DISTANCE DETERMINATIONS AT THE ACTIVE SITE OF KIDNEY ($NA^+ + K^+$)-ATPase BY Mn(II) ION ELECTRON PARAMAGNETIC RESONANCE

Sally E. O'CONNOR and Charles M. GRISHAM
Department of Chemistry, University of Virginia, Charlottesville, VA 22901, USA

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1. Introduction

Sodium and potassium ion-activated adenosine triphosphatase (($Na^+ + K^+$)-ATPase) is a plasma membrane-bound enzyme which is responsible for extrusion of sodium and uptake of potassium in animal cells [1]. Information relating to the detailed molecular architecture of the active site of this enzyme will be essential to understanding the mechanisms of ATP hydrolysis and ion transport. However, the particulate, lipoprotein state and high molecular weight of this enzyme will surely preclude the application of X-ray diffraction techniques in the near future. Magnetic resonance methods, on the other hand, offer another approach to studies of structure and mechanism at the active site, particularly when paramagnetic probes can be effectively substituted at activator and substrate sites on the enzyme. Several groups have employed the Leigh theory [2] to determine the distance between two sources of unpaired spin on a macromolecule by EPR measurements [3–6]. We have used the paramagnetic substrate analog, CrATP, to determine the distance between the bound Mn^{2+} and the Cr^{3+} of CrATP at the ATP site of the ($Na^+ + K^+$)-ATPase.

2. Experimental

2.1. *Materials*

($Na^+ + K^+$)-ATPase from sheep kidneys (Pel-Freeze Biologicals, Rogers, AR) was purified as in [7]. The final specific activity of these preparations were typically 30–38 μmol ATP hydrolyzed . mg protein^{-1} . min^{-1}.

The β,γ-bidentate isomer of CrATP was prepared by the method in [8]. To 250 ml 20 mM Na_2ATP at 0°C is added with stirring, 250 ml 20 mM $[CrCl_2(H_2O)_4]Cl$. The resulting solution is slowly adjusted to pH 5.7 using saturated $KHCO_3$ and then allowed to stand at room temperature for ~30 min (maintained at pH $\ll$ 6). After cooling to 0°C the mixture is adjusted to pH 2.5 with $HClO_4$ and then absorbed onto a Dowex 50 W (H^+ form, 2% cross-linkage) column (4 × 11 cm) at 4°C. The column is washed with H_2O until all of the bidentate CrATP (apple green band) has focused below the Na^+ band (total loading and elution time should not exceed 2–3 h). After removal of the resin above the bidentate CrATP band, the column is washed with 0.3 M $HClO_4$. The eluate is quickly adjusted to pH 4–5 using saturated $KHCO_3$, filtered, then stored at 4°C (60% yield).

2.2. *EPR measurements*

A Varian E-109 EPR spectrometer was used for X-band EPR measurements, as in [7]. EPR spectra at 35 GHz were collected in a similar manner on an E-12 EPR spectrometer at Pennsylvania State University.

2.3. *EPR theory for spin–spin relaxation – The Leigh theory*

The uses of EPR measurements to measure the distance between a Mn(II) site on an enzyme and either a nitroxide spin label [9,10] or another metal ion site [3,4] have been based on the theory of Leigh [2]. This theory describes the interaction of two unpaired spins imbedded in a rigid lattice. In previous cases described, as well as in this case, the macromolecule itself is the 'rigid lattice'. If the correlation time, τ_c, which dominates the dipolar interaction is short with respect to the rotation of the macromole-

cule, one does not observe a broadening of the EPR signal. Instead, only an apparent decrease in spectral amplitude with no change in linewidth is observed. According to [2], the linewidth of the observed spin is:

$$\delta H = C(1 - 3\cos^2\theta_{R'})^2 + \delta H_0 \quad (2)$$

Here δH_0 is the linewidth of the observed spin in the absence of dipolar broadening, while $\theta_{R'}$ is the angle between the vector joining the two interacting spins and the applied magnetic field. C is a coefficient describing the interaction and it is defined as:

$$C = g\beta\mu^2\tau_c / \hbar r^6 \quad (3)$$

where g is the electronic g-value, β is the Bohr magneton, μ and τ_c are the magnetic moment and electronic spin relaxation time, respectively, of the perturbing spin, $\hbar$ is Planck's constant divided by 2π and r is the distance between the two spins. The angular dependence of the first term in eq. (2) is the source of the apparent loss in signal intensity which is observed experimentally. For most angles $\theta_{R'}$, the dipolar contribution to δH is very large compared to the unperturbed linewidth and the resulting lines are too broad (typically 2000 G) to be observed. However for a very small fraction of the observed spins, $\theta_{R'} \cong 54°44'$ so that $(1 - 3\cos^2\theta_{R'}) = 0$ and $\delta H = \delta H_0$, resulting in no line broadening for this fraction of the spins. Thus spins having $\theta_{R'}$ within a small range of 54°44′ will give observable signals which are indistinguishable in line shape from the unperturbed signal. On the other hand, spins having $\theta_{R'}$ outside this range will give signals which are unobservably broad. It also follows that the larger the value of C in eq. (2), the smaller the range of $\theta_{R'}$ for which observable signals are obtained. From the resulting fraction decrease in the spectral amplitude, one obtains the interaction constant C, and thence the distance r. Here, the value of C was read from fig.3 of Leigh's original paper [2].

3. Results and discussion

3.1. *Interaction of CrATP with ATPase–Mn(II)*

Previous studies have established that CrATP binds to $(Na^+ + K^+)$-ATPase [11], interacts with a K^+ site observed by $^7Li^+$ NMR [12], and competes with ATP and MgATP at the nucleotide sites on the enzyme. If the binding sites for CrATP and Mn(II) are close together on the enzyme, binding of CrATP should effect a diminution of the enzyme-bound Mn(II) EPR spectrum. Such an effect is displayed in the spectra of fig.1. The addition of β,γ-bidentate CrATP to a solution of MN(II) and $(Na^+ + K^+)$-ATPase results in a decrease in the amplitude of the spectrum with no apparent change in linewidth. The effect saturates at high levels of CrATP, as shown in a plot of signal intensity vs CrATP in fig.2. Similar titrations with α,β,γ-tridentate CrATP (not shown) had no effect on the Mn(II) signal amplitude, indicating either that tridentate CrATP does not bind to the enzyme or that it binds in a manner which precludes a dipolar interaction with enzyme-bound Mn(II). Titrations with diamagnetic β,γ-bidentate $Co(NH_3)_4ATP$ likewise produced no effect on the Mn(II) EPR spectrum (fig.3), demonstrating that the effect observed with CrATP is purely a dipolar interaction. Titrations of similar Mn(II) solutions in the absence of enzyme showed no decrease in amplitude of the Mn(II) EPR signal. In the absence of enzyme, the Mn(II) and CrATP species interact only infrequently in solution. On the enzyme, however, these two species are bound rigidly and can interact strongly.

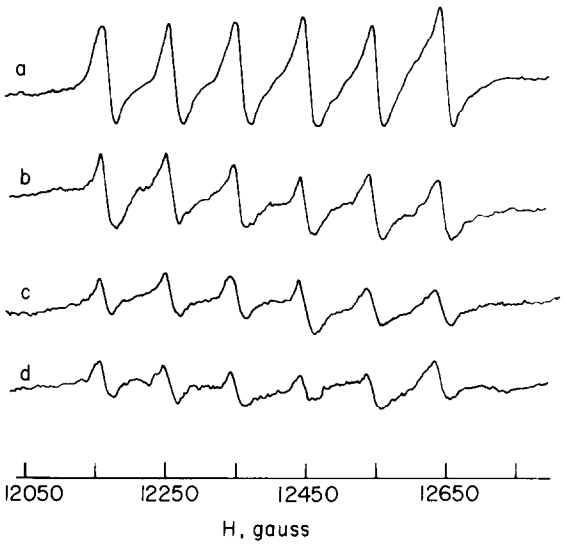

Fig.1. Manganese (II) EPR spectra at 35 GHz for complexes of $(Na^+ + K^+)$-ATPase and by β,γ-bidentate CrATP. All solutions contained 20 mM Tes–TMA (pH 7.5), 0.15 mM ATPase and 0.1 mM $MnCl_2$. CrATP was: (a) 0.0 mM; (b) 0.1 mM; (c) 1.0 mM; (d) 5.0 mM. T = 23°C.

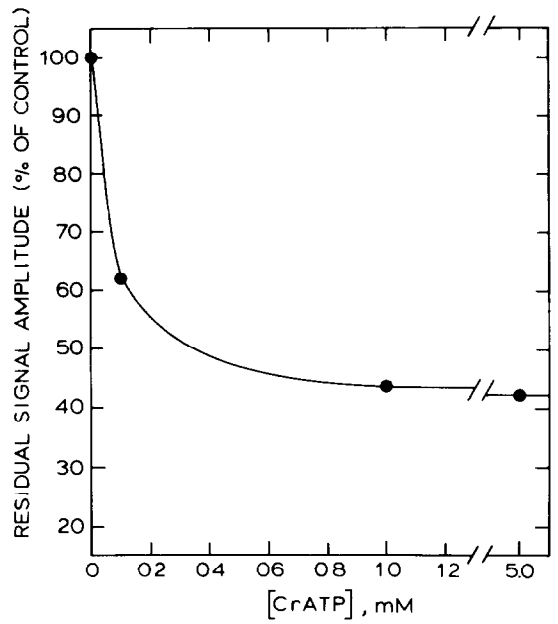

Fig.2. Effect of β,γ-bidentate CrATP on signal intensity of $(Na^+ + K^+)$-ATPase–Mn^{2+} complex. At each level of CrATP the intensities of the 6 hyperfine lines of the Mn^{2+} spectrum were averaged. Conditions were as in fig.1.

