Vol. 93, No. 4, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 29, 1980 Pages 1146-1151

SUBSTRATE-METAL INTERACTIONS AT THE ACTIVE SITE OF KIDNEY ($Na^+ + K^+$)-ATPase CHARACTERIZED BY Mn(II) ELECTRON PARAMAGNETIC RESONANCE

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Received January 14,1980

SUMMARY

Electron paramagnetic resonance spectra at 35 GHz of Mn^{2+} ion bound to highly purified membrane-bound ($Na^+ + K^+$)-ATPase from sheep kidney medulla are much narrower than the corresponding spectra at 9 GHz. As a result, the sensitivity of the enzyme-Mn^{2+} spectrum to added substrates is much greater at the higher frequency. ATP and AMP-PNP, which caused very little broadening at low frequency, effect dramatic decreases in intensity of the Mn^{2+} EPR signal at 35 GHz. On the other hand, virtually no changes are observed upon addition of ADP and AMP, suggesting that the γ-phosphate of ATP plays a key role in the interaction between Mn^{2+} and ATP on the enzyme. The data indicate that ATP and AMP-PNP, binding at low affinity substrate sites, induce a severe distortion of the Mn^{2+} coordination geometry. The data also support the suggestion that the enzyme-bound Mn^{2+} does not enter into a typical M^{2+}-ATP complex in this system.

INTRODUCTION

Recently we have used the X-band EPR spectra of bound Mn^{2+} ion in various enzyme-metal-substrate complexes to characterize the active site of sheep kidney medulla ($Na^+ + K^+$)-ATPase (1-3). While useful, these X-band spectra possessed an inherent limitation. Since the EPR spectrum of the binary ATPase-Mn^{2+} complex at X-band is extremely broad, approaching the rigid limit, the additional broadening induced by ATP and ATP analogues such as AMP-PNP is not easily characterized.

*Research Career Development Awardee (NIH AM00613) of the United States Public Health Service

Abbreviations used: EPR, electron paramagnetic resonance; AMP-PNP, β,γ-imidoadenosine-5'-triphosphate; TES, N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; TMA, tetramethylammonium.

0006-291X/80/081146-06$01.00/0

On the other hand, the 35 GHz EPR spectrum of Mn^{2+} is characteristically much narrower than its 9 GHz counterpart in a variety of macromolecular systems (4). For this reason, we undertook a study at 35 GHz of the interactions of substrates and analogues with ATPase-bound Mn^{2+}. We have observed a substrate-induced diminution of the 35 GHz Mn^{2+} signal which is unusually severe, and which is consistent with an unprecedented distortion of Mn^{2+} coordination geometry by a substrate.

MATERIALS AND METHODS

The $(Na^+ + K^+)$-ATPase used in these studies was prepared from sheep kidney medulla as previously described (1) and was 90-95% pure. The X-band EPR measurements were made with a Varian E-109 EPR spectrometer equipped with a Varian E-257 variable temperature accessory and a Digital PDP 11V03 computer. The 35 GHz EPR spectra were measured on a Varian E-9 EPR spectrometer in the Department of Chemistry at the Pennsylvania State University. The enzyme samples were centrifuged out of the appropriate solutions of buffer, salts, substrates, etc., and the enzyme pellet was mixed with a minimum of the same buffer solution and taken up in ultrapure quartz capillary tubes (Amersil). Sample volumes of up to 70 μL could be accomodated in the cavity in this way. The spectra were taken at 23°C.

RESULTS AND DISCUSSION

As shown in Figure 1, the X-band EPR spectrum of the ATPase-bound Mn^{2+} ion exhibits a "powder" line shape, with the EPR transitions spread over a considerable range of magnetic field. At this frequency, the spectrum shows only small amounts of broadening by added ATP, ADP and AMP-PNP, while AMP effects a narrowing of the spectrum. On the other hand, the EPR spectrum of the binary Mn^{2+}-ATPase complex at 35 GHz (Figure 2) is much narrower than the corresponding spectrum at 9 GHz. Such a narrowing at high frequency has been observed routinely for protein-Mn^{2+} complexes (5-7). The spectrum is relatively unaffected by ADP and AMP at levels which were found to broaden or narrow, respectively, the 9 GHz spectrum.

