ELSEVIER

Biochimica et Biophysica Acta 1232 (1995) 225–229

BBA Biochimica et Biophysica Acta

Aerobic and anaerobic functioning of superoxide-producing cytochrome *b*-559 reconstituted with phospholipids

Vasilij Koshkin *

The Julius Friedrich Cohnheim Center for Phagocyte Research, Tel-Aviv University, Tel-Aviv 69978, Israel

Received 29 March 1995; revised 7 August 1995; accepted 1 September 1995

Abstract

Cytochrome *b*-559 reconstituted with phospholipids and FAD represents the simplest model of the respiratory burst NADPH oxidase and reproduces the main catalytic features of this system (Koshkin, V. and Pick, E. (1993) FEBS Lett. 327, 57–62; (1994) FEBS Lett. 338, 285–289). In the present report it is shown that activation by oxygen, characteristic of the NADPH oxidase complex, is an intrinsic property of flavocytochrome *b*-559, in principle independent of its complexation with the other components of NADPH oxidase. Facilitation of electron transfer from NADPH to FAD is found to be the reason for this phenomenon. Kinetic studies of anaerobic operation of flavocytochrome *b*-559 revealed the functional heterogeneity of two hemes, manifested as a dramatic difference in their reducibility under these conditions.

Keywords: Respiratory burst; NADPH oxidase; Flavocytochrome *b*-559; Oxygen activation

1. Introduction

Superoxide production in phagocytic cells, known as respiratory burst, is catalyzed by an NADPH oxidase complex, consisting of a membrane-bound cytochrome *b*-559 and three cytosolic proteins (p47-*phox*, p67-*phox* and the small G-protein *rac*1 p21 or *rac*2 p21) [1–5]. Recent studies of this complex in the cell-free system [6,7] led to the concept that cytochrome *b*-559 is the only catalytic component of NADPH oxidase, containing, in addition to two hemes, FAD- and NADPH-binding domains, and catalyzing electron transfer in the sequence NADPH → FAD → heme → O_2 [8–10]. The function of cytosolic proteins, according to this concept, is to bring this electron transport chain from the dormant to the active state. On the other hand, it was found that the cytochrome *b*-559 heme can also be reduced by foreign flavoproteins [11–13], including one from neutrophil membranes [14,15].

Recently we demonstrated the cytochrome *b*-559 ability to function as an independent generator of O_2^- in vitro, provided that it is incorporated into phospholipid vesicles of a certain composition [16]. This offers a direct functional proof for the catalytic competence of cytochrome *b*-559 and a useful model to study the enzymology of the respiratory burst [17–19]. It is known that oxygen is a requirement for efficient electron flow within the NADPH oxidase complex, and the rate of anaerobic heme reduction accounts for less than 10% of the aerobic steady-state turnover [20,21]. Possible explanations include a conformational change in NADPH oxidase caused by oxygen binding [20], or such kinetic organization of this system that the presence of oxidant is necessary for its rapid functioning [20,22]. In the present work I undertook an investigation of the influence of oxygen on the operation of flavocytochrome *b*-559 reconstituted with phospholipids. It is shown that activation by oxygen is characteristic of flavocytochrome *b*-559 itself, and its behaviour in aerobic and anaerobic conditions is explored.

2. Materials and methods

Cytochrome *b*-559 in catalytically active state was obtained essentially as described earlier [19,23]. Cytochrome *b*-559 was solubilized from high-speed-sedimented fraction of prewashed guinea-pig peritoneal macrophage mem-

Abbreviations: O_2^-, superoxide; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; LiDS, lithium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; SOD, superoxide dismutase.

* Corresponding author. Fax: +972 3 6429119.

SSDI 0005-2728(95)00123-9

branes [19] with 40 mM octyl glucoside in buffer A (0.05 M sodium phosphate (pH 7.4), 1 mM $MgCl_2$, 1 mM EGTA, 20% glycerol) in the presence of 1 mM PMSF and 1 μg/ml of leupeptin. Further purification included affinity chromatography on heparin agarose and resulted in cytochrome *b*-559 preparations with a specific content of 4–6 nmol heme/mg protein. The concentration of cytochrome *b*-559 heme was calculated from differential reduced *minus* oxidized spectra using the extinction coefficient (427–411 nm) = 200 mM^{-1} cm^{-1} [24]. Protein concentration was measured by the method of Bradford [25] with bovine serum albumin as the standard.

