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Effect of ethoxyformic anhydride on the Rieske iron–sulfur protein of bovine heart ubiquinol: cytochrome *c* oxidoreductase

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Abstract Treatment of bovine heart ubiquinol-cytochrome *c* oxidoreductase (complex III, bc_1 complex) with ethoxyformic anhydride (EFA) inhibits electron transfer between cytochromes *b* and c_1 [Yagi et al., Biochemistry 21 (1982) 4777–4782]. This paper shows that EFA alters the EPR lineshape of the Rieske iron–sulfur cluster in complex III and in the isolated Rieske protein without a significant decrease of spin concentration. The effect of EFA on the Rieske iron–sulfur cluster is competitive with that of Q_o site inhibitors, such as stigmatellin, and is completely reversed by hydroxylamine. These results are consistent with the possible ethoxyformylation by EFA of histidine ligands of the Rieske iron–sulfur cluster at the non-iron binding imidazole nitrogens.

Key words: Ethoxyformic anhydride[1]; Rieske iron–sulfur cluster; EPR spectra; Bovine ubiquinol-cytochrome *c* oxidoreductase; bc_1 complex; Complex III

1. Introduction

Quinol-cytochrome *c* (*c_2 or* plastocyanin) oxidoreductases from mitochondria, chloroplasts, and photosynthetic or non-photosynthetic bacteria contain three redox proteins, a bis-heme cytochrome *b*, cytochrome c_1 (or cytochrome *f* in chloroplasts), and a binuclear iron–sulfur protein [1,2]. This iron– sulfur protein, generally referred to as the Rieske iron–sulfur protein, differs from typical ferredoxin-type iron–sulfur proteins in the following respects: (i) the Rieske iron–sulfur cluster is ligated by two histidine and two cysteine residues [3–7], (ii) it shows a unique EPR spectrum with average *g* value of 1.91 [1,2], and (iii) its redox midpoint potential is higher than +150 mV (see [1] and references therein).

According to the modified Q-cycle hypothesis [2,8,9], two separate quinone reaction sites, Q_o and Q_i, are present on opposite sides of the membrane [1,10,11], and an electrogenic transmembraneous electron transfer occurs between these two sites [12,13]. At the Q_o site, two electrons from QH_2 are split; the first electron reduces the Rieske iron–sulfur cluster, while the second electron cycles back via cytochrome b_L (low potential *b*) and b_H (high potential *b*) to reduce Q at the Q_i site [1,8,9]. The reduced Rieske iron–sulfur cluster is oxidized by the *c*-type cytochromes.

The inhibitors of the enzyme (hereafter referred to as complex III or bc_1 complex) are divided into two groups depending on their assigned reaction site near the Q_i or the Q_o site. Examples of the former are antimycin, HQNO (2-n-heptyl-4-hydroxyquinoline-*N*-oxide) and diuron, and examples of the latter are myxothiazol, mucidin, stigmatellin and UHDBT (undecylhydroxydioxobenzothiazole) [1,14]. Phenomenologically, however, all of these reagents inhibit the oxidation of the *b* and the reduction of the *c* cytochromes in steady state kinetic analyses.

Another reagent that has the same effect on bovine complex III is ethoxyformic anhydride (EFA), which is capable of ethoxyformylating nucleophilic amino acid residues of proteins, including the imidazole nitrogens of histidyl residues [15]. Yagi et al. [16] showed that the inhibitory modification of complex III by EFA did not alter the spectra of cytochromes *b* and c_1, was complementary to the inhibition by antimycin A, resulted in the development of a peak at 238 nm, and was readily reversed by addition of hydroxylamine. While it was recognized that the peak at 238 nm and the ready reversibility by hydroxylamine are characteristic of ethoxyformylation of histidyl residues at one imidazole nitrogen, the site of EFA modification was not identified. This paper shows that EFA treatment of bovine complex III results in modification of the Rieske iron–sulfur protein, and the data suggest that ethoxyformylation of complex III or the isolated bovine Rieske protein involves the histidine ligands of the iron–sulfur cluster.

2. Materials and methods

Bovine heart complex III was prepared as described in [17]. The Rieske iron–sulfur protein was isolated from bovine heart cytochrome bc_1 complex as detailed in Ref [18]. The EFA treatment method is described in the figure legends, and follows the procedure reported in [16]. EPR spectra were recorded as described in [11].

3. Results

The effect of EFA on the EPR spectrum of the Rieske cluster in bovine heart complex III is shown in Fig. 1. The Rieske cluster reduced by Q_2H_2 (reduced ubiquinone-2) exhibits an EPR spectrum with $g_{x,y,z}$ = 1.76, 1.90, 2.023 (Fig. 1A). Upon treatment with EFA (Fig. 1B), the Rieske lineshape became less rhombic, with the g_x and g_z shifting to 1.80 and 2.017, respectively, and with considerable broadening of the g_x signal. These changes could be reversed by incubating the EFA-treated complex with hydroxylamine (Fig. 1C), as a result of which the spectral features returned essentially to that in Fig. 1A. The

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[1]Ethoxyformic anhydride is also refered to as diethylpyrocarbonate.

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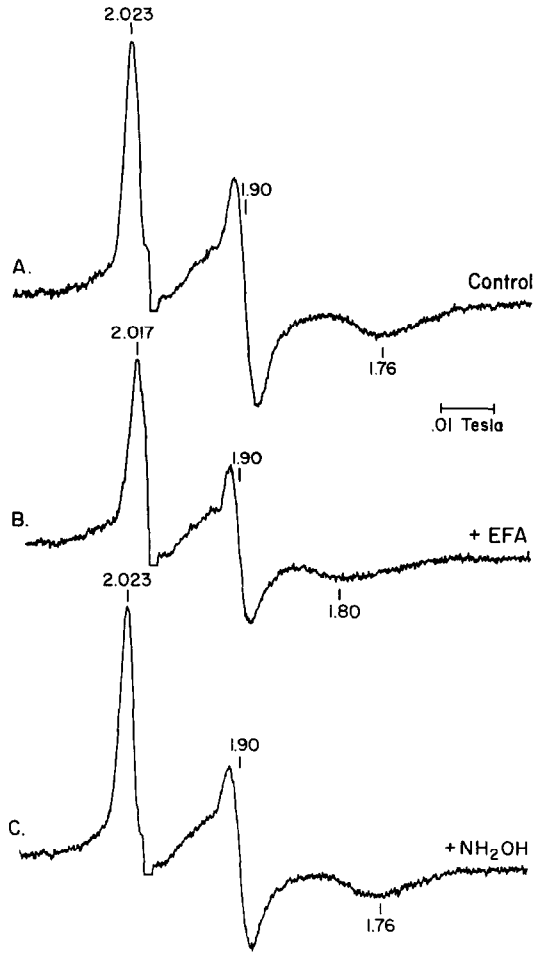

Fig. 1. Effect of ethoxyformic anhydride (EFA) on the EPR signature of the Rieske iron–sulfur cluster and the reversal of this effect by hydroxylamine. (A) 41 μM complex III was reduced with 1 mM ubiquinol-2. (B) 94.3 μM complex III was incubated with 7.6 mM EFA at 1°C for 10 min, then diluted to 41 μM and reduced as in (A). (C) EFA treated complex III (94.3 μM) was treated with 30 mM hydroxylamine for 30 min. at 0°C before dilution and reduction as in (B). EPR conditions: microwave power, 1 mW; modulation amplitude, 1×10^{-3} tesla; time constant, 0.128 s; sample temperature, 15 K.

total spin concentration of the Rieske cluster in Fig. 1B did not show a significant decrease from that of Fig. 1A, indicating that EFA does not modify the δ-nitrogens of the two histidine imidazoles, which directly coordinate an iron of the Rieske iron–sulfur cluster [19]. The ready reversibility of this modification by hydroxylamine is consistent with the possibility that EFA ethoxyformylates one or both of these imidazole groups at their ε-nitrogen(s). However, this conclusion does not preclude the possibility that the effect on the Rieske cluster lineshape originates from EFA modification of histidine residues not directly involved in cluster formation.