3.2. *Calculation of Mn(II)–Cr(III) distance on $(Na^+ + K^+)$-ATPase*

The decrease in the amplitude of the Mn(II) EPR signal measured in the presence of CrATP can be used to calculate a MN(II)–Cr(III) distance as in section 2. The essence of such a distance calculation is the determination of the dipolar interaction constant C. In practice it is necessary to perform a computer simulation of the lineshape and signal amplitudes arising for various values of C. A plot of such simulated amplitudes as a function of $C/\delta H_0$ is given in fig.3 in [2]. For the present case, the data of our fig.2 yield an interaction constant C of 22.5 ± 5.0 G. From eq. (3) we then calculate a Mn(II)–Cr(III) distance to 8.1 ± 0.5 Å, using a correlation time τ_c of $2.7 \pm 0.3 \times 10^{-10}$ s. This value has been determined from the frequency dependence of water proton relaxation in solutions of the ATPase, CrATP and Mg(II) [13].

Our Mn^{2+} EPR studies [7,14] with a variety of diamagnetic substrates and substrate analogs suggest, but do not prove, that the Mn^{2+} site on this enzyme is close to at least one ATP site. However, these EPR studies do not necessarily rule out actions at a distance by the nucleotides in question, and without the availability of paramagentic CrATP, the question might remain unanswered. However, the changes in Mn^{2+} EPR signal intensity produced by CrATP in solutions with the enzyme permit a precise determination of the Mn^{2+}–Cr^{3+} distance and thus provide a more quantitative estimate of the distance between the Mn^{2+} site and the ATP site(s) on the enzyme.

It is worth noting that only the β,γ-bidentate CrATP effects a decrease in amplitude of the Mn^{2+} signal. The α,β,γ-tridentate CrATP, on the other hand, causes no such decrease, either because it cannot bind to the ATPase or because it binds 'incorrectly'. Also to be considered is the fact that the β,γ-coordinated CrATP is a mixture of diastereomers. It is not yet

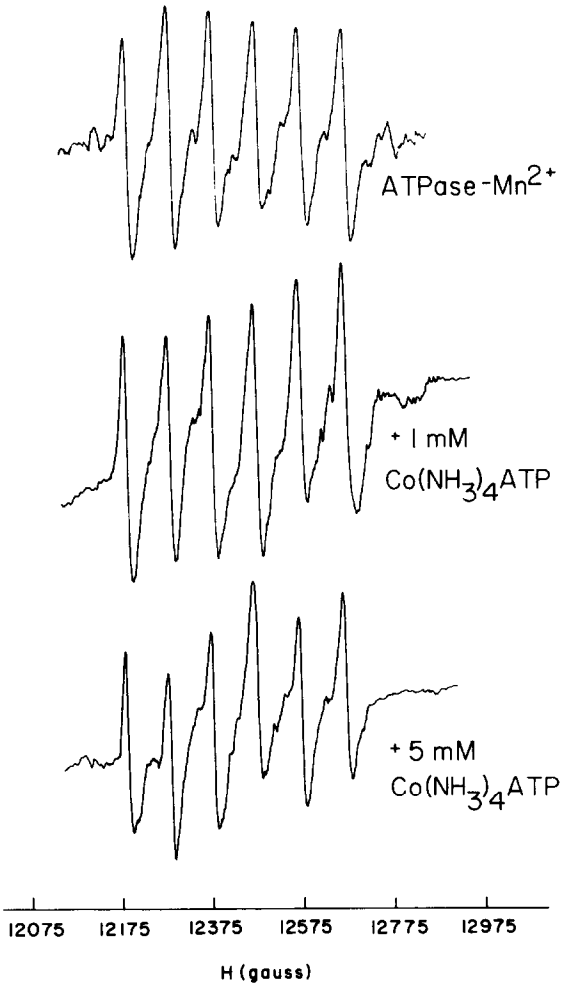

Fig.3. Effect of β,γ-bidentate $Co(NH_3)_4ATP$ on the EPR spectrum of the $(Na^+ + K^+)$-ATPase–Mn^{2+} complex. Solutions were as in fig.1, with the $[Co(NH_3)_4ATP]$ shown.

known with certainty whether only one or more isomers bind to this system. Such uncertainty need not affect our confidence in the calculations of r, the distance, since the value of C calculated from the dipolar relaxation effect is independent of K_d and unaffected by the presence of non-interacting species in the titrant solution (at saturating [CrATP]).

