Proc. Natl. Acad. Sci. USA
Vol. 78, No. 1, pp. 234–237, January 1981
Biochemistry

Cytochrome oxidase (a_3) heme and copper observed by low-temperature Fourier transform infrared spectroscopy of the CO complex

(photolysis/mitochondria/beef heart)

J. O. ALBEN, P. P. MOH, F. G. FIAMINGO, AND R. A. ALTSCHULD

Department of Physiological Chemistry, The Ohio State University, Columbus, Ohio 43210

Communicated by Hans Frauenfelder, October 10, 1980

ABSTRACT **Carbon monoxide bound to iron or copper in substrate-reduced mitochondrial cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) from beef heart has been used to explore the structural interaction of the a_3 heme–copper pocket at 15 K and 80 K in the dark and in the presence of visible light. The vibrational absorptions of CO measured by a Fourier transform infrared interferometer occur in the dark at 1963 cm^{-1}, with small absorptions near 1952 cm^{-1}, and are due to a_3 heme–CO complexes. These disappear in strong visible light and are replaced by a major absorption at 2062 cm^{-1} and a minor one at 2043 cm^{-1} due to Cu–CO. Relaxation in the dark is rapid and quantitative at 210 K, but becomes negligible below 140 K. The multiple absorptions indicate structural heterogeneity of cytochrome oxidase in mitochondria. The Cu–CO absorptions (ν_{CO}) are similar to those in hemocyanin–CO complexes from molluscs (ν_{CO} = 2062 cm^{-1}) and crustaceans (ν_{CO} = 2043 cm^{-1}). The 2062 cm^{-1} Cu–CO absorption of cytochrome oxidase is split into two bands at 15 K. Analysis of spectral data suggest the presence of a very nonpolar heme–Cu pocket in which the heme–CO complex is highly ordered, but in which the Cu–CO complex is much more flexible, especially above 80 K. A function for these structures in oxygen reduction is proposed.**

Cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) is the terminal respiratory enzyme that catalyzes the controlled reduction of dioxygen to water with concomitant production of chemical energy in the form of adenosine triphosphate. Mammalian cytochrome oxidase contains 2 moles of heme *a* and 2 moles of copper. In the aerobic resting (oxidized) oxidase, electron paramagnetic resonance (EPR) signals are observed for only one heme and one copper, which are designated as components of cytochrome *a*. The EPR "invisible" heme and copper are designated as components of cytochrome a_3. The absence of an EPR signal for the a_3 metal centers in the resting enzyme has been ascribed to antiferromagnetic coupling of the unpaired spins (1, 2). In the fully reduced enzyme, iron and copper have even numbers of electrons and yield no EPR signal.

Carbon monoxide has been used extensively as a structural probe for oxygen-binding enzymes because it is a competitive inhibitor for many of them. Heme and copper enzymes were classically distinguished by the sensitivity of the former, but not the latter, to reversal of CO inhibition by visible light (3). Photolysis of CO from the reduced heme a_3 results in high-spin iron, according to the temperature dependence of magnetic circular dichroism (4). Recombination of photolyzed CO has been studied over the temperature range 185–295 K and noted to be much slower at low temperatures than in corresponding complexes of hemoglobin or myoglobin (5, 6). Our recent brief report (7) that CO photolyzed from heme a_3 in beef heart mitochondria is bound reversibly to the a_3 copper would appear to explain these results. Here, we extend the observations to lower temperatures, illustrate the structural differences between heme and copper CO complexes in cytochrome a_3, and show how this may be related to the functional contributions of these metal centers.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Mitochondria, prepared from fresh beef heart by use of Nagarse (8), were kindly donated by G. P. Brierley (Department of Physiological Chemistry, Ohio State University). They were saturated with carbon monoxide to form the endogenous substrate-reduced CO complex and dehydrated with CO-saturated glycerol (100%) to yield a highly concentrated wax-like glass that transmitted light in both the visible and midinfrared regions of interest. Visible spectra obtained with a Cary 17DX spectrophotometer yielded a shoulder at 590 nm due to reduced cytochrome a_3–CO complex. Infrared spectra were obtained with a Digilab FTS-14D infrared interferometer fitted with an InSb detector cooled by liquid nitrogen. Mitochondrial samples with a 0.2-mm optical pathlength were held between CaF_2 windows in the cryostat. Interferograms were collected at 1 cm^{-1} resolution through a 15-bit A/D converter and signal averaged into 32-bit computer words, which were used for all further computations. Single-beam spectra result from Fourier transforms of the (4096) signal-averaged interferograms. No additional averaging or smoothing of the data was required. Absorbance difference spectra (dark minus light) were computed from single-beam spectra obtained in the absence and in the presence of continuous visible radiation at the indicated temperatures. Low temperatures (11–280 K) were conveniently obtained by use of a Lake Shore Cryotronics helium refrigerator, model LTS-21-D70C. Cryostat cell temperature was measured by use of a Lake Shore Cryotronics digital thermometer (model DRC-70) with a calibrated silicon diode probe. Photolysis of heme–CO at 80 K and 15 K was obtained by use of continuous illumination from a 500-W tungsten lamp focused through a slide projector and optically filtered through water. Replications were conducted after relaxation of the CO in the dark at 210 K for 30 min. The observations have been quantitatively reproduced many times with the same sample and with a variety of preparations. Some differences in relative amounts of major and minor CO absorptions with preparation have been noted.