Fig. 2 shows the effect of EFA on the binding of various inhibitors to complex III. Fig. 2A presents the spectrum of the Rieske cluster in the EFA-treated bc_1 complex. In this case the $g_{x,y,z}$ values are 1.80, 1.90, and 2.015. Subsequent antimycin treatment did not give rise to any alteration of the Rieske spectrum of the EFA-treated complex (Fig. 3B), as is expected for a Q_i site inhibitor. Likewise, myxothiazol, which is known to bind to the cytochrome b domain of the Q_o site [1,14] caused no spectral alteration of the Rieske cluster (not shown). Stigmatellin is known to exert a strong effect on both the lineshape and the redox midpoint potential of the Rieske cluster, as well as to induce a large spectral shift of cytochrome b_L [20]. However, when stigmatellin was added to the EFA-treated complex, the spectrum of the Rieske cluster again remained unaltered as in the case of antimycin treatment (Fig. 2C). For comparison, Fig. 2D shows the effect of stigmatellin on the Rieske lineshape when it was added to the bc_1 complex not pretreated with EFA. In this case a much sharper EPR lineshape with $g_{x,y,z}$ = 1.79, 1.88, 2.021 is seen, which is the specific signature of the effect of stigmatellin on the Rieske cluster (Fig. 2D). Similarly, no spectral alteration was observed with UHDBT (data not presented). These results suggested that modification of the Rieske

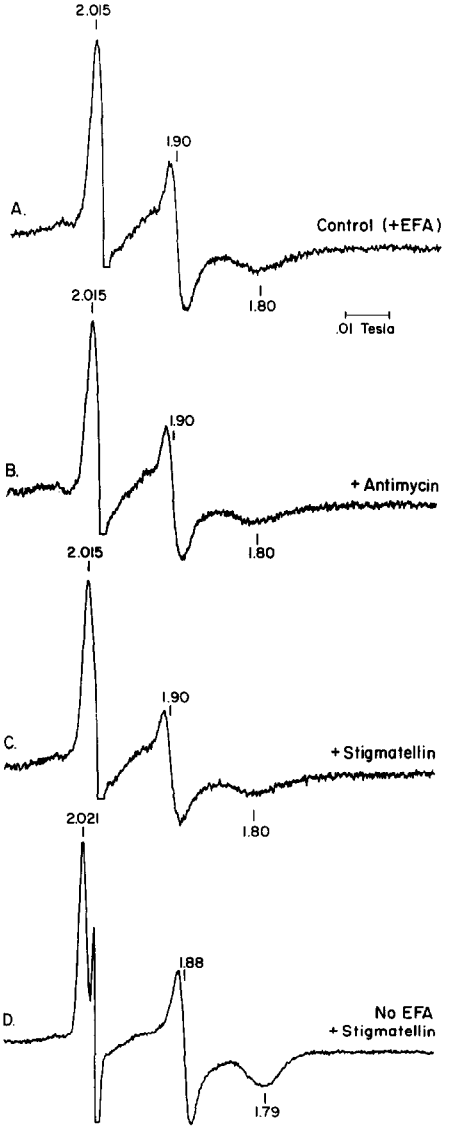

Fig. 2. Pretreatment of complex III with EFA prevents the effect of stigmatellin on the EPR line shape of the Rieske iron–sulfur cluster. (A) EFA treated complex III, same as in Fig. 1B. (B) Sample A was treated with 12.5 μM antimycin. (C) Sample A was treated with 125 μM stigmatellin. (D) EFA untreated complex III was reduced with 2.5 mM ascorbate, then treated with 125 μM stigmatellin. EPR conditions were the same as in Fig. 1.

