activators (as nucleophiles) of the HRP-catalyzed peroxidation of dianisidine, offers a second line of support for the existence of a dianisidine radical moiety in the HRP–dianisidine complex.

Acknowledgments

One of the authors (A.C.) thanks Doug Malinowski and Darrell McCaslin for helpful discussions during the course of this work.

References

Araiso, T., Miyoshi, K., & Yamazaki, I. (1976) *Biochemistry 15*, 3059–3063.
Bender, M. L., & Turnquest, B. W. (1957) *J. Am. Chem. Soc. 79*, 1652–1655.
Brill, A. S. (1966) *Compr. Biochem. 14*, 447–479.
Brown, H. C., & Kanner, B. (1953) *J. Am. Chem. Soc. 75*, 3865.
Bruice, T. C., & Benkovic, S. (1966) *Bioorganic Mechanisms*, Vol. 1, pp 27–46, W. A. Benjamin, New York.
Chance, B. (1949) *Arch. Biochem. Biophys. 22*, 224–252.
Chance, B. (1952a) *Arch. Biochem. Biophys. 41*, 404–415.
Chance, B. (1952b) *Arch. Biochem. Biophys. 41*, 416–424.
Chance, B., & Fergusson, R. R. (1954) in *The Mechanism of Enzyme Action* (McElroy, W. D., & Glass, B., Eds.) pp 389–398, Johns Hopkins Press, Baltimore, MD.
Claiborne, A., & Fridovich, I. (1979) *Biochemistry 18* (preceding paper in this issue).
Cowgill, R. W., & Clark, W. M. (1952) *J. Biol. Chem. 198*, 33–61.
Dolphin, D., Forman, A., Borg, D. C., Fajer, J., & Felton, R. H. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 614–618.
Edwards, J. O. (1954) *J. Am. Chem. Soc. 76*, 1540–1547.
Fridovich, I. (1963) *J. Biol. Chem. 238*, 3921–3927.
George, P. (1952) *Nature (London) 169*, 612–613.
Hildebrandt, A. G., & Roots, I. (1975) *Arch. Biochem. Biophys. 171*, 385–397.
Jencks, W. P., & Carriuolo, J. (1960) *J. Am. Chem. Soc. 82*, 1778–1786.
Lumry, R., Smith, E. L., & Glantz, R. R. (1951) *J. Am. Chem. Soc. 73*, 4330–4340.
Lund, H. (1957) *Acta Chem. Scand. 11*, 1323–1330.
Maehly, A. C. (1955) *Methods Enzymol. 2*, 801–813.
Manning, G., Parker, V. D., & Adams, R. N. (1969) *J. Am. Chem. Soc. 91*, 4584–4585.
Møller, K. M., & Ottolenghi, P. (1966) *C. R. Trav. Lab. Carlsberg 35*, 369–389.
Piette, L. H., Ludwig, P., & Adams, R. N. (1962) *Anal. Chem. 34*, 916–921.
Roman, R., & Dunford, H. B. (1972) *Biochemistry 11*, 2076–2082.
Roman, R., & Dunford, H. B. (1973) *Can. J. Chem. 51*, 588–596.
Staab, H. A. (1957) *Ber. 90*, 1320–1325.
Talbot, N. B., Wolfe, J. K., MacLachlan, E. A., Karush, F., & Butler, A. M. (1940) *J. Biol. Chem. 134*, 319–330.

Mechanism of *Lactobacillus leichmannii* Ribonucleotide Reductase Studied with *Coα*-[*α*-(Aden-9-yl)]-*Coβ*-adenosylcobamide (Pseudocoenzyme B_{12}) as Coenzyme†

