Biochimica et Biophysica Acta 850 (1986) 423–428
Elsevier

BBA 42053

Evidence for two independent pathways of electron transfer in mitochondrial NADH:Q oxidoreductase. II. Kinetics of reoxidation of the reduced enzyme

S.P.J. Albracht and P.T.A. Bakker

Laboratory of Biochemistry, University of Amsterdam, P.O. Box 20151, 1000 HD Amsterdam (The Netherlands)

(Received February 27th, 1986)

Key words: NADH:Q oxidoreductase; Electron transfer; pH effect; (Pre-steady-state kinetics, Bovine heart mitochondria)

The pre-steady-state kinetics of reoxidation of NADH:Q oxidoreductase present in submitochondrial particles has been studied by the freeze-quench method. It was found that at pH 8 only 50% of the Fe-S clusters 2 and 4 and 75% of the clusters 3 were rapidly reoxidised after transient and complete reduction by a pulse of NADH in the presence of excess NADPH. Thus, NADPH keeps 50% of the clusters 2 and 4 and 25% of the clusters 3 permanently reduced at this pH. Since NADH oxidation is nearly optimal at this pH, whereas NADPH oxidation is virtually absent, it was concluded that these permanently reduced clusters were not involved in the NADH oxidation activity. Incomplete reoxidation of the clusters 2, 3 and 4 after a pulse of NADH was also found in the absence of NADPH, both at pH 6.5 and at pH 8. A pulse of NADPH given at pH 6.5, where NADPH oxidation by oxygen is nearly optimal, caused a slow reduction of 50% of clusters 2 and 4 and 30% of the clusters 3, which persisted for a period of at least 15 s. It was concluded that these clusters were not involved in the oxidation of NADPH by oxygen, as catalysed by the particles. As a working hypothesis a dimeric model for NAD(P)H:Q oxidoreductase is proposed, consisting of two different protomers. One of the protomers, containing FMN and the Fe-S clusters 1–4 in stoichiometric amounts, only reacts with NADH, and its oxidation by ubiquinone is rapid at pH but slow at pH 6.5. The other protomer, containing FMN and the clusters 2, 3 and 4, reacts with both NADH and NADPH and has a pH optimum at 6–6.5 for the reaction with ubiquinone.

Introduction

NADH:Q oxidoreductase (EC 1.6.99.3) from bovine heart mitochondria is a large and complicated enzyme. Its molecular weight is about 700 kDa per FMN molecule [1]; its hydrodynamic properties [2] suggest that it could well be a dimer. The enzyme contains flavin and at least four different Fe-S clusters as carriers of reducing equivalents [3].

Freeze-quench experiments with the enzyme present in submitochondrial particles, reported in Ref. 4 and the accompanying paper [5], have revealed that 50% of the clusters 2, 3 and 4 can be rapidly reduced by NADPH, whereas the other 50% and also cluster 1 are reduced slowly. The kinetics of reduction by NADPH of the various clusters at pH 6.5 hardly differed from those at pH 8.0. This has led us to propose a dimeric model for the reduction of the enzyme by NAD(P)H (see Fig. 10 of the preceding paper [5]). Although 50% of the clusters 2 were rapidly reduced by NADPH at pH 8.0, the oxidation of NADPH by oxygen, as catalysed by submitochondrial particles, is almost negligible at this pH. This suggests that under these conditions reoxidation of part of the reduced NADH:Q oxidoreductase, in particular reoxidation of 50%

of the clusters 2 by ubiquinone, is virtually absent.

In the past, several experiments demonstrating incomplete reoxidation of the reduced enzyme have been published. Some 15 years ago it was first shown by Bois and Estabrook [6], and later also by Gutman et al. [7], that a pigment giving rise to bleaching at 470 nm upon reduction is reoxidised incompletely in submitochondrial particles which were partly inhibited by rotenone and transiently reduced by an amount of NADH substoichiometric to the oxygen concentration in the reaction mixture. Later, this permanently bleached chromophore was identified as Fe-S cluster 2 [8]. In submitochondrial particles still capable of oxidative phosphorylation, reoxidation of cluster 2 could be induced by ATP, probably as a result of reversed electron flow to NAD^+ [9]. Even in the absence of inhibitors, part of the clusters 2 were not oxidised when reduced submitochondrial particles were oxidised with oxygen [10]. Lebanidze [11] has also reported that in mitochondria which were reoxidised at $-12°C$ part of the clusters 2 remained reduced. It was indicated that the temperature dependence of the EPR signal of these slowly reoxidising clusters differed from that of the quickly reoxidised clusters 2 measured under fully reduced conditions [11].

