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Quantitative amino acid analysis of bovine NADH: ubiquinone oxidoreductase (Complex I) and related enzymes. Consequences for the number of prosthetic groups

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Abstract

Bovine-heart NADH:ubiquinone oxidoreductase (EC 1.6.5.3; Complex I) is the first and most complicated enzyme in the mitochondrial respiratory chain. Biochemistry textbooks and virtually all literature on this enzyme state that it contains one FMN and at least four iron–sulfur clusters. We show here that this statement is incorrect as it is based on erroneous protein determinations. Quantitative amino acid analysis of the bovine Complex I, to our knowledge the first reported thus far, shows that the routine protein-determination methods used for the bovine Complex I overestimate its protein content by up to twofold. The FMN content of the preparations was determined to be at least 1.3–1.4 mol FMN/mol Complex I. The spin concentration of the electron paramagnetic resonance (EPR) signal ascribed to iron–sulfur cluster N2 was determined and accounted for 1.3–1.6 clusters per molecule of Complex I. These results experimentally confirm the hypothesis [FEBS Lett. 485 (2000) 1] that the bovine Complex I contains two FMN groups and two clusters N2. Also the protein content of preparations of the soluble NAD^+-reducing [NiFe]-hydrogenase (EC 1.12.1.2) from *Ralstonia eutropha*, which shows clear evolutionary relationships with Complex I, scores too high by the colorimetric protein-determination methods. Determination of the FMN content and the spin concentration of the EPR signal of the [2Fe–2S] cluster shows that this hydrogenase also contains two FMN roups. A third enzyme (Ech), the membrane-bound [NiFe]-hydrogenase from *Methanosarcina barkeri* which shows an even stronger evolutionary relationship with Complex I, behaves rather normal in protein determinations and contains no detectable acid-extractable FMN in purified preparations.

Keywords: Bovine complex I; Protein determination; FMN content; Iron–sulfur cluster

1. Introduction

Bovine-heart NADH:ubiquinone oxidoreductase (EC 1.6.5.3; Complex I) is located in the inner membrane of mitochondria. It catalyzes the transfer of electrons from NADH to ubiquinone and couples this to the extrusion of protons from the mitochondrial matrix (for review, see e.g. Refs. [1,2]). The resulting proton-motive force drives the synthesis of ATP from ADP and inorganic phosphate. Malfunctioning of Complex I can give rise to severe diseases in man, for example, Parkinson's disease and several mitochondrial encephalomyopathies [3,4]. Moreover, one of its subunits is probably involved in apoptotic cell death [5].

The bovine Complex I consists of 43 different polypeptides [1,5] with a total molecular mass of over 900 kDa. A crystal structure is not available. Chemical analyses of purified preparations for FMN and Fe by two pioneering groups in this field [6–11] showed the presence of 16–18 non-haem Fe atoms per FMN. As such Complex I preparations are often contaminated with some Complex II (succinate:ubiquinone oxidoreductase) and Complex III (ubiquinol:ferricytochrome *c* oxidoreductase), which also contain Fe–S clusters but no FMN, these numbers are upper values. Electron paramagnetic resonance (EPR) studies of the NADH-reduced enzyme showed the presence of four different EPR signals. One signal could be ascribed to a [2Fe–2S] cluster, whereas the other three signals were

Abbreviations: AA method, protein determined via quantitative amino acid analysis; SH, the soluble NAD^+-reducing [NiFe]-hydrogenase from *Ralstonia eutropha*; Ech, the membrane-bound [NiFe]-hydrogenase from *Methanosarcina barkeri*; BSA, bovine serum albumin

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demonstrated to be due to [4Fe–4S] clusters. EPR spectra of the purified preparations were identical to those of the enzyme as present in submitochondrial particles (for overview, see Ref. [12]). There is a general agreement in the field that the total number of spins represented by these signals is 3.5–4 per FMN [12–14]. Therefore, it has been assumed early on [12] that the bovine Complex I contains one FMN, one [2Fe–2S] cluster (called N1b), and three [4Fe–4S] clusters (N2, N3 and N4). An additional EPR signal, due to a [2Fe–2S] cluster, could be detected only at very low redox potentials [15], but in the membrane-bound enzyme or the Hatefi-type Complex I, this cluster (N1a) is not reducible by NADH. Magnetic circular dichroism (MCD) studies eliminated the possibility of paramagnetic, EPR-silent Fe–S clusters [9]. So, these EPR and MCD data predicted the presence of two 2Fe clusters and three 4Fe clusters, resulting in 16 Fe atoms per FMN, in good agreement with the earlier data from chemical analyses. This is why Biochemistry textbooks as well as most research papers on Complex I, including those on Complex I from bacterial origin, state that this enzyme contains one FMN and four to five Fe–S clusters.

Flavine determinations of an enzyme with a molecular mass of over 900 kDa [5], containing one FMN molecule, should result in no more than 1.1 nmol FMN/mg protein. The classical preparations of bovine Complex I were already reported to contain considerably more flavine: 1.2–1.5 nmol FMN/mg protein [6–8,10,11]. A more recent purification procedure [16] resulted in preparations with 1.5–1.6 nmol FMN/mg protein, but this preparation, termed 1α, contained at most 29 of the 43 subunits of the parent Complex I.

As these values (1.2–1.5 nmol FMN/mg protein for the full complex) considerably exceed the value (1.1 nmol/mg) for an enzyme of 900 kDa with one FMN group, but are lower than the value (2.2 nmol/mg) for such an enzyme with two FMN groups, we decided to closely inspect the reliability of the routine protein-determination methods for obtaining the concentration of Complex I. We found that these methods grossly overestimate the protein content of the bovine enzyme. We show that the bovine Complex I contains clearly more than one FMN group and up to 1.6 clusters N2. A number of puzzling literature data on Complex I and some consequences of this finding are discussed. The evolutionary related enzyme, the soluble NAD^+-reducing [NiFe]-hydrogenase (EC 1.12.1.2) from *Ralstonia eutropha* also shows too high proteins contents by the routine protein-determination methods. This enzyme likewise contains two FMN groups.