Raymond L. Blakley,* William H. Orme-Johnson, and John M. Bozdech

ABSTRACT: *Coα*-[*α*-(Aden-9-yl)]-*Coβ*-adenosylcobamide (pseudocoenzyme B_{12}) purified from *Clostridium tetanomorphum* has been reacted with ribonucleotide reductase purified from *Lactobacillus leichmannii* under various conditions, and the properties of the products obtained have been compared by electron paramagnetic resonance (EPR) with those previously reported for products formed from the normal coenzyme (adenosylcobalamin). The rapidly formed intermediate and the slowly formed "doublet" species from the pseudocoenzyme have EPR spectra identical with those formed from the normal coenzyme. This and other considerations make it less likely that the unusual magnetic properties of the rapidly formed intermediate are due to strongly distorted octahedral symmetry about Co(II) as previously postulated. Instead it is probable that the EPR spectrum is due to interaction of the radical pair by both exchange coupling and magnetic dipole–dipole coupling. Although *Coα*-[*α*-(aden-9-yl)]cob(II)amide in solution does not show superhyperfine splitting in the EPR spectrum because of its base-off configuration, the cob(II)amide formed by degradation of the pseudocoenzyme within the catalytic site of the enzyme did show triplets due to a nitrogen axially coordinated to cobalt. This suggests that binding of the cob(II)amide to the reductase catalytic site causes a shift to the base-on form.

The ribonucleoside-triphosphate reductase of *Lactobacillus leichmannii* (EC 1.17.4.2) differs from the reductase in *Escherichia coli* and in cells of vertebrate tissues in several important respects (Blakley, 1978; Hogenkamp & Sando, 1974; Follmann, 1974). Inter alia the *Lactobacillus* enzyme requires adenosylcobalamin as coenzyme and has no metal component or other requirement, whereas the *E. coli* and vertebrate enzymes contain nonheme iron and have no B_{12} requirement. Nevertheless, evidence is accumulating that these two classes of ribonucleotide reductase share some important features in common since both appear to catalyze reactions involving a radical mechanism.

In the presence of dGTP or other allosteric activators (which

† From the Department of Biochemistry, College of Medicine, University of Iowa, Iowa City, Iowa 52242, and the Department of Biochemistry, College of Agricultural and Life Science, University of Wisconsin, Madison, Wisconsin 53706. *Received November 17, 1978.* The research was supported in part by U.S. Public Health Service Research Grants CA11165 from the National Cancer Institute, National Institutes of Health, and GM17170 from the National Institute of General Medical Sciences, National Institutes of Health. R.L.B. gratefully acknowledges support by a John Simon Guggenheim Memorial Foundation Fellowship during the period in which most of this work was carried out.

0006-2960/79/0418-2335$01.00/0

promote coenzyme binding: Singh et al., 1977) and a reducing substrate (e.g., dihydrolipoate) the *Lactobacillus* enzyme rapidly cleaves the cobalt–carbon bond in the coenzyme to generate a relatively stable radical pair presumably consisting of cob(II)alamin and 5′-deoxyadenos-5′-yl (Tamao & Blakley, 1973; Orme-Johnson et al., 1974). This process, which has a half-time of about 17 ms at 37 °C, is readily reversible by dropping the temperature to 5 °C. Addition of a ribonucleotide substrate (e.g., ATP) to the radical pair at 37 °C causes a rapid, large decrease in the concentration of the radical pair. Since the rate of formation of the radical pair and of its disappearance in the presence of ribonucleotide indicates kinetic competence, we consider that this stabilized radical pair is a true intermediate in ribonucleotide reduction. As further evidence of this, only analogues of adenosylcobalamin that generate detectable amounts of the radical pair show significant coenzyme activity (Sando et al., 1975).

Recently reported data indicate that the *E. coli* reductase also has a radical mechanism of action. The isolated B2 subunit contains a stable, free organic radical which shows a typical electron paramagnetic resonance (EPR) spectrum (Sjöberg et al., 1977). The radical is dependent on the presence of iron in the enzyme and can be destroyed selectively (e.g., by hydroxyurea) with concomitant loss in activity. Different preparations of the pure protein may contain different amounts of radical, and the specific activity of the enzyme is then directly related to the amount of radical present. Thus the catalytic function of B2 is completely dependent on the radical. Isotope substitution experiments localized the radical to a tyrosine residue of the polypeptide chain, and the evidence now suggests that the unpaired electron is localized on the benzene ring of this side chain (Reichard, 1978).

Although the way in which radicals participate in either reaction is still unclear, radical formation nevertheless seems to be essential, and the structure of the radical pair formed by the *Lactobacillus* reductase is therefore of considerable interest. The EPR spectrum of this intermediate indicates that is has unusual magnetic properties, which we originally interpreted (Coffman, et al., 1976) as being due to strongly distorted sixfold coordination about the Co(II). If this interpretation of the magnetic characteristics of the radical pair is correct, then the corresponding radical pair formed from *Coα*-[α-(aden-9-yl)]-*Coβ*-adenosylcobamide (i.e., pseudocoenzyme B_{12}) should have different magnetic properties, since the adenine base in the nucleotide portion of the pseudocoenzyme, unlike dimethylbenzimidazole in the normal coenzyme, does not coordinate to cobalt.