The present study provides evidence for the heterogeneity of the clusters 2 with respect to the kinetics of reoxidation. A tentative working mechanism for the electron flow through NADH:Q oxidoreductase is proposed which explains the pH dependence of both the NADPH and the NADH oxidation. A preliminary account of this work was presented at a recent meeting [12].

Materials and Methods

Freeze-quench experiments were carried out as described in the preceding article [5] in the Materials and Methods section under (ii). The reduction levels of the various clusters at $t > 400$ ms were obtained by transferring the reaction mixture from the mixer directly to an EPR tube. After a certain period of time, the tube was quickly immersed in cold isopentane (133 K). Where indicated, the submitochondrial particles were treated with trypsin, as described in Ref. 5.

Results

In the preceding paper [5] it was shown that the kinetics of reduction by NADPH of NADH:Q oxidoreductase in submitochondrial particles at pH 8.0 were very similar to those at pH 6.5. Since the NADPH oxidation activity was about 0.3 μmol NADPH/min per mg of protein at pH 6.5, but only about 10 nmol NADPH/min per mg at pH 8.0, it became of interest to examine the reoxidation kinetics of the reduced enzyme. A pilot experiment was carried out as follows. A suspension of submitochondrial particles in 0.25 M sucrose/70 mM Tris-HCl (pH 8.0) was made anaerobic by adding 35 mM ethanol, 30 μM NAD^+ and so much alcohol dehydrogenase that the oxygen in

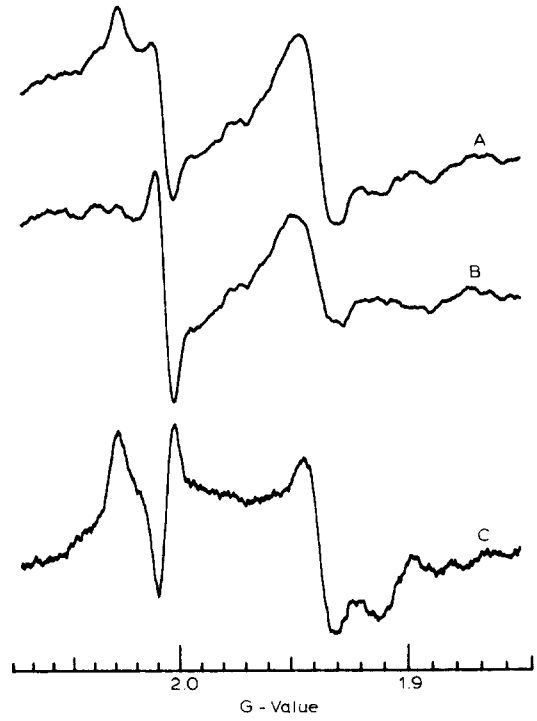

Fig. 1. Oxidation of cluster 1 of NADH:Q oxidoreductase present in anaerobic, reduced submitochondrial particles. (A) Reduced, anaerobic submitochondrial particles were mixed with anaerobic buffer in the rapid-mixing rapid-freezing machine and frozen after 42 ms. This sample served as a reduced control sample. (B) Mixing with aerobic buffer, reaction time 100 ms. (C) Difference spectrum A minus B, enlarged 2-fold. EPR conditions: microwave frequency, 9266 MHz; temperature, 48 K; incident microwave power, 9 mW; modulation amplitude, 1.25 mT. See text for other experimental details.

the suspension was consumed in about 10 min. The suspension was transferred to syringe A of the freeze-quench machine. The other syringe was filled with aerobic buffer. The experiment was performed within a nitrogen-flushed glove box. All components of the respiratory chain, except for some of the clusters of NADH : Q oxidoreductase specified below, were oxidised within 4 s after mixing. Half of the signal of cluster 2 disappeared during this time (not shown). The remaining half persisted for at last 30 s. Likewise the signals due to clusters 3 and 4 did not disappear completely (see below). Although the line at $g = 1.94$, indicative of reduced cluster 1, did not diminish to zero amplitude, it was concluded that cluster 1 was fully oxidised within 200 ms (Fig. 1). The line shape of the remaining signal (Fig. 1B), which was also still present after 4 s, strongly resembled that of the Fe-S clusters present in mitochondrial outer membranes ($g_{x,y} = 1.89$, 1.93–1.94) [13], which are also present in preparations of submitochondrial particles [14] and are slowly reduced by NADH. The difference spectrum (Fig. 1C) represents the Fe-S clusters which were rapidly reoxidised. From comparison with the line shape in the $g = 1.9$–2 region of pure mitochondrial inner membranes in the reduced state [14] it is concluded that both cluster 1 of NADH : Q oxidoreductase and cluster 1 of succinate : Q oxidoreductase were rapidly reoxidised.