2. Materials and methods

2.1. Protein samples

Two bovine mitochondrial Complex I samples were used. One [17] was a kind gift from Dr. L.A. Sazanov (Cambridge, UK) while the other sample was from a previous study [16] and had been stored in liquid nitrogen. Soluble, NAD^+-reducing hydrogenase (SH) from *R. eutropha* was purified as described [18]; the cells were kindly provided by the group of Prof. B. Friedrich (Berlin, Germany). The samples of the membrane-bound [NiFe]-hydrogenase (Ech) from *Methanosarcina barkeri* [19] were gifts from Dr. R. Hedderich (Marburg, Germany).

2.2. Protein determinations

Four different routine protein-determination methods were used: the biuret method [20,21], the Bradford method [22], the Lowry method [23] and the bicinchoninic acid (BCA) method [24] (Pierce). Ovalbumin or bovine serum albumin (BSA) were used as standards; their concentrations were obtained from the optical absorption at 279 nm (BSA; $A_{279\ nm}=6.67$ for a 1% solution [25]) or 280 nm (ovalbumin; $A_{280\ nm}=7.37$ for a 1% solution, based on an extinction coefficient of 3.15 M^{-1} cm^{-1} [25] and a molecular mass of 42 755 Da). Quantitative amino acid analysis was performed as described [26] (EuroSequence bv, Groningen, The Netherlands). For this method, all protein solutions were extensively dialyzed against 5 mM potassium phosphate buffer (pH 7.0). Samples were recovered from the dialysis bag and their volumes and protein concentrations (Bradford) were measured. This accounted for dilution and possible loss of protein. Subsequently, known amounts were freeze-dried for the analysis. As the proteins were hydrolyzed in 50% acetic acid, Asn and Gln were deamidated into Asp and Glu, respectively. As this does not influence the mass, no corrections were made for this. Trp and Cys could not be determined after this treatment, but here a correction was made on the basis of the sequences of 41 subunits of the bovine Complex I (5.3%), or the sequences of *R. eutropha* SH (5.6%), *M. barkeri* Ech (4.4%), ovalbumin (2.7%) and BSA (6.0%).

2.3. EPR spectroscopy

EPR spectra at X-band (9 GHz) were obtained with a Bruker ECS 106 EPR spectrometer at a field-modulation frequency of 100 kHz. Cooling was performed by an Oxford Instruments ESR 900 cryostat with a ITC4 temperature controller. The sample-temperature indication from this instrument was correct from 4.2 to 100 K within ±2% as ascertained from the Curie dependence of a copper standard (10 mM $CuSO_4{\cdot}5H_2O$, 2 M $NaClO_4$, 10 mM HCl). The magnetic field was calibrated with an AEG Magnetic Field Meter. The X-band frequency was measured with a HP 5350B microwave frequency counter. The microwave power incident to the cavity was measured with a HP 432 B power meter (260 mW at 0 dB). Manipulations and simulations of

EPR spectra were carried out with homemade software [12]. Quantification of EPR signals from the membranes was performed by double integration of well-fitting simulations [27,28]. Spectra from the copper standard were directly double integrated and used as a reference [29].

2.4. Other methods

Acid-extractable flavine was determined fluorometrically [30], using FMN (synthetic, from Sigma) as a standard, in a Shimadzu RF-5001PC spectrofluorometer. The concentration of the standard (in a buffer solution of pH 6.9) was calculated from the difference in absorption at 450 nm before and after addition of excess dithionite using an extinction coefficient of 11.2 mM^{-1} cm^{-1} [31].

3. Results

3.1. Protein determinations

We have determined the protein content of bovine Complex I preparations by quantitative amino acid analysis and by four conventional methods (Table 1). The routine methods overestimate the protein content of this enzyme by between 1.3 and 2.1 times. The albumin samples showed that our overall method was highly reliable. We have also included into this study two enzymes that have clear evolutionary links to Complex I, namely the soluble NAD^+-reducing [NiFe]-hydrogenase (SH) from *R. eutropha* [13,32–35] and the membrane-bound [NiFe]-hydrogenase (Ech) from *M. barkeri* [19,36]. The routine protein-determination methods overestimated the protein content of the *R. eutropha* SH by between 1.4 and 1.9 times. The Bradford protein-determination method, used for all enzymes in Table 1, overestimated the protein content of bovine Complex I by 1.76 times, that of the *R. eutropha* SH by 1.53 times, but slightly underestimated (0.85 times) the protein content of the *M. barkeri* Ech.

We have also compared the experimentally determined amino acid composition of the samples with the theoretically predicted ones. This is depicted in Fig. 1a. Deviations, calculated as explained in the legend to Fig. 1, were plotted for each individual amino acid (except for Trp and Cys). The residues Asn and Asp, as well as Gln and Glu were taken together, due to the conversion of the amide form into the acid form during the amino acid analysis procedure. It can be seen that the deviations for the several enzyme preparations are of the same magnitude as the deviations found for the pure albumin samples. When the theoretical values of the enzyme preparations were taken as basis for a comparison with the experimentally determined values for the albumins (Fig. 1b), it can be seen that, as expected, the deviations are much (about five times) larger. Hence, the method clearly detects that the amino acid composition of the albumins is quite different from that of each of the enzymes. The data thus show that the amino acid composition of the samples agreed very well with that calculated from the amino acid sequences of the subunits.

Table 1
Protein determinations of bovine Complex I and two related enzymes

Preparation	Method						
	Bradford (mg/ml)	Lowry (mg/ml)	Biuret (mg/ml)	BCA (mg/ml)	AA[a] (mg/ml)	Ratio[b] (Br/AA)	Range[c] (Col/AA)
Complex I[d]	23.4	27.4	26.4	24.7	13.3	1.76	1.76–2.06
Complex I (MQ)[e]	20.5	24.1	15.3	17.2	11.6	1.77	1.32–2.08
R.e. SH (a)[f]	15.8	20.7	16.2	16.1	10.7	1.48	1.48–1.93
R.e. SH (b)[f]	22.2	26.3	19.5	21.9	14.1	1.57	1.38–1.87
Ech[g]	4.5	–	–	–	5.2	0.87	–
Ech[g]	3.4	–	–	–	4.6	0.74	–
Ech[g]	3.6	–	–	–	3.8	0.95	–
Ovalbumin[h]	(2.0 mg/ml; from $A_{280\ nm}$)				2.0	(1.00)[i]	
BSA[h]	(5.0 mg/ml; from $A_{279\ nm}$)				4.9	(1.02)[i]	
BSA[h]	(4.5 mg/ml; from $A_{279\ nm}$)				4.6	(0.98)[i]	
BSA[h]	(4.6 mg/ml; from $A_{279\ nm}$)				4.3	(1.07)[i]	

[a] Amino acid analysis.
[b] Ratio of the protein content determined by the Bradford method (Br) and that determined with amino acid analysis (AA).
[c] Ratio of the protein content determined by the colorimetric methods (Col) and that determined with amino acid analysis (AA).
[d] Bovine mitochondrial Complex I (gift from Dr. L.A. Sazanov).
[e] A sample used in a previous study [16], prepared according to Ref. [68].
[f] Two different preparations of the soluble, NAD^+-reducing hydrogenase from *R. eutropha*.
[g] Three different preparations of the membrane-bound [NiFe]-hydrogenase (Ech) from *M. barkeri* (gifts from Dr. R. Hedderich).
[h] Control samples to validate the overall method.
[i] Ratio of the protein content determined from the absorbance at 279 or 280 nm and that determined by amino acid analysis.

3.2. FMN content

Our findings have important implications for the FMN content of both Complex I and the *R. eutropha* SH. When corrected for the systematic error in the protein determination, the FMN contents (1.2–1.5 nmol FMN/mg protein) reported in literature for the most purified preparations of bovine Complex I should be raised to values around 2.2 nmol/mg, as expected for pure Complex I with two FMN molecules. The *R. eutropha* SH was reported to contain 1.1–1.4 mol FMN/mol enzyme, using the Lowry and biuret methods to determine the protein content [37]. Our results (Table 1) show that these values should be multiplied by about 1.6 times and then become 1.8–2.2 mol FMN/mol enzyme. Hence, we predict that also the *R. eutropha* SH contains two FMN groups.

To experimentally verify this for the preparation used in Table 1, we have determined acid-extractable FMN in these preparations. The results are summarized in Table 2. The two inspected samples of the complete Complex I contained 1.5–1.6 nmol FMN/mg protein (AA method). Assuming a molecular mass of 900 kDa or more, this means that these preparations contained at least 1.3–1.4 mol FMN/mol Complex I. The two samples of the *R. eutropha* SH contained 10.6 and 8.8 nmol FMN/mg protein (AA method). With a mass of 171 kDa for this four-subunit enzyme, this results in 1.8 and 1.5 mol FMN/mol enzyme, respectively. We could not detect any acid-extractable FMN in the *M. barkeri* Ech.

We have also compared the FMN content with an internal standard (Table 2). The area of the left-half of the g_z peak (at $g=2.05$) of the EPR signal at 17 K from iron–sulfur cluster N2 is a reliable measure for the concentration of this cluster in NADH-reduced Complex I [12,27]. When applying this method to the preparations used in Table 2, we found 1.3–1.6 mol spins/mol Complex I. This means that the FMN/N2 ratio was 1.05 for the Sazanov preparation [17] and 0.86 for Finel's MQ preparation [16]. These ratios are in full agreement with values found by other workers. The present data demonstrate, however, that the number of spins detected in the EPR signal ascribed to iron–sulfur