For activation, cytochrome *b*-559 was incorporated into vesicles consisting of azolectin-phosphatidic acid in the following way: cytochrome-containing fractions, after affinity chromatography, were concentrated by ultrafiltration to a concentration of 4–6 μM, supplemented with phospholipids (L-α-phosphatidylcholine, type IV, and phosphatidic acid from egg yolk lecithin, both from Sigma, 130 μg/ml of each) and frozen at −75°C. After thawing, cytochrome *b*-559-containing vesicles possessing superoxide producing activity were formed by dialysis against detergent-free buffer A. This material was kept until use at −75°C.

FAD binding properties of this system were studied by the fluorometric titration with FAD. As we have shown earlier, binding to cytochrome *b*-559 results in quenching of FAD fluorescence [17], specificity of this quenching is confirmed by the fact that cytochrome *b*-559 in inactive state, i.e., devoid of activating phospholipids, does not quench fluorescence of added FAD. The normalized fluorescence quenching ($q_{norm} = q/q_{max}$), that can serve as a measure of protein liganding [26], was calculated by comparison with the control cytochrome-free vesicles. The plots of this value against total FAD added were fitted to the equation of equilibrium binding $K = (b - bf)(f - bf)/bf$, that was rearranged to the form:

$$bf/b = (K + b + f)/2b - \sqrt{[(K + b + f)/2b]^2 - f/b} \quad (1)$$

in order to express fractional saturation of cytochrome *b*-559 with FAD as a function of the total FAD concentration (K denotes the dissociation constant, b and f total concentrations of cytochrome *b*-559 and FAD, and bf concentration of the cytochrome *b*-559-FAD complex).

Electron transport in this system in the presence of NADPH, superoxide dismutase (SOD) and catalase was followed by spectral scanning in the range 360–600 nm with 10 s intervals using a diode-array spectrophotometer (HP-8452A; Hewlett-Packard). A time-course of NADPH oxidation and oxidoreduction of heme was derived from these spectra at the wavelengths 366 and 558–540 nm, respectively (contribution of FAD to NADPH absorbance was no more than 0.2%). Separate samples of identical composition were used to monitor oxidoreduction of FAD

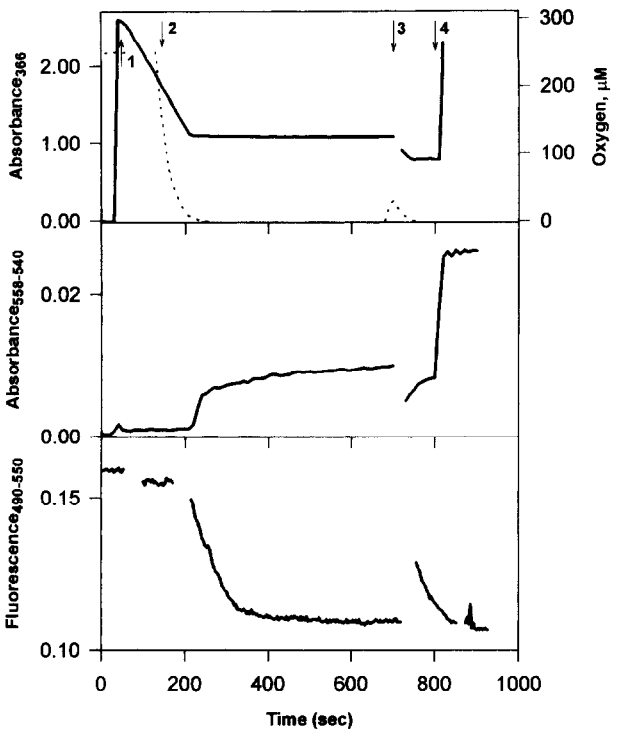

Fig. 1. Reaction time course for NADPH (upper panel, solid line, left *Y*-axis), O_2 (upper panel, dotted line, right *Y*-axis), heme *b*-559 (middle panel) and FAD (lower panel) during aerobic and anaerobic operation of flavocytochrome *b*-559. Phospholipid-reconstituted cytochrome *b*-559 in buffer A (1.19 μM of heme, 130 μg/ml of phosphatidylcholine, 130 μg/ml of phosphatidic acid) was supplemented with 0.58 μM of FAD, 30 μM of LiDS, 75 U/ml of SOD, 257 U/ml of catalase and 150 mM of glucose. The reaction was started by the addition of 0.66 mM of NADPH (arrow marked 1), aerobiosis was maintained by the stirring of the cuvette content between spectral scans, anaerobiosis was achieved by the addition of 12 U/ml of glucose oxidase and covering of the cuvette content by the paraffin oil (arrow marked 2). This was followed by the oxygen pulse (addition of 20 μl of buffer A bubbled with oxygen for 10 min, arrow marked 3); finally the system was completely reduced by dithionite (arrow marked 4).