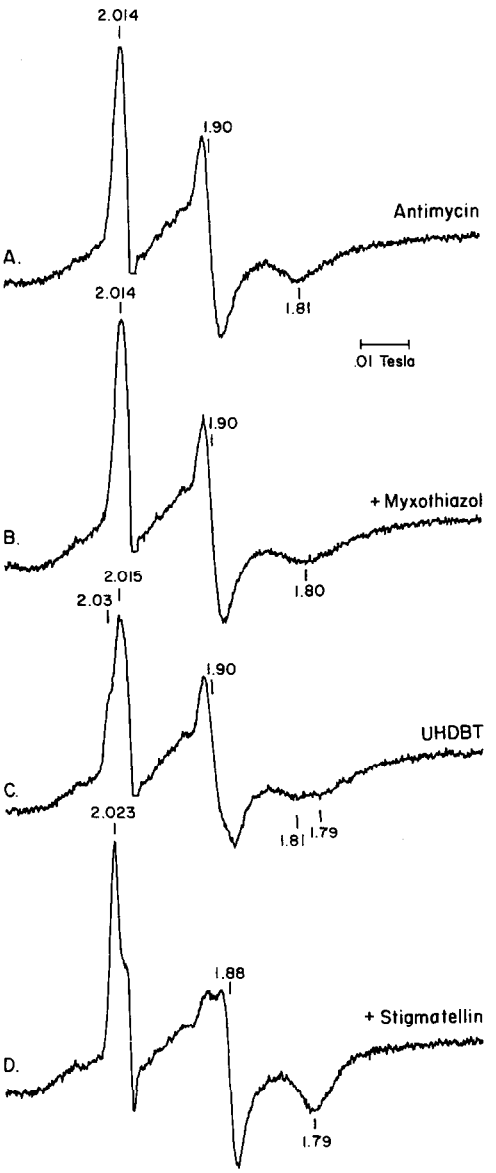

Fig. 3. Effect of EFA on the EPR spectral features of the Rieske iron–sulfur cluster after pretreatment of complex III with various inhibitors. The inhibitors shown were added to complex III (26 mg protein/ml) at the final concentration of 125 μM. Subsequently EFA was added to a final concentration of 7.8 mM and incubated at O°C for 10 min. After incubation, the Rieske cluster was reduced with ferrocytochrome *c* at the final concentration of 1.33 mM: A., antimycin; B., myxothiazol; C., UHDBT; D., stigmatellin. EPR conditions were the same as in Fig. 1.

iron–sulfur protein by EFA prevents stigmatellin and UHDBT from inducing changes in the Rieske lineshape, and are consistent with the possibility that EFA reacts directly with the Rieske cluster.

Fig. 3 shows the effect of EFA after pretreatment of the bc_1 complex with various inhibitors. When antimycin was added prior to EFA (Fig. 3A), the lineshape of the Rieske iron–sulfur cluster was essentially the same as that effected by EFA alone (see Fig. 2A). A similar spectrum, with only a slight broadening of the g_x lineshape, was obtained when myxothiazol was added prior to EFA. On the other hand, when complex III was pre-treated with UHDBT, which is a weak inhibitor of the Rieske domain of the Q_o site, prior to addition of EFA, then the resulting spectrum appeared to be composed of contributions from two iron–sulfur species, one species being the EFA modified type as indicated by peaks at $g_{x,z}$ = 1.81 and 2.015 (Fig. 3C), the other species being the remaining UHDBT bound form as suggested by the peaks at $g_{x,z}$ = 1.79 and 2.03. However, when the stigmatellin effect was examined, the major species appeared to be the stigmatellin-bound form, with $g_{x,y,z}$ = 1.79, 1.88, 2.023, plus only a small contribution from the EFA modified species as suggested by the small shoulder to the left of the g = 1.88 peak (Fig. 3D). Competitive binding studies of various Q_o site inhibitors have shown that stigmatellin has a much stronger affinity for the complex than UHDBT [14]. Therefore, it appears from the results in Fig. 3 that the ability of an inhibitor to prevent the modification of the Rieske lineshape by EFA correlates with its binding affinity. In previous studies of site specific inhibition, it was shown that those compounds which induced a lineshape change in the Rieske cluster of bovine heart bc_1 complex also altered its midpoint redox potential [21]. We attempted to investigate this possibility in EFA-treated bc_1 complex. However, when EFA-treated complex III was