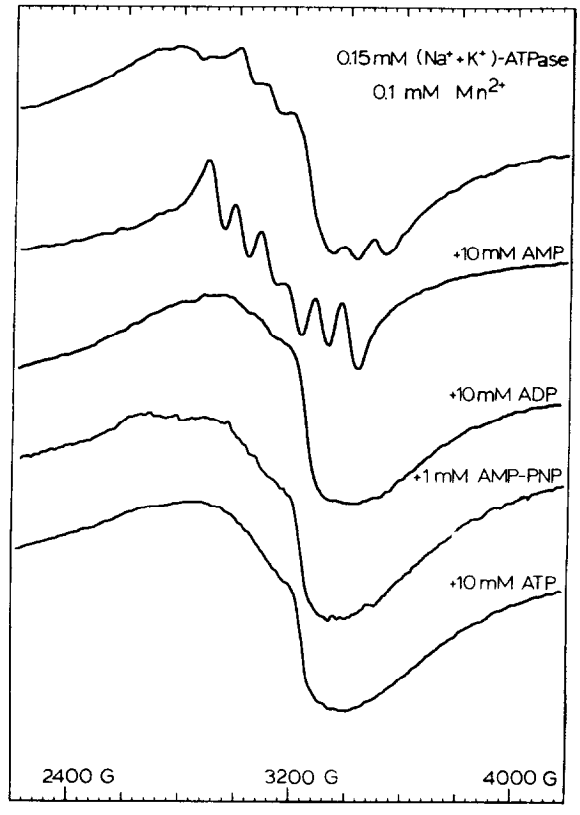

Figure 1. X-band EPR spectra for Mn^{2+} complexes of $(Na^+ + K^+)$-ATPase. All solutions contained 20 mM TES-TMA, pH 7.5, 0.15 mM ATPase, 0.1 mM $MnCl_2$, and the indicated concentrations of the substrates shown. T = 23°C.

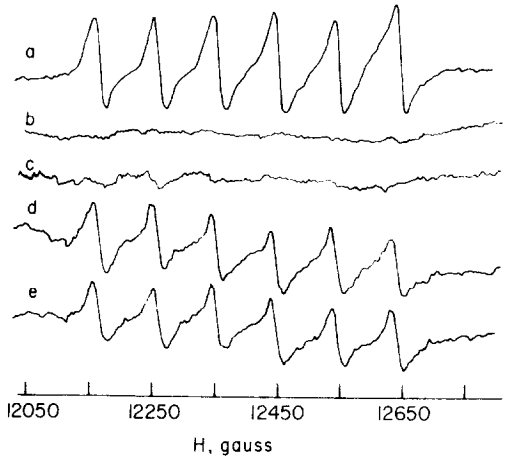

Figure 2. 35 GHz EPR spectra for Mn^{2+} complexes of $(Na^+ + K^+)$-ATPase. Conditions were the same as in Figure 1, with a) no additions, b) 5 mM ATP, c) 5 mM AMP-PNP, d) 5 mM ADP and e) 5 mM AMP.

However, ATP and AMP-PNP both effect a dramatic decrease in intensity of the spectrum. When the system is saturated with either of these latter agents, the resulting Mn^{2+} spectrum is essentially unobservable at the same conditions of concentration and spectrometer gain. The magnitude of this change at 35 GHz is unusual in the literature of Mn^{2+}-protein interactions. The spectra are similar to those observed by Villafranca et al. (7) for complexes of glutamine synthetase-Mn^{2+}-methionine (SR)-sulfoximine-Mg^{2+}-ATP, but even in this latter case, the broadened Mn^{2+} spectrum is still sufficiently narrow to be observed.

The spectra for the Mn^{2+}-ATPase complexes with ATP or AMP-PNP suggest that Mn^{2+} is in a greatly distorted environment. If the substrate-induced distortions were purely axial, a doublet pattern of splitting would be expected for each $-\frac{1}{2} \leftrightarrow \frac{1}{2}$ transition at 35 GHz, whereas more complex effects are anticipated for rhombic distortions (7). While the spectra for the Mn^{2+}-ATPase-(ATP) and Mn^{2+}-ATPase-(AMP-PNP) complexes demonstrate that the bