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Activation of the Anaerobic Ribonucleotide Reductase by *S*-Adenosylmethionine

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In all living organisms, deoxyribonucleotides, the precursors of DNA, are produced by reduction of the corresponding ribonucleotides. The reaction is catalyzed by an enzyme called ribonucleotide reductase (RNR), which is thus absolutely essential for growth and survival.[1] A number of facultative and strict anaerobes depend on a class III RNR, which is characterized by the presence of a catalytically essential and oxygen-sensitive glycyl radical in the active site.[2] The introduction of the radical into the RNR protein is initiated by a second protein (the activase), which is a member of the recently discovered "radical-SAM" enzyme superfamily.[3] The enzymes of the "radical-SAM" family are characterized by a (4 Fe–4 S) center, which is chelated by the three cysteines of the conserved Cys-X_3-Cys-X_2-Cys motif and serving for binding, reducing, and cleaving *S*-adenosylmethionine (SAM) into methionine and a putative 5′-deoxyadenosyl radical (Ado°).[4] It is now generally accepted that, in all these systems, a cluster–SAM complex is formed as a reaction intermediate, since such a complex has been directly observed by ENDOR spectroscopy in the cases of pyruvate-formate lyase activase (PFL) and lysine aminomutase (LAM)[5] and by X-ray crystallography in the cases of biotin synthase (BioB), coproporphyrinogen oxidase (HemN), and MoaA, an enzyme involved in the biosynthesis of the Mo cofactor.[6]

It is likely that a cluster–SAM complex is also generated in the activase of the RNR as a precursor of the Ado° radical. Glycyl radical formation implies radical transfer from one protein (activase) to the other (RNR). In this work, we investigate the question of whether this transfer occurs by direct attack of Ado° onto the glycyl residue of the RNR active site or through radical relays along a radical-transfer chain connecting the activase to the RNR. For this purpose, we used HYSCORE (Hyperfine Sublevel Correlation) spectroscopy[7] to demonstrate the intermediate formation of a cluster–SAM complex in the activase and label-transfer experiments with RNR preparations

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containing ^{2}H-labeled glycine residues. Our results are consistent with the intermediate involvement of Ado$^{\circ}$ as the direct glycine Hα atom-abstracting agent.

The addition of SAM to an anaerobic solution of the activase in the reduced state, thus with a $S=^{1}/_{2}$ $(4\,Fe\text{–}4\,S)^{+}$ cluster, resulted in a significant change of the axial EPR spectrum, as previously reported.[8] The ^{1}H ENDOR spectrum recorded in the presence of SAM at a field corresponding to the $g_{\|}$ region of the EPR spectrum exhibited a well-defined intense ^{1}H resonance line ($a_{H}=7.6$ MHz) that was absent in the spectrum of the activase without SAM (see Supporting Information). These data provided a strong indication for SAM binding to the protein. In this report, we present evidence for a cluster–SAM complex using X-band HYSCORE spectroscopy.

HYSCORE is a two-dimensional pulsed ESR technique that is used to study hyperfine coupling of nuclei with low gyromagnetic moments in orientationnally disordered systems.[7] One of the main advantages of HYSCORE is its ability to sort three types of nuclei: the strongly ($|a|/2>\nu_{n}$) and weakly ($|a|/2<\nu_{n}$) coupled ones and the "distant" nuclei, which are characterized by very low hyperfine constants. In the last case, the corresponding peaks lie on the diagonal of the (+,+) quadrant, whereas the strongly and weakly coupled nuclei appear in the (−,+) and (+,+) quadrants, respectively. There are no peaks in the (−,+) quadrant of the HYSCORE spectrum of the reduced activase in the absence of SAM (Figure 1 A). Upon addition of SAM, the HYSCORE spectrum was dramatically modified (Figure 1 B). Whereas the (+,+) quadrant was not affected, a new symmetrical set of cross features appeared in the (−,+) quadrant, whose number and positions indicated the presence of a strongly coupled ^{14}N nucleus (an $I=1$ nucleus with quadrupolar coupling). The general shapes and positions of the two features (labeled dq in Figure 1 B) are characteristic of a pair of double-quanta correlation peaks in the case of a strong hyperfine coupling constant ($|a_{N}|/2\gg\nu_{N}$). They extend along the antidiagonal up to (−9.16, +5.25) and (−5.25, +9.16) MHz; this indicates that there is a small anisotropic hyperfine contribution in addition to the main isotropic hyperfine constant. It is possible to obtain a good estimation of the latter value by using