RESULTS

The infrared absorption spectrum of the CO complex of cytochrome oxidase was first reported by Caughey and coworkers

Abbreviation: EPR, electron paramagnetic resonance.

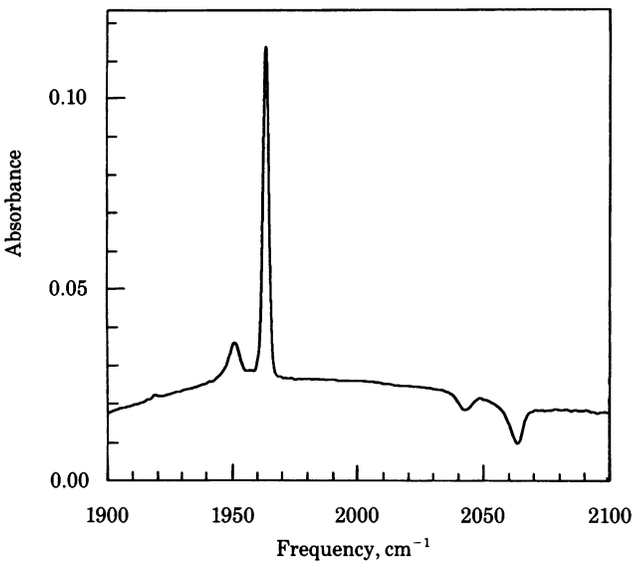

FIG. 1. Absorbance difference spectrum (dark minus light) of CO in glycerol-extracted mitochondria at 80 K. The cytochrome a_3 heme–CO absorbs near 1963 cm^{-1}, with a small band near 1952 cm^{-1}; photolyzed CO absorbs mainly at 2062 cm^{-1}, with a minor fraction at 2043 cm^{-1}.

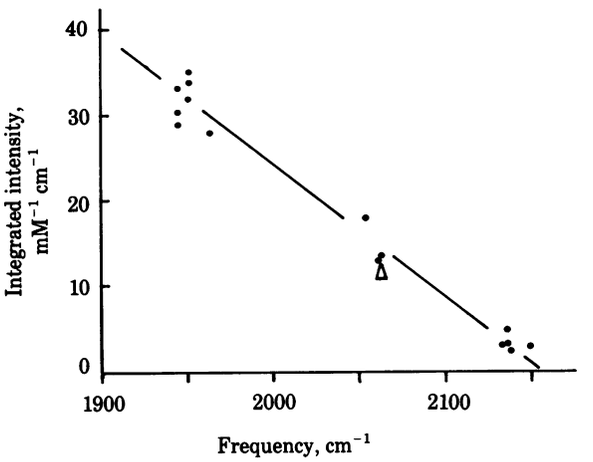

FIG. 2. Correlation of vibrational absorption frequencies with integrated absorptivities (B, mM^{-1}cm^{-2}) for CO at low temperatures (calculated from ref. 15 and CO complexes with hemocyanins (12), cytochrome oxidase (11), hemoglobin (11, 16, 17), and myoglobin (16), where $B = (1/cl) \int \log(I_0/I)d\nu$. The point indicated by the triangle (△) is calculated for cytochrome oxidase–CuCO as described in the text.

(9–11) at 1963 cm^{-1}. This frequency for ν_{CO} is similar to that for other heme carbonyl complexes and is clear evidence that this CO is bound in a nonbridging complex to iron. We have confirmed this observation many times. The CO complex of glycerol-extracted beef heart mitochondria also shows a sharp absorption band at 1963 cm^{-1} due to the cytochrome oxidase (a_3)–CO complex. This is illustrated in Fig. 1 by the absorbance difference spectrum (dark minus light) obtained at 80 K for the CO vibrational region. The small absorption band at 1919.5 cm^{-1} is due to the 1% natural abundance of ^{13}CO, corresponding to the $^{12}C^{16}O$ heme complex at 1963 cm^{-1}. The ratio of band areas is 0.01, confirming the assignment to CO. The photolyzed CO absorbs at 2062 cm^{-1}, with a smaller absorption near 2043 cm^{-1}. Photolysis is reversible in the dark at temperatures above 140 K. Reversal is rapid and quantitative at 210 K, at which temperature the CO was routinely allowed to reassociate with the a_3 heme. Relaxation overnight at 140 K allows only a few percent of the CO to return to the iron.

We assign the main photolyzed CO absorption at 2062 cm^{-1} and the smaller one at 2043 cm^{-1} to Cu–CO complexes. Fager and Alben (12) observed Cu–CO complexes at 2062 and 2063 cm^{-1} for molluscan carboxyhemocyanins from squid and limpet and at 2043 cm^{-1} for crustacean hemocyanins from marine crab and fresh-water crayfish. The photolyzed CO absorptions are at sufficiently lower frequency than the absorption of the free gas at 2143.8 cm^{-1} to require the CO to be covalently coordinated to copper, rather than weakly associated to a polar group in the heme pocket as in photolyzed carboxymyoglobin. The relatively high temperature required to dissociate this complex (>140 K) confirms this interpretation. CO photolyzed from myoglobin or hemoglobin recombines with heme in the dark at significant rates at temperatures as low as 10–20 K (refs. 13 and 14 and unpublished data). We interpret these data to show that in cytochrome *c* oxidase, the a_3 heme directly communicates with the a_3 copper at all temperatures that we have measured (>10 K) by a pocket (or space) that may be readily traversed by small molecules such as carbon monoxide.

The question of what fraction of the photolyzed CO is recovered in the absorption bands at 2062 cm^{-1} and 2043 cm^{-1} is more difficult to answer. Absorptivities for CO complexes of heme and copper proteins and for CO gas condensed into an argon matrix are correlated with the CO vibrational absorption frequency in Fig. 2. If we assume that the integrated absorptivity for cytochrome oxidase–CO complex given by Yoshikawa *et al.* (11) also applies to glycerol-extracted mitochondria at low temperature, then from the ratio of observed integrated absorbances (dark at 1963 cm^{-1}/light at 2062 cm^{-1} in Fig. 1) the integrated absorptivity of the photolyzed CO is nearly the same as that for hemocyanin copper carbonyl (12).

The absorption of the photolyzed CO can provide considerable information about its local molecular environment. The heme–CO complex shows a very narrow bandwidth of 2.5 cm^{-1} ($\Delta\nu_{1/2}$ = full width at half-maximal absorbance), indicating a very nonpolar environment or highly ordered surroundings (or both). The photolyzed CO has broader bandwidths ($\Delta\nu_{1/2}$ = 6 cm^{-1}) at 80 K and at 140 K (Fig. 1, and ref. 7), indicating that it sees more polar surroundings or a less ordered and more flexible environment (or both) than at the heme. Some of these structural possibilities are explored by examination of the spectra at a lower temperature. Heme a_3–CO (dark) is not significantly different at 15 K. The bandwidth is unchanged, and no additional interactions with nearest neighbors are "frozen out." Conversely, the a_3 Cu–CO absorption at 2062 cm^{-1} is split into two bands, at 2054.8 and 2065.1 cm^{-1} (Fig. 3), but without significant differences in $\Delta\nu_{1/2}$ for the individual bands.