This initial result prompted us to study the reoxidation reaction in more detail, using a different approach. When aerobic, trypsin-treated particles were mixed at pH 8 with an excess of NADPH and 25 μM of NADH, a very similar result was obtained (Fig. 2). After an initial rapid (5 ms) and complete reduction of all clusters of NADH : Q oxidoreductase by NADH, a rapid oxidation started when the small amount of NADH had been consumed. Cluster 1 was completely oxidised within 400 ms (not shown), whereas the clusters 2, 3 and 4 were oxidised only to a certain extent. The levels reached after 1 s remained constant for at least 30 s. Cluster 2 remained reduced by precisely 50%, the clusters 3 and 4 were reoxidised to a greater extent. The final level reached by cluster 4 was found to be dependent on the NAD^+ concentration. Fig. 3 shows the result when particles were reduced by

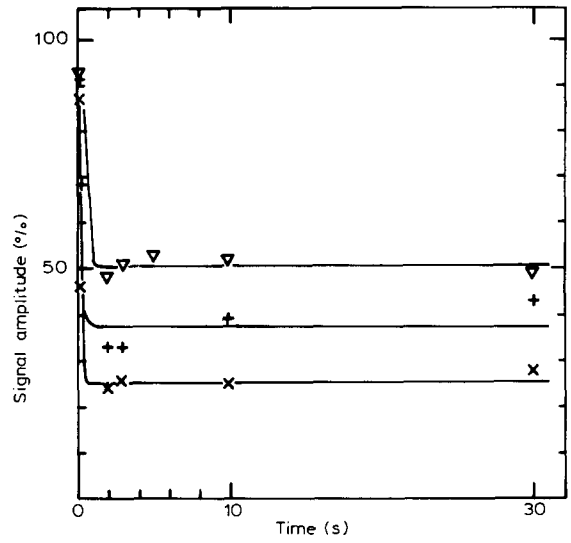

Fig. 2. Kinetics of reduction and reoxidation of the clusters 2, 3 and 4 in trypsin-treated submitochondrial particles at pH 8.0 after mixing with NADPH (final concentration 5 mM) plus NADH (25 μM). ▽———▽, cluster 2; ×———×, cluster 3; +———+, cluster 4. Cluster 1 was completely reoxidized within 400 ms.

NADPH in the presence of variable amounts of NAD^+. When extrapolated to zero-NAD^+ concentration, the permanent reduction level of clus-

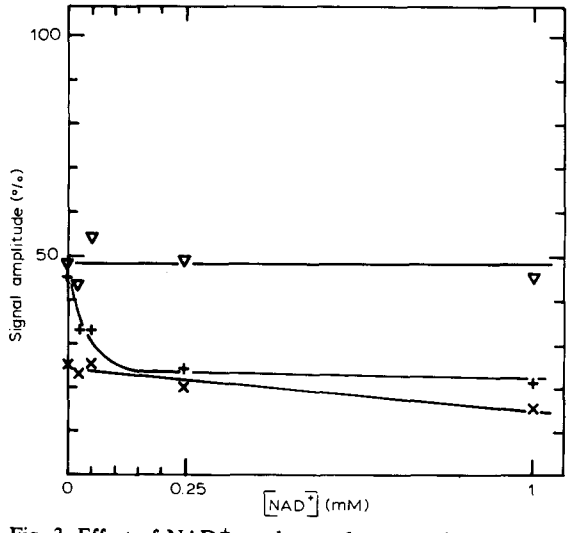

Fig. 3. Effect of NAD^+ on the steady-state reduction level of the clusters 2, 3 and 4 in trypsin-treated submitochondrial particles, after reduction by excess (5 mM) NADPH at pH 8.0 in the presence of variable amounts of NAD^+. The reaction was quenched after 5 s by cooling to 133 K. The symbols used are the same as in Fig. 2.