We have, therefore, examined the various paramagnetic species formed from pseudocoenzyme B_{12} in the ribonucleotide reductase system. We also report some other properties of this coenzyme analogue.

Experimental Procedure

Materials. Pseudocoenzyme B_{12} was purified from approximately 8 lb of cell paste of *Clostridium tetanomorphum* (ATCC 15920) harvested from a 1000-L fermentation which was carried out by Grain Processing, Muscatine, IA, according to the procedure of Barker et al. (1960). The coenzyme analogue was isolated from the cells by the general procedure of Barker et al. (1960), with only slight modifications. Instead of the second phenol extraction, the combined fractions from the chromatography on Dowex 50 at pH 3 in step 5 were concentrated by rotary evaporation under reduced pressure in dim light, and the concentrate was passed through a Bio-Gel P2 column which had been equilibrated with 0.05 M NH_4OH.

Table I: Absorption Maxima for Adeninylcobamides

compd	wavelength max (nm)	mM extinction coeff
adeninyl-*Coβ*-adenosylcobamide	264	43.4
	303	21.8
	375	9.12
	457	8.57
adeninyl-*Coβ*-hydroxocobamide	262	35.6
	356	23.8
	412	4.16
	(512)[a]	(7.84)
	534	7.93
adeninylcob(II)amide	313	25.1
	400	7.08
	469	9.45

[a] Shoulder.

Approximately 5 μmol of pseudocoenzyme B_{12} was loaded on a 1.0 × 150 cm column and eluted with 0.05 M NH_4OH. The fractions containing the main cobamide peak were combined and the concentration of pseudocoenzyme B_{12} was calculated from the absorbance and $\epsilon_{458} = 8.75 \times 10^3$ (Barker et al., 1960). A measured volume of the solution was then evaporated to dryness and the residue dissolved in water to give a 1.0 mM solution. The total yield of purified pseudocoenzyme B_{12} was 20 μmol.

Ribonucleotide reductase was purified from *L. leichmannii* by the previously used procedure (Blakley, 1978). In most experiments, the enzyme was from the final affinity chromatography step and had a specific activity of 2.3–2.5 international units/mg.

Other materials were as previously used (Orme-Johnson et al., 1974).

EPR Spectral Measurements. Rapid reaction with freeze-quenching followed by EPR recording from the packed snow was carried out as previously described (Orme-Johnson et al., 1974). Mixtures of enzyme, pseudocoenzyme, dihydrolipoate, dGTP, and buffer were incubated at 37 °C for 12.5–157 ms before freeze-quenching. Recording conditions for EPR spectroscopy were as follows: microwave frequency, 9.113 GHz; time constant, 0.3 s; microwave power, 1.0 mW; modulation amplitude, 10 G; modulation frequency, 100 KHz; scan rate, 1000 G min^{-1}; temperature, 13 K.

In the case of photolyzed pseudocoenzyme, of the enzyme-degraded coenzyme analogue, and of the doublet spectrum derived from the coenzyme analogue, spectra were recorded at 77 K after freezing the sample in the EPR tube by immersion in liquid nitrogen. Recording was performed on a Varian V-4500 spectrometer under the following conditions: microwave frequency, 9.093 GHz; time constant, 1 s; microwave power, 25 mW; modulation amplitude, 5 G; modulation frequency, 100 kHz; scan rate, 250 G min^{-1}.

Photolysis of Adenosylcobamides. Reaction mixtures were photolysed under nitrogen at 5 cm from a 150-W tungsten lamp for the periods of time indicated.

Spectrophotometric Measurements. These were performed on a Cary 14 recording spectrophotometer.

