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Comparison of energization of complex I in membrane particles from *Paracoccus denitrificans* and bovine heart mitochondria

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Abstract

The results of preliminary studies of the effects of energization on the catalytic and EPR properties of complex I in tightly coupled membrane vesicles of *Paracoccus denitrificans* (SPP) are presented. They are compared to those observed in submitochondrial particles from bovine heart (SMP). All signs of energization of complex I detected by EPR in SMP (uncoupler-sensitive splitting of the g_z lines of the clusters 2 and a broadening of their g_{xy} lines, a fast-relaxing, piericidin-sensitive ubiquinone-radical signal, and a broad signal around $g=1.94$) were also observed with the bacterial enzyme. There were some prominent differences, though. The signal of the fast-relaxing radicals could be evoked both in the presence or absence of reduced clusters 2, suggesting that enhancement of its spin-relaxation rate is caused by coupling to another paramagnet. The signal was hardly affected by the presence of gramicidin. The slow-relaxing radical signal did not disappear upon anaerobiosis, but was detectable for at least another 30 s. The fast-relaxing signal vanished immediately upon anaerobiosis. The activity of the bacterial enzyme during oxidation of NADH by oxygen or reduction of NAD induced by succinate oxidation, was 5–6 times higher than that of the mitochondrial enzyme. Unlike the mitochondrial enzyme, the bacterial enzyme was not inactivated by incubation at 35°C. The spin concentration of the NADH-reducible [2Fe–2S] cluster (1b) was half that of the clusters 2, indicating no difference with the mitochondrial enzyme.

Keywords: NADH:Q oxidoreductase; Complex I; Energization; *Paracoccus denitrificans*; Mitochondrion

1. Introduction

NADH:ubiquinone oxidoreductase (complex I, EC 1.6.99.3) is the most complicated and least understood energy-coupling device in the electron transfer chain of many organisms. The smallest form of the enzyme has so far been found in *Escherichia coli* and consists of 14 subunits [1,2]. The mitochondrial enzyme contains at least 42 polypeptides [3], FMN and several Fe–S clusters. In an effort to understand some 30 years of research on the enzyme, one of us has come to the conclusion that the mitochondrial complex I quite likely contains two FMN molecules and eight Fe–S clusters [4].

In 1991 it was recognized [5] that three Fe–S-containing polypeptides of complex I (24, 51 and 75 kDa) show considerable sequence similarity with the NADH-dehydrogenase (diaphorase) part of the solu-

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ble, NAD^+-reducing [NiFe]-hydrogenase from *Alcaligenes eutrophus* [6]. The Fe–S-binding sequence of the 75-kDa subunit shows also a remarkable similarity with the N-terminal region of [Fe]-hydrogenases [4,7]. It was therefore proposed that the Fe–S clusters 1a, 1b, 3 and 4 (being six Fe–S clusters) are solely involved in electron transfer, and are not required for the electrogenic proton pump of complex I [4]. The two clusters 2, assumed to be anchored in the TYKY subunit, were proposed to be essential for coupled electron transfer to Q-10 [4].

It was also recognized that the PSST and the 49-kDa subunits, are probably evolutionarily related to the small and the large subunits, respectively, of [NiFe]-hydrogenases [7,8]. As complex I has no hydrogenase properties [9], it was postulated that the protein structure of [NiFe]-hydrogenases, with its intrinsic proton transfer channels [10,11], has been conserved in the PSST/49-kDa subunit couple in complex I and is somehow used to translocate protons linked to electron transfer [7,12].

In submitochondrial particles (SMP) reduced with NADPH the two clusters 2 are not in redox equilibrium at pH 8, but only at pH 6 [13]. NADH reduces both clusters but their $S=1/2$ systems, assumed to be present in the TYKY subunit, show no spin–spin interaction. Only under energized conditions, in tightly coupled SMP, do the two clusters 2 show an EPR-detectable exchange coupling [14]. At the same time a fast-relaxing complex I-bound Q_I radical [15,16], weakly interacting with a cubane cluster (perhaps one or both of the clusters 2) [14], is observed, as well as a broad [2Fe–2S] signal around $g=1.94$ [14].

