*MAGNETIC RESONANCE STUDIES OF SPIN-LABELED CREATINE KINASE SYSTEM AND INTERACTION OF TWO PARAMAGNETIC PROBES**

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Abstract.—Creatine kinase from rabbit muscle has been specifically labeled with a derivative of a nitroxide free radical on the two essential sulfhydryl groups, one per active site. The conformation in the environment of the enzyme-bound free radical and the change in conformation induced upon binding of the substrate, metal adenosine diphosphate, has been monitored by two parameters of the bound spin label, its electron paramagnetic resonance spectrum and its contribution to the proton relaxation rate of water. The specificity in activation of the enzymic reaction of the series of alkaline earth ions, Mg, Ca, Sr, Ba, was not reflected by differences in conformation of the ternary enzyme metal substrate complexes observable with the spin probe. However, with the inhibitory zinc ion, the conformation of ternary complex was distinctly different. The electron paramagnetic resonance spectra of complexes with two paramagnetic probes, namely, spin-labeled enzyme with paramagnetic metal adenosine diphosphate complexes (Mn(II), Ni(II), and Co(II)) revealed a spin-spin interaction between the protein-bound free radical and the paramagnetic metal ions. From the magnitude of the interaction, the distance between the manganous ion and the electron spin of the nitroxide moiety in the MnADP-spin-labeled creatine kinase was estimated to be in the range of 7–10 Å.

Previous investigations on the enzyme-metal-substrate complexes of creatine kinase in this laboratory[1–3] have made use of the effect of the paramagnetic manganous ion on the proton relaxation rate of water as a probe of the local environment at the active site of the enzyme. With the introduction of stable free radical "spin labels" by McConnell and co-workers,[4] it seemed feasible to use another paramagnetic probe for this enzyme system covalently linked by reaction of the essential sulfhydryl groups with the nitroxide radical, N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide (I). The stoichiometry of the reaction with iodoacetamide had been established,[5] and it had been shown that although the modified enzyme was inactive, it retained the ability to bind metal nucleotide substrates and free nucleotide inhibitors.[6] It seemed likely that reaction with stoichiometric amounts of a free radical derivative of iodoacetamide would yield a modified enzyme with similar properties, and this was indeed the case.

The present study consists of two parts: (1) investigation of the rotational mobility in the region of the essential sulfhydryl groups of creatine kinase (1 SH per subunit) in the "spin-labeled" enzyme itself and in its metal nucleotide complexes with various diamagnetic metal ions. The parameters observed were the

electron paramagnetic resonance (EPR) spectrum and the proton relaxation rate (PRR) of water due to the covalently bound free radical and (2) an estimation of the distance between manganese and the nitroxide radical in the ternary complex of MnADP-spin-labeled creatine kinase from the interaction of the two paramagnetic probes as deduced from EPR spectra.

Materials and Methods.—Chemicals: Adenosine diphosphate (ADP) was purchased from P-L Biochemicals, Inc. Divalent metal salts were spectrographically analyzed reagents from Johnson, Matthey and Company, Ltd., London. N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide (I) was synthesized by the method of Rozantzev and Krinitzkaya.[7] The six-membered analog, N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide (II) was purchased from Varian Associates. N-Ethylmorpholine was purchased from K and K Laboratories, Inc. and redistilled under vacuum.

Spin-labeled creatine kinase: Creatine kinase was prepared from rabbit muscle by method B of Kuby, Noda and Lardy.[8] Spin-labeled enzyme was prepared by reacting 20 mg/ml protein (0.5 mM active sites) with a 15% molar excess of (I) at pH 8, 4°C. The spin label reacted with the enzyme at the same rate as iodoacetamide.[5] The number of moles of enzyme sites inactivated, calculated from loss of enzyme activity[9] as spin-labeling proceeded, paralleled the decrease in the number of moles of free spin label, determined from the amplitude of the EPR spectrum of (I). At the completion of the reaction, the reaction mixture was passed through a column of Sephadex G-25, and was dialyzed overnight to remove all traces of (I). An attempt to repeat the preparation with the analogous free radical with a six-membered ring (II) was unsuccessful. Only a very slow, nonspecific labeling occurred in this case.

Magnetic resonance measurements: Pulsed nuclear magnetic resonance (NMR) measurements at 24.3 MHz of the proton relaxation rate of water were made as described previously.[1] EPR spectra were measured with a Varian E-3 spectrometer. The EPR spectra due to protein-bound radical in enzyme-manganese nucleotide complexes were obtained by storing the complete spectrum in a Varian C-1024 time-averaging computer, and then subtracting the EPR signal due to the unbound manganese-nucleotide complex. Computer simulation studies of the "spin-label" spectra were done on a PDP-6 computer, under the auspices of the University of Pennsylvania Medical School Computer Facility.

Results.—Ternary complexes of spin-labeled enzyme with ADP and diamagnetic metal ions: The spectra of (I) before and after reaction with a stoichiometric amount of creatine kinase (one mole of free radical per subunit of 41,000 mol wt) are shown in Figure 1. The spectrum of the nitroxide radical covalently bound to the enzyme corresponds to a "highly immobilized" species[4] with a separation of the outer peaks of ~58 gauss at 22°C. This value may be compared to ~31 gauss for the free radical in H_2O and ~64 gauss for the solid powder spectrum.[4] It is estimated that the spectrum of the bound label corresponds to a rotational correlation time, τ, of the order of 5×10^{-8} sec compared to $\sim 10^{-11}$ sec for (I) itself.[4]

Titration of the enzyme in the presence of excess metal ion with ADP leads to further immobilization of the nitroxide radical as shown in Figure 2 for Ca and ADP. The same type of spectra are observed in ADP titrations with the alkaline earth ions, Mg, Ca, Sr, and Ba. The downfield shift of the low field peak, the upfield shift of the high field peak, and the decrease in amplitude of the center peak are approximately the same at saturating concentrations of metal ADP for all four ions. However, Sr and Ba were found to have less than 2 per cent of the activation effect of Mg on the velocity of the enzymatic reaction. The

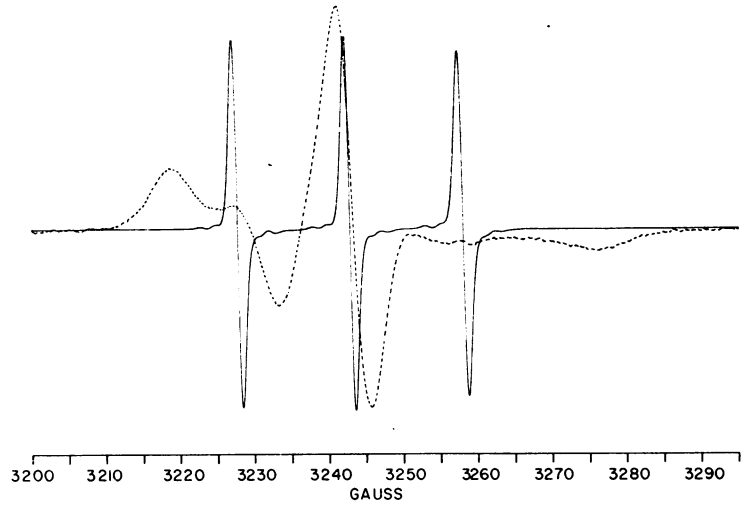

FIG. 1.—Comparison of EPR spectra of free N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide (I) (*solid line*), and of (I) bound to creatine kinase (*dashed line*), in 0.05 *M* N-ethylmorpholine-Cl, pH 8.0, room temperature. At equal concentrations and gain, the amplitude of the center peak of the free radical spectrum is 14 times that of the protein-bound radical.

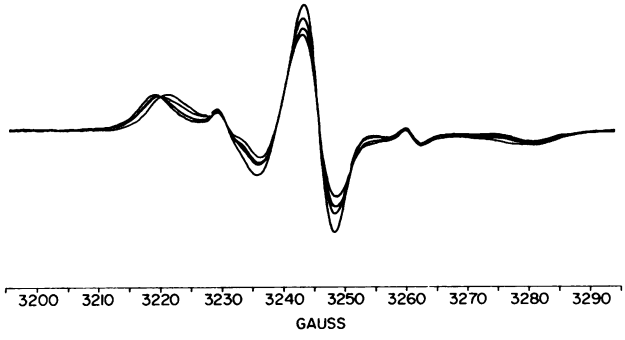

FIG. 2.—Titration of spin-labeled enzyme with CaADP. The solutions contained enzyme, 3.8 mg/ml; $CaCl_2$, 5.0 mM; 0.05 *M* N-ethylmorpholine-Cl, pH 8.0. The curve with the highest amplitude of the center peak contained no ADP. The ADP concentrations were 0.168, 0.452, and 3.61 mM, respectively, in order of decreasing amplitude of the center peak. $T = 20°C$.

effect of metal ADP on the spectrum may be simulated by lowering the temperature of the spin-labeled enzyme from 25°C to 3°C, implying that only a change in mobility, i.e., in the rotational correlation time, has occurred. However with Zn, which is an irreversible inactivator of the native enzyme,[10] the EPR spectrum of the ternary complex has a different pattern; the shift in the low field peak is the same, but decreases in the amplitude of the center peak are ~20 per cent and ~40 per cent for Mg and ZnADP, respectively. It seems likely that ZnADP forms a complex with the enzyme structurally different from the alkaline earth metal ions. Furthermore, in the presence of Zn and ADP, the enzyme is not

stable as evidenced by the gradual conversion of the highly immobilized spectrum to a weakly immobilized spectrum with peaks in approximately the same position found for unbound (I) but somewhat broadened. The latter spectrum is typical of denatured spin-labeled creatine kinase.

The paramagnetic contribution of the different free-radical species to the proton relaxation rate of water, as well as their EPR spectra has been measured. A comparison of the molar relaxivities and the EPR spectral changes between spin-labeled creatine kinase and its ternary metal-ADP complexes is given in Table 1. It should be noted that the attachment of the spin label to the enzyme

TABLE 1. *Comparison of the molar relaxivities and EPR spectral amplitudes of the free-spin label and spin-labeled enzyme species.*

	Molar relaxivity ($1\ moles^{-1}\ sec^{-1}$)	Increase* in molar relaxivity (%)	Amplitude† of center line	Decrease* in EPR amplitude (%)
Compound I	245	—	5,070	—
E·	1,500	(0)	362	(0)
E·MgADP	3,600	140	289	20
E·CaADP	4,300	190	280	23
E·SrADP	4,200	180	297	18
E·BaADP	3,200	110	306	15
E·ZnADP	2,600	73	225	38

* Referred to spin-labeled enzyme (E·).

† Arbitrary units.

The solutions contained N-ethylmorpholine, 0.05 *M*, pH 8; enzyme, 5.7 mg/ml; ADP, 3 mM; $ZnCl_2$, 3 mM; Mg, Ca, Sr, Ba chlorides, 5 mM; T = 22°C.

increases its effect on the proton relaxation rate of water by a factor ~6 and upon binding of metal ADP to the enzyme, there is a further increase by an additional factor ~2.5. The EPR spectrum, on the other hand, changes drastically when the free radical is bound to the enzyme, and there is only a small change of the order of 20 per cent when the ternary E-MADP complex is formed. The ternary Zn complex is exceptional; not only does the change in the EPR spectrum differ qualitatively and quantitatively from the other metal complexes, but its molar relaxivity is considerably smaller than for other metal complexes (cf. Table 1). No further change in EPR spectrum or proton relaxation rate of water could be observed upon addition of creatine to any ternary E-MADP complex.

Ternary complexes of spin-labeled enzyme with ADP and paramagnetic ions: The addition of MnADP drastically affects the EPR spectrum of the spin-labeled enzyme as shown in Figure 3. At saturating concentration of MnADP, the amplitude of the signal falls about 95 per cent. The effects of the Co and Ni complexes of ADP are smaller but qualitatively similar (cf. Fig. 3). That the effect of MnADP is due to its binding to the enzyme in close proximity to the nitroxide group and not merely to the presence of the paramagnetic manganous ion in the solution is established by the experiment graphically presented in Figure 4. With constant Mn concentration, upon successive additions of ADP, the amplitude of the center peak as plotted on the ordinate in Figure 4 first decreases and then begins to rise towards its initial value as free ADP displaces MnADP from the binding sites on the enzyme. A similar curve was observed in

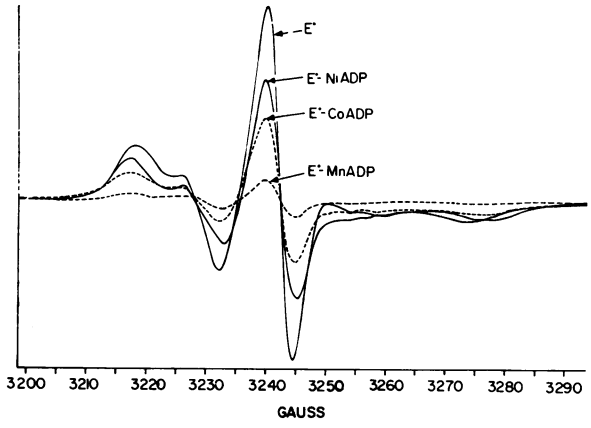

FIG. 3.—Effect of paramagnetic metal nucleotide complexes on EPR spectrum of spin-labeled creatine kinase. The solutions contained enzyme, 9 mg/ml in 0.05 *M* N-ethylmorpholine-Cl, pH 8.0, (E·), no metal nucleotide; (E·NiADP), 1 mM $NiCl_2$ and 1 mM ADP; (E·CoADP), 3 mM $CoCl_2$ and 3 mM ADP; (E·MnADP), 2.5 mM MnCl and 2.5 mM ADP. $T = 22°C$.

an ADP titration of the proton relaxation rate enhancement of the native enzyme and manganese (cf. Fig. 4 of ref. 11).

One might have expected that the interaction of the free radical with a paramagnetic ion at close range would lead to significant broadening of the free-radical EPR spectrum. However (cf. Fig. 3), practically no broadening is observed, only an apparent decrease in the amplitude of the unperturbed signal. Similar changes in EPR spectra have been observed upon binding of a Cu complex to spin-labeled hemoglobin.[12] The contrast between the effects of diamagnetic and paramagnetic metal ion complexes is particularly striking on the low-field peak since its amplitude is unaffected by diamagnetic complexes. The explanation of this phenomenon becomes apparent upon theoretical analysis of the line shape. A calculation of the line shape of the free radical has been made for a completely immobilized system with two unlike spins, e.g., a free radical and manganous ion, taking into account only the secular part of the dipolar interaction. The details of the calculation, the assumptions, and nature of the approximations will be published elsewhere. The treatment leads to the following equation for the line width of the free radical:

$$\delta H = C(1-3\cos^2\theta'_R)^2 + \delta H_0 \qquad (1)$$

where δH_0 is the residual line width of the free radical in the absence of paramagnetic ion, θ'_R is the angle between the applied magnetic field and the line joining the two electron spins and

$$C = \frac{(g\beta)_1\mu_2^2}{r^6}\tau \qquad (2)$$

where r is the distance between the two electron spins, the subscripts 1 and 2 refer to the free radical and the paramagnetic ion, respectively, and τ is the correlation time for the dipolar interaction, i.e., the electron-spin relaxation time of the manganese.

A computer simulation of the line shape for different values of C for a particular configuration of the two paramagnetic species on the protein molecule is shown in Figure 5. The computed curves are gratifyingly similar qualitatively to the

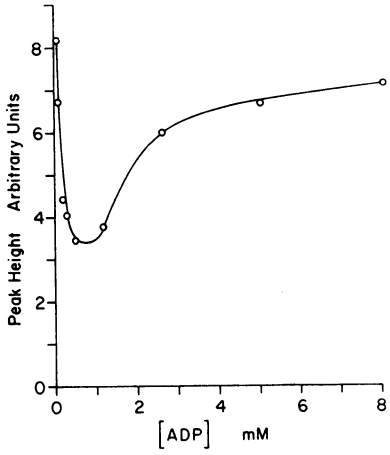

FIG. 4.—Titration of the spin-labeled enzyme at constant Mn(II) with ADP. The amplitude of the center EPR line is plotted against ADP concentration. The solutions contained enzyme, 4.2 mg/ml; $MnCl_2$, 1 mM; 0.05 *M* N-ethylmorpholine-Cl, pH 8.0. T = 22°C.

experimentally observed curves illustrated in Figure 3. The underlying physical reason that the dipolar "broadening" results in a decreased amplitude with no observable broadening resides in the fact that the two spins are embedded in a rigid lattice, so that with a random orientation of molecules in the magnetic field, in a certain fraction of the orientations, $C(1-3\cos^2\theta_R')^2$ will be small compared to δH_0, and the signal from this fraction will yield the unperturbed spectrum. On the other hand, for most other orientations, $C(1-3\cos^2\theta_R')^2$ is large compared to δH_0, and the resulting spectra are too broad to be observable. Consequently, one may observe a fraction of the original signal, practically unperturbed in shape; the magnitude of the fraction depends on the value of C as illustrated in Figure 4. The value of C for the ions tested falls in the order Mn > Co > Ni.

The analysis of the line shape given above is based on the assumption that the two interacting spins are in a rigid lattice. The high degree of immobilization of

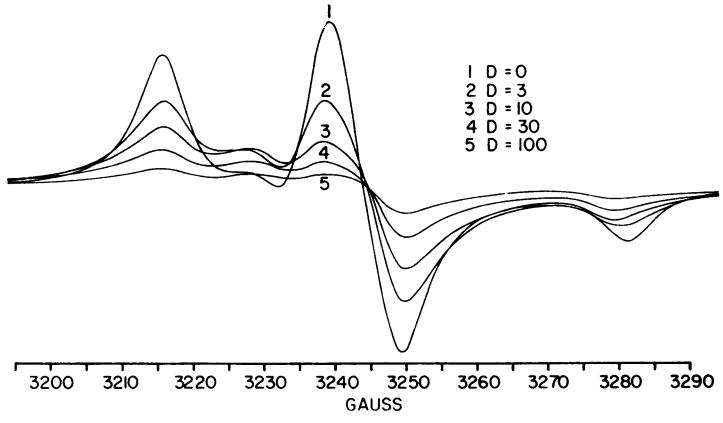

FIG. 5.—Computer simulated spectra of enzyme-bound spin-label resulting from interaction with paramagnetic ions. The curves represent increasing values of C, defined in Eq. (2) (denoted as D in the Figure).