group by fluorometry (at 490 → 550 nm to avoid interference from excess of NADPH) and oxygen consumption by polarography. Steady-state aerobic functioning of cytochrome *b*-559 was maintained in the samples open to atmospheric oxygen by periodic stirring (every 10 s), to achieve anaerobic state the samples were supplemented with the oxygen-scavenging system glucose-glucose oxidase and covered by the paraffin oil [27] (kinetics of the oxygen depletion due to combined process of glucose and NADPH oxidation is shown in Fig. 1). Kinetic curves of heme and FAD reduction were fitted to the single or double exponential kinetics:

$$\text{Absorbance(fluorescence)} = a_1[1 - \exp(-k_1 t)] + a_2[1 - \exp(-k_2 t)] \quad (2)$$

from which first-order rate constants (k) and amplitudes (a) were obtained.

Experiments were carried out in the presence of 30 μM of lithium dodecyl sulfate (LiDS), which is known as a moderate activator of this system [16]. O_2^- production was quantitated by the initial rate of SOD-inhibitable cytochrome *c* reduction, essentially as described before [16]. All fluorometric measurements were performed with a FP-770 spectrofluorometer (Jasco); for routine absorbance measurements a Uvikon 860 spectrophotometer (Kontron) was used and oxygen consumption was measured with the micro probe of a YSI 53 oxygen monitor (Yellow Springs Instruments). Fitting and plotting procedures were performed using SigmaPlot scientific graphing software (Jandel Scientific Co.).

3. Results and discussion

Fig. 1 demonstrates the time course of NADPH oxidation, kinetics of oxygen depletion and redox state of FAD and heme centers during aerobic and anaerobic operation of cytochrome *b*-559. The first aerobic part of this diagram shows the process of steady-state NADPH oxidation with the low stationary level of FAD and heme reduction (9 and 3%, respectively), so that electron flows on the separate steps are $V_{NADPH \rightarrow FAD} = V_{FAD \rightarrow heme} = V_{heme \rightarrow oxygen} = k[heme^{2+}]$ [20], where k is the pseudo-first-order rate constant for heme oxidation. The value of 186 s^{-1} can be calculated for the last parameter (from the rate of NADPH oxidation of 5.59 μequiv/s and the steady-state concentration of reduced heme of 0.03 μM), which is close to the value of 147 s^{-1} reported earlier for the whole NADPH oxidase complex [20,28]. K_m for O_2 was estimated by a separate run of oxygen consumption (data not shown) and found to be about 10 μM, which is also close to the corresponding parameter of the native system [29,30].

When oxygen was exhausted, the steady-state electron flow through the system ceased and the reduction of flavin and heme above the stationary level started with initial rates of 0.005 and 0.009 μequiv/s, respectively. When this reduction was nearly completed a small amount of oxygen was introduced into the system, and after its depletion dithionite was added.

The discrepancy between velocities of aerobic functioning (5.59 μequiv/s) and anaerobic reduction (0.014 μequiv/s) of flavocytochrome prosthetic groups suggests that in anaerobic state electron transfer within this system is strongly inhibited. This inhibition was reversible judging by the effect of the small oxygen pulse on NADPH oxidation. As both FAD and heme undergo only slow reduction under anaerobic conditions, the site in the electron transport chain affected by anaerobiosis is situated before these centers, namely, at the step of binding or oxidation of NADPH. The lowering of NADPH concentration to the level of K_m for the aerobic state had a minor effect on the rate of anaerobic heme reduction; hence, in the anaerobic state the system can be saturated with