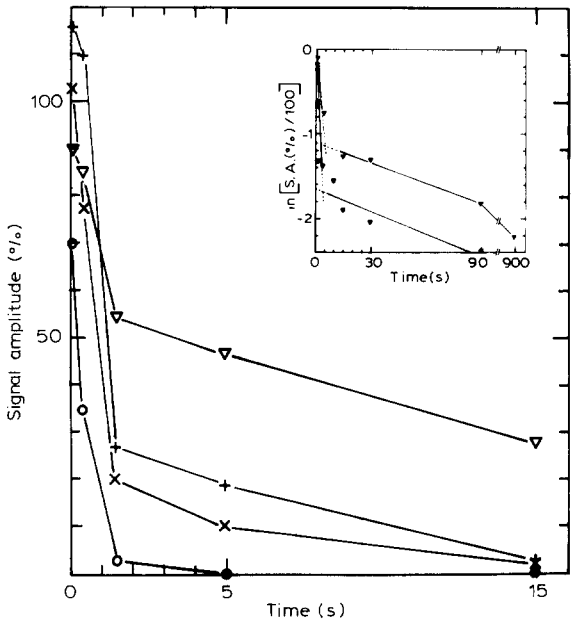

Fig. 4. Redox behaviour of NADH:Q oxidoreductase present in submitochondrial particles after a pulse of NADH (75 μM) at pH 8. O——O, cluster 1; ▽——▽, cluster 2; ×——×, cluster 3; +——+, cluster 4. The inset is a semi-logarithmic plot of the reoxidation of cluster 2. Closed symbols, pH 6.5; open symbols, pH 8.0 (S.A., signal amplitude).

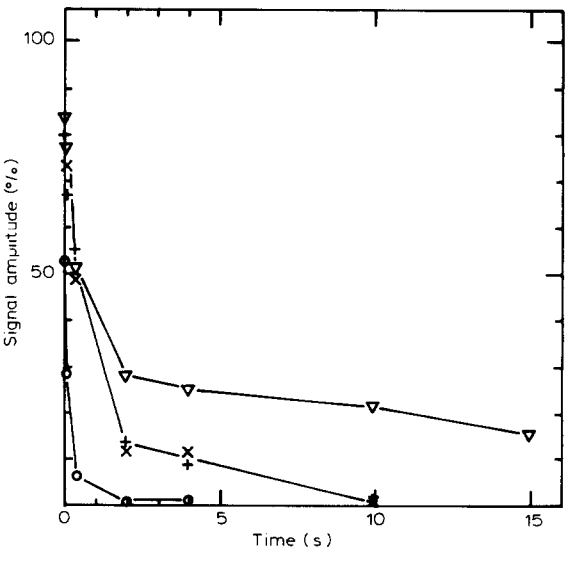

Fig. 5. Transient reduction of NADH:Q oxidoreductase in trypsin-treated submitochondrial particles mixed with a pulse of NADH (75 μM) at pH 6.5. The symbols used were the same as in Fig. 4.

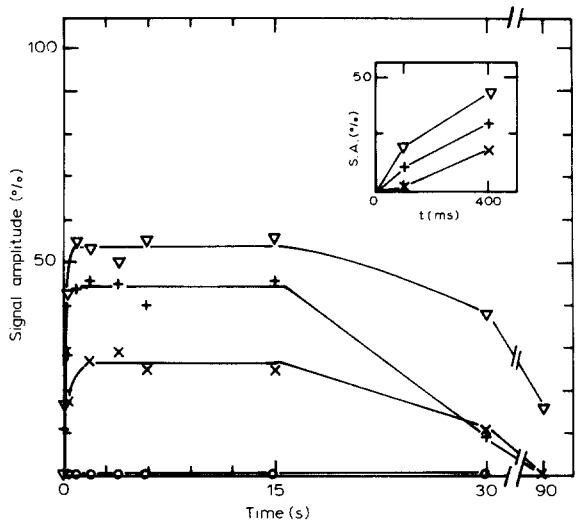

Fig. 6. Behaviour of NADH:Q oxidoreductase in trypsin-treated submitochondrial particles after mixing with a pulse of NADPH (40 μM) at pH 6.5. O——O, cluster 1; ▽——▽, cluster 2; ×——×, cluster 3; +——+, cluster 4. The inset shows the reduction at 100 and 400 ms (S.A., signal amplitude).