the free radical in ternary complexes is apparent from the EPR spectra. The degree of immobilization of the magnanese was investigated by determining the proton relaxation rate of water of the ternary E-MnADP complex by methods described previously.[1] The value of the proton relaxation rate enhancement of the ternary E-MnADP complex was calculated to be 19; a small correction for the free radical determined from the E-MgADP complex was made. If one considers that probably only three molecules of water remain in the hydration shell, the rotational correlation time has increased at least 40-fold over that of the manganous aquocation, indicating a highly immobilized site for MnADP.

Discussion.—The free radical covalently bound to the essential SH group at the active sites of creatine kinase has been demonstrated to be immobilized by two criteria, the dramatic change in the EPR spectrum and a six fold enhancement of the proton relaxation rate of water. Further immobilization of the free radical occurs upon binding metal ADP to the active site. It might be noted parenthetically that the proton relaxation rate enhancement proved to be a more sensitive probe than EPR of the conformational change in the region of the radical induced by substrate binding. Thus, the ability of the native enzyme to undergo conformational change in the region of the sulfhydryl group upon binding of metal nucleotide[13] is retained in the spin-labeled enzyme. What has been lost apparently is the ability of undergoing further conformational change at the SH group upon addition of creatine in the presence of metal ADP since no change was observed in the EPR spectrum or the proton relaxation rate of water. In the native enzyme this conformational change was reflected by a change in the reactivity towards iodoacetate and iodoacetamide[13] and a concomitant change in the proton relaxation rate of water due to addition of creatine to the E-MnADP complex.[3]

It may be concluded that both the metal nucleotide binding site and the sulfhydryl group of the active site of creatine kinase are in a rigid region of the molecule, probably in a cleft. This may explain why, unlike the five-membered ring nitroxide derivative, the analogous six-membered ring compound does *not* react with the sulfhydryl at the active site. The limitation may be the thickness of the chair conformation of the six-membered ring[14]; the five-membered ring derivative which is more planar[15] reacts at the same rate as iodoacetamide.

Although the alkaline earth ions differ greatly in the magnitude of their activation of the enzymic reaction, this difference is not reflected in any large differences in the EPR spectra or in the proton relaxation rates of water in the enzyme-metal-substrate (EMS) complexes of the spin-labeled enzyme. By these criteria, the conformation of the Mg, Ca, Sr, and Ba complexes in the region of the sulfhydryl group are very similar. These results are consistent with the finding that the binding of either BaADP or of MgADP to native enzyme increases the sulfhydryl group reactivity towards iodoacetate to a similar degree.[6] On the other hand, a difference between the Ba and Mg quaternary complexes with native enzyme, ADP, and creatine is observed: in the Mg complex, the sulfhydryl group is protected from attack by iodoacetate, whereas this protection is greatly diminished for the Ba complex.[6] One of the shortcomings of the spin-labeled enzyme is the lack of observable differences be-

tween the quaternary complexes with creatine and the ternary complexes. It should be noted that the ternary complexes of the irreversible inhibitor, Zn, does show significant differences by both EPR spectra and proton relaxation rate of water.

The distance between the metal ion and the spin label on the sulfhydryl group may be estimated from the EPR spectrum of the ternary MnADP-spin-labeled creatine kinase complex. From equation (2), C is a function of two variables, τ and r, where τ can be identified with the electron-spin relaxation time of manganese, τ_s, and r is the distance between manganese and the unpaired electron of the nitroxide radical. The experimentally determined diminution of signal, 95 per cent for the MnADP complex corresponds to a value of C equal to 600 gauss from theoretical curves of Figure 5. A value for τ_s may be estimated from the enhancement of the proton relaxation rate of water due to Mn in the ternary complex. The indicated 40-fold increase in the Mn-H_2O correlation time means that $\tau_s \geq 1.2 \times 10^{-9}$ sec where a value of 3×10^{-11} sec has been assumed for the correlation time of the unbound Mn aquocation. This leads to a value $r \sim 7$ Å, which is of course a lower limit. An upper limit of $r \sim 10$ Å may be obtained by assuming that τ_s is unchanged from its value 1×10^{-8} sec[16] in the free ion.

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