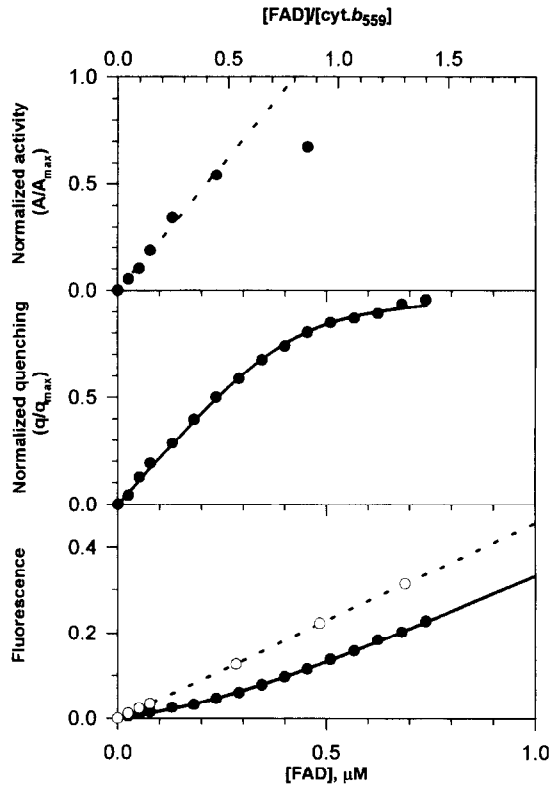

Fig. 2. Cytochrome *b*-559 titration with FAD. Phospholipid-reconstituted cytochrome *b*-559 in buffer A (1.08 μM of heme, 130 μg/ml of phosphatidylcholine, 130 μg/ml of phosphatidic acid) was supplemented with 30 μM of LiDS and titrated with FAD with the parallel measurement of the fluorescence (lower panel, filled circles-titration of cytochrome *b*-559, open circles-titration of the control cytochrome-free phospholipid vesicles) and superoxide-producing activity (upper panel). The normalized fluorescence quenching derived from fluorescence titration was fitted to the equation of equilibrium binding (Materials and methods, Eq. (1)) (middle panel, dots – experimental data; line – theoretical curve with $b = 0.42$ μM and $K = 0.024$ μM).

NADPH more easily and anaerobiosis hinders NADPH oxidation rather than its binding.

It is noteworthy that, as apparent in Fig. 1, when the reduction of FAD was practically complete, the heme reduction did not exceed half-maximal level. A similar situation is known for the complete NADPH oxidase complex and in order to explain it, the existence of inactive fraction of cytochrome *b*-559 was supposed [28,31]. In the present system, the state of cytochrome *b*-559 can be characterized precisely by its titration with FAD, which provides an estimation of its FAD-binding capacity and of the catalytic efficiency of its reflavinated form. Typical data illustrating this approach are presented in Fig. 2. Fluorometric titration of cytochrome *b*-559 with FAD was performed (lower panel) and a plot of the normalized fluorescence quenching against added FAD (middle panel) was fitted to dependence of fractional protein liganding on total ligand added (see Materials and methods, Eq. (1)). This gives a K_{dis} of 0.024 μM and the concentration of binding sites of 0.42 μM, which corresponds to approxi-

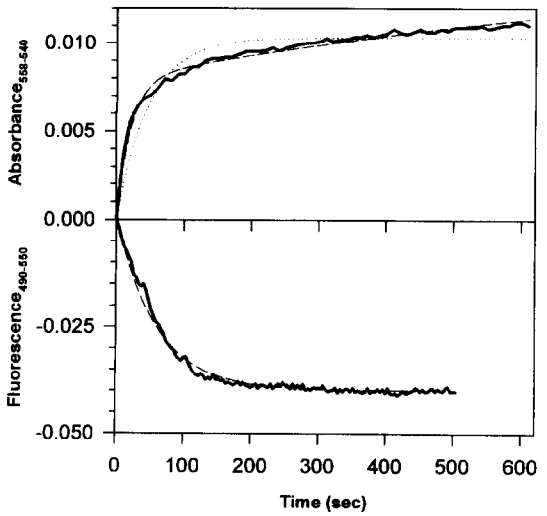

Fig. 3. Kinetics of the anaerobic heme and FAD reduction in flavocytochrome *b*-559. Phospholipid-reconstituted cytochrome *b*-559 (1.20 μM of heme, 130 μg/ml of phosphatidylcholine, 130 μg/ml of phosphatidic acid) in the same medium as in Fig. 1 was brought into anaerobic state with the aid of glucose oxidase, the reaction was started by the addition of deoxygenated NADPH solution to a final concentration of 0.70 mM at zero time. The FAD reduction (lower panel, solid line) was fitted to the single exponential kinetics (dashed line), and the heme reduction (upper panel, solid line) to the single and double exponential kinetics (dotted and dashed lines, respectively).