Results

Absorption Spectra. Figure 1 shows the spectra of pseudocoenzyme B_{12}, the corresponding hydroxo derivative, *Coα*-[α-(aden-9-yl)]-*Coβ*-hydroxocobamide, and the reduced derivative, adeninylcob(II)amide. Table I gives the wavelength maxima and extinction coefficients for the three compounds. It should be noted that the adenosylcobamide and the cob(II)amide have very similar spectra so that the enzymatic

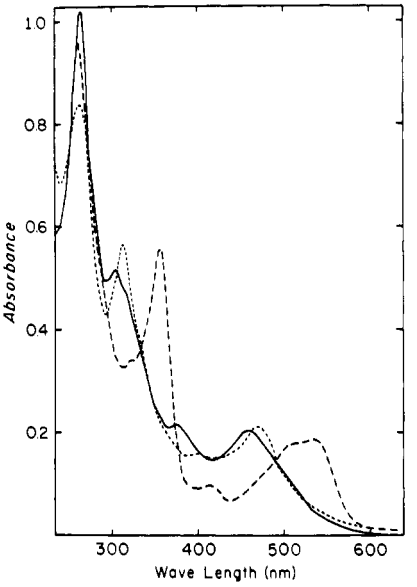

FIGURE 1: Spectra of pseudovitamin B_{12} derivatives. The cobamides were dissolved in 0.167 M sodium dimethylglutarate buffer, pH 7.3, and the concentration, as calculated from the molar extinction coefficients for the pseudocoenzyme of Barker et al. (1960), was 0.27 mM. Spectra are (—) *Coα*-[α-(aden-9-yl)]-*Coβ*-adenosylcobamide; (– – –) *Coα*-[α-(aden-9-yl)]-*Coβ*-hydroxocobamide; (- - -) *Coα*-[α-(aden-9-yl)]cob(II)amide. The hydroxocobamide was produced by photolysis of the adenosylcobamide solution in the spectrophotometer cuvette for 10 min. The cob(II)amide solution was prepared by the addition, under a stream of humidified nitrogen, of 30 μL of 0.5 M sodium dihydrolipoate (final concentration 0.03 M).

conversion of the pseudocoenzyme to the cob(II)amide cannot be followed spectrophotometrically in the manner previously used for the coenzyme (Yamada et al., 1971; Tamao & Blakley, 1973).

Radical Pair Formation. We have previously shown that *Coα*-[α-(aden-9-yl)]-*Coβ*-adenosylcobamide does function as a coenzyme for the *L. leichmannii* ribonucleotide reductase but with a K_m three- to fourfold higher than that of the normal coenzyme (Vitols et al., 1967). It was also found that the reductase rapidly generated a radical pair from the bound pseudocoenzyme. In these experiments one syringe of the rapid reaction apparatus contained 1.22 mM ribonucleotide reductase and 0.2 M sodium dimethylglutarate buffer, pH 7.3, 1.46 mM dGTP, and 30 mM sodium dihydrolipoate. The other syringe of equal volume to the first contained 1.76 mM adeninyladenosylcobamide and 0.2 M sodium dimethylglutarate buffer, pH 7.3. After short periods of incubation at 37 °C after mixing, followed by freeze-quenching, the EPR recordings from the packed snow gave a spectrum exactly the same as that previously recorded for reaction mixtures containing the normal coenzyme (Orme-Johnson et al., 1974). There is, therefore, no difference in the magnetic properties of the radical pairs formed by the coenzyme and pseudocoenzyme, respectively. Moreover, the rate of formation of the radical pair by the pseudocoenzyme was very similar to that previously reported for the coenzyme. $T_{1/2}$ for the former was found to be 19 ms as compared with 17 ms for the coenzyme (Orme-Johnson et al., 1974).

"Doublet" Formation with the Pseudocoenzyme. When adenosylcobalamin is incubated with the reductase, dGTP, and either ATP or *ara*-ATP in the presence of a monothiol, a different type of EPR signal is subsequently recorded from the frozen reaction mixture (Hamilton et al., 1972). The signal at X band is characterized by two major peaks of different intensity with effective **g** values of 2.032 and 1.965, with a smaller broad peak at low field. Subsequently it was shown

