 0.3 (4)
antimycin	2.21[b] ± 0.09 (4)	6.7 ± 0.3 (4)
myxothiazol	3.5[b] ± 0.3 (4)	6.5 ± 0.4 (4)
cyanide	3.5[b] ± 0.3 (4)	10.0 ± 0.4 (4)
CCCP	0.26[b] ± 0.01 (7)	8.9 ± 0.3 (4)
succinate	0.67[b] ± 0.05 (10)	11.6 ± 0.6 (4)
propyl gallate	0.35 ± 0.08 (5)	6.3 ± 0.6 (4)
SKF-525A	0.33 ± 0.03 (8)	9.2 ± 0.6 (4)

[a] The unit for the rate is 10^7 molecules $cell^{-1}$ min^{-1}. The concentrations of these agents were identical with the concentrations used in Table I. The bulk concentration of 5-doxylstearate is 0.05 mM. The results are given as means ± SEM (number of independent measurements in parentheses). [b] Rates of reduction at $[O_2]_{int}$ = 118 μM were significantly different ($P < 0.05$) from the control value by Student's *t* test.

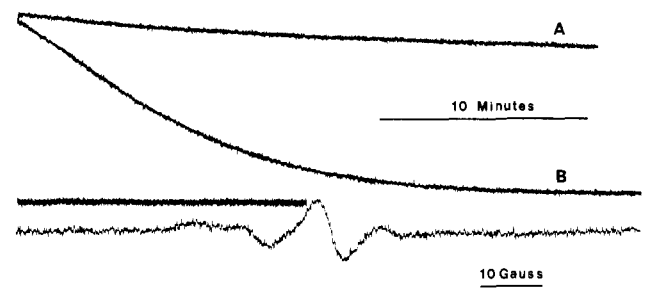

FIGURE 4: Effect of antimycin A on the rate of reduction of 5-doxylstearate. The time-dependent changes in the concentration of 5-doxylstearate are shown at 118 μM intracellular oxygen, without (A) and with (B) the addition of 0.05 mM antimycin A; the initial value is the peak height with 100% nitroxide (and no hydroxylamine). The initial bulk concentration of 5-doxylstearate is 0.05 mM. A steady state was reached in about 30–45 min; the ESR spectrum of the state in the presence of antimycin A also is shown.

Discussion

Sites of Reduction of Nitroxides and Oxidation of Hydroxylamines. The specific sites of action of the inhibitors used in this study, along with the data on reduction rates provided in Table II, can be used to determine at what point along the electron transport chain reduction of nitroxides occurs. The first column of data in Table II gives reduction rates in the presence of 118 μM oxygen, representing the average oxygen concentration experienced by untreated TB cells perfused with air. When rotenone and malonate together are added to the cells, electron flow beyond complex I and complex II is blocked, and the corresponding reduction rates are low. In contrast, when electron flow is blocked at the level of complex III (by the addition of antimycin A or myxothiazol) or complex IV (by the addition of cyanide), the reduction rates are much higher. Incubation with excess succinate also increases the reduction rate above the control value. These observations are consistent with the conclusion that reduction of these doxylstearate occurs at the level of ubiquinone in the respiratory chain in mitochondria.

A similar investigation of oxidation rates in the presence of various inhibitors has led to the conclusion that cytochrome *c* oxidase (complex IV) is principally responsible for the oxidation of hydroxylamines of doxystearates in TB cells (Chen & Swartz, 1988).

Steady State of Reduction and Oxidation. The inhibitors of complex III (i.e., antimycin A and myxothiazol) keep the

site of reduction in the reduced state and do not affect the activity of cytochrome *c* oxidase. As shown in Figure 4, upon addition of antimycin A, the reduction of 5-doxylstearate reaches a steady state of about 14% of the initial concentration of the nitroxide in the presence of oxygen, while in the control sample the level was 83%. One explanation of these results is that 5-doxylstearate is reduced at the level of ubiquinone and then diffuses to cytochrome *c* oxidase to become oxidized. Eventually, these two reactions reach equilibrium so that the steady-state concentration of 5-doxylstearate is maintained by equivalent reduction and oxidation rates.