mately 80% of cytochrome *b*-559 present ([cytochrome *b*-559 heterodimer] = [heme *b*-559]/2). A gradual growth of superoxide producing activity of cytochrome *b*-559 in the course of titration was measured, normalized and plotted as a function of the FAD/cytochrome *b*-559 ratio (upper panel). The initial linear part of this curve, representing the region of stoichiometric FAD binding, has a slope of 0.8 and indicates that 80% of cytochrome *b*-559 is accessible to activation. Thus, both FAD-binding capacity and catalytic activity show 80% degree of cytochrome *b*-559 activation; at the same time only 38% of heme in this preparation was readily reducible under anaerobic conditions (Fig. 1). Therefore it seems reasonable to suppose that the cause for incomplete heme reduction is heterogeneity of two hemes in the cytochrome *b*-559 molecule, only one of them being easily accessible to reduction in an anaerobic state.

More detailed kinetic investigation of anaerobic reduction of redox centers in flavocytochrome *b*-559 is shown in Fig. 3, where kinetic traces of FAD fluorescence and heme absorbance are fitted to the single or double exponential kinetics (see Materials and methods, Eq. (2)). The reduction of FAD looks like a single exponential reaction with a first-order rate constant of 0.017 s^{-1}, although this is only an apparent value reflecting a complex process of FAD reduction by NADPH and its oxidation by hemes. The heme reduction is clearly better approximated by the double than by the single exponential kinetics with equal amplitudes of both phases and rate constants of 0.0476 and 0.0009 s^{-1}. Inequivalence of hemes in cytochrome *b*-559 was suggested first by Iizuka et al. [32] from the low-temperature absorbance spectra and by Hurst et al. [33] from the data on cytochrome *b*-559 photoreduction. On other hand, cytochrome *b*-559 hemes behave as a thermodynamically and kinetically homogeneous pool in reduction by dithionite [28,33–35] and oxidation by oxygen and ferricyanide [13,20,28,33]. One can suppose that two hemes are differently located in the protein molecule, so that they are equally accessible to external reductants and oxidants but differ in accessibility to reduction by the bound FAD, at least under anaerobic conditions.

In conclusion, the data presented show that activation by oxygen (as well as the kinetics of interaction with it) is an intrinsic property of flavocytochrome *b*-559, in principle independent of its complexation with other components of NADPH oxidase. In this relation it resembles such redox enzymes, as cytochrome *c* oxidase [36], ascorbate oxidase and laccase [37], although underlying mechanisms in each case could be different. This activation is due to acceleration of FAD reduction, and suggests that interaction of some kind with oxygen precedes an efficient electron transfer within flavocytochrome *b*-559 and results in its conversion from the slow to the fast operating state. In the slow state of cytochrome *b*-559, the two hemes differ greatly in their ability to accept electrons from FAD; the question whether there is any difference between hemes in the fast aerobic state requires further investigation.

Acknowledgements

This work was supported by: a Ministry of Absorption grant, to Dr. V. Koshkin; grant No.10/94 from the Israel Science Foundation, to Prof. Edgar Pick; a grant from the Israel Cancer Research Fund, to Prof. Edgar Pick, and by the David and Natalie Roberts Chair in Immunopharmacology (Incumbent: Prof. Edgar Pick). The author is grateful to Professor Edgar Pick for support and encouragement.

References

[1] Morel, F., Doussiere, J. and Vignais, P.V. (1991) Eur. J. Biochem. 201, 513–546.
[2] Cross, A.R. and Jones, O.T.G. (1991) Biochim. Biophys. Acta 1057, 281–298.
[3] Rotrosen, D. (1992) in Inflammation – Basic Principles and Clinical Correlates (Gallin, J.I., Goldstein, I.M. and Snyderman, R., eds.), pp.589–601, Raven Press, New York.
[4] Babior, B.M. (1992) in Advances in Enzymology (Meister, A., ed.), Vol. 65, pp. 49–95, John Wiley & Sons, New York.
[5] Segal, A.V. and Abo, A. (1993) Trends Biochem. Sci. 18, 43–47.
[6] Bromberg, Y. and Pick, E. (1984) Cell. Immunol. 89, 213–221.
[7] Bromberg, Y. and Pick, E. (1985) J. Biol. Chem. 260, 13539–13545.
[8] Rotrosen, D., Yeung, C.L., Leto, C.L., Malech, H.L. and Kwong, C.H. (1992) Science 256, 1459–1462.
[9] Segal, A.W., West, J., Wientjes, F., Nugent, J.H.A., Chavan, A.J.,

