residues to be smaller; the larger the number of sequences in a set, the extrapolated fraction being about 5 percent.
51. A computer model has been constructed that follows the divergence of mutated protein sequences under various circumstances of constraint (R. F. Doolittle and D. F. Feng, in preparation).
52. One of the earliest estimates made about the prokaryote-eukaryote divergence concluded, on the basis of a relatively small number of transfer RNA sequences, that the split occurred about twice as long ago as the divergence of plants, animals, and fungi (*6*).
53. There will be some who will remind us of alternative scenarios concerning the origin of eukaryotic organisms, and especially of the possibility that some of the sequences discussed here were actually imported by an archaebacterial symbiont destined to become the nucleus. The fusion of a eubacterial "prokaryote" and an archaebacterium has been widely discussed (*54*). Although we are skeptical of such models on other grounds, we should point out that such an occurrence would not affect our findings, except that the time we are reporting as a divergence time for eukaryotes and eubacteria would instead chronicle the alleged fusion event.
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RESEARCH ARTICLE

Thiyl Radicals in Ribonucleotide Reductases

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The ribonucleoside triphosphate reductase (RTPR) from *Lactobacillus leichmannii* catalyzes adenosylcobalamin (AdoCbl)-dependent nucleotide reduction, as well as exchange of the 5′ hydrogens of AdoCbl with solvent. A protein-based thiyl radical is proposed as an intermediate in both of these processes. In the presence of RTPR containing specifically deuterated cysteine residues, the electron paramagnetic resonance (EPR) spectrum of an intermediate in the exchange reaction and the reduction reaction, trapped by rapid freeze quench techniques, exhibits narrowed hyperfine features relative to the corresponding unlabeled RTPR. The spectrum was interpreted to represent a thiyl radical coupled to cob(II)alamin. Another proposed intermediate, 5′-deoxyadenosine, was detected by rapid acid quench techniques. Similarities in mechanism between RTPR and the *Escherichia coli* ribonucleotide reductase suggest that both enzymes require a thiyl radical for catalysis.

Although the reactivity of free radicals has often been associated with mutagenesis and molecular degradation, sophisticated methods have evolved to harness this reactivity to effect difficult reactions with remarkable selectivity. The past few years have witnessed a renaissance in the detection of protein-derived radicals that have been proposed to play essential roles in metabolism, from DNA biosynthesis and repair to prostaglandin biosynthesis and acetyl–coenzyme A production (*1*–*4*). The *Escherichia coli* ribonucleotide reductase (RNR), which has served as a prototype for these enzymes, was demonstrated, in 1977, to contain a stable tyrosyl radical that plays an essential role in the conversion of all nucleotides to deoxynucleotides (*5*). This reduction is accompanied by oxidation of two cysteines to a disulfide (Scheme 1), and additional turnovers require re-reduction of the enzyme by a reducing system such as thioredoxin (TR), thioredoxin reductase (TRR), and nicotinamide adenine dinucleotide phosphate reduced (NADPH) (Scheme 1) (*6*). Ribonucleotide reductases, despite their central role in deoxynucleotide formation in all organisms, have been shown over the past decades to contain metallo-cofactors that are structurally and chemically distinct (Fig. 1) (*7*–*9*). The reductase from *Lactobacillus leichmannii* requires adenosylcobalamin (AdoCbl) as a cofactor, which can generate cob(II)alamin and a putative 5′-deoxyadenosyl radical (5′-dA·) in a kinetically competent fashion (*10*, *11*). The reductase from *E. coli* grown under anaerobic conditions uses an iron-sulfur cluster and *S*-adenosylmethionine to generate a glycyl radical essential for nucleotide reduction (*12*), and a reductase from *Brevibacterium ammoniagenes* uses a manganese cluster to generate a putative protein radical (*13*). All of these reductases are associated with metallo-cofactors that are thought to generate, in the protein environment, an organic radical that initiates the nucleotide reduction process. However, in no case has a protein radical in a reductase system been demonstrated to disappear and reappear with a rate faster than the turnover of the enzyme (*7*).