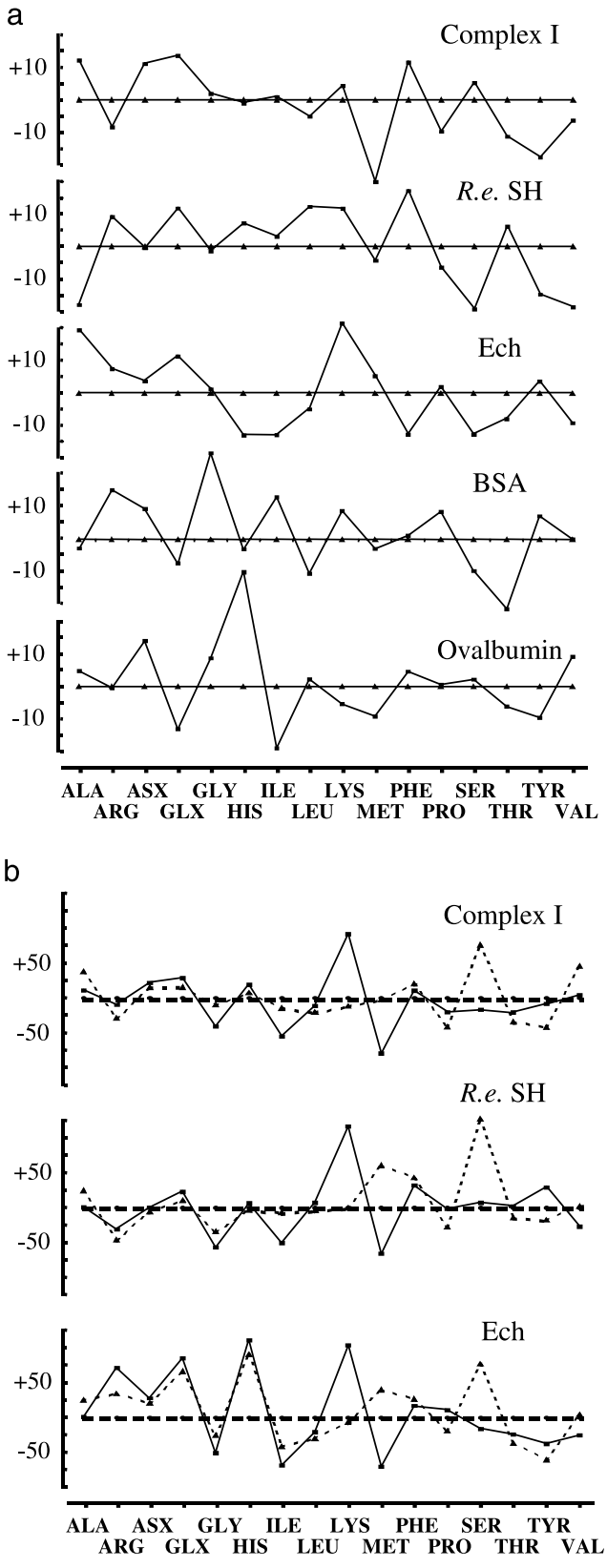

Fig. 1. Comparison of the experimental amino acid composition of the samples with the theoretical composition. (a) For each amino acid, the theoretical number of residues per mol was derived from the amino acid sequences; this was called n_t. Likewise, the number of experimentally determined residues per mol was called n_e. These values were then expressed as percentage of the total number of amino acids N, as $p=(n_t/N)$ and $q=(n_e/N)$. Subsequently, the value $r=\{(q-p)/q\}(100\%)$ was plotted in the figure for each individual amino acid. Asn and Gln residues were deamidated into Asp and Gly, respectively, during the experimental procedure; therefore, only values for Asx and Glx are given. Trp and Cys residues could not be determined. The values for Complex I, the SH from *R. eutropha*, Ech and BSA are averages from the different analyses shown in Table 1. (b) The experimental values (r) of BSA (solid lines) and ovalbumin (dashed lines) were compared with the theoretical values of Complex I, *R. eutropha* SH or Ech. In this case, the experimental values q were from BSA or ovalbumin, whereas the theoretical values p were from the reference enzymes mentioned. Note that the y-scaling in B is approximately five times reduced as compared with A.

Table 2
FMN content of bovine Complex I and the NAD^+-reducing [NiFe]-hydrogenase (SH) from *R. eutropha*

Preparation	FMN nmol/mg[a] (mol/mol)	N2 nmol/mg[a,b] (mol/mol)	FMN per N2[c]	[2Fe–2S] nmol/mg[a,d] (mol/mol)	FMN per [2Fe–2S] cluster[e]
Complex I	1.48 (1.33)	1.42 (1.28)	1.05	–	–
Complex I (MQ)	1.55 (1.40)	1.80 (1.62)	0.86	–	–
R.e. SH (a)	10.6 (1.82)	–	–	5.86 (1.0)	1.81
R.e. SH (b)	8.8 (1.51)	–	–	5.83 (1.0)	1.51

The FMN content was also compared with an internal standard, that is, the spin concentration of the EPR signal ascribed to cluster N2 (Complex I) or the [2Fe–2S] cluster (SH).

[a] Protein determined by amino acid analysis.

[b] From the spin concentration of the EPR signal at 17 K ascribed to cluster N2 in the NADH-reduced preparations.

[c] Ratio of the concentrations of FMN and the Fe–S cluster N2.

[d] From the spin concentration of the EPR signal of the [2Fe–2S] cluster at 45 K as determined in NADH-reduced enzyme.

[e] Ratio of the concentrations of FMN and the [2Fe–2S] cluster.

cluster N2 for the preparations investigated in this study (1.3–1.6 mol/mol Complex I; Table 2) warrants the conclusion that there is clearly more than one cluster N2 in these preparations.

With the SH, the EPR signal at 45 K of the NADH-reducible [2Fe–2S] cluster is a reliable measure of the enzyme concentration, provided that a good-fitting simulation is used for the double integration procedure [27]. In this case, 1.0 mol spins/mol SH was found in both preparations and hence the value of the FMN/[2Fe–2S] ratio was 1.82 and 1.51 for the preparations a and b, respectively.

4. Discussion

4.1. The number of prosthetic groups in bovine Complex I

To our knowledge, this report is the first study that combines quantitative amino acid analysis of bovine Complex I with quantitative determinations of the FMN content and the spin concentration of the EPR signal ascribed to cluster N2. The data clearly show that the bovine Complex I contains more than one FMN group: (i) at least 1.4 FMN for the intact preparations used in this study; (ii) close to two for the best preparations reported in the literature, when corrected for the error in the protein determination. We therefore conclude that bovine Complex I contains two FMN groups.

Our data likewise demonstrate that 1.3–1.6 $S = 1/2$ systems per Complex I contribute to the EPR signal ascribed to cluster N2. As Complex I preparations are never '100% pure', these values are lower limits. We conclude from this that bovine Complex I contains two clusters N2 with highly similar EPR signals. As the spin concentrations determined from the EPR signals ascribed to the clusters N3 and N4 are the same as the spin concentration of the signal ascribed to cluster N2, it follows that also the individual signals ascribed to N3 and N4 each receive contributions from two separate $S = 1/2$ systems.

4.2. There are two FMN groups in the SH

Table 2 shows that the amount of spins represented in the EPR signal of the [2Fe–2S] signal in the NADH-reduced SH is 1.0 mol/mol enzyme in both preparations. This shows that both the method of determination of the spin concentration as well as the method of protein determination (AA method) are reliable. The amount of FMN was 1.5–1.8 mol/mol enzyme. We therefore conclude that the SH contains two FMN groups. We recently found that under certain conditions, one of the FMN groups in the *Ralstonia* enzyme can be specifically released, whereby the physiological activity of the enzyme, the reduction of NAD^+ by H_2, was abolished. One FMN group remained firmly bound to the enzyme and the NADH-dehydrogenase activity was not perturbed (Van der Linden, E., Faber, B., Bleijlevens, B., Burgdorf, T., Bernard, B., Friedrich, B. and Albracht, S.P.J., manuscript in preparation).

4.3. Proposed binding site for the second FMN

One of the two FMN groups is bound to the 51-kDa subunit of Complex I, which contains an FMN-binding motif [38]. The binding site of the other FMN is discussed hereafter. On the basis of extensive comparisons of the amino acid sequences of [NiFe]-hydrogenases and Complex I [36], it has been found that the basic elements of the typical flavodoxin fold observed in the X-ray structures of [NiFe]-hydrogenases are also present in the evolutionary related PSST subunits of all Complex I enzymes known. Hence, a flavodoxin fold was proposed to be present in the PSST subunit. In looking for a binding site for the extra FMN group uncovered in the present report, the PSST subunit in the bovine Complex I is the obvious choice. For the soluble [NiFe]-hydrogenase from *R. eutropha*, the HoxF subunit contains an FMN-binding motif and binds the FMN involved in the NADH-dehydrogenase reaction. We propose that the HoxY subunit binds the second FMN.