Haley, B., Garcia, R.C., Rosen, H. and Scarce, G. (1992) Biochem. J. 284, 781–788.
[10] Sumimoto, H., Sakamoto, N., Nozak, M., Sakaki, Y., Takeshige, K. and Minakami, S. (1992) Biochem. Biophys. Res. Commun. 186, 1368–1375.
[11] Sakane, F., Kojima, H., Takahashi, K. and Koyama, J. (1987) Biochem. Biophys. Res. Commun. 147, 71–77.
[12] Isogai, Y., Shiro, Y., Nasudo-Kouyama, A. and Iizuka, T. (1991) J. Biol. Chem. 266, 13481–13484.
[13] Isogai, Y., Iizuka, T., Makino, R., Iyanagi, T. and Orii, Y. (1993) J. Biol. Chem. 268, 4025–4031.
[14] Laporte, F., Doussiere, J., Mechin, V. and Vignais, P.V. (1991) Eur. J. Biochem. 196, 59–66.
[15] Miki, T., Yoshida, L.S. and Kakinuma, K. (1992) J. Biol. Chem. 267, 18695–18701.
[16] Koshkin, V. and Pick, E. (1993) FEBS Lett. 327, 57–62.
[17] Koshkin, V. and Pick, E. (1994) FEBS Lett. 338, 285–289.
[18] Leusen, J.H.W., Bolscher, B.G.J.M., Hilarius, P.M., Weening, R.S., Kaulfersch, W., Seger, R.A., Roos, D. and Verhoeven, A.J. (1994) J. Exp. Med. 180, 2329–2334.
[19] Koshkin, V. (1995) Biochim. Biophys. Acta 1229, 329–333.
[20] Cross, A.R., Parkinson, J.F. and Jones, O.T.G. (1985) Biochem. J. 226, 881–884.
[21] Ellis, J.A., Cross, A.R. and Jones, O.T.G. (1989) Biochem. J. 262, 575–579.
[22] Cross, A.R., Yarchover, J.L. and Curnutte, J.T. (1994) J. Biol. Chem. 269, 21448–21454.
[23] Knoller, S., Shpungin, S. and Pick, E. (1991) J. Biol. Chem. 266, 2795–2804.
[24] Light, D.R., Walsh, C., O'Callaghan, A.M., Goetzl, E.J. and Tauber, A.J. (1981) Biochemistry 20, 1468–1476.
[25] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[26] Edsall, J.T. and Gutfreund, H. (1983) Biothermodynamics, pp. 157–165, John Wiley & Sons, Chichester.
[27] Englander, S.W., Calhoun, D.B. and Englander, J.J. (1987) Anal. Biochem. 161, 300–306.
[28] Cross, A.R., Higson, F.K., Jones, O.T.G., Harper, A.M. and Segal, A.W. (1982) Biochem. J. 204, 479–485.
[29] Morel, F., Doussiere, J., Stasia, M-J. and Vignais, P.V. (1985) Eur. J. Biochem. 152, 669–679.
[30] Green, T.R. and Wu, D.E. (1986) J. Biol. Chem. 261, 6010–6015.
[31] Cross, A.R., Parkinson, J.F. and Jones, O.T.G. (1984) Biochem. J. 223, 337–344.
[32] Iizuka, T., Kanegasaki, S., Makino, R., Tanaka, T. and Ishimura, Y. (1985) J. Biol. Chem. 260, 12049–12053.
[33] Hurst, J.K., Loehr, T.M., Curnutte, J.T. and Rosen, H. (1991) J. Biol. Chem. 266, 1627–1634.
[34] Cross, A.R., Jones, O.T.G., Harper, A.M. and Segal, A.W. (1981) Biochem. J. 194, 599–606.
[35] Aviram, I. and Sharabani, M. (1986) Biochem. J. 237, 567–572.
[36] Antonini, E., Brunori, M., Colosimo, A., Greenwood, C. and Wilson, M.T. (1977) Proc. Natl. Acad. Sci. USA 74, 3128–3132.
[37] Tollin, G., Meyer, T.E., Cusanovich, M.A., Curir, P. and Marchesini, A. (1993) Biochim. Biophys. Acta 1183, 309–314.