The two reductases whose mechanisms have been examined in the greatest detail are those from *E. coli* and *L. leichmannii*. Even though each of these proteins possesses a characteristic primary and quaternary structure and a distinct metallo-cofactor, an in-depth examination of these proteins with mechanism-based inhibitors and site-directed mutants has revealed an extensive congruence in catalytic details (*7*, *8*, *14*). The role of the metallo-cofactor appeared to be even more complex than originally hypothesized, and, in 1990, the proposal was made that the function of the tyrosyl radical in the *E. coli* reductase and the putative 5′-dA· in the *L. leichmannii* reductase was to generate a thiyl radical, which initiated the nucleotide reduction process by abstraction of the 3′ hydrogen atom from the nucleotide substrate (*7*, *8*). Direct evidence in support of this proposal, however, has remained elusive.

We now describe the direct evidence for the intermediacy of a thiyl radical (C^{408}) in the nucleotide reduction process catalyzed by the *L. leichmannii* reductase. Even though there is no statistically significant sequence similarity between the *E. coli* and the *L. leichmannii* reductases (*15*), the sequence context surrounding the putative

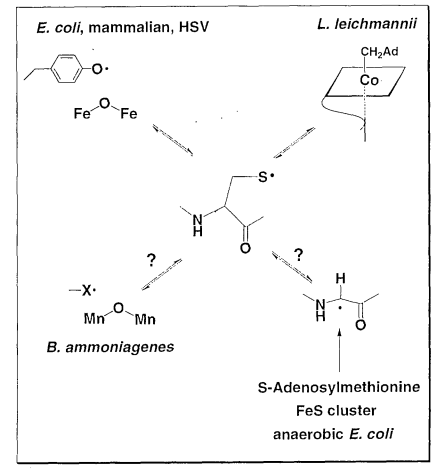

Fig. 1. Metallo-cofactors of RNRs required for the generation of the putative thiyl radical essential for the nucleotide reduction process.

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thiyl radical in the two systems is strikingly similar (*16*):

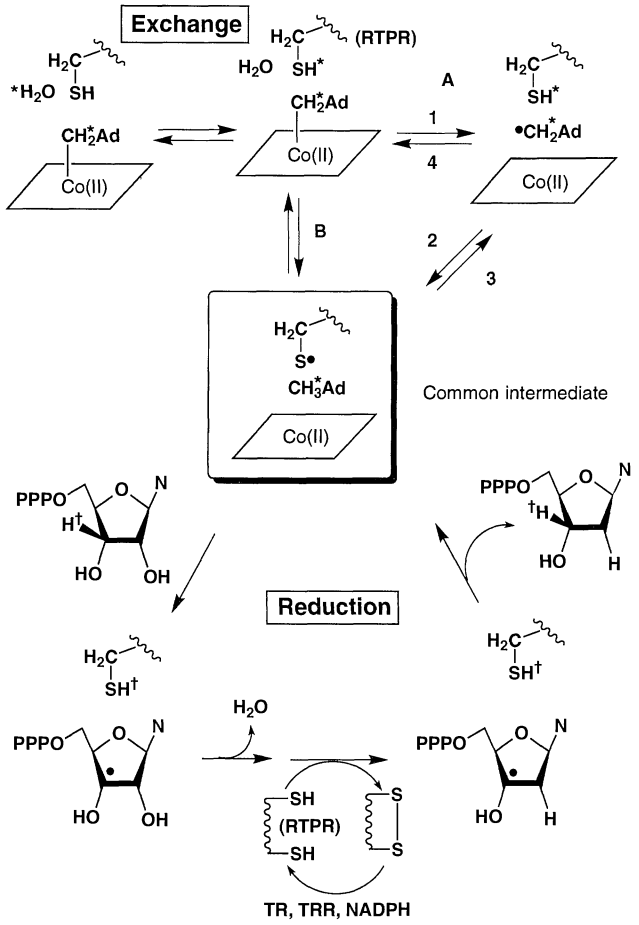
TNPC^{408}GEISLA *L. leichmannii*
SNLC^{439}LEIALP *E. coli*

Thus, our data provide strong support for the involvement of thiyl radicals in the reactions catalyzed by two classes of reductases.

Fate of the axial ligand of AdoCbl. In addition to nucleotide reduction (Scheme 1), ribonucleoside triphosphate reductase (RTPR) was shown (in the 1960s) to catalyze ^3H_2O formation when the enzyme was incubated with [5′-^{3}H]AdoCbl, an allosteric effector such as deoxyguanosine triphosphate (dGTP), and a reductant such as dihydrolipoate (*17*, *18*). However, the rate constants for this process were not determined, and the relevance of this result to the mechanism of nucleotide reduction was at that time enigmatic. On the basis of recent studies (*14*, *19*), however, tritium exchange can be formulated as indicative of the mechanism by which AdoCbl generates the thiyl radical (C^{408}) essential for both the exchange and reduction processes (Scheme 1).