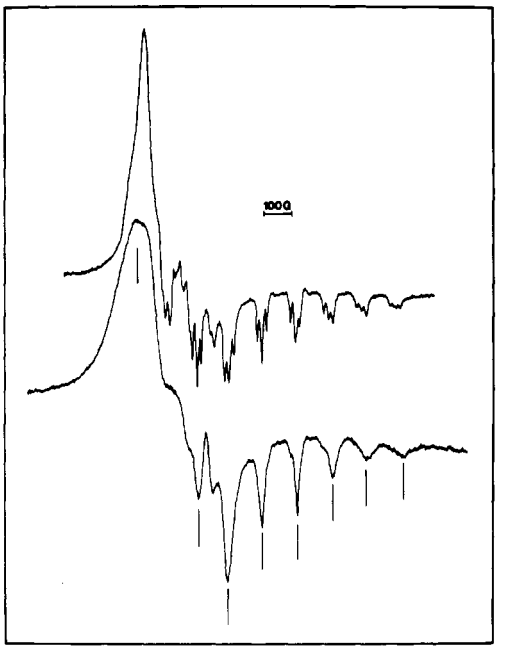

FIGURE 2: EPR spectra of *Coα*-[α-(aden-9-yl)]cob(II)amide. The solution used to obtain the lower curve was prepared by irradiating (for 1.5 h with a 150-W tungsten lamp at a distance of 20 cm) a solution containing 0.4 mM adeninyladenosylcobamide in 0.19 M sodium dimethylglutarate buffer, pH 7.3. The solution in an EPR tube was maintained at 0–5 °C during irradiation, and after irradiation the cob(II)amide was formed by anaerobic addition of 25 mM sodium dihydrolipoate. The solution was frozen in liquid nitrogen for spectrum recording. The upper spectrum was obtained from a sample which initially contained in solution: 0.5 mM adeninyl adenosylcobamide, 0.175 M sodium dimethylglutarate buffer, pH 7.3, 1.5 mM dGTP, 20 mM dihydrolipoate, and 0.41 mM ribonucleotide reductase. After incubation in an EPR tube under nitrogen in the dark at 37 °C for 6 h, the solution was frozen for spectrum measurement. The indicators correspond to g values of 2.376 (for the maximum) and 2.196, 2.122, 2.043, 1.963, 1.891, 1.824, and 1.759 for the minima.

that this spectrum results from exchange coupling and dipole–dipole spin coupling between the unpaired electrons of cob(II)alamin and the deoxyadenosyl radical (Buettner & Coffman, 1977; Boas et al., 1978). The data indicated that the dipole–dipole interaction was over a distance of about 10 Å, with the radical located 34° off the principal $\mathbf{g}_{zz}$ axis of the Co(II). When the pseudocoenzyme was allowed to react in a similar system, and the reaction mixture was subsequently frozen, the EPR spectrum recorded was the same as that obtained with the coenzyme, indicating that this "loose" radical pair was formed in precisely the same way as for the coenzyme.

Enzymatic Degradation of Pseudocoenzyme. As remarked above, it was not possible to follow the degradation of the pseudocoenzyme by reductase, dihydrolipoate, and dGTP spectrophotometrically because of the similarity of the *Coα*-[α-(aden-9-yl)]-*Coβ*-adenosylcobamide and *Coα*-[α-(aden-9-yl)]cob(II)amide absorption spectra (Figure 1). However, by freezing samples of the reaction mixture at intervals and determining the peak-to-peak EPR signal amplitude (corrected for the gain setting), the approximate rate of decay of the radical pair could be determined. The X-band spectrum for adeninylcob(II)amide in the presence of buffer and dihydrolipoate is shown in Figure 2 (lower curve). Although the spacing of the minima is a little irregular probably because of overlapping of the signals corresponding to the $\mathbf{g}_x$, $\mathbf{g}_y$, and $\mathbf{g}_z$ tensors, part of the eight-line hyperfine splitting by the Co nucleus is clearly present in the upfield portion of the spectrum. Superhyperfine splitting of these maxima into triplets is conspicuously absent, whereas it is a prominent

feature of the EPR spectrum of cob(II)alamin, where it is due to the dimethylbenzimidazole nitrogen coordinated to the β side of the cobalt atom. This confirms that, at neutral pH, the adenine base of the adeninylcob(II)amide is not coordinated to cobalt.

During prolonged incubation of the pseudocoenzyme (0.5 mM) with reductase (0.5 mM), sodium dimethylglutarate buffer, pH 7.3 (0.175 M), sodium dihydrolipoate (38 mM), and dGTP (1.8 mM) at 37 °C under nitrogen in the dark, the pseudocoenzyme was degraded slowly to the cob(II)amide as shown by the EPR of frozen samples taken at intervals. The rate of cob(II)amide formation from the pseudocoenzyme was about half the rate of cob(II)alamin formation from the normal coenzyme under the same conditions. The EPR spectrum of the cob(II)amide formed by degradation on the enzyme catalytic site during 4 h of incubation is shown in Figure 2 (upper curve). An unexpected feature of this spectrum is that superhyperfine splitting of the lines into triplets is clearly seen.