A schematic representation of the prosthetic group in bovine Complex I, based on these new experimental results, is given in Fig. 2.

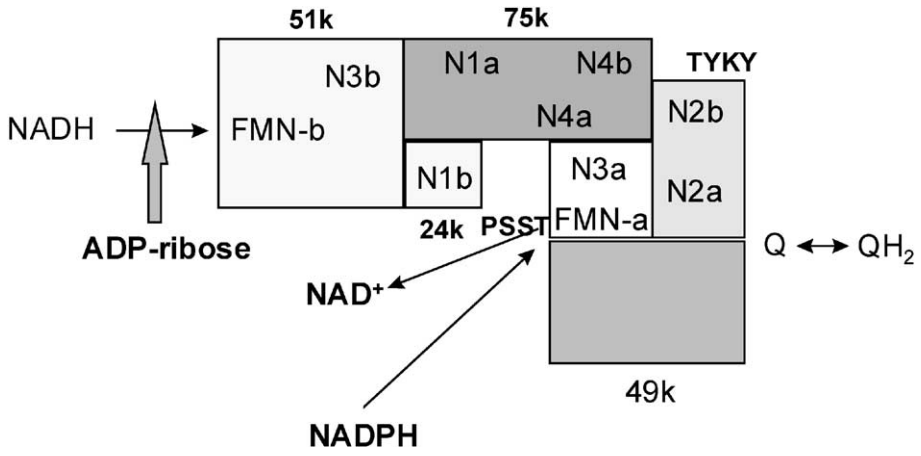

Fig. 2. Schematic representation of the five subunits of bovine Complex I (the 24-, 51- and 75-kDa, PSST and TYKY subunits) containing the prosthetic groups, plus the 49-kDa subunit. The clusters N1a and N1b are [2Fe–2S] clusters; the clusters N2a, N2b, N3a, N3b, N4a and N4b are [4Fe–4S] clusters. NADPH can reduce FMN-a and the clusters N2a, N3a and N4a, but the electrons cannot reach ubiquinone (Q) [28]. NADH can reduce all prosthetic groups except cluster N1a. ADP-ribose is an inhibitor of NADH oxidation, but has no effect on the energy-driven reduction of NAD^+ [52].

4.4. Puzzling observations on Complex I can now be explained

At this point, it is useful to recall a number of experimental findings in the long literature on the bovine Complex I, that can now be more easily understood.

(A) About three decades ago, Hatefi [39] noted an optical bleaching of purified Complex I (measured at 460–510 or 475–510 nm) when NADH was added. Curiously, in the presence of the inhibitor rotenone, only half of this bleaching was observed. When NADPH was used, then also only about 50% of the bleaching induced with NADH was observed [40]. Subsequent addition of NADH immediately resulted in the full bleaching. When NAD^+ was added instead, a complete bleaching was only obtained in a slow reaction; this reaction, in fact a transhydrogenase activity of the purified Complex I, could be inhibited by palmitoyl-CoA. It was later established that rotenone did not inhibit the reduction of any of the EPR-detectable Fe–S clusters in the bovine complex [41]. Hence, it can be concluded that the redox component responsible for about 50% of the total bleaching of bovine Complex I is apparently not one of the EPR-detectable Fe–S clusters or the flavine (FMN-b) required for the oxidation of NADH. The presence of a second flavine group (FMN-a), which can specifically react with NADPH, solves this long-standing puzzle. Incidentally, these classical experiments, in combination with the present knowledge on Complex I, suggest that (i) the FMN-a group may be the site where the transhydrogenase activity of Complex I is catalyzed (reduction by NADPH, oxidation by NAD^+) and (ii) that rotenone interferes with electron transfer from the Fe–S clusters to the FMN-a group. In this respect, it is worthwhile to recall that the PSST subunit specifically binds a photoaffinity derivative of pyridaben [42], a potent inhibitor of Complex I. This binding is counteracted by other inhibitors like rotenone, piericidin A, bullatacin and rolliniastatin I [42].

(B) Quantification by our laboratory of the EPR signals ascribed to the Fe–S clusters N1b and N2 clearly showed that the relative intensities of these signals (a direct measure of their relative concentrations) in the purified enzyme, as well as in the enzyme in submitochondrial particles, differ by a factor of 2 [27,43–45]. It was demonstrated a decade ago [45] that the direct double integration of the experimental EPR signal of cluster N1b at around 50 K, as commonly used in the Complex I field up to that time, results in a considerable overestimation of the concentration of this cluster, due to the broad underlying signals of the relaxation-broadened EPR spectra of the [4Fe–4S] clusters. The correct way to determine the spin concentration for the signal due to cluster N1b is to use a good-fitting computer-simulated line shape for the double integration [27,45]. It then turned out that the intensity of this signal was half that of the signal ascribed to cluster N2 (and half of the intensities of the signals ascribed to the cluster N3 and cluster N4). The number of spins represented by the signal ascribed to cluster N2 was close to one per FMN, as also determined for the Complex I preparations used in the present study. This prompted this laboratory already more than two decades ago [27,43] to propose: (i) that each of the EPR signals ascribed to the clusters N2, N3 and N4 must receive contributions from two different clusters with very similar EPR line shapes (N2a, N2b, N3a, N3b, N4a, N4b); (ii) that the minimal enzymatic unit of the bovine Complex I must at least contain one cluster N1b, two FMN groups and one of the six clusters mentioned above.

(C) Kinetic studies (steady state and pre-steady state) with aerobic submitochondrial particles at pH 7.5 or higher showed that NADPH can rapidly (within 50 ms) evoke maximally 50% of the EPR signals ascribed to the clusters N2, N3 and N4. This reduction was interpreted as a specific reduction of the clusters N2a, N3a and N4a. The reduction persisted for minutes without noticeable oxygen consumption [28,46,47]. Cluster N1b was not reduced. As electrons

did not leave Complex I, the apparent point of entrance for electrons from NADPH was assumed to be different from the point of entrance for electrons from NADH. The clusters N1b, N2a, N2b, N3a, N3b, N4a and N4b are all reduced within 6 ms when NADH is used [12]. For each reaction, the reaction with NADPH and the reaction with NADH, a flavine was assumed to be a prerequisite. These data can now be more easily understood. They showed, however, that there is apparently no redox equilibrium between the clusters when NADPH is used as the electron donor. This still remains a mystery.