The rate constant for ^3H_2O release has now been found to be 0.16 s^{-1} when dGTP was used as the allosteric effector and TR-TRR-NADPH was the reductant (*20*). This value was ~10 percent that of the turnover for nucleotide reduction, which is 1.6 s^{-1}. Given that 5′-dA (as shown below) is an intermediate in this exchange process, and assuming a selection effect of 10 on cleavage of the carbon-^{1}H bond relative to the carbon-^{3}H bond (due to mass differences) [Scheme 1, A(3) or B] and a statistical effect (5′-dA has three equivalent hydrogens), we estimate a rate constant for the exchange of the 5′ hydrogens with solvent of at least 4.8 s^{-1}. Thus, this exchange reaction is rapid, and can be used to examine protein radical formation (the first step in the nucleotide reduction process) in the absence of the later steps of nucleotide reduction.

The mechanism in Scheme 1 predicts that AdoCbl reacts with RTPR to form a thiyl radical, cob(II)alamin and 5′-dA. This hypothesis can be tested with stopped-flow ultraviolet-visible (UV-vis) spectroscopy to monitor cob(II)alamin formation (*21*), rapid chemical quench with acid to monitor the formation of 5′-dA or 5′-dA· (*22*), and rapid freeze quench (RFQ) EPR spectroscopy to monitor thiyl radical formation and cob(II)alamin formation (*23*). In previous studies, Tamao and Blakley (*10*) and Orme-Johnson *et al.* (*11*) examined the fate of cobalt in this exchange reaction using stopped-flow UV-vis and RFQ EPR spectroscopies. Their experiments and all of our exchange reaction experiments were carried out by rapidly mixing the contents of one syringe containing RTPR, reductant, and allosteric effector with the contents of a second syringe containing AdoCbl and effector at 37°C. Their early studies revealed by both spectroscopic methods that cob(II)alamin was formed with a rate constant of 35 to 50 s^{-1}. They postulated at that time that the axial ligand of AdoCbl was converted to a 5′-dA· [Scheme 1, A(1)]. We have repeated these experiments using the protein reductants TR, TRR, and NADPH in place of dihydrolipoate (used in their experiments) and obtained a similar rate constant for cob(II)alamin formation by stopped flow (42 s^{-1}) (Fig. 2) (*21*). In a parallel set of experiments, the fate of the axial ligand was established with [5′-^{3}H]AdoCbl in the second syringe and a third syringe containing 2 percent perchloric acid to rapidly quench the reaction (*22*). Only 5′-dA and H_2O were detected as tritium-labeled species, and the rate constant for their formation ([5′-^{3}H]dA + ^3H_2O) was identical to that observed for formation of cob(II)alamin (Fig. 2). Stoichiometric isolation of 5′-dA relative to cob(II)alamin supports the mechanism in Scheme 1 and provides direct evidence for the fate of the adenosyl moiety of AdoCbl during the RTPR-catalyzed reaction.



Scheme 1

RFQ-EPR: Evidence for thiyl radical formation. The models in Scheme 1 predict that rapid quenching of the reaction in liquid isopentane at −140°C could trap cob(II)alamin and a 5′-dA·, a thiyl radical, or both. Cob(II)alamin is EPR-active (*S*, the electron spin quantum number, is 1/2), and the hyperfine interaction with the cobalt nucleus (*I*, the nuclear spin quantum number, is 7/2) is evident in its spectrum (*24*). The appearance of the actual EPR spectrum of the intermediate is difficult to predict because of the possibility of coupling of the cob(II)alamin unpaired spin to one or more radical species *via* spin exchange interactions, dipolar interactions, or both. Orme-Johnson *et al.* performed a RFQ-EPR experiment in 1974 (*11*) and identified a new species identical to that shown in Fig. 3A (our data, where we used TR, TRR, and NADPH in place of dihydrolipoate used in their earlier experiments) (*23*). They observed a broad signal with an "effective" electron Zeeman splitting constant g_{eff} of 2.12 and hyperfine splitting of ~50 gauss assigned to the cobalt nuclear spin. Their expectation at the time was that, if this intermediate contained a 5′-dA· and cob(II)alamin, replacement of AdoCbl with [5′-^{2}H]AdoCbl or [5′-^{13}C]AdoCbl would result in hyperfine interactions al-

tered with respect to the [^{1}H]AdoCbl features. Use of [5′-^{2}H]AdoCbl, for example, would be expected to give narrowed features in the EPR spectrum because of the difference in gyromagnetic ratios between ^{1}H and ^{2}H, which result in a difference in hyperfine interactions (*25*). They reported that these isotopic substitutions had no effect on the EPR spectrum of their observed intermediate. The results of a similar experiment that we carried out using [5′-^{2}H]AdoCbl (*26*) also indicate no apparent difference from those shown in Fig. 3A. Thus, in contrast to expectations in 1974, a 5′-dA· does not appear to be a component of the observed EPR signal.

Alternatively, the models in Scheme 1 predict that cob(II)alamin could be interacting with a thiyl radical rather than a 5′-dA·. To test this hypothesis, RTPR was grown under conditions that allow incorporation of β-(^{2}H)cysteine (*15, 27–29*). The expectation is that this substitution would narrow the features of the EPR signal as described above for the [5′-^{2}H]AdoCbl experiments (Fig. 3B). The sharpening of the features associated with the cobalt hyperfine structure relative to Fig. 3A is readily apparent. The effect of ^{2}H-labeling of cysteine on the cobalt hyperfine features defines unambiguously that a cysteine is adjacent to and interacting with cob(II)alamin. These results, in conjunction with the stopped flow and acid quench experiments described above, support the proposal (Scheme 1) that the function of the AdoCbl is to generate 5′-dA, cob(II)alamin, and a thiyl radical (*30*).

The model predicts that the intermediate observed by EPR spectroscopy should be produced with the same rate constant as cob(II)alamin observed by stopped-flow and should contain two spins per equivalent of cob(II)alamin. The half-time for formation of the EPR signal under exchange conditions is 10 to 20 ms, consistent with the rate constant of 49 s^{-1} ($t_{1/2}$ = 14 ms) observed by stopped-flow UV-vis spectroscopy under identical conditions. Stopped-flow experiments also reveal formation of 48 μM cob(II)alamin (0.18 equivalent per equivalent of RTPR). The amount of spin formed has been quantitated by comparing the RFQ EPR samples to a $CuSO_4$ standard at 100 K (*31, 32*). If the paramagnetic intermediate is assumed to have $S = 1/2$, the average spin concentration in the steady state is 80 ± 20 μM, a value consistent with the formation of two equivalents of spin per equivalent of cob(II)alamin, as was reported by Orme-Johnson *et al.* (*11*).

However, it is possible that the intermediate species comprises two spins that are coupled strongly enough to be described as a triplet. The spin state of the intermediate was further investigated; the temperature dependence of its EPR signal intensity was determined by recording spectra from 4 to 99 K, with the use of a non-saturating power at each temperature, and with the intensities being normalized for power as necessary. As a control, a similar temperature dependence was determined for the cob(II)alamin-(5′-dA)-RTPR complex (*24*). The EPR signal intensity of this complex follows the Curie-Weiss law from 4 K to 99 K (*25*). In contrast, the signal intensity of the intermediate generated from quenching deviates from Curie-Weiss law behavior below 10 K; the intensity at 5.4 K is 53 ± 6 percent of the intensity

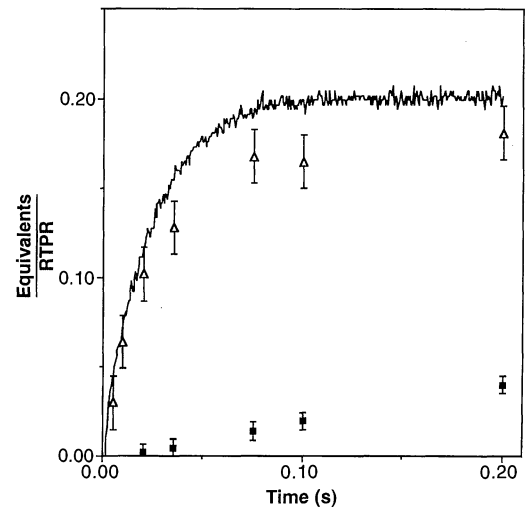


Fig. 2. Formation of cob(II)alamin and 5′-dA by RTPR. The reaction mixture consisted of 100 μM RTPR, 20 μM TR, 1 μM TRR, 2 mM NADPH, and 1 mM dGTP in 200 mM sodium dimethylglutarate, pH 7.3, along with an equal volume of the same reaction buffer containing 50 μM AdoCbl and 1 mM dGTP; the formation of cob(II)alamin was measured by monitoring change of A_{525} at 37°C. The solid trace shows the equivalents of cob(II)alamin formed, calculated directly from the stopped-flow absorbance trace and the difference in extinction coefficients between AdoCbl and cob(II)alamin (4800 M^{-1} cm^{-1}) (*21*). A point-by-point spectrum assembled from traces at different wavelengths showed the expected isosbestic point at 490 nm. The open triangles represent equivalents of 5′-dA formed, and the closed squares represent equivalents of ^3H_2O as measured after a rapid chemical quench. For this reaction 100 μM RTPR, 20 μM TR, 1 μM TRR, 2.6 mM NADPH, 1 mM dGTP, and 100 mM sodium dimethylglutarate, pH 7.3, were rapidly mixed at 37°C with an equal volume of [5′-^{3}H]AdoCbl (100 μM, 1.2 × 10^6 cpm/μmol) in the reaction buffer. After the specified time, the reaction was quenched with 2 percent perchloric acid (60 to 220 μl), collected in tubes containing 5′-dA (55 nmol) and 5′,8-cycloadenosine (15 nmol), neutralized with equal volumes of 0.4 M KOH and 0.5 M sodium dimethylglutarate, pH 7.3 (50 to 200 μl each), and rapidly frozen. For analysis, each sample was thawed and loaded onto a Sep-Pak Classic (Millipore); ^3H_2O was eluted with H_2O and analyzed by scintillation counting. The nucleosides were eluted with a mixture of methanol and water (1:1 CH_3OH/H_2O), and the solvent was removed by lyophilization. Each lyophilized mixture was dissolved in 450 μl of H_2O and analyzed by reversed phase–C_{18} HPLC, with a linear gradient. Solvent A was H_2O, and solvent B was CH_3OH: 0 to 5 min, 0 to 20 percent B; 5 to 24 min, 20 percent B; 24 to 34 min, 20 to 100 percent B; 34 to 40 min, 100 percent B. The flow rate was 1 ml/min, and the elution profile was monitored by absorbance at 260 nm. The fractions eluting at 19 and 21 min contained 5′, 8-cycloadenosine and 5′-dA, which were quantitated by UV-vis spectroscopy (ε_{264} = 15,100 M^{-1} cm^{-1} and ε_{260} = 15,200 M^{-1} cm^{-1}, respectively) and scintillation counting (8.5 ml of Scint-A per milliliter of eluate).

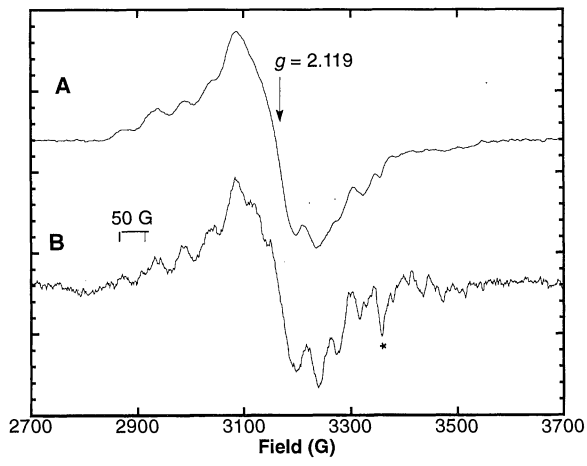


Fig. 3. Effect of deuteration of the cysteines of RTPR on the EPR signal of the intermediate. (**A**) The EPR spectrum that resulted when RTPR (525 μM) and 50 μM TR, 3 μM TRR, 1.7 mM NADPH, 1 mM dGTP in 100 mM sodium dimethylglutarate, pH 7.3, were mixed with an equal volume of 400 μM AdoCbl and 1 mM dGTP in the reaction buffer, and the reactions were quenched in isopentane (−140°C) at 175 ms (*23*). (**B**) is the spectrum observed in the presence of [β-$^2H_2$-cysteine]RTPR (250 μM) and AdoCbl (300 μM). This labeled RTPR was prepared with *E. coli* JM105 containing plasmid pSQUIRE (*15, 28*). The EPR spectra were recorded at 100 K with spectrometer frequency 9.41 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 4 gauss; time constant, 1.3 s; and scan time, 671 s. In each case, 10 scans were recorded. The feature at g = 2 (*) was observed in variable amounts in both unlabeled and isotopically labeled samples. Although thawing of the sample resulted in disappearance of the intermediate species, there was a g = 2 signal that remained after thawing and re-freezing of the sample, suggesting that this feature is unrelated to the spectrum of the intermediate.

predicted by the Curie-Weiss law. These data are consistent with a model in which the intermediate is composed of two exchange-coupled spins, with a singlet ground state and a thermally accessible triplet excited state. At low temperatures, the triplet state is depopulated, and the EPR signal intensity decreases (*25*).