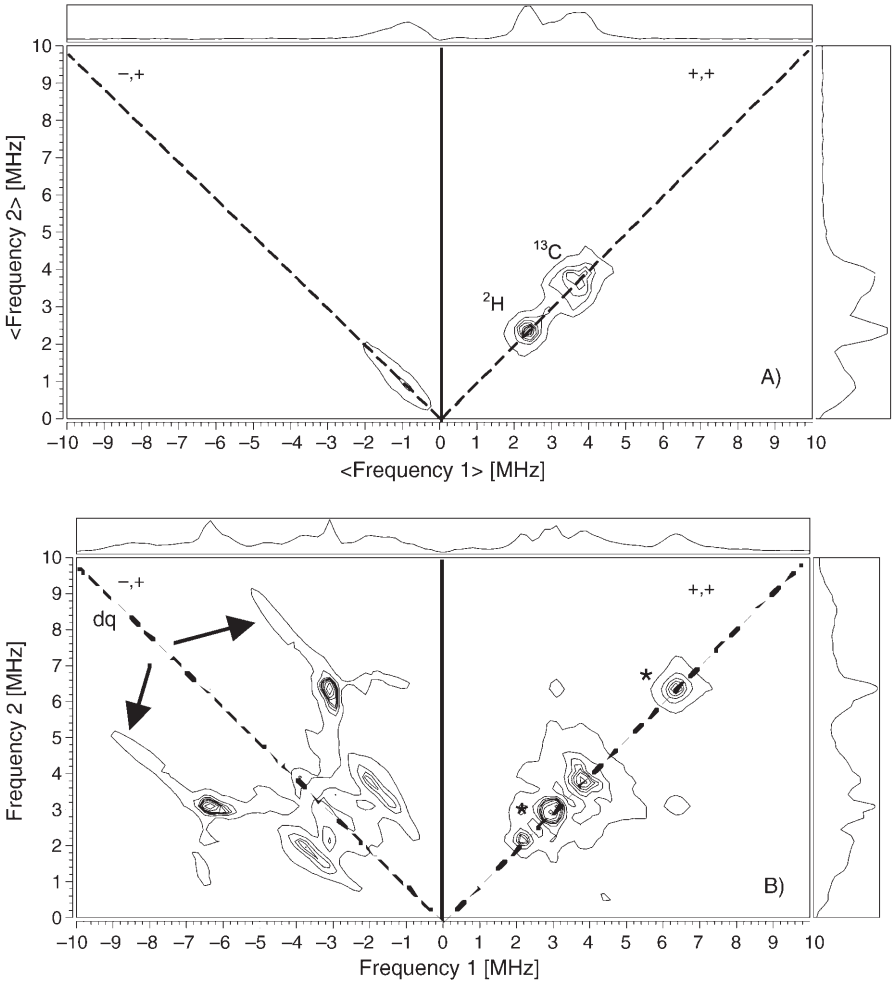


Figure 1. Low-frequency region of the X-band HYSCORE spectra of the activase (0.8 mM) reduced with sodium dithionite in the presence of DTT (5 mM) A) before and B) after addition of SAM (2 mM). In the (+,+) quadrant, spots due to distant ^{13}C and ^{2}H nuclei are indicated. In the (−,+) quadrant of (B), the peaks are characteristic of a strongly coupled ^{14}N nucleus and those labeled "dq" are assigned to a pair of a double-quanta correlation peaks. The marked features (*) in (B) are artefactual and due to nonideal pulses.