At least three types of phenomena might contribute to the observed differences in $\Delta\nu_{1/2}$ between Cu–CO and Fe–CO absorption bands and to the low-temperature splitting of the Cu–CO band near 2062 cm^{-1}. Vibrational and rotational absorption bands are very narrow in the gas phase at low partial pressures. They are collision-broadened at higher pressures and in condensed phases where rotational structure is lost and only vibrational absorptions persist. Further broadening depends upon the thermal distribution of interactions with local dipoles or charged groups. This distribution may be partially frozen into multiple energy minima at low temperatures to maintain a broadened vibrational absorption band. Splitting of a band below 40 K may be caused by an exchange process between two states into which the system is frozen as kT is lowered below the energy barrier that separates them or by stabilization of a noncovalent association with a dipole or charged group. Other similar models are also possible. An exchange process is predicted

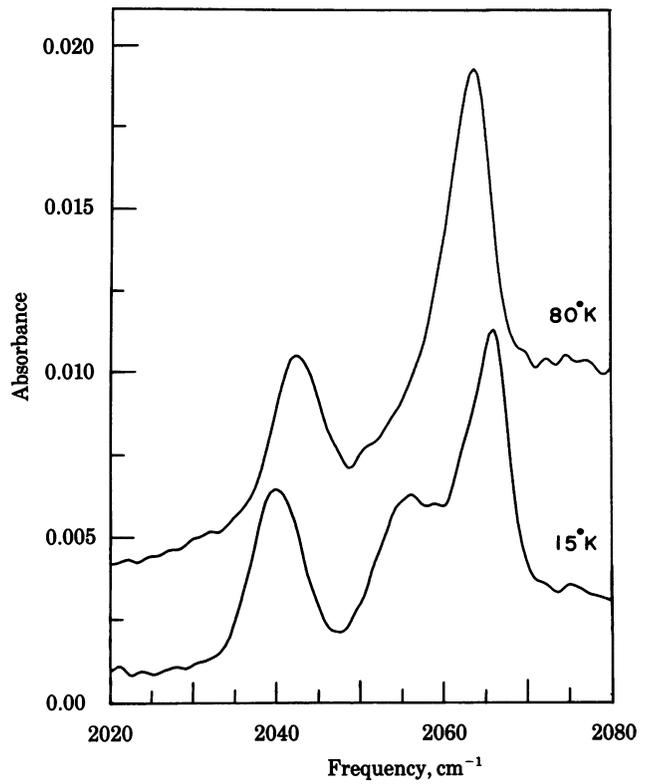

FIG. 3. Absorbance difference spectra (light minus dark) of mitochondria from Fig. 1 at 80 K and 15 K.

to produce frequencies of the split bands that are shifted from that observed at high temperatures in inverse proportion to the equilibrium amounts (band areas) present at low temperatures. Conversely, stabilization of a molecular pair at low temperatures should result in a shifted frequency for the associated pair, with the remainder unchanged. Unfortunately, other changes such as stabilization of a distribution of conformational states of the protein are expected to vary with temperature and must be distinguished from processes that cause splitting of the Cu–CO vibrational absorption. Thus, a much more detailed study is required to distinguish the molecular processes that produce these observations. However, certain aspects are clear.

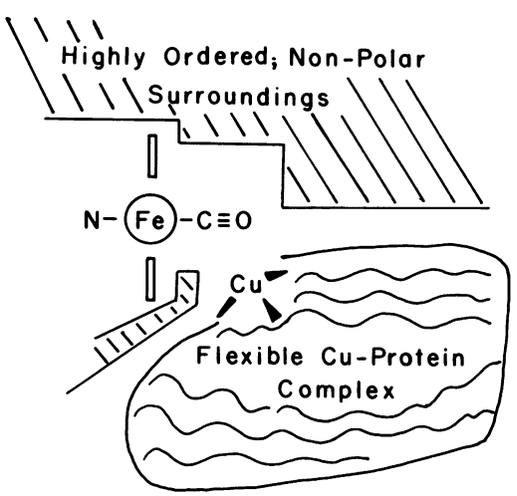

FIG. 4. Representation of local interactions of CO in cytochrome oxidase.