A rigorous analysis of the temperature dependence of the signal intensity is difficult given the possibility that the singlet-triplet splitting is of the order of the microwave frequency. An approximate treatment (*25*) suggests that the magnitude of the exchange coupling (J) is of the order of 1 cm^{-1}. However, uncertainty in the temperature of the helium-cooled sample prevents accurate measurement of this value by this method.

We have interpreted these data as indicating that a thiyl radical is adjacent to cob(II)alamin, a contention that is consistent with the [^{2}H]cysteine labeling experiments, the kinetics of formation of the new signal, and the spin quantitation and its temperature dependence. Preliminary spectral simulations incorporating dipolar coupling (60×10^{-4} to 600×10^{-4} cm^{-1}) and exchange coupling ($|J_{ex}| > 0.3$ cm^{-1}) have yielded a reasonable fit to the data for both unlabeled and [^{2}H]cysteine-labeled RTPR (*33*).

RFQ-EPR: Evidence for thiyl radical formation in the presence of adenosine triphosphate (ATP). A similar set of experiments was performed under conditions identical to those above with the exception that ATP (1 mM) was also added to both syringes. The kinetics in the presence of substrate are much faster than those observed for the exchange reaction. Stopped-flow experiments show that cob(II)alamin is formed with a $t_{1/2}$ of less than 3 ms. RFQ-EPR experiments were carried out and spectra were obtained from quenching the reaction at 20 ms. The spectrum in Fig. 4A is qualitatively similar to that obtained under the conditions of the exchange reaction although differences, particularly in the high field region around $g = 2$, are evident. Furthermore, incorporation of [β-^{2}H]cysteine into RTPR (*34*) (Fig. 4B) gives rise to a dramatic sharpening of the spectrum, indicating that the paramagnetic intermediate formed under turnover conditions also includes a thiyl radical interacting with cob(II)alamin. These results support the proposal (Scheme 1) that the function of the AdoCbl is to generate 5′-dA, cob(II)alamin, and a thiyl radical.

A thiyl radical intermediate in the exchange and turnover reactions. A model for RTPR-catalyzed transfer of ^{3}H from [5′-^{3}H]AdoCbl to solvent, in which the thiyl radical of C^{408} is generated as an intermediate in this process by AdoCbl, is presented in Scheme 1. Rapid kinetics experiments have provided evidence in support of this model and, as outlined below, for a similar sequence of events in the reduction process. In addition, direct evidence for the fate of the axial ligand of AdoCbl has now been obtained.

Using [5′-^{3}H]AdoCbl and rapid acid quench technology, we examined the fate of the axial ligand from 10 to 150 ms. All of the ^{3}H label was found in either 5′-dA or in H_2O. No other products such as 5′, 8-cycloadenosine, proposed as an intermediate in the exchange process by Hogenkamp and co-workers (*35*) were observed. These results support the proposed mechanisms for the exchange reaction and allow us to favor a concerted pathway (Scheme 1, B).

Additional evidence in favor of these exchange models was obtained with RFQ-EPR technology. Specific deuteration of the β hydrogens of the cysteines in RTPR resulted in sharpening of the features associated with the cobalt hyperfine structure of the observed intermediate (*27*). Analogous experiments with [5′-^{2}H]AdoCbl had no effect on the EPR spectrum. This signal was produced with a rate constant similar to that observed by stopped-flow UV-vis spectroscopy [monitoring cob(II)alamin formation], and spin quantitation and temperature dependence of the observed signal suggest production of two coupled spins for each equivalent of cob(II)alamin. These two results are consistent with formation of cob(II)alamin and a thiyl radical as an interacting spin system. This spin-spin interaction requires further investigation with additional biophysical methods and further spectral simulations (*33*). However, the data presented here provide direct evidence that the cofactor generates a protein-based thiyl radical with a rate constant sufficiently large for the radical to be an intermediate in both the exchange and the reduction processes.