Discussion

The major purpose of this project was to obtain further information about the radical pair rapidly formed from coenzyme B_{12} by its interaction with ribonucleotide reductase in the presence of dihydrolipoate and dGTP. This paramagnetic species is of special interest because, of the three such species that are generated by the reductase under various conditions, this alone behaves kinetically like a true intermediate of ribonucleotide reduction. Previous simulation studies (Coffman et al., 1976) indicated that most aspects of the line shape could be generated on the assumption of hyperfine splitting by a Co(II) nucleus, provided decreased anisotropy of both the **g** values and the hyperfine tensors was assumed in comparison with cob(II)alamin. Comparison with spectral data for other Co(II) species led us to suggest that the marked differences in the **g** and **A** values were attributable to strongly distorted octahedral symmetry about the cobalt in the intermediate. However, the above results show that precisely the same spectrum is obtained for the intermediate formed from *Co*α-[α-(aden-9-yl)]-*Co*β-adenosylcobamide where the nucleotide base is adenine and is not normally coordinated to cobalt at neutral pH. This finding makes the suggested explanation of the intermediate structure less likely, although the evidence below that *Co*α-[α-(aden-9-yl)]cob(II)amide may be bound to the enzyme in the base-on configuration makes the argument less conclusive.

Additional considerations also make the original hypothesis unlikely. As we previously reported, no choice of parameters gave a line shape for a single Co(II) species having rhombic symmetry **g** and **A** tensors and anisotropic line width, which closely fitted the experimental spectrum. A better fit was obtained by the summation of two line shapes corresponding to slightly different magnetic parameters. However, we have no independent evidence for the existence of two alternative Co(II) species on the enzyme active site. Thus, the other enzyme-bound paramagnetic species give spectra which can be closely simulated by a single spin-Hamiltonian (Pilbrow & Winfield 1973; Buettner & Coffman, 1977; Boas et al., 1978). A second difficulty with the previous explanation of the spectrum is the absence of the organic radical signal from the spectrum.

Further investigation of the spectra generated by two interacting $S = 1/2$ systems, where the coupling is due both to exchange coupling and magnetic dipole–dipole coupling, has shown that in principle all the major features of the experimental spectrum can be simulated by the proper spin-Hamiltonian (Buettner & Coffman, 1977) in which appropriate parameters are chosen (R. E. Coffman, personal communication). It thus appears that both the rapidly formed intermediate and the species giving rise to the doublet spectrum are radical pairs, the members of which are the 5′-deoxyadenos-5′-yl radical and cob(II)alamin. They apparently differ only in the relative contributions of the exchange interaction and magnetic dipole–dipole interaction to the electron spin–electron spin coupling. This in turn probably reflects a different spatial arrangement of the members of the radical pair with respect to each other within the catalytic site of the enzyme.

This conclusion is consistent with other mechanistic information, since only *Co*β-nucleosidylcobalamins that are coenzymatically active and rapidly form an intermediate can also form the "doublet" species under appropriate conditions (Sando et al., 1975). It may be noted that formation of a species giving a "doublet" type spectrum has been observed with a number of other adenosylcobalamin-dependent enzyme reactions: glyceroldehydrase (Cockle et al., 1972); dioldehydrase (Valinsky et al., 1974a,b); and ethanolamine deaminase (Babior et al., 1974). However, in these rearrangement reactions, the organic radical appears to be a substrate radical rather than the 5′-deoxyadenos-5′-yl radical (Valinsky et al., 1974b; Babior et al., 1974). While experiments with deuterated substrate and deuterated coenzyme were inconclusive (Hamilton et al., 1972), it is unlikely that a ribonucleotid-2′-yl radical is involved rather than the 5′-deoxyadenos-5′-yl radical in the production of the doublet spectrum by ribonucleotide reductase since the approach of the 2′-deoxyribonucleotide radical to the Co(II) corrin would be hindered.