(D) Piericidin A is a well-known potent inhibitor of the bovine Complex I. It was shown that the oxidation of NADH by submitochondrial particles can be completely blocked when only one piericidin molecule per two clusters N2 (N2a+N2b) is bound to the enzyme, provided that the particles were preincubated with the inhibitor in the presence of NADPH [48]. Again, this pointed to a minimal enzymatic unit with two clusters N2 and two FMN groups. With a preincubation in the presence of NADH, however, two piericidin molecules per two clusters N2 were required [48]. The latter result is in agreement with findings of other groups who, by using labelled inhibitors, have shown that the bovine complex in submitochondrial particles has two binding sites for rotenone, piericidin A and 1-methyl-4-phenylpyridinium (MPP^+) [49–51] and that (in experiments without a preincubation with NADPH) both inhibitor sites must be occupied for the complete inhibition of NADH oxidation. Initially, our laboratory explained the findings under B, C and D by proposing a dimeric structure of Complex I [28]. The dimeric model could be replaced by a monomeric one [13] once information on the amino acid sequences of the subunits from the bovine enzyme became available from the group of Walker (see below) [1,2,38].

(E) From the amino acid sequences of the subunits of bovine Complex I, the binding sites for two 2Fe clusters, six 4Fe clusters and one FMN were predicted [38]. Assuming that all Fe–S clusters but one, cluster N1a, would be NADH reducible, this would lead to seven spins and a total of 28 Fe atoms per FMN. Both of these numbers are nearly twofold higher than those (3.4–4 spins and 16–18 Fe atoms per FMN) determined by the EPR-spectroscopical and chemical analyses mentioned in the introduction. The presence of a second FMN, however, reduces these numbers to 3.5 spins and 14 Fe atoms per FMN, in much better agreement with the experimental data.

(F) From studies on coupled submitochondrial particles with the inhibitor ADP-ribose [52], it was concluded that the site for the reduction of NAD^+, in the energy-induced reversal of electron transfer in Complex I, is not the same as that for NADH oxidation. The latter reaction is inhibited by ADP-ribose, whereas the former reaction is not. It is reasonable to assume that a flavine is required for each of these reactions. The results can be easily understood in a Complex I with two FMN groups with a different function.

4.5. Possible reason for the overestimation of the protein content of Complex I

The reason for the overestimation of the protein content by the routine methods is not quite clear. Lowry et al. [23] already noticed that, when using pure proteins, their method and the biuret method can result in protein contents deviating considerably from true values. This also holds for the Bradford assay [53]. The amino acid sequences of the six subunits from the *M. barkeri* Ech are closely similar to those of six of the subunits from Complex I [36], but these subunits are apparently not the cause for the overestimation of the protein content. Two of the four subunits of the *R. eutropha* SH have homologues in both Ech and Complex I. The other two, HoxF and HoxU, form the NADH-dehydrogenase module in this enzyme and are related to the NADH-dehydrogenase part of Complex I [33]. The N-terminal sections of the HoxU subunit in SH and of the 75-kDa subunit in Complex I are similar to each other and to the N-terminal part of the sequence from a number of [Fe]-hydrogenases [13]. These sections contain common motifs for the binding of one [2Fe–2S] cluster and two [4Fe–4S] clusters. The X-ray structure of the [Fe]-hydrogenase from *Clostridium pasteurianum* shows that one of these 4Fe clusters has a His residue as a ligand [54]. This His residue is conserved in the sequence of the 75-kDa subunit of Complex I, but not in that of the HoxU subunit of the SH [13]. Incidentally, the routine protein determination methods used for the [Fe]-hydrogenase from *C. pasteurianum* also resulted in a large overestimation of the protein content [55]. This points to the 75-kDa subunit in Complex I and the HoxU subunit in SH as possible contributors to this deviation.

5. Conclusions and implications

The results in this paper require a reinterpretation of a number of observations in the recent literature on Complex I. Three examples are discussed.

5.1. Bovine Complex I does not contain reducible, EPR-silent Fe–S clusters

Rasmussen et al. [56] have proposed the presence of EPR-undetectable, reducible Fe–S clusters in non-bovine Complex I. From experiments with Complex I purified from *Neurospora crassa* and *Escherichia coli*, it was concluded that the TYKY subunit contains two [4Fe–4S] clusters (termed N6a and N6b), both with a pH-independent midpoint potential of −270 mV. The reduction of these clusters could be detected with UV/Vis spectroscopy but caused no detectable contribution to the EPR spectrum. The experiments of Kowal et al. [9] and the results from the present study exclude such a possibility for the bovine Complex I.

5.2. In bovine Complex I, cluster N2 is not located in the PSST subunit

Another example is the location of 'the cluster N2'. On the basis of studies on Complex I from *Yarrowia lipolytica* [57] and *N. crassa* [58], where point mutations were generated in analogues of the PSST and 49 kDa subunits, it was concluded that the PSST subunit would be the location of 'cluster N2'. Because we show here that in the bovine enzyme the EPR signal ascribed to cluster N2 receives contributions from two $S = 1/2$ systems (clusters N2a and N2b) and because the PSST subunit contains only one possible motif for the binding of a 4Fe cluster, this proposal cannot hold for the bovine enzyme.

5.3. The clusters N2 in Complex I are located in the TYKY subunit

The TYKY subunit has two classical amino-acid sequence motifs (four-Cys motifs) for the binding of a 4Fe cluster [59] and it was proposed that this subunit might hold cluster N2. Our group has advocated the presence in this subunit of two EPR-detectable clusters N2 [13,36]. It was assumed [13,36] that these clusters have virtually identical EPR spectra in the bovine enzyme. The paramagnetic clusters show a clear exchange coupling under energized conditions in coupled submitochondrial particles [60,61]. Under non-energized conditions, as well as in isolated Complex I, no such magnetic interaction has been observed, however.