Complex I from *Paracoccus denitrificans* is almost identical to its mitochondrial counterpart in terms of EPR, thermodynamic properties of the Fe–S clusters, and the sensitivity to the specific inhibitors [17–20]. The Fe–S clusters seem to be present in stoichiometric amounts [19,20]. It has been described [17] that the rate of NADH oxidation, catalyzed by the inside-out vesicles prepared from *P. denitrificans* cells increased 3-fold by addition of ADP and up to 10 times by the addition of most uncouplers.

Here we report on the effects of energization on Complex I in tightly coupled membrane vesicles from *P. denitrificans* and compare them with those observed in the enzyme from beef heart mitochondria. All the signs of energized complex I demonstrated in our previous studies [14] for the mitochondrial system (uncoupler-sensitive splitting of the g_z lines of the clusters 2 and the broadening of the g_{xy} lines, a fast-relaxing radical signal, and a broad signal around $g=1.94$), were also found in the bacterial enzyme. In addition, it could be unequivocally shown that the spin concentrations of the NADH-reducible [2Fe–2S] cluster (1b) was half that of the clusters 2, just like in the mitochondrial system [21–24], suggesting that the bacterial enzyme also contains eight Fe–S clusters.

2. Catalytic properties of coupled particles from mitochondria and *P. denitrificans*

The growth of *P. denitrificans* and the preparation of membrane particles was carried out as reported [17], except that Mn was omitted from the trace element solution. The particles were suspended in 10 mM Tris-acetate buffer (pH 7.3), 5 mM Mg-acetate and 1 mg/ml bovine serum albumin. SMP were prepared as described [25]. All experiments were carried out at 25°C.

The catalytic properties of the SMP and the particles prepared from the *P. denitrificans* cells (SPP, sub-*Paracoccus* particles) are given in Table 1. The piericidin-sensitive NADH oxidation was strongly stimulated by uncoupler, indicating that the particles from the two sources were tightly coupled. Both

Table 1
Catalytic properties of membrane particles from mitochondria (SMP) and *P. denitrificans* (SPP)

Preparation	Rate[a] (μmol NAD(H)/min per mg protein)		
	NADH oxidation[b]		NAD^+ reduction[c]
	−uncoupler	+uncoupler[d]	−uncoupler
SMP[e]	0.21	1.25	0.24
SMP[e] (10 min, 37°C)	0.00	0.10	0.00
SPP	0.41	2.51	0.53
SPP (10 min, 37°C)	0.40	2.46	0.52

[a]Initial rates of the reactions were measured 3 s after the addition of particles to the assay mixtures.
[b]Measured at 25°C in: 0.25 M sucrose, 20 mM Hepes (pH 7.8), 1 mg/ml BSA, 0.1 mM EDTA, 2 mM $MgCl_2$ and 100 μM NADH.
[c]Measured at 25°C in: 0.25 M sucrose, 20 mM Hepes (pH 7.8), 1 mg/ml BSA, 0.1 mM EDTA, 20 mM succinate and NAD^+. No NAD^+ reduction could be measured in the presence of gramicidin.
[d]Gramicidin (0.25 μM) plus 15 mM ammoniumacetate.
[e]The particles were pretreated with oligomycin (0.4 μg/mg).

preparations catalyzed the $\Delta\mu H^+$-dependent, piericidin-sensitive, reversed electron transfer from succinate to NAD^+. The rate of NAD^+ reduction catalyzed by the bacterial preparation was twice that of SMP. A special pre-treatment of SMP was needed to obtain high rates of the reversed electron transfer in the absence of ATP. The procedure included coupling of the vesicles with oligomycin to reduce the proton conductivity of the membranes and activation of succinate dehydrogenase by malonate [26] to provide succinate-oxidation rates sufficient to maintain a high steady-state $\Delta\mu H^+$ level. No such pre-treatment was required to drive efficient reversed electron transfer in SPP. The time course of the aerobic succinate–NAD^+-reductase reaction is shown in Fig. 1. The lag phase in the kinetics of SMP is due to the initial, inactive state of mitochondrial complex I. As SMP contain about 3 times more complex I than SPP (see below), the activity of the enzyme in SPP is about 5–6 times as high. It was previously shown [19] that the relative ratios of complexes I–IV in SMP and SPP are comparable.