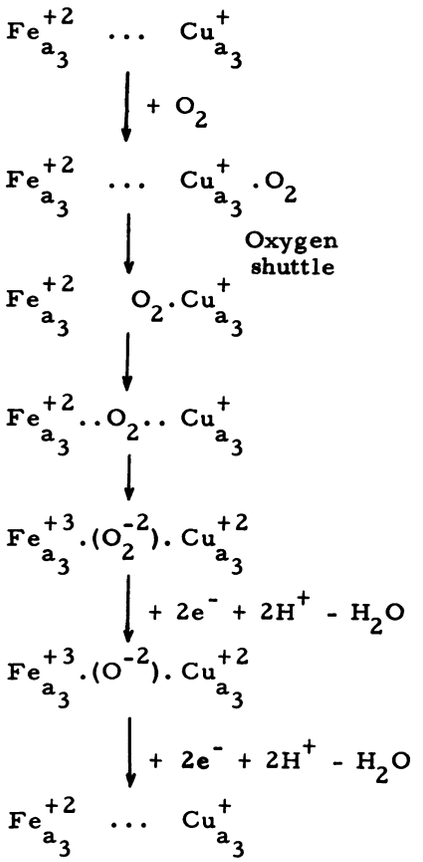

FIG. 5. Proposed reaction sequence for oxygen reduction by cytochrome a_3. The last two reactions represent reduction, proton shuttle, and water shuttle.

(*i*) The Fe–CO absorption at 1963 cm^{-1} is nearly unchanged with temperature, with center frequency, $\Delta\nu_{1/2}$, and band intensity remaining nearly constant. The minor Fe–CO band near 1952 cm^{-1} differs in frequency, bandwidth, and temperature dependence from the 1963 cm^{-1} band, but is similar in photolysis and recombination, and is probably due to altered heme a_3–CO.

(*ii*) The copper–CO complexes are also found to have vibrational absorptions with different characteristics. The major absorption near 2062 cm^{-1} is split at low temperatures by an undefined molecular process, whereas the smaller band near 2043 cm^{-1} exhibits no splitting. Both absorptions reversibly disappear at 210 K in the dark with reformation of the Fe–CO bands and represent two populations of cytochrome a_3 copper. It is not known whether the Fe–CO populations are independent of the Cu–CO populations. However, all Cu–CO bands are more than twice the width of the major Fe–CO band and are much more affected by broadening processes. However, the narrower observed bandwidth (2.5 cm^{-1} at 1963 cm^{-1}) for a_3 heme–CO requires a more highly ordered structure that is not broadened by variations of nearest-neighbor polar contacts or conformational transitions of heme–CO.

We can now describe cytochrome a_3 as containing an iron–copper center that may bind one molecule of CO to either the iron or the copper. CO may be photolyzed from the iron but not from the copper by visible light. The Cu–CO complex dissociates at measurable rates above 140 K. The a_3 heme–CO complex is surrounded by a highly ordered nonpolar molecular environment, whereas the Cu–CO is subject to several temperature-dependent broadening processes. Cytochrome a_3 heme and copper are connected by a channel, perhaps 4–6 Å long be-

cause it is readily traversed by CO at low temperatures, but it must be sufficiently large that the heme–CO absorption (ν_{CO}) is not broadened by the copper–protein. These features are illustrated in Fig. 4.

DISCUSSION

Our observation that one molecule of carbon monoxide may be bound to cytochrome a_3 at either the heme or the copper and that it moves reversibly from one to the other depending upon conditions of light and temperature clearly distinguishes which may be correct among the model structures that have been proposed to explain many spectroscopic and kinetic observations. Those that include bridging CO complexes (18) are ruled out inasmuch as the observed values of ν_{CO} are for nonbridging CO complexes with heme Fe or Cu. Conversely, the reversible movement of CO between iron and copper in the a_3 pocket suggests that the metals are close enough to accommodate a bridging oxygen molecule or peroxide anion. The proximity of the CO-binding heme and copper, which we designate as belonging to cytochrome a_3, limits other possible metal centers to the EPR-visible heme and copper that belong to cytochrome *a*. Models that include an imidazole bridge (2) between a_3 heme and Cu are ruled out because the a_3 Cu must be on the same side of the heme a_3 as that to which CO is bound and the proposed imidazole bridge is *trans* to this position. Similarly, a bridge between Cu_a and Fe_{a_3} is unlikely because this would provide three electrons for reduction of molecular oxygen rather than two, as suggested by the low and high oxidation potentials of the cytochrome *a* and a_3 metals (19).