Effects of Microsomal Inhibitors. As shown in Table II, the lack of effects of SKF-525A and propyl gallate on the rate of reduction of 5-doxylstearate with and without oxygen suggests that cytochrome P-450 plays little role in the reduction of doxylstearates in these cells.

Primary and Secondary Effects of Oxygen. While the redox state of the cells (as reflected in the redox state of various enzymes in the electron transport chain) is certainly affected by $[O_2]_{int}$, these two variables do not necessarily correlate closely and therefore need to be studied independently. It is necessary to differentiate between a primary effect of oxygen (e.g., oxygen competing directly with nitroxides for electrons at the same site) and a secondary oxygen effect (e.g., changes in the redox state of the enzymes of the respiratory chain) on the rates of metabolism of nitroxides. The addition of inhibitors or an uncoupler changes the redox potential of the enzymes in the electron transport chain, but, as is shown in Figure 3, $[O_2]_{int}$ can change as well, by more than a factor of 4. In order to study the effect of redox potential on reduction rates, we held $[O_2]_{int}$ constant by adjusting the oxygen concentration of the perfusing gas. This situation is illustrated by the horizontal line at 118 μM $[O_2]_{int}$ in Figure 3. The converse arrangement also is desirable, i.e., to hold the redox potential constant while varying $[O_2]_{int}$. This is achieved by adding NaCN, which allows full reduction of the mitochondrial enzymes independent of $[O_2]_{int}$.

Sites and Mechanisms of Effects of Oxygen on Metabolism of Nitroxides. Compared to controls, inhibitors of cytochrome *c* oxidase (cyanide) and cytochrome *c* reductase (antimycin A and myxothiazol) increased considerably the rate of reduction of doxylstearates when $[O_2]_{int}$ was held at 118 μM, indicating that oxygen affects the reduction of doxylstearates through cytochrome *c* oxidase and reductase.

The data obtained with inhibitors and other agents show that the rate of reduction can vary by a factor of >15 at the same $[O_2]_{int}$, indicating that it is the redox state of the enzymes which is the principal factor in nitroxide reduction. This is supported by the effects of CCCP, which can bring the components in the electron transport chain to a more oxidized state in the presence of oxygen. In this case, the rate of reduction of doxylstearates is decreased.

When the site of nitroxide reduction was kept in the reduced state by using cyanide, antimycin A, or myxothiazol, the rate of reduction became less dependent on $[O_2]_{int}$ (Table II); e.g., the ratio of the rates of nitroxide reduction in the presence and absence of oxygen is 2.9 with addition of cyanide, compared to 27.5 for the control sample. These results also suggest that oxygen affects the rate of reduction of nitroxides principally by changing the redox state of the enzymes in the respiratory chain in these cells. The residual dependence on oxygen concentration of the reduction in the presence of cyanide, antimycin A, or myxothiazol might be due to either incomplete inhibition at the cellular level by these inhibitors or some direct dependence on $[O_2]_{int}$ of the reduction of doxylstearates.

Effects of Concentration of Oxygen on the Rates of Metabolism of Nitroxides. The doxyl moiety of 10-doxylstearate is deep in the membrane whereas it is close to the membrane surface for 5-doxylstearate (Jost et al., 1971). It has been shown previously that the rate of reduction of 10-doxylstearate is limited by the diffusion of reducing equivalents within or into the membrane under severely hypoxic conditions (Chen et al., 1988). The rate of reduction of 10-doxylstearate is always less than that of 5-doxylstearate at any $[O_2]_{int}$ (Figure 1). In contrast, the relation between effects of $[O_2]_{int}$ and oxidation rates of the hydroxylamines is similar for 5- and 10-doxylstearates (Figure 2). These results are consistent with cytochrome *c* oxidase being principally responsible for the oxidation of the hydroxylamines of both doxystearates and with the conclusion that diffusion in the membrane is not the rate-limiting step for this oxidation (Chen & Swartz, 1988).