The two basic criteria for applicability of the Leigh theory for interacting spins bound to a rigid lattice are:

(1) The longitudinal electron spin relaxation time T_{1e} of the perturbing spin (Cr^{3+} here) must be short compared with the transverse electron spin relaxation time T_{2e} of the observed spin (Mn^{2+} here);

(2) The paramagnetic contribution to the observed effect must be separated from any diamagnetic effects. With respect to the first criterion, for kidney ($Na^+ + K^+$)-ATPase, T_{2e} for bound Mn^{2+} is $1.7-2.0 \times 10^{-9}$ s in the various complexes [15], while T_{1e} for Cr(III) is $2.7 \pm 0.3 \times 10^{-10}$ s [13]. Thus, if it depended upon this point alone, it would be valid to use the Leigh theory to compute metal-metal distances in this system.

The experiment with $Co(NH_3)_4ATP$ (fig.3) provides the appropriate diamagnetic control for the CrATP titrations. Since no net decrease in the intensity of the Mn^{2+} EPR signal is observed even at 5 mM $Co(NH_3)_4ATP$, the entire effect observed with CrATP can be ascribed to paramagnetic origins.

These new structural data, as well as measurements from our previous NMR and EPR studies, can be combined to provide a picture of the active site of the ($Na^+ + K^+$)-ATPase as it is currently understood. We have characterized binding sites at the active center of the enzyme for Na^+ (by $^{205}Tl^+$ NMR [16]), K^+ (by $^7Li^+$ NMR [12]) and phosphate (by ^{31}P NMR [17]), and also characterized the fast-exchanging water protons on enzyme-bound Mn^{2+} [15]. Interactions between Li^+ and the K^+ site and enzyme-bound, paramagnetic CrATP have also been observed by $^7Li^+$ NMR [12]. This information is summarized in fig.4. Molecular models constructed with typical $Mn-OH_2$, P–O, O–H and Na^+–O bond lengths indicate that the Mn^{2+}–P and Mn^{2+}–Na^+ distances from NMR are consistent with the complex shown here. In a survey of several phosphoryl- and nucleotidyl-transfering enzymes, including phosphoglucomutase, pryuvate kinase, and DNA polymerase I, it was suggested that Mn^{2+}–P second sphere complexes (e.g. that in fig.4)

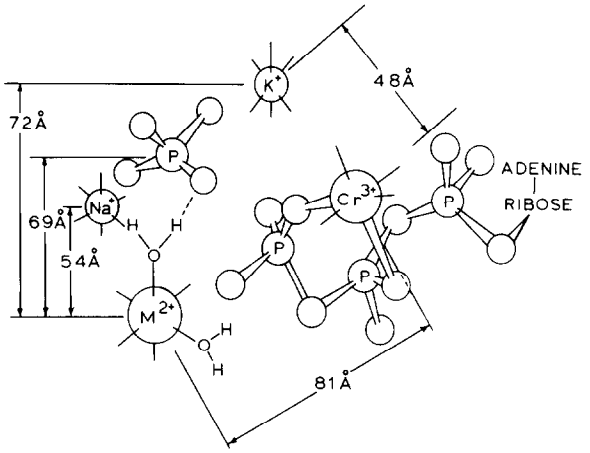

Fig.4. Active site structure of ($Na^+ + K^+$)-ATPase as determined by ^1H, $^{205}Tl^+$, ^{31}P, and $^7Li^+$ NMR, Mn^{2+} EPR, and kinetic studies (from [7,11–17]).

as a general mode of activating phosphoryl groups for nucleophilic attack in the phosphoryl-transfer reaction [18]. To distinguish between this and other possible mechanisms, it will be important to determine the structure and location of ATP itself on the ATPase. This work constitutes an initial effort in this direction. Clearly 35 GHz Mn^{2+} EPR studies should provide part of the desired information, using ATP and CrATP to probe the nucleotide sites of the enzyme. However, we have also initiated ^{31}P and ^1H NMR studies of the conformation of $Co(NH_3)_4$ ATP on the ($Na^+ + K^+$)-ATPase. Since Mn^{2+} binding to this analog is weak, it provides an unambiguous probe of ATP sites on the ATPase. These studies will be the subject of future communications.

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