We now propose (Fig. 5) a series of reactions for binding of oxygen and release of water that appear to be consistent with properties of cytochrome oxidase observed with the carbon monoxide complex. This series is similar to reactions proposed by others (e.g., ref. 20) except that we propose a "pumping" function for a_3 Cu to help transport dioxygen and protons into the heme pocket and water out of it (shuttle mechanism). Such an action would be consistent with a highly ordered nonpolar surrounding for a_3 heme and a flexible structure for a_3 Cu.

We thus propose that cytochrome a_3 copper facilitates O_2 transport, participates equally with a_3 heme in oxygen reduction, stabilizes a peroxide bridge from half-reduced dioxygen, and facilitates removal of water from the a_3 heme pocket.

We thank Prof. Howard S. Mason, who suggested the importance of cytochrome *c* oxidase to one of us (J.O.A.) many years ago; H. Frauenfelder and coworkers, who introduced us to low-temperature spectroscopy; and G. Brierley, who furnished mitochondrial preparations that were used in this work. This work was supported in part by grants from the National Institutes of Health (HL 17839) and the American Heart Association (78-1089).

1. Van Gelder, B. F. & Beinert, H. (1969) *Biochim. Biophys. Acta* **189**, 1–24.
2. Tweedle, M. F., Wilson, L. J., Garcia-Iniquez, L., Babcock, G. T. & Palmer, G. (1978) *J. Biol. Chem.* **253**, 8065–8071.
3. Kubowitz, F. (1938) *Biochem. Z.* **299**, 32–57.
4. Thomson, A. J., Brittain, T., Greenwood, C. & Springall, J. P. (1977) *Biochem. J.* **165**, 327–336.
5. Sharrock, M. & Yonetani, T. (1976) *Biochim. Biophys. Acta* **434**, 333–344.
6. Sharrock, M. & Yonetani, T. (1977) *Biochim. Biophys. Acta* **462**, 718–730.
7. Alben, J. O., Altschuld, R. A., Fiamingo, F. G. & Moh, P. P. (1980) in *Interaction Between Iron and Proteins in Oxygen and Electron Transport*, ed. Ho, C. (Elsevier/North-Holland, New York), in press.
8. Jung, D. W., Chavez, E. & Brierley, G. P. (1977) *Arch. Biochem. Biophys.* **183**, 452–459.
9. Caughey, W. S., Bayne, R. A. & McCoy, S. (1970) *J. Chem. Soc. D*, 950–951.
10. Volpe, J. A., O'Toole, M. C. & Caughey, W. S. (1975) *Biochem. Biophys. Res. Commun.* **62**, 48–53.
11. Yoshikawa, S., Choc, M. G., O'Toole, M. C. & Caughey, W. S. (1977) *J. Biol. Chem.* **252**, 5498–5508.
12. Fager, L. Y. & Alben, J. O. (1972) *Biochemistry* **11**, 4786–4792.
13. Alberding, N., Austin, R. H., Beeson, K. W., Chan, S. S., Eisenstein, L., Frauenfelder, H. & Nordlund, T. M. (1976) *Science* **192**, 1082–1104.
14. Alben, J. O., Beece, D., Bowne, S. F., Eisenstein, L., Frauenfelder, H., Good, D., Marden, M. C., Moh, P. P., Reinisch, L., Reynolds, A. H. & Yue, K. T. (1980) *Phys. Rev. Lett.* **44**, 1157–1160.
15. Jiang, G. J., Person, W. P. & Brown, K. G. (1975) *J. Chem. Phys.* **62**, 1201–1211.
16. Yen, L. (1971) Dissertation (Ohio State Univ., Columbus, OH).
17. Bare, G. H. (1973) Dissertation (Ohio State Univ., Columbus, OH).
18. Lindsay, J. G. & Wilson, D. F. (1974) *FEBS Lett.* **48**, 45.
19. Schroedl, N. A. & Hartzell, C. R. (1977) *Biochemistry* **16**, 4966–4971.
20. Erecinska, M. & Wilson, D. F. (1978) *Arch. Biochem. Biophys.* **188**, 1–14.