ter 4 approached 50%. Thus, in the experiment of Fig. 2 the permanent reduction level of cluster 4 of 30% was due to the NAD^+ which was formed after the oxidation of the added NADH. The level of permanent reduction of cluster 2 was independent of the presence of NAD^+ and remained 50%. The reduction level of cluster 3 was only slightly affected. During the experiments of Fig. 3 cluster 1 was not reduced at all (not shown). NADH:Q oxidoreductase in submitochondrial particles that were pulsed with 75 μM NADH but now in the absence of NADPH, displayed biphasic reoxidation kinetics (Fig. 4). The reduction of the clusters 2, 3 and 4 was rapid and virtually complete. About 50% of the clusters 2 were reoxidised quickly, as in the previous experiment, but the remaining fraction was reoxidised slowly. The reoxidation kinetics of the clusters 3 and 4 showed the same pattern, but the extent of the rapid phase of cluster 4 was greater, due to the formation of 75 μM NAD^+. Cluster 1 was reduced incompletely and was fully reoxidised within 1 s. A semi-logarithmic plot of the reoxidation kinetics (inset Fig. 4) indicates that 50–65% of cluster 2 is reoxidised quickly, whereas 35% is reoxidised slowly ($k' = 8 \cdot 10^{-3}\ s^{-1}$). The same experiment, but now carried

out at pH 6.5, resulted in the rapid disappearance of about 70% of the signal of cluster 2 (Fig. 5). The remaining fraction was reoxidised slowly ($k' = 9 \cdot 10^{-3}\ s^{-1}$), like at pH 8.0. The kinetics of the reoxidation of the clusters 3 and 4 (Fig. 5) could not be distinguished from monophasic kinetics. Cluster 1 was completely reoxidised within 1 s. A different result was obtained when the particles were pulsed with NADPH at pH 6.5 (Fig. 6): the clusters 2 and 4 were slowly reduced, up to about 50%. This reduction level was stable for at least 15 s, after which a slow reoxidation occurred, presumably due to exhaustion of NADPH. The behaviour of cluster 3 followed the same pattern, except for the steady-state reduction level which was only 25%. Cluster 1 was not reduced at all.

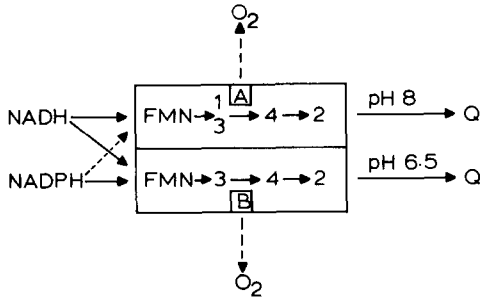

Fig. 7. Schematic representation of the oxidation and reduction reactions carried out by NAD(P)H:Q oxidoreductase under various conditions. The solid arrows represent fast reactions, the dashed arrows stand for slow reactions. Reoxidation of the upper protomer by ubiquinone is proposed to be fast at pH 8, but slow at pH 6.5. In contrast, reoxidation of the lower protomer is proposed to be optimal at pH 6–6.5, but slow at pH 8.

Discussion

The reduction kinetics of the enzyme with NADPH (cf. the preceding paper [5]) did not explain the low pH optimum for the oxidation of this substrate by submitochondrial particles in air. The results presented in this paper demonstrate that it is the reoxidation of part of the reduced enzyme that is dependent on pH. In the experiment of Fig. 1, reoxidation of the reduced enzyme at pH 8.0 in the presence of a low rate of supply with NADH (via the ethanol/NAD^+/alcohol dehydrogenase system) resulted in permanent reduction of 50% of the clusters 2. It could be calculated that the oxidising capacity of the thick suspension of particles was three orders of magnitude greater than the reducing capacity of the NADH-regenerating system. Consequently, the reoxidation of 50% of the clusters 2 was extremely slow. In terms of the model developed in the preceding paper, which has been extended in Fig. 7, the most plausible explanation would be that only one protomer (the upper one in Fig. 7) can be quickly oxidised at pH 8. This would also explain the results obtained in the experiment of Fig. 2, where trypsin-treated submitochondrial particles were mixed at pH 8.0 with excess NADPH and an amount of NADH sub-stoichiometric to the oxygen present. NADH reduced the enzyme rapidly and completely but was consumed after about 400 ms. NADPH is able to rapidly reduce only one of the protomers [5], but the reoxidation of this part of the enzyme (the lower protomer in Fig. 7) by ubiquinone is very slow at this pH. This results in permanent reduction of the clusters 2, 3 and 4 in the lower protomer. The slow reoxidation ($k' = 8 \cdot 10^{-3}\ s^{-1}$) of cluster 2 in the lower protomer could be monitored when only one pulse of NADH was applied (Fig. 4). It is concluded that at pH 8 only the upper protomer is involved in active NADH oxidation. Note that the prosthetic groups in this active NADH-oxidising protomer are present in stoichiometric amounts.