The K_m of cytochrome oxidase for oxygen is about 0.5 μM for the nonenergized state and 0.05 μM for the energized state of mitochondria (Wikström et al., 1981). Because the components of the respiratory chain are tightly coupled, one can expect that the other enzymes also become oxidized at fairly low oxygen concentrations. This could explain why the apparent K_m for oxygen calculated from Figure 1 is less than 1 μM, since the rate of reduction of doxylstearates is greatly affected by the redox state of the respiratory chain (see Table II and the discussion below). It is not clear, however, why in the case of the oxidation of hydroxylamines the apparent K_m for oxygen is as high as 30 μM (Figure 2). One possible explanation is that the high concentration of hydroxylamines (compared with the concentration of cytochrome *c*) provides electrons to cytochrome *c* oxidase in addition to the electrons from cytochrome *c* (Chen & Swartz, 1988), so that more oxygen is required to fully oxidize cytochrome *c* oxidase.

Relevance to in Vivo NMR and ESR. The dependence of nitroxide metabolism on the concentration of oxygen could be a valuable tool to measure oxygen concentrations in vivo when nitroxides are used as metabolically responsive contrast agents or imaging agents for in vivo NMR or ESR: the rate of reduction could be used to determine the presence of severely hypoxic cells while the rate of oxidation could be used to measure intermediate levels of oxygen.

ACKNOWLEDGMENTS

We thank Maoxin Wu for preparing the cell cultures and Dr. A. R. Crofts for helpful comments.

Registry No. O_2, 7782-44-7; 5-doxylstearate, 29545-48-0; 10-doxylstearate, 50613-98-4; 5-doxylstearate hydroxylamine, 118418-10-3; 10-doxylstearate hydroxylamine, 118418-11-4; cytochrome *c* oxidase, 9001-16-5.

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New Protein Cross-Linking Reagents That Are Cleaved by Mild Acid

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Received June 23, 1988; Revised Manuscript Received October 31, 1988

ABSTRACT: New homo- and heterobifunctional cross-linking reagents have been synthesized. These reagents are based on ortho ester, acetal, and ketal functionalities that undergo acid-catalyzed dissociation but are base stable. The protein-reactive group in all the homobifunctional reagents is a maleimide group; the heterobifunctional acetal cross-linker has a maleimide group at one end and an *N*-hydroxysuccinimide ester at the other. These reagents have been used to cross-link diphtheria toxin (DT) to itself to give covalently cross-linked DT dimer or to conjugate DT monomer to the anti-CD5 antibody, T101. The hydrolysis of these cross-linked proteins was studied as a function of pH. Cleavage rates vary from minutes to hours at the pH of acidified cellular vesicles (~pH 5.4), ortho esters being the fastest, acetals the slowest, and ketals intermediate, but the cross-linked products are approximately 100 times more stable at the vascular pH of 7.4 and 1000 times more stable at a storage pH of 8.4 in all cases. The utility of these reagents in the reversible blockade of a toxic protein functional domain was demonstrated by using cross-linked DT dimer where the blocking and unblocking of toxin binding sites correlates with cellular toxicity. Of the different cross-linkers described, the acetone ketal, bis(maleimidoethoxy)propane (BMEP), appears to be the most promising in the construction of highly efficacious immunotoxins.

Bifunctional cross-linking reagents have found widespread application in protein chemistry in recent years. Many aspects of protein structure and function and relationship to surrounding proteins have been investigated by using these reagents (Davies & Stark, 1970; Hucho et al., 1975; Hadju et al., 1976; Peters & Richards, 1977). A variety of cross-linkers are commercially available—homobifunctional, heterobifunctional, noncleavable, and cleavable. Most commonly used are heterobifunctional reagents that have maleimide and *N*-hydroxysuccinimidyl ester functionalites separated by a spacer group (Kitagawa & Aikawa, 1976; Kitagawa et al., 1981; Yoshitake et al., 1982; Gitman et al., 1985). Specific

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