THE STOICHIOMETRY OF THE IRON–SULPHUR CLUSTERS 1a, 1b AND 2 OF NADH:Q OXIDOREDUCTASE AS PRESENT IN BEEF-HEART SUBMITOCHONDRIAL PARTICLES

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1. Introduction

NADH:Q oxidoreductase as purified from beef-heart mitochondria contains 6 prosthetic groups: FMN and 5 different Fe-S clusters. The EPR signals of three of these Fe-S clusters, the clusters 2, 3 and 4, are only detectable at $T < 25$ K [1–5]. At 80 K the enzyme shows an EPR signal of an apparent axial symmetry that has been known since 1960 [6]. Although this was at one time [5] assigned to a single Fe-S cluster, cluster 1, a more detailed analysis of this signal, recorded at a sample temperature of 50 K where optimal resolution is obtained, demonstrated that it is a representation of two overlapping equally intense axial signals [7]. Moreover, the relative intensity of this EPR signal was found [7] to be only half the value reported earlier by Orme-Johnson et al. [5] although the relative concentrations of cluster 2 determined by both groups were in agreement. For isolated NADH:Q oxidoreductase (Complex I) this means that only *one* cluster 1a and *one* cluster 1b are present per *four* clusters 2. On basis of the similarity of the overall EPR line shape at 10–20 K [7], it was concluded that the same is true for submitochondrial particles (SMP), but no data were presented at that time.

Abbreviation: SMP, submitochondrial particle

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As the stoichiometry of the clusters 1a, 1b and 2 is of essential importance for our knowledge of the basic enzymic unit of NADH:Q oxidoreductase and since purification procedures appear to affect the stoichiometry of the prosthetic groups of inner-membrane enzyme complexes [8,9] we have determined the stoichiometry of the above mentioned clusters directly in *ten* different SMP preparations.

2. Materials and methods

2.1. *Submitochondrial particles*

These were prepared from beef-heart mitochondria by sonication in a medium containing 0.25 M sucrose and 50 mM Tris–HCl (pH 8.0). After removal of remaining mitochondria at 15 000 × *g*, SMP were obtained at 100 000 × *g*. They were washed once and then resuspended in the sonication medium. This procedure was carried out ten times using one batch of mitochondria, so that *ten* individual preparations of SMP could be investigated.

2.2. *EPR samples*

SMP were incubated with 60 mM succinate in an EPR tube for 15 min at 30°C. The sample was frozen in liquid nitrogen and an EPR spectrum was recorded at 45 K. The tube was then warmed up to 0–4°C and the sample was mixed with 4 mM NADH for 30 s at this temperature. After freezing in liquid nitrogen a second EPR spectrum was recorded under identical conditions. The whole procedure was carried out for all ten preparations of SMP.

2.3. *EPR spectra*

Spectra were recorded on a Varian E-9 EPR spectrometer connected to a HP 2100 minicomputer via a PDP 11–03 microprocessor-based data acquisition front-end septem. In this way the spectra (1000 data points were taken) could be directly stored on magnetic disc for later processing. Other experimental details were as described previously [7].

2.4. *Signal quantitation*

The signal of cluster 2 of NADH:Q oxidoreductase was quantitized using the area of the low-field half of the g_z line recorded at 14 K (fig.1) and relating twice this area to the signal intensity with the formulae given by Aasa and Vänngård [10].

Direct quantitation of the signals of cluster 1a and 1b is not possible since many overlapping spectra are present. Therefore, the line shape was obtained as a difference of the spectra at 45 K of SMP reduced with succinate plus NADH and reduced with succinate alone. Before doing so, the spectra were transformed to the same g-value scale, since the microwave frequencies at which the EPR spectra are obtained always differ slightly. Since the resonance field is proportional to the microwave frequency, direct subtraction would give erroneous results. A typical example of this procedure is shown in fig.2. It appears that all the [2Fe-2S] clusters of the contaminating mitochondrial outer membranes [11,12] are fully reduced by succinate, under the incubation conditions used, so their signals are absent in the difference spectra.

It is obvious that the resulting difference spectrum (fig.2, lower trace) is not suitable for direct double integration. Instead, this spectrum was compared with a simulation of the spectrum of the clusters 1a plus 1b as present in isolated complex I [7]. The

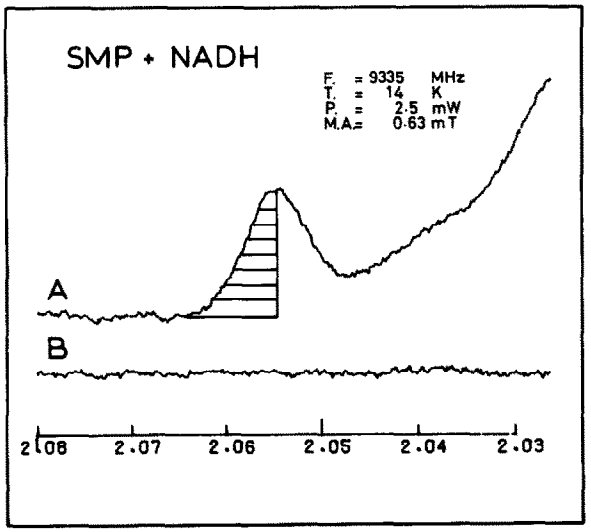

Fig.1. Experimental trace of the g_z line of cluster 2 of NADH:Q oxidoreductase as present in SMP reduced with succinate plus NADH (see Materials and methods). The hatched area was used for quantitation. The lower trace is a base line run with water under the same conditions. EPR conditions: microwave frequency (F), 9336 MHz; temperature (T), 14 K; microwave power (P), 2.5 mW; modulation amplitude (MA), 0.63 mT; scanning rate (SR), 5 mT/min. The modulation frequency for these and other spectra was 100 kHz. The X-axis scale refers to g-values.

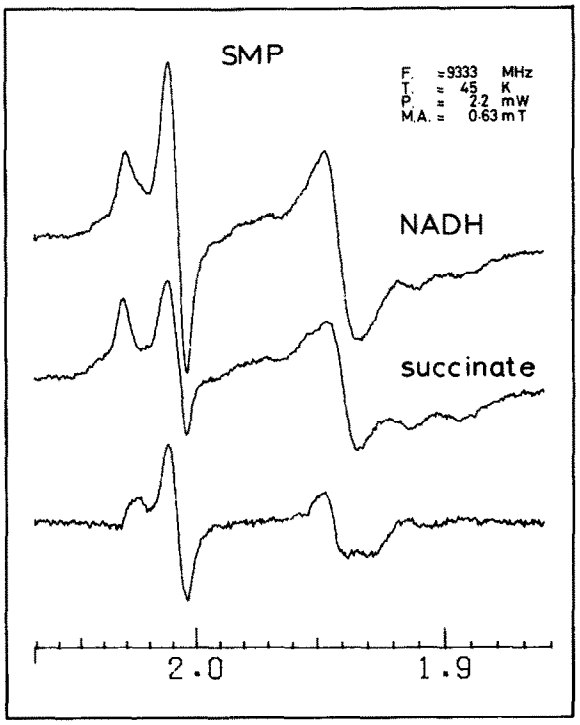

Fig.2. Quantitation of the EPR signals of the clusters 1a plus 1b of NADH:Q oxidoreductase as present in SMP: isolation of the signal. Upper trace: spectrum of SMP reduced with succinate plus NADH. Middle trace: spectrum of SMP reduced with succinate. Lower trace: difference spectrum. EPR conditions: F, 9333 MHz; T, 45 K; P, 2.2 mW; MA, 0.63 mT; SR, 10 mT/min.

intensity of the simulated spectrum was varied until a difference spectrum gave a flat base line in the $g_{\parallel}$ and $g_{\perp}$ region. An example is given in fig.3. The double integral value of the simulated trace was taken to compute the concentration of the sum of the clusters 1a and 1b. A standard of 10 mM copper perchlorate was used as a reference both at 14 and 45 K.

All manipulations with the spectra, including the quantitations were carried out on a Tektronix 4010 computer display terminal connected to a HP 2100 minicomputer. Simulations of EPR spectra were performed as before [7] on the minicomputer.

3. Results and discussion

The absolute concentrations of the clusters 1a plus 1b and cluster 2 in the ten different SMP preparations are compiled in table 1. The important number is the mean value of the ratios of the concentrations of the clusters 1a plus 1b and cluster 2. This is precisely 0.5, meaning that the individual clusters 1a and 1b in SMP are present in a concentration *one quarter* of that of cluster 2. This agrees with our earlier findings on isolated complex I [7] and indicates that the stoichiometry is not altered by the purification procedure for this complex.