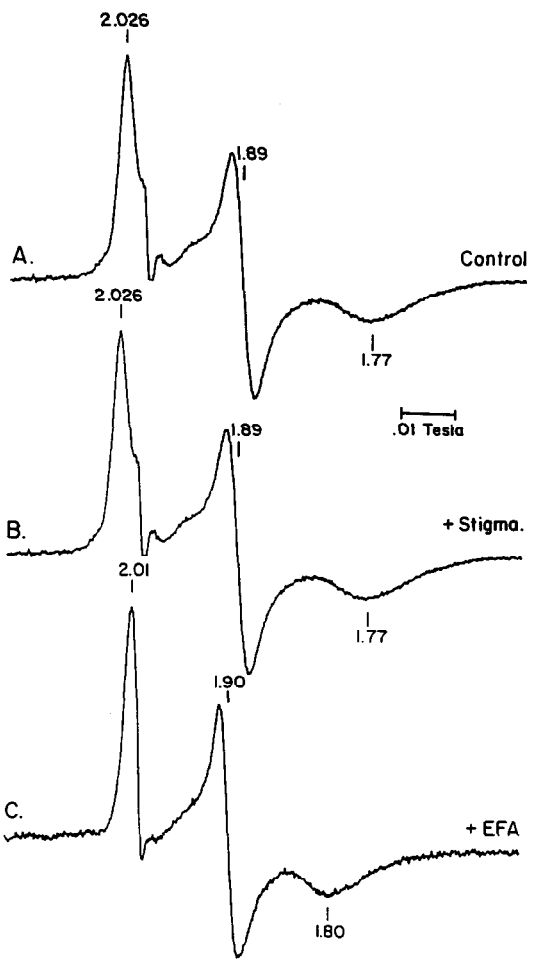

Fig. 4. Effect of stigmatellin and EFA on the EPR spectrum of the isolated Rieske iron–sulfur protein. (A) 27 μM isolated Rieske iron–sulfur protein in 50 mM 3-(*N*-Morpholino)propanesulfuric acid (MOPS) (pH 7.2) was reduced with 5 mM dithionite. (B) Sample A treated with 130 μM stigmatellin. (C) Sample A incubated with 7.6 mM EFA form 10 min on ice.

reduced with sodium dithionite in the presence of redox mediators, the effects of EFA were readily reversed. Consequently, we were unable to measure any shift in the redox midpoint potential that might be caused by EFA treatment. It has been reported that EFA inhibits the electron transfer activity of complex III more strongly in its reduced state than in its oxidized state [16]. Therefore, it is possible that EFA modification results in a positive E_m shift of the Rieske iron–sulfur cluster, similar to that produced by stigmatellin.

In order to further pinpoint the effect of EFA on the Rieske iron–sulfur protein itself, we have examined the effect of this compound on isolated bovine heart Rieske iron–sulfur protein (Fig. 4). Isolated Rieske iron–sulfur protein [18] reduced with dithionite exhibits an EPR spectrum with $g_{x,y,z} = 1.77, 1.89, 2.026$ (Fig. 4A). Stigmatellin does not induce any spectral lineshape alteration in the isolated Rieske iron–sulfur protein (Fig. 4B). However, upon treatment of the isolated Rieske protein with EFA, the EPR lineshape of the dithionite-reduced iron–sulfur cluster changed to $g_{x,y,z} = 1.80, 1.90, 2.010$ (Fig. 4C), which is similar to the spectral change observed with the Rieske iron–sulfur protein localized within the bc_1 complex.