Superhyperfine Splitting in Bound Coα-[α-(*Aden-9-yl*)]-*cob(II)amide.* The triplet superhyperfine structure in the EPR spectrum of adeninylcob(II)amide formed by degradation of the pseudocoenzyme within the active site of the reductase (Figure 2, upper curve) is unexpected since adeninylcobamides do not normally have nitrogen in the axial coordination positions of cobalt. This is confirmed by the absence of the superhyperfine structure from adeninylcob(II)amide in the absence of reductase (Figure 2, lower curve). The triplet structure seen in the upper curve in Figure 2 could be explained in either of two ways. One possibility is that, when the pseudocoenzyme binds in the active site, the constraints imposed on the conformation of the cobamide by the protein side chains force the adenine into a position where N(7) is coordinated to cobalt. In agreement with this interpretation, the values of $A_{\|Co}$ ($111 \pm 3 \times 10^{-4}$ cm^{-1}) and $A_{\|N}$ ($15.8 \pm 0.7 \times 10^{-4}$ cm^{-1}) were very similar to the values for enzyme-bound cob(II)alamin, $A_{\|Co}$ ($103 \pm 2 \times 10^{-4}$ cm^{-1}) and $A_{\|N}$ ($16.9 \pm 0.5 \times 10^{-4}$ cm^{-1}) (Hamilton et al., 1971). Alternatively a nitrogen from an enzyme side chain might become coordinated as the base-off pseudocoenzyme binds in the active site, but this seems unlikely because of the steric hindrance such a side chain would provide for adenosylcobalamin binding.

Acknowledgments

We acknowledge assistance received from William Hamilton and Dr. Robert E. Coffman in obtaining the EPR spectra. We are also indebted to Dr. Coffman for helpful advice.

References

Babior, B. M., Moss, T. A., Orme-Johnson, W. H., & Beinert, H. (1974) *J. Biol. Chem. 249*, 4537–4544.

Barker, H. A., Smyth, R. D., Weissbach, H., Munch-Peterson, A., Toohey, J. I., Ladd, J. N., Volcani, B. E., & Wilson, R. M. (1960) *J. Biol. Chem. 235*, 181–190.

Blakley, R. L. (1978) *Methods Enzymol. 51*, 246–259.
Boas, J. F., Hicks, P. R., Pilbrow, J. R., & Smith, T. D. (1978) *J. Chem. Soc., Faraday Trans. 2 74*, 417–431.
Buettner, G. R., & Coffman, R. E. (1977) *Biochim. Biophys. Acta 480*, 495–505.
Cockle, S. A., Hill, H. A. O., Williams, R. J. P., Davies, S. P. & Foster, M. A. (1972) *J. Am. Chem. Soc. 94*, 275–277.
Coffman, R. E., Ishikawa, Y., Blakley, R. L., Beinert, H., & Orme-Johnson, W. H. (1976) *Biochim. Biophys. Acta 444*, 307–318.
Follmann, H. (1974) *Angew. Chem., Int. Ed. Engl. 13*, 569–579.
Hamilton, J. A., Yamada, R., Blakley, R. L., Hogenkamp, H. P. C., Looney, F. D., & Winfield, M. E. (1971) *Biochemistry 10*, 347–355.
Hamilton, J. A., Tamao, Y., Blakley, R. L., & Coffman, R. E. (1972) *Biochemistry 11*, 4696–4705.
Hogenkamp, H. P. C., & Sando, G. N. (1974) *Struct. Bonding (Berlin) 20*, 23–58.
Orme-Johnson, W. H., Beinert, H., & Blakley, R. L. (1974) *J. Biol. Chem. 249*, 2338–2343.
Pilbrow, J. R., & Winfield, M. E. (1973) *Mol. Phys. 25*, 1073–1092.
Reichard, P. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 9–14.
Sando, G. N., Blakley, R. L., Hogenkamp, H. P. C., & Hoffmann, P. J. (1975) *J. Biol. Chem. 250*, 8774–8779.
Singh, D., Tamao, Y., & Blakley, R. L. (1977) *Adv. Enzyme Regul. 15*, 81–100.
Sjöberg, B.-M., Reichard, P., Gräslund, A., & Ehrenberg, A. (1977) *J. Biol. Chem. 252*, 536–541.
Tamao, Y., & Blakley, R. L. (1973) *Biochemistry 12*, 24–34.
Valinsky, J. E., Abeles, R. H., & Mildvan, A. S. (1974a) *J. Biol. Chem. 249*, 2751–2755.
Valinsky, J. E., Abeles, R. H., & Fee, J. E. (1974b) *J. Am. Chem. Soc. 96*, 4709–4710.
Vitols, E., Brownson, C., Gardiner, W., & Blakley, R. L. (1967) *J. Biol. Chem. 242*, 3035–3041.
Yamada, R., Tamao, Y., & Blakley, R. L. (1971) *Biochemistry 10*, 3959–3968.