$$\nu_{dq\pm}=2\,[(\nu_{N}\pm a_{N}/2)^{2}K^{2}(3+\eta^{2})]^{1/2}$$

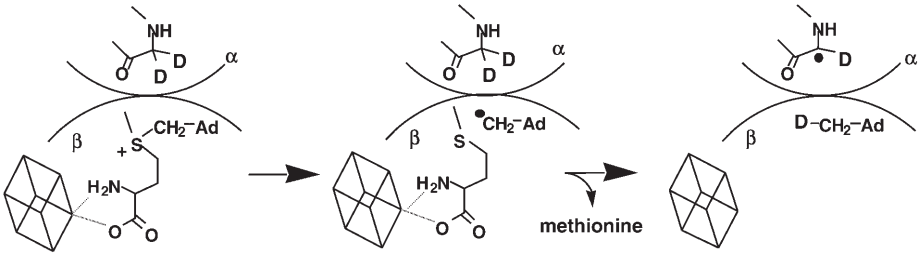
where K is the quadrupole coupling constant and η is the asymmetry parameter.[9] The value obtained ($a_{N}=6.4$ MHz) when scaled by ($\gamma_{^{15}N}/\gamma_{^{14}N}$) gives a coupling constant of 8.9 MHz, which is in the range of those obtained by ^{15}N-ENDOR spectroscopy for LAM (9.1 MHz) and PFL activase (5.8 MHz). We suggest that the amino group of SAM is coordinated to the singular Fe of the Fe–S center of the RNR activase in a manner similar to that demonstrated in related enzymes.[5] In view of these similarities, it is unlikely that the observed N atom is associated with the polypeptide chain rather than with SAM.

It was important to show that a cluster–SAM complex is also formed within a RNR–activase complex. This is not possible in this configuration due to the very short half-life of the EPR-active reduced cluster upon addition of SAM. However, the re-

duced cluster is much more stable in the presence of SAM when the glycine radical site of RNR is changed to alanine.[10] Indeed, the same HYSCORE spectrum as that shown in Figure 1 could be observed with this mutant (data not shown). We suggest that HYSCORE can be used as a simple method to check SAM binding to the cluster with any "Radical-SAM" enzyme.

Radical transfer was then studied by using RNR in which the glycine residues were replaced by deuterated 2,2′(^{2}H)$_2$-glycine and purified by standard procedures (see Supporting Information). The activase was reduced with dithionite and desalted anaerobically by Sephadex G-25 chromatography. The reduced activase (31 μM spin) was then incubated with SAM (2 mM) and the deuterated RNR (0.1 mM) under anaerobic conditions. After 8 min reaction, analysis of the mixture by EPR spectroscopy showed that the cluster was fully oxidized and that a glycyl radical was generated (13 μM spin, 42% yield). In agreement with the deuterated state of the residue, the radical appeared as a singlet signal in the EPR spectrum.[11] The 5′-deoxyadenosine produced in the reaction (35 μM, corresponding to one adenosine per oxidized cluster as expected) was isolated by HPLC and analyzed by electospray mass spectrometry for its deuterium content (see Supporting Information). It was found that 11 μM of 5′-deoxyadenosine had incorporated one deuterium atom (32%, corresponding to one AdoD per glycyl radical formed). These data are thus consistent with an Ado$^\circ$ intermediate radical and with a direct H-atom abstraction at the target glycine α-carbon by Ado$^\circ$. A similar conclusion was reached from comparable experiments in the case of PFL, another glycyl radical enzyme.[12] As shown by the formation of unlabeled AdoH (24 μM) in other systems (PFL, BioB, lipoate synthase), nonproductive cleavage of SAM also occurs. The observation that glycyl radical formation does not proceed with a 100% yield under the in vitro conditions is not surprising considering the great reactivity of Ado$^\circ$ and the complexity of the system.

The results reported here can be rationalized in terms of the mechanism for class III RNR activation shown in Scheme 1.



Scheme 1. Direct hydrogen atom abstraction by the 5′-deoxyadenosyl radical.

SAM binds to the subsite iron of the reduced cluster of the activase in complex with RNR, then electron transfer to SAM results in the formation of the 5′-deoxyadenosyl radical in close proximity to the glycine of the RNR active site so that it selectively reacts with that residue. This is an intriguing conclusion considering that: i) the 5′-deoxyadenosyl radical is produced in one protein and the glycyl radical is on a different one, ii) the 5′-deoxyadenosyl radical is highly energetic; and iii) the glycine site in RNR displays low accessibility, as shown by the three-dimensional structure of the bacteriophage T4 RNR.[2,13] A structure of the RNR–activase complex would be a major breakthrough for understanding the molecular details of this biologically critical process.

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Keywords: ENDOR spectroscopy · HYSCORE spectroscopy · radicals · ribonucleotide reductase · *S*-adenosylmethionine

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