4. Discussion

It has been shown in this study that EFA treatment of bovine complex III or the isolated Rieske iron–sulfur protein results in a specific lineshape alteration of the Rieske iron–sulfur cluster, which can be reversed by subsequent treatment with hydroxylamine. These data are consistent with the interpretation that EFA ethoxyformylates the non-iron binding ε-nitrogen(s) of the histidine ligands of the Rieske iron–sulfur cluster (see Fig. 5). Lorusso et al. [22] have also observed an effect of EFA on the EPR g_x feature of the Rieske iron–sulfur cluster (their data was not shown). They have concluded that the subtle spectral change of the Rieske cluster in EFA-treated preparations is caused by conformational changes transmitted via the EFA-modified core protein II of the bc_1 complex. However, it is clear from our results that the EPR lineshape changes of the iron–sulfur cluster are the consequence of the direct modification of the Rieske iron–sulfur protein by EFA.

We have shown that in complex III stigmatellin and UHDBT, but not myxothiazol and antimycin, compete with EFA for modification of the EPR spectrum of the Rieske iron–sulfur cluster. However, when the isolated Rieske protein was used, stigmatellin had no effect, whereas the effect of EFA was essentially the same. Brandt et al. [23] have demonstrated that once the Rieske protein is completely deleted from the bc_1 complex, stigmatellin loses its tight binding capacity observed with intact bc_1 complex. These results together with our data suggest that the stigmatellin binding site covers both the Rieske and the cytochrome *b* domains of the Q_o pocket.

Based on quantitative analysis of electron transfer kinetics, quinone occupancy at the Q_o site, and the redox state of Q in *Rhodobacter capsulatus* wild-type and mutant chromatophores, Dutton and his colleagues [24,25] have proposed that the Rieske histidine-ligands may also function as direct hydrogen-bond donors to quinone and/or quinol at the Q_o site, in a manner analogous to the Q_A and Q_B sites in the bacterial photosynthetic reaction center of which high-resolution structural information is available [26,27]. It would be of interest to investigate this point by quantitative analysis of EFA modification of the Rieske iron–sulfur protein in the *Rb. capsulatus* complex III, using both EPR and ENDOR analysis [4]. Bovine heart Rieske iron–sulfur protein contains 6 histidines; 3 non-conserved histidines may be located in the vicinity of the Rieske iron–sulfur cluster [28]. *Rb. capsulatus* Rieske iron–sulfur protein contains five histidines; one is very close to the two ligand-histidines near the C-terminus and the remaining two histidines are located in the hydrophobic N-terminal region [6]. Mutants of *Rb. capsulatus* are available, in which the former histidine is replaced with alanine or serine. These mutants have wild-type properties [6]. EFA modification of the Rieske protein isolated from one of these mutants should allow a quantitative analysis of the EFA-modified histidine residues that alter the Rieske cluster spectrum.

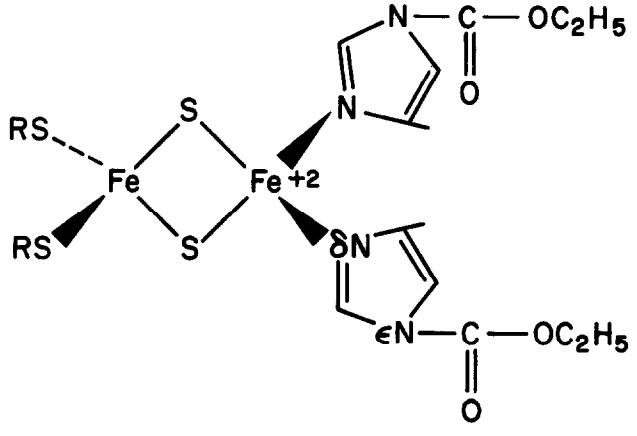

Fig. 5. A possible model of the Rieske iron–sulfur cluster ethoxyformylated at the imidazole nitrogens of the histidyl ligands.