The first experimental evidence for the presence of [4Fe–4S] clusters in the TYKY subunit came from studies on an overexpressed, truncated form of the Nqo9 (TYKY) subunit from *Paracoccus denitrificans*, reconstituted with iron and sulfide [62]. The *g* values of one of the two signals in the EPR spectrum ($g_{xyz} = 1.92, 1.92, 2.05$) were similar to those found in the membrane-bound complex [63,64]. The midpoint potentials of the clusters (< -600 mV) were, however, very much lower than that of the N2 EPR signal in the membrane-bound complex [62].

Quite recently, an extensive study focused at the effect of point mutations in the NuoI (TYKY) subunit from Complex I in *Rhodobacter capsulatus* has been completed (Ref. [65], accompanying paper). Mutants in the NuoI (TYKY) subunit were constructed in which five out of the eight conserved Cys residues in NuoI were replaced by other residues. EPR analysis of membrane preparations showed a specific, 50% decrease of the signal attributed to cluster N2, when a particular Cys residue in one or the other 'four-Cys' motif was replaced by a Ser residue. The EPR signals of the other clusters, as well as the activity of the complex in the isolated membranes and its function in the growing cells, were hardly perturbed. Replacement of the Cys residue by an Arg residue abolished the biosynthesis of intact Complex I in the membranes. This study provides the first direct demonstration that point mutations in the TYKY subunit specifically alter the properties of half of the EPR signal ascribed to cluster N2. It shows that in this bacterium, the clusters N2 are in the NuoI (TYKY) subunit and that an intact Complex I cannot be formed if one of the [4Fe–4S] clusters in the TYKY subunit is missing. This raises questions about the interpretation of the effects of mutations leading to the partial loss or the complete absence of the EPR signal of cluster N2 in purified, apparently completely intact preparations of Complex I from *Y. lipolytica* [57] and *N. crassa* [58]. This will be further discussed in the accompanying paper [65].

In summary, the information presented here and in the accompanying paper provide strong experimental evidence that all the Fe–S clusters predicted by the amino-acid sequence information [38] are EPR detectable when reduced and that Complex I contains two FMN molecules. Reduction of the second FMN group (proposed to be in the PSST subunit) might (partly) explain the UV/Vis-detected reduction of EPR-undetected redox groups in Complex I from bovine heart [39,40], *N. crassa* and *E. coli* [56,66,67]. In view of the high similarities of the amino acid sequences of the subunits of Complex I from many different sources, with their conserved motifs for the binding of Fe–S clusters and FMN, and the proposed flavodoxin fold in the PSST subunits, we feel that a reinvestigation of the FMN and protein contents for the non-bovine enzymes is called for.

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References

[1] J.E. Walker, Q. Rev. Biophys. 25 (1992) 253–324.
[2] J.E. Walker, J.M. Skehel, S.K. Buchanan, Methods Enzymol. 260 (1995) 14–34.
[3] A.H. Schapira, Biochim. Biophys. Acta 1364 (1998) 261–270.
[4] R.H. Triepels, L.P. Van Den Heuvel, J.M. Trijbels, J.A. Smeitink, Am. J. Med. Genet. 106 (2001) 37–45.
[5] I.M. Fearnley, J. Carroll, R.J. Shannon, M.J. Runswick, J.E. Walker, J. Hirst, J. Biol. Chem. 276 (2001) 38345–38348.
[6] Y. Hatefi, A.G. Haavik, D.E. Griffiths, J. Biol. Chem. 237 (1962) 1667–1680.
[7] T. Cremona, E.B. Kearney, J. Biol. Chem. 239 (1964) 2328–2334.
[8] C.J. Lusty, J.M. Machinist, T.P. Singer, J. Biol. Chem. 240 (1965) 1804–1810.
[9] A.T. Kowal, J.E. Morningstar, M.K. Johnson, R.R. Ramsay, T.P. Singer, J. Biol. Chem. 261 (1986) 9239–9245.
[10] T. Ohnishi, H. Blum, Y.M. Galante, Y. Hatefi, J. Biol. Chem. 256 (1981) 9216–9220.
[11] C. Paech, J.R. Reynolds, T.P. Singer, R.H. Holm, J. Biol. Chem. 256 (1981) 3167–3170.

[12] H. Beinert, S.P.J. Albracht, Biochim. Biophys. Acta 683 (1982) 245–277.
[13] S.P.J. Albracht, A.M.P. De Jong, Biochim. Biophys. Acta 1318 (1997) 92–106.
[14] T. Ohnishi, Biochim. Biophys. Acta 1364 (1998) 186–206.
[15] C.I. Ragan, Y.M. Galante, Y. Hatefi, T. Ohnishi, Biochemistry 21 (1982) 590–594.
[16] M. Finel, J.M. Skehel, S.P.J. Albracht, I.M. Fearnley, J.E. Walker, Biochemistry 31 (1992) 11425–11434.
[17] L.A. Sazanov, S.Y. Peak-Chew, I.M. Fearnley, J.E. Walker, Biochemistry 39 (2000) 7229–7235.
[18] C.G. Friedrich, K. Schneider, B. Friedrich, J. Bacteriol. 152 (1982) 42–48.
[19] J. Meuer, S. Bartoschek, J. Koch, A. Kunkel, R. Hedderich, Eur. J. Biochem. 265 (1999) 325–335.
[20] A.G. Gornall, C.J. Bardawill, M.M. David, J. Biol. Chem. 177 (1949) 755–766.
[21] K.W. Cleland, E.C. Slater, Biochem. J. 53 (1953) 547–556.
[22] M.M. Bradford, Anal. Biochem. 100 (1976) 201–220.
[23] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[24] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimato, N.M. Goeke, B.J. Olson, D.C. Klenk, Anal. Biochem. 150 (1985) 76–85.
[25] G.D. Fasman, Handbook of Biochemistry and Molecular Biology, CRC Press Inc., Cleveland, OH, 1976.
[26] R. Schuster, J. Chromatogr. 431 (1988) 271–284.
[27] S.P.J. Albracht, F.J. Leeuwerik, B. van Swol, FEBS Lett. 104 (1979) 197–200.
[28] R. Van Belzen, S.P.J. Albracht, Biochim. Biophys. Acta 974 (1989) 311–320.
[29] R. Aasa, T. Vänngård, J. Magn. Reson. 19 (1975) 308–315.
[30] J. Kozol, Methods Enzymol. 18b (1971) 253–285.
[31] R.M.C. Dawson, D.C. Elliot, W.H. Elliot, K.M. Jones, Data for Biochemical Research, Oxford Science Publications, Clarendon Press, Oxford, UK, 1986.
[32] A. Tran-Betcke, U. Warnecke, C. Bocker, C. Zaborosch, B. Friedrich, J. Bacteriol. 172 (1990) 2920–2929.
[33] S.J. Pilkington, J.M. Skehel, R.B. Gennis, J.E. Walker, Biochemistry 30 (1991) 2166–2175.
[34] S.P.J. Albracht, Biochim. Biophys. Acta 1144 (1993) 221–224.
[35] S.P.J. Albracht, Biochim. Biophys. Acta 1188 (1994) 167–204.
[36] S.P.J. Albracht, R. Hedderich, FEBS Lett. 485 (2000) 1–6.
[37] K. Schneider, H.G. Schlegel, Biochem. Biophys. Res. Commun. 84 (1978) 564–571.
[38] I.M. Fearnley, J.E. Walker, Biochim. Biophys. Acta 1140 (1992) 105–134.
[39] Y. Hatefi, Proc. Natl. Acad. Sci. U. S. A. 60 (1968) 733–740.
[40] Y. Hatefi, W.G. Hanstein, Biochemistry 12 (1973) 3515–3522.
[41] N.R. Orme-Johnson, R.E. Hansen, H. Beinert, J. Biol. Chem. 249 (1974) 1922–1927.
[42] F. Schuler, T. Yano, S. DiBernardo, T. Yagi, V. Yankovskaya, T.P. Singer, J.E. Casida, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 4149–4153.
[43] S.P.J. Albracht, G. Dooijewaard, F.J. Leeuwerik, B.V. Swol, Biochim. Biophys. Acta 459 (1977) 300–317.
[44] D.O. Hearshen, W.R. Dunham, S.P.J. Albracht, T. Ohnishi, H. Beinert, FEBS Lett. 133 (1981) 287–290.
[45] R. Van Belzen, A.M.P. De Jong, S.P.J. Albracht, Eur. J. Biochem. 209 (1992) 1019–1022.
[46] P.T. Bakker, S.P.J. Albracht, Biochim. Biophys. Acta 850 (1986) 413–422.
[47] S.P.J. Albracht, P.T. Bakker, Biochim. Biophys. Acta 850 (1986) 423–428.
[48] R. Van Belzen, M.C. van Gaalen, P.A. Cuypers, S.P.J. Albracht, Biochim. Biophys. Acta 1017 (1990) 152–159.
[49] M. Gutman, T.P. Singer, J.E. Casida, J. Biol. Chem. 245 (1970) 1992–1997.
[50] R.R. Ramsay, M.J. Krueger, S.K. Youngster, M.R. Gluck, J.E. Casida, T.P. Singer, J. Neurochem. 56 (1991) 1184–1190.
[51] M.R. Gluck, M.J. Krueger, R.R. Ramsay, S.O. Sablin, T.P. Singer, W.J. Nicklas, J. Biol. Chem. 269 (1994) 3167–3174.
[52] T.V. Zharova, A.D. Vinogradov, Biochim. Biophys. Acta 1320 (1997) 256–264.
[53] G.L. Peterson, Methods Enzymol. 91 (1983) 95–119.
[54] J.W. Peters, W.N. Lanzilotta, B.J. Lemon, L.C. Seefeldt, Science 282 (1998) 1853–1858.
[55] M.W.W. Adams, E. Eccleston, J.B. Howard, Proc. Natl. Acad. Sci. U.S.A. 86 (1989) 4932–4936.
[56] T. Rasmussen, D. Scheide, B. Brors, L. Kintscher, H. Weiss, T. Friedrich, Biochemistry 40 (2001) 6124–6131.
[57] N. Kashani-Poor, K. Zwicker, S. Kerscher, U. Brandt, J. Biol. Chem. 276 (2001) 24082–24087.
[58] M. Duarte, H. Populo, A. Videira, T. Friedrich, U. Schulte, Biochem. J. 364 (2002) 833–839.
[59] A. Dupuis, J.M. Skehel, J.E. Walker, Biochemistry 30 (1991) 2954–2960.
[60] A.M.P. De Jong, A.B. Kotlyar, S.P.J. Albracht, Biochim. Biophys. Acta 1186 (1994) 163–171.
[61] R. Van Belzen, A.B. Kotlyar, N. Moon, W.R. Dunham, S.P.J. Albracht, Biochemistry 36 (1997) 886–893.
[62] T. Yano, S. Magnitsky, V.D. Sled, T. Ohnishi, T. Yagi, J. Biol. Chem. 274 (1999) 28598–28605.
[63] S.P.J. Albracht, H.W. van Verseveld, W.R. Hagen, M.L. Kalkman, Biochim. Biophys. Acta 593 (1980) 173–186.
[64] S.W. Meinhardt, T. Kula, T. Yagi, T. Lillich, T. Ohnishi, J. Biol. Chem. 262 (1987) 9147–9153.
[65] M. Chevallet, A. Dupuis, J.-P. Issartel, J. Lunardi, R. Van Belzen, S.P.J. Albracht, Biochim. Biophys. Acta 1557 (2002) this issue.
[66] U. Schulte, A. Abelmann, N. Amling, B. Brors, T. Friedrich, L. Kintscher, T. Rasmussen, H. Weiss, BioFactors 8 (1998) 177–186.
[67] T. Friedrich, B. Brors, P. Hellwig, L. Kintscher, T. Rasmussen, D. Scheide, U. Schulte, W. Mantele, H. Weiss, Biochim. Biophys. Acta 1459 (2000) 305–309.
[68] Y. Hatefi, J.S. Rieske, Methods Enzymol. 10 (1967) 235–239.