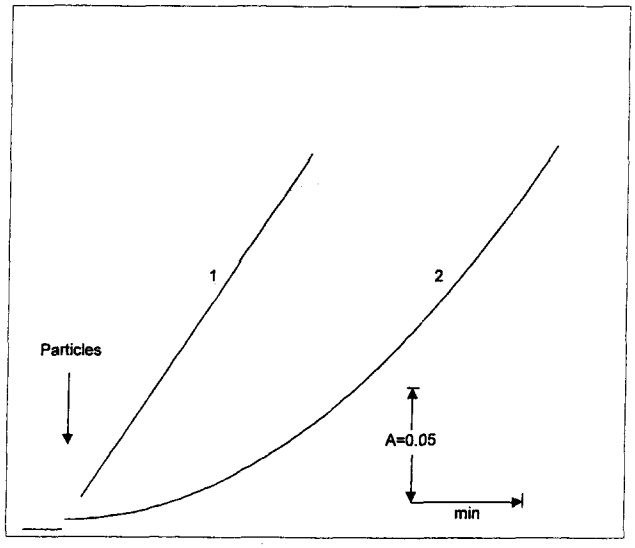

Fig. 1. Time course of aerobic succinate-NAD^+ reductase in tightly coupled membrane vesicles from mitochondria (SMP) and *P. denitrificans* (SPP). SMP (10 mg/ml) were pretreated with 0.4 μg/mg of oligomycin and pre-incubated for 10 min at 37°C in 0.25 M sucrose, 1 mg/ml BSA, 0.1 mM EDTA, 20 mM succinate and 50 mM Hepes (potassium salt, pH 7.8). SPP were pre-incubated under the same conditions as SMP in Tris–HCl buffer (pH 7.3), 1 mg/ml BSA, 20 mM succinate and 5 mM $MgCl_2$. The succinate-supported NAD^+ reduction was measured at 25°C in an assay mixture containing 0.25 M sucrose, 20 mM Hepes (pH 7.8), 1 mg/ml BSA, 0.1 mM EDTA, 20 mM succinate and NAD^+ (potassium salts). The reaction was started by addition of 50 μg/ml of SPP (curve 1) and 100 μg/ml of SMP (curve 2) and the reaction was followed at 340 nm.

In earlier reports [26] we have demonstrated that the deactivated state of complex I is formed by a short-term pre-incubation of the enzyme at near-physiological temperatures (35–40°C). Pre-incubation of the bacterial enzyme, even for more than 30 min at 37°C, caused no deactivation and the enzyme retained maximum initial NAD^+-reductase activity (Fig. 1). The difference in temperature-induced inactivation of bacterial and mammalian enzymes raises the question about the significance of this process for a possible regulation of complex I in mammals. The equilibrium between the active and inactive forms of the mammalian enzyme may be affected by a variety of endogenous components, e.g. bivalent metal ions [27], and can be observed only at temperatures higher than 35°C (see also Ref. [28]). The high activation energy makes this mechanism less relevant for the bacteria, which normally live at lower and variable temperatures.

3. A closer look at EPR properties of coupled submitochondrial particles

3.1. Piericidin blocks energization of the reduced clusters 2

As observed before [14], gramicidin (50 μM) completely abolished succinate-induced reduction of complex I. When piericidin (15 μM) was firmly bound to complex I, via temporary reduction of the clusters 2 by a pulse (100 μM) of NADH [23], the clusters 2 remained largely (77%) reduced upon subsequent flushing with oxygen, whereas the other clusters were completely oxidized [29]. When energization of SMP was then started by either succinate, or a mixture of succinate plus NADH, no signs of energization of complex I could be observed. Also, no oxidation of the clusters 2 was induced by the $\Delta\mu H^+$ generated by succinate oxidation. This means that binding of piericidin to complex I with reduced clusters 2 fixes the complex in its non-energized state. The concentration of the clusters 2, determined as described before [24], amounted to 0.12 nmol per mg of protein.

3.2. Reduction of cluster 1a

The EPR spectra enabled inspection of the apparent line shape of the 'broad feature', detectable in the $g=1.94$ region only under coupled conditions during steady-state oxidation of NADH or succinate. Difference spectra at 45 K of SMP respiring succinate in the absence or presence of piericidin gave results shown in Fig. 2. The difference could even be observed at 70 K and so we conclude that the spectra are due to a [2Fe–2S] cluster. The broad signal has a rhombic nature (approximate values $g_{xy}=1.922$, 1.940; g_z not uncovered, but presumably close to $g=2$. The species could not be induced by succinate oxidation in the presence of piericidin, and so, in contrast to our earlier suggestion [14], it is assigned to be a member of complex I. The experimental conditions used here are better defined than those used previously [14], where succinate oxidation could evoke a broad signal in SMP with inactive complex I. Addition of a mixture of 4 mM NAD^+, 0.1–10 mg/ml lactate dehydrogenase and 10 mM pyruvate to oxidize complex I at the NAD^+-reacting site, eliminated this extra signal virtually completely. This mixture also considerably diminished the signals of the clusters 1b, 3 and 4, whereas the reduction of the clusters 2 halved. These experiments show that the unknown [2Fe–2S] cluster is oxidized by NAD^+. The signal was not observed 5 s after mixing of SMP with NADH in the presence of piericidin. We ascribe the signal to cluster 1a, a cluster discovered by Ohnishi et al. [30] and detectable only under special conditions.

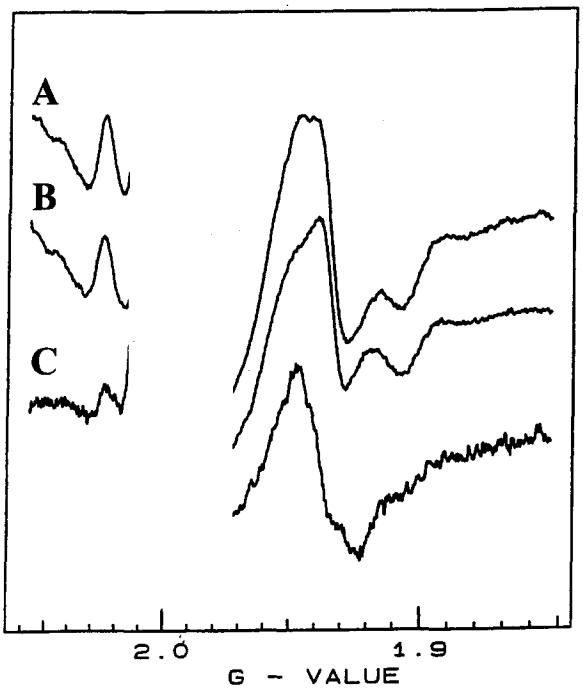

Fig. 2. Approximate line shape of the broad $g=1.94$ signal, transiently observed in tightly coupled SMP. (A) SMP (25 mg/ml) in an EPR tube were flushed with oxygen and then mixed with 40 mM succinate during 5 s, whereafter the tube was shock-frozen in cold (−140°C) isopentane. (B) As in (A), but the SMP were first pretreated by mixing with 15 μM piericidin, and then with 100 μM NADH to obtain optimal binding of the inhibitor. (C) Difference: (A) minus (B); enlarged 2 times. EPR conditions: microwave frequency, 9413 MHz; microwave power incident to the cavity, 2 mW; modulation amplitude, 1.27 mT; temperature, 45 K.

4. *P. denitrificans* membrane particles

The results of steady-state experiments with SPP showed similarities, as well as differences with the results obtained with SMP. The batches of SPP all contained some contaminating Mn^{2+} and so EPR spectra shown were corrected with a spectrum of contaminating Mn^{2+} in 1 M Mg-acetate.

4.1. The radicals

Five seconds after mixing with either NADH or succinate, the same amount of radicals were observed (non-saturating, as well as fast-relaxing; Fig. 3, inset). The total spin concentration (non-saturating signal) was 60–70% of that of the clusters 2. Piericidin completely abolished the NADH-induced radicals, but diminished the amount of succinate-induced radicals, both the intensity (non-saturating conditions) as well as the contribution of the fast-relaxing radicals, only to 40%. The fast-relaxing radicals induced by NADH disappeared as soon as the system reached anaerobiosis (observed via the $g_z=3$ line of cytochrome a in cytochrome c oxidase). However, unlike in SMP, the total intensity of the Q_I radical (slow relaxing) was stable for up to 30 s, but eventually (1 min) vanished. Gramicidin (200 μM) plus ammonium acetate (15 mM), conditions described to induce optimal rates of NADH oxidation [17], did not affect the amount of NADH-induced radicals, but only diminished the proportion of the fast-relaxing population by 20%. It has been demon-