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References

[1] Gennis, R.B., Barquera, B., Hacker, B., Van Doren, S.R., Arnaud, S., Crofts, A.R., Davidson, E., Gary, K.A. and Daldal, F. (1993) J. Bioenerg. Biomembr. 25, 195–209.
[2] Trumpower, B.L. (1990) Microbiol. Rev. 54, 101–129.
[3] Gurbiel, R.J., Batie, C.J., Sivaraja, M., True, A.E., Flee, J.A., Hoffman, B.M. and Ballou, D. (1989) Biochemistry 28, 4861–4871.
[4] Gurbiel, R.J., Ohnishi, T., Robertson, D., Daldal, F. and Hoffman, B.M. (1991) Biochemistry 30, 11579–11584.
[5] Britt, R.D., Sauer, K., Klein, M.P., Knaff, D.B., Krianciunas, A., Yu, C.A., Yu, L. and Malkin, R. (1991) Biochemistry 30, 1892–1901.
[6] Davidson, E., Ohnishi, T., Atta-Asafo-Adjei, E., Tokito, M. and Daldal, F. (1992) Biochemistry 31, 3342–3351.
[7] Van Doren, S.R., Yun, C.-H., Crofts, A.R. and Gennis, R.B. (1993) Biochemistry 32, 628–636.
[8] Mitchell, P. (1976) J. Theoret. Biol. 62, 327–367.
[9] Crofts, A.R., Meinhardt, S.W., Jones, K.R. and Snozzi, M. (1983) Biochim. Biophys. Acta 723, 202–218.
[10] Ohnishi, T., Schägger, H., Meinhardt, S.W., LoBrutto, R., Link, T.A. and von Jagow, G. (1989) J. Biol. Chem. 264, 735–744.
[11] Meinhardt, S.W. and Ohnishi, T. (1991) Biochim. Biophys. Acta 1100, 67–74.
[12] Glaser, E.G. and Crofts, A.R. (1984) Biochim. Biophys. Acta 766, 322–333.
[13] Robertson, D.E. and Dutton, P.L. (1988) Biochim. Biophys. Acta 935, 273–291.

[14] von Jagow, G. and Link, T.A. (1986) Methods Enzymol. 126, 253–271.
[15] Miles, E.W. (1977) Methods Enzymol. 47, 431–442.
[16] Yagi, T., Vik, S.V. and Hatefi, Y. (1982) Biochemistry 21, 4777–4782.
[17] Hatefi, Y. (1978) Methods Enzymol. 53, 35–40.
[18] Engel, W.D., Michalski, C. and von Jagow, G. (1983) Eur. J. Biochem. 132, 395–402.
[19] Hoffman, B.M., Depose, V.C., Dan, P.E., Gurbiel, R.J., Houseman, A.L.P. and Telser, J. (1993) in: Biological Magnetic Resonance, Vol. 13: EMR of Paramagnetic Molecules (Berliner L.J. and Reuben J. Eds.) Plenum Press, New York, pp. 151–219.
[20] von Jagow, G. and Ohnishi, T. (1985) FEBS Lett. 185, 311–315.
[21] Ohnishi, T., Brandt, U. and von Jagow, G. (1988) Eur. J. Biochem. 176, 385–389.
[22] Lorusso, M., Gatti, D., Marzo, M., Boffoli, D., Cocco, T. and Papa, S. (1987) Eur. J. Biochem. 162, 231–238.
[23] Brandt, U., Haase, U., Schägger, H. and von Jagow, G. (1991) J. Biol. Chem. 266, 19958–19964.
[24] Robertson, D.E., Daldal, F. and Dutton, P.L. (1990) Biochemistry 29, 11249–11260.
[25] Ding, H., Robertson, D.E., Daldal, F. and Dutton, P.L. (1992) Biochemistry 31, 3144–3158.
[26] Paddock, M.L., Rongey, S., Avresch, E.C., Feher, G. and Okamura, M. (1988) Photosynth. Res. 17, 75–96.
[27] Sinning, I., Michel, H., Mathis, P. and Rutherford, W. (1989) Biochemistry 28, 5544–5553.
[28] Nishikimi, M., Hosokawa, Y., Toda, H., Suzuki, H. and Ozawa, T. (1989) Biochem. Biophys. Res. Commun. 159, 19–25.