A key experiment of Ashley *et al.* (*19*) provides the link between the exchange reaction and the reduction reaction. They incubated [3′-^{3}H]NTP (N is adenosine or uridine 5′-triphosphate) with RTPR and examined the fate of the ^{3}H under single turnover conditions; that is, conditions in which there was no external reductant, and hence only a single NTP was converted to dNTP. They showed that under such conditions no ^{3}H was transferred from the substrate to either AdoCbl or H_2O, even though the 3′ carbon-hydrogen bond of the [3′-^{3}H]NTP was cleaved. If 5′-dA· were the hydrogen atom abstractor from the substrate, then $^{-3}$H should have been detected in AdoCbl under these conditions. These studies required that the function of AdoCbl be the generation of a protein radical that initiates the nucleotide reduction process by 3′-hydrogen atom abstraction (Scheme 1). The observation that the hydrogen atom abstracted from the 3′ position is returned quantitatively to the same position in the deoxynucleotide product requires that this protein residue be monoprotic. RFQ experiments now furnish direct spectroscopic evidence for the link between the exchange process and nucleotide reduction. Furthermore, EPR analysis of a freeze-quenched reaction mixture containing substrate (ATP) revealed the signal we interpret as a thiyl radical coupled to cob(II)alamin, the common intermediate in both the exchange reaction and turnover (Fig. 4).

Recent site-directed mutagenesis studies show that C^{408} is necessary for nucleotide reduction and thus support the assignment of C^{408} as the monoprotic protein residue that reacts to form a protein radical (*14*). Mutagenesis also demonstrates that C^{408} is necessary for the exchange reaction. On the

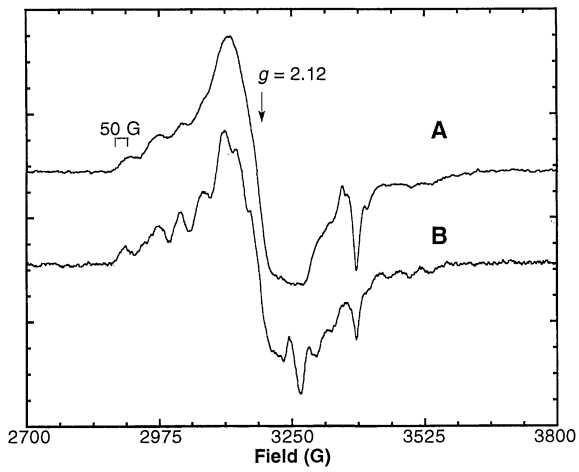


Fig. 4. Effect of deuteration of the cysteines of RTPR on the EPR signal of the intermediate generated in the presence of substrate. Reaction and quenching conditions were identical to those described in Fig. 3A except that ATP (1 mM) was present in both syringes, and the reaction was quenched at 20 ms (*23*). (**A**) Spectrum observed when unlabeled RTPR was used. (**B**) Spectrum observed in the presence of [β-$^2H_2$-cysteine]RTPR. In this experiment, [β-$^2H_2$]cysteine was incorporated into RTPR by transforming the *E. coli* cysteine auxotroph JM15 with plasmid pSQUIRE, growing the bacteria on minimal media supplemented with amino acids, including the labeled cysteine (*34*), and inducing expression by addition of isopropylthiogalactoside. The EPR spectra were recorded at 6 K with spectrometer frequency 9.47 GHz; microwave power, 1 mW; modulation frequency, 100 kHz; modulation amplitude, 4 gauss; time constant, 1.3 s; and scan time, 671 s. In each case, eight scans were recorded.

basis of the remarkable similarity between the *L. leichmannii* and *E. coli* reductases with respect to the nucleotide reduction process, reaction with mechanism-based inhibitors, and phenotypes of site-directed cysteine to serine mutants (*7–9, 14*), we propose that a thiyl radical also plays an essential role in the *E. coli* reductase.

This contention is supported by analysis of the three-dimensional structure of the subunit of the *E. coli* RNR on which the reduction process occurs (*36*). Modeling shows that C^{439}, the sequence equivalent of C^{408} in the *L. leichmannii* RNR (Scheme 1), is poised to remove the 3′-hydrogen atom from the nucleotide substrate. Thus, the data are consistent with the proposal that the function of the cofactors (Fig. 1) in both reductases is to generate a thiyl radical which initiates the reduction process.