Nuclear Magnetic Resonance Studies of the Conformation of Tetraamminecobalt(III)–ATP Bound at the Active Site of Bovine Heart Protein Kinase†

Joseph Granot, Hiroki Kondo, Richard N. Armstrong,‡ Albert S. Mildvan,* and E. T. Kaiser

ABSTRACT: The binding of metal–nucleotide substrates and substrate analogues to the catalytic subunit of cAMP-dependent protein kinase from bovine heart has previously been shown to induce the appearance of an additional tight, inhibitory, divalent cation binding site on the enzyme [R. N. Armstrong et al. (1979) *Biochemistry 18*, 1230]. The location of this inhibitory metal and the conformation of the metal–nucleotide substrate on the catalytic subunit have been studied by using β,γ-bidentate tetraammineCo(III)–$(NH_3)_4$ATP, a substitution inert analogue of MgATP, which is shown to be a linear competitive inhibitor ($K_I = 151 \pm 10$ μM) with respect to MgATP ($K_m = 2.8 \pm 0.3$ μM). From the paramagnetic effects of Mn^{2+} bound at the inhibitory site of the enzyme on the longitudinal relaxation rates of the protons (at 100 and 360 MHz) and phosphorus nuclei (at 40.5, 72.9, and 145.8 MHz) of $Co(NH_3)_4$ATP at the substrate site, nine distances from Mn^{2+} to $Co(NH_3)_4$ATP are determined. The distances indicate either bidentate α,γ or tridentate α,β,γ coordination of the triphosphate chain of both the Δ and Λ stereoisomers of $Co(NH_3)_4$ATP by the enzyme-bound Mn^{2+}. This location of Mn^{2+} is shown to be consistent with the known thermodynamic and kinetic properties of complexes of protein kinase with nucleotides and metal ions. In the absence of enzyme, Mn^{2+} binds weakly to the adenine ring of $Co(NH_3)_4$ATP.

Cyclic adenosine 3′,5′-monophosphate (cAMP)[1] dependent protein kinase (EC 2.7.1.37; ATP:protein phosphotransferase) provides a major pathway, whereby cAMP can exert its effect on processes of cell regulation and metabolism (Krebs, 1972). This enzyme is composed of regulatory and catalytic subunits which form an inactive tetrameric holoenzyme. The activation of the enzyme by cAMP occurs through its dissociation into a dimeric regulatory subunit–cAMP complex and two active catalytic subunit monomers.

We have recently studied the interaction of Mn^{2+} and substrates with the catalytic subunit of protein kinase by magnetic resonance and kinetic methods (Armstrong et al., 1979a). While the enzyme alone was found to bind Mn^{2+} very weakly, enzyme complexes of nucleotides, such as ADP or the nonhydrolyzable β,γ-methylene analogue of ATP, bind 2 Mn^{2+} ions/mol of complex tightly. With the substitution-inert metal–nucleotide β,γ-bidentate complex $Co(NH_3)_4$ATP, only one tight Mn^{2+} binding site was detected. These findings

† From the Departments of Chemistry (R.N.A., H.K., and E.T.K.) and Biochemistry (E.T.K.), University of Chicago, Chicago, Illinois 60637, and the Fox Chase Cancer Center (J.G. and A.S.M.), Philadelphia, Pennsylvania 19111. *Received January 22, 1979.* Supported by National Institutes of Health Research Grants GM-19037 (E.T.K.) and AM-13351 (A.S.M.), National Science Foundation Grant PCM-74-03739 (A.S.M.), National Institutes of Health Postdoctoral Fellowship GM-05516 (R.N.A.), a Chaim Weizmann Fellowship (J.G.), and an appropriation from the Commonwealth of Pennsylvania to the Institute for Cancer Research. Support for the computation was provided by National Institutes of Health Grant CA-22780. The 145.8- and 360-MHz studies were done at the Middle Atlantic Regional NMR Facility which is supported by National Institutes of Health Grant RR-542.

‡ Present address: National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, MD 20014.

[1] Abbreviations used: cAMP, adenosine 3′,5′-monophosphate; Co-$(NH_3)_4$ATP, tetraamminecobalt(III)-β,γ-phosphate–ATP.

0006-2960/79/0418-2339$01.00/0