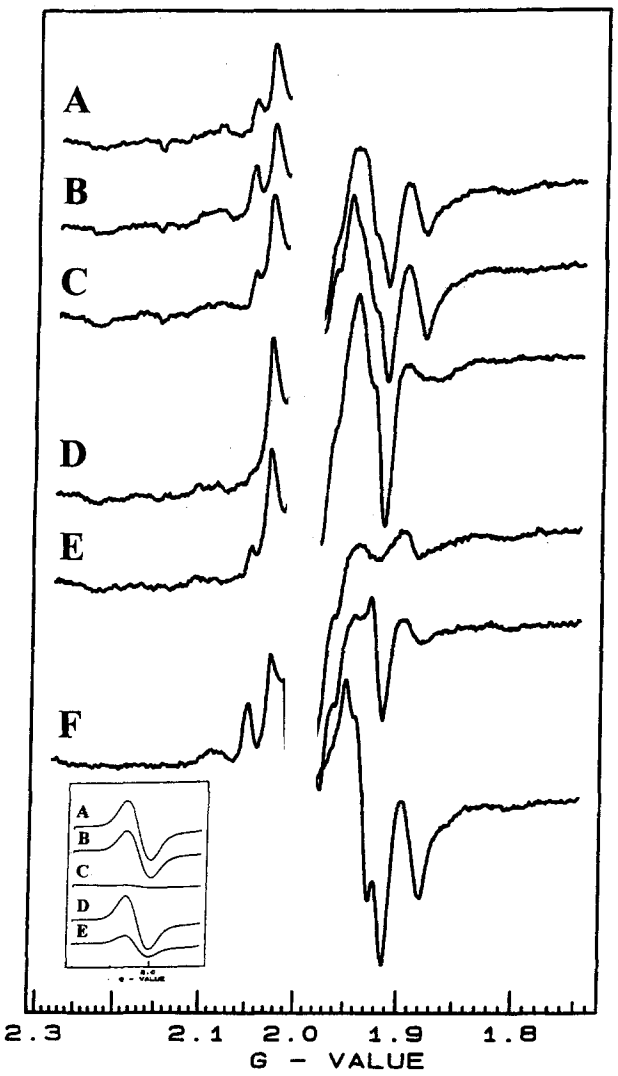

Fig. 3. Effect of energization on EPR spectra of tightly coupled membrane particles (SPP) from *P. denitrificans*. The inset shows the fast-relaxing Q_f radical. (A) SPP (20 mg/ml) in an EPR tube were flushed with oxygen, mixed with 5 mM NADH for 5 s and then shock-frozen. (B) As in (A), but the SPP were first mixed with 100 μM gramicidin, 15 mM ammonium acetate. (C) As in (A), but 20 μM piericidin was added before starting the treatment. (D) As in (A), but the SPP were mixed with 40 mM succinate for 5 s. (E) As in (D), but 20 μM piericidin were added before flushing with oxygen. (F) As in (A), but the tube was frozen after 60 s. EPR conditions: microwave frequency, 9416 MHz; microwave power incident to the cavity, 2 mW; modulation amplitude, 1.27 mT; temperature, 16 K. The contributions to the spectra of contaminating Mn^{2+} were removed by subtraction of a spectrum of contaminating Mn^{2+} in a solution of 1 M Mg-acetate, recorded under the same conditions. Inset: microwave power, 20 mW; temperature, 40 K.

strated earlier [17], that most uncouplers are unable to completely decouple the *Paracoccus* vesicles. Presently we do not understand the observed low sensitivity to gramicidin of the fast-relaxing Q_f radical.

4.2. Fe–S clusters

Although the EPR spectra from SPP were not as nice as those from SMP, similar effects of energization could be observed (Fig. 3). During NADH oxidation a broad $g=1.94$ feature was detectable, and the amplitude of the lines of the clusters 2 seemed rather small. We conclude that this is due to energy-induced broadening/splitting of these lines, just as in SMP. In the presence of gramicidin, the clusters 2 were more clearly detectable (due to sharpening) and also the reduction of complexes II and III (e.g. the line at $g=1.89$) was clearly enhanced (due to faster, uncoupled electron transfer). Trace C shows the Fe–S clusters 1b and 2 reduced by NADH in the presence of piericidin. The lack of reduction of complex III showed that the inhibitor stopped electron transfer. In trace F, no inhibitor was present and the reaction was stopped only after 1 min (anaerobic). Optimal reduction by NADH of all components of the respiratory chain was now observed.

4.3. Effect of $\Delta\mu H^+$ induced by succinate oxidation

Although succinate led to a set of radical signals just as those observed during steady-state NADH oxidation (Fig. 3, inset), no apparent reduction of Fe–S cluster of complex I was noticed. This would imply that the presence of the fast-relaxing radical is independent of the reduction of the clusters 2. In addition, virtually no reduction of the [2Fe–2S] signal of succinate dehydrogenase, and only a small reduction of the Rieske Fe–S cluster could be observed. When piericidin was present (Fig. 3, trace E), some reduction of the clusters 2 of complex I was clearly observed and there was a decrease (60%) of the amount of fast-relaxing radicals. As piericidin completely inhibited succinate-induced reversal, we presently do not understand why no EPR signals due to reduced clusters 2 showed up in the absence of this inhibitor. The reversal activity of SPP was twice as high as that of SMP (Table 1). Clearly more studies are required.

4.4. There is one cluster 1b per two clusters 2

In Fig. 4, the EPR spectrum (17 K) is shown, obtained 5 s after mixing SPP with NADH in the presence of piericidin. At 17 K, the main contributions are from the clusters 1b and 2 (the clusters 3 and 4 have quite broad signals at this temperature). Two simulations to interpret the spectrum are also shown. It is quite clear that the clusters 1b and 2 are present in a 1:2 stoichiometry, just like in mitochondrial complex I [21–24]. Spectra taken after longer times of incubation with NADH showed a larger $g=1.94$ line (see Fig. 3, trace F), and this may explain why previous estimates of the 1b/2 ratio gave larger (but erroneous) values [19]. The present analysis suggests that complex I from *Paracoccus* contains eight Fe–S clusters, just like the mitochondrial enzyme [4]. The concentration of the clusters 2 was 0.043 nmol per mg of protein.

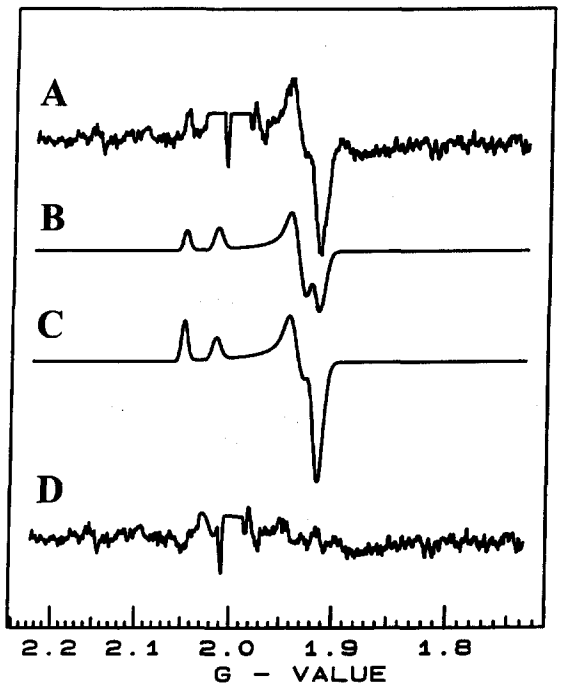

Fig. 4. EPR line shape of the clusters 1b and 2 in membrane vesicles from *P. denitrificans* (SPP), 5 s after mixing with 5 mM NADH in the presence of piericidin. (A) SPP (20 mg/ml), mixed with 20 μM piericidin, were flushed with oxygen and then mixed with NADH. From the resultant EPR spectrum a spectrum of untreated SPP was subtracted. (B) Simulation as a 1:1 (spin concentration) addition of the simulated spectra of the clusters 1b and 2, respectively. Parameters: cluster 1b (g_{xyz}=1.9280, 1.9382, 2.0158; $W(xyz)$=1.5, 1.79, 1.45 mT; weight=1); clusters 2 (g_{xyz}=1.9186, 1.1986, 2.04839; $W(xyz)$=2.1, 2.1, 1.03 mT; weight=1). (C) Simulation as a 1:2 addition of the clusters 1b and 2, respectively. (D) Difference: (A) minus (C). EPR conditions: microwave frequency, 9413 MHz; microwave power incident to the cavity, 0.2 mW; modulation amplitude, 1.27 mT; temperature, 17 K.

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