While amino acid radicals, including those generated from tyrosines, tryptophans, glycines, and cysteines, have been detected directly or indirectly in enzymes that play central roles in metabolism, only the tyrosyl radicals (*37, 38*) have been shown to be oxidized and reduced in a kinetically competent fashion. The observation of a kinetically competent thiyl radical intermediate is therefore a landmark in our efforts over the past decade to demonstrate the feasibility and the importance of protein radical involvement in catalysis, and in particular the importance of thiyl radicals.

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16. Abbreviations for the amino acid residues are: A, Ala; C, Cys; E, Glu; G, Gly; I, Ile; L, Leu; N, Asn; P, Pro; S, Ser; and T, Thr.
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20. A typical assay contained (in a final volume of 305 μl) 50 mM potassium phosphate (pH 7.5), 300 μM dGTP, 4 mM EDTA, 50 to 300 nM RTPR, 50 to 200 μM [5′-^{3}H]AdoCbl (7 × 10^5 to 100 × 10^5 cpm/μmol), 0.2 mM NADPH, 65 μM TR, 0.5 μM TRR. These reaction mixtures were incubated in dim light at 37°C. For the detailed kinetics of the exchange reaction and its requirements, see S. Booker, thesis, Massachusetts Institute of Technology (1994).
21. An Applied Photophysics DX.17MV spectrophotometer was used for the stopped-flow spectroscopy. The loading and drive syringes were filled with 50 mM dithionite, septum-sealed, and left overnight. The sample lines were then flushed with 20 ml of 0.2 M sodium dimethylglutarate (pH 7.3), which had been deoxygenated by bubbling argon through it for 3 hours. The syringes and lines were maintained at 37°C in a circulating bath. Argon was bubbled through the bath for 3 hours preceding the acquisition of data and through the entire course of the experiments. All reactants were deoxygenated by stirring under an argon atmosphere for 20 minutes. The samples consisted of 100 μM RTPR, 20 μM TR, 1 μM TRR, 2 mM NADPH, and 1 mM dGTP in 200 mM sodium dimethylglutarate, pH 7.3, which was mixed with an equal volume of the same reaction buffer containing 100 μM AdoCbl and 1 mM dGTP. The formation of cob(II)alamin was measured by monitoring change in A_{525} at 37°C.
22. A Kin-Tek Instruments model RQF-3 was used.
23. An Update Instruments System 1000 was used. The RAM velocity was 1 cm/sec. The EPR spectra were recorded on a Bruker ESP-300. The cooling system consisted of the quartz sample holder and temperature controller from a Bruker VT 1000 system connected to a transfer line delivering N_2 (at 25 liter/min) through a copper heat exchanger maintained at 77 K with liquid N_2. The samples were maintained at 100 K. For recording at temperatures below 100 K, an Oxford Instruments ESR 900 continuous-flow liquid helium cryostat was used.
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26. [5′-^{2}H]AdoCbl was prepared by the procedure of Hogenkamp *et al.* (*18*) and characterized by electrospray mass spectrometry. The isotopic enrichment was >95 percent.
27. The incorporation of [β-$^2H_2$]cysteine into RTPR was carried out by the procedure of Hibler *et al.* (*28*). Gas chromatography–mass spectrometry analysis of the iodoacetamide-modified cysteine derivatized with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (*29*) revealed that 60 percent of the cysteine in RTPR was dideuterated and the remaining cysteine was diprotonated.
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30. RFQ methods could result in detection of a species that is an artifact of the quenching process. In fact, it has been suggested that the spectral narrowing observed on incorporation of [β-^{2}H]cysteine (Figs. 3B and 4B) could also indicate a thiolate ligated to cob(II)alamin. Two experiments have shown this not to be the case. (i) If this formulation were correct, an additional EPR-active species should be detected, because an amino acid residue would have to be oxidized in order to reduce a thiyl radical to a thiolate. No such species was observed. (ii) If the signal was due to a kinetically competent intermediate, then warming the sample under anaerobic conditions should result in re-formation of the carbon-cobalt bond, generating AdoCbl. However, if the observed signal is the result of a quenching artifact, then cob(II)alamin, which is stable under anaerobic conditions, should still be present and readily detected by EPR spectroscopy. When this experiment was performed, the spectrum revealed no cob(II)alamin.
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