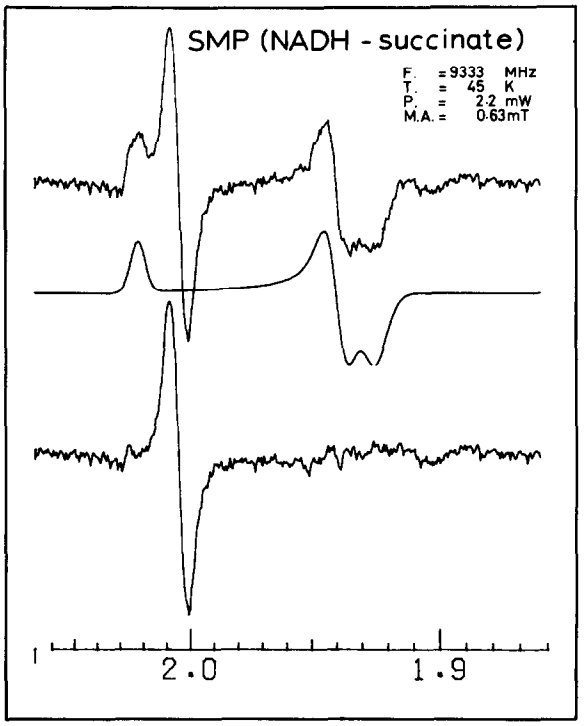

Fig.3. Quantitation of the EPR signal of the clusters 1a plus 1b of NADH:Q oxidoreductase as present in SMP: comparison with a simulated spectrum. Upper trace: same as lower trace from fig.2. Middle trace: simulation of the line shape of the clusters 1a and 1b as present in isolated complex I. For parameters, see [7]. The intensity was adjusted such that a difference spectrum (lower trace) showed no intensity in the $g_{\parallel}$ and $g_{\perp}$ region.

Table 1
Concentrations of the Fe-S clusters 1a, 1b and 2 of NADH:Q oxidoreductase as present in beef-heart submitochondrial particles

Sample	Concentration (μM)		Ratio
	Clusters 1a + 1b	Cluster 2	Cluster 1a + 1b / Cluster 2
1	3.78	6.52	0.58
2	3.51	6.70	0.52
3	4.29	7.81	0.55
4	3.17	6.41	0.49
5	2.53	6.34	0.40
6	3.18	6.29	0.51
7	2.91	5.90	0.49
8	3.71	7.97	0.47
9	3.83	7.33	0.52
10	3.01	6.74	0.45
Mean value	–	–	0.50 (0.05)[a]

[a] The number in parentheses is the standard deviation using $N-1$ weighting

In our opinion this finding has an important bearing on the composition of the basic enzymic unit of NADH:Q oxidoreductase as present in SMP and in isolated complex I. Although the molecular weight of the purified enzyme complex has been based for a long time on the FMN content, a recent direct determination of the molecular weight of complex I gives a value at least twice that number [13], suggesting that one molecule of the enzyme contains 2 FMN molecules and two clusters 2. (The FMN content equals that of cluster 2 [5,7].) Such a molecule cannot be meaningful, however, since twice that molecular mass is needed to accomodate *one* of each of the clusters 1a and 1b.

A similar dilemma has recently been encountered

in a study on the stoichiometry of the prosthetic groups of QH_2 : cytochrome *c* oxidoreductase [8], where two [2Fe-2S] clusters and two *b*-cytochromes (*b*-566 and *b*-558) are present in a concentration only half that of the cytochromes c_1 and *b*-562. The molecular weight determined thus for the beef-heart enzyme by two independent methods [14] agrees with the value based on the cytochrome c_1 content. Also in this case such a molecule is not realistic, since double this mass is needed to accomodate *one* of the cytochromes *b*-566 and *b*-558 and *one* of the Fe-S clusters 1 and 2.

We see two possibilities to understand these findings for both NADH:Q oxidoreductase and QH_2 : cytochrome *c* oxidoreductase.

(1) The basic enzymic unit of NADH:Q oxidoreductase contains *one* cluster 1a, *one* cluster 1b and four molecules or clusters of the other prosthetic groups. The unit of QH_2 : cytochrome *c* oxidoreductase would then contain two molecules of cytochrome c_1 and of *b*-562 and one species of the other prosthetic groups. In this case the molecular weight determinations on both complexes would be incorrect.

(2) There are two types of NADH:Q oxidoreductase molecules in equal amounts, both containing two FMN molecules and two of the clusters 2, 3 and 4. One molecule would then contain cluster 1a and the other cluster 1b. The physico-chemical properties of both complexes would be such that they cannot be separated with the preparative techniques thus far used for isolation of complex I or the type I NADH-dehydrogenase. A similar reasoning holds for the b–c_1 complex. This possibility is in agreement with the molecular weight determinations on both the complexes I and III.

At present we are not able to discriminate between the two possibilities.

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