When at pH 6.5 the trypsin-treated particles were mixed with a pulse of NADPH (Fig. 6), cluster 2 in the lower protomer was reoxidised so quickly that it could not become reduced by the input of reducing equivalents from NADPH. This input was slow, since the concentration of this substrate was less than one-tenth of its K_m value. Cluster 2 in the upper protomer was now slowly reduced and could hardly be reoxidised at this pH. The same holds for clusters 3 and 4. Thus we conclude that at this pH only the lower protomer is active in the oxidation of substrate, which is either NADPH or NADH. There is also a major difference between the two protomers in substrate specificity. The upper one is specific for NADH, but the lower one can react with both NADH and NADPH.

Transient reduction of the enzyme at pH 6.5 with a pulse of NADH resulted in biphasic reoxidation of the clusters 2 (Fig. 5). Only 30% was

reoxidised slowly. This was also observed at pH 8, but here the reoxidation of 50% of the clusters 2 was slow (Fig. 4). Within the simple model of Fig. 7 it cannot be understood why the extents of the fast reoxidation phases of the clusters 2, 3 and 4 at pH 6.5 significantly exceeded the expected 50%. Attempts to demonstrate heterogeneity of the two types of cluster 2 by other means were, as yet, not successful; no significant difference in spin-relaxation behaviour of the two types could be demonstrated.

Although the model presented in Fig. 7 can explain the majority of the experiments in this and in the preceding article, not all findings can be interpreted with this model. The reduction levels of cluster 3, both in the reduction [5] and in the reoxidation experiments (this paper), are difficult to reconcile with the model. It must be mentioned, however, that the actual reduction level of cluster 3 was usually difficult to determine due to overlap with other signals [5]. Also the incomplete reduction of cluster 1 by NADPH at pH 6.5 (or pH 8), even under anaerobic conditions, is not understood. In experiments where a pulse of NADPH was given at pH 6.5, this cluster was not reduced at all, whereas cluster 2 in the upper protomer was reduced after 400 ms. If one allows for internal electron transfer from one protomer to the other, then there would be several possibilities to explain such results. More experiments are required to develop further insight in the mechanism of reduction of the enzyme by substrates, the pH regulation of its oxidation by ubiquinone, and the effects of NAD^+.

Acknowledgements

We thank Professor E.C. Slater for his stimulating interest during this research. This work was supported by grants from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.).

References

1 Hatefi, Y. and Rieske, J.S. (1967) Methods Enzymol. 10, 235–239
2 Dooijewaard, G., De Bruin, G.J.M., Van Dijk, P.J. and Slater, E.C. (1978) Biochim. Biophys. Acta 501, 458–469
3 Beinert, H. and Albracht, S.P.J. (1982) Biochim. Biophys. Acta 683, 245–277
4 Albracht, S.P.J. (1982) in Flavins and Flavoproteins (Massey, V. and Williams, C.H., eds.), pp. 759–762, Elsevier/North-Holland, Amsterdam
5 Bakker, P.T.A. and Albracht, S.P.J. (1986) Biochim. Biophys. Acta 850, 413–422
6 Bois, R. and Estabrook, R.W. (1969) Arch. Biochem. Biophys. 129, 362–369
7 Gutman, M., Mayr, M., Oltzik, R. and Singer, T.P. (1970) Biochem. Biophys. Res. Commun. 41, 40–44
8 Gutman, M., Singer, T.P. and Beinert, H. (1971) Biochem. Biophys. Res. Commun. 44, 1572–1578
9 Gutman, M., Singer, T.P. and Beinert, H. (1972) Biochemistry 11, 556–562
10 Albracht, S.P.J. and Slater, E.C. (1971) Biochim. Biophys. Acta 245, 503–507
11 Lebanidze, A.V. (1977) Biofizika 22, 730–731
12 Albracht, S.P.J. and Bakker, P.T.A. (1985) 13th International Congress of Biochemistry, Amsterdam, Abstract Tu-494, p. 355
13 Heidrich, H.-G., Albracht, S.P.J. and Bäckström, D. (1978) FEBS Lett. 95, 314–318
14 Albracht, S.P.J. and Heidrich, H.-G. (1975) Biochim. Biophys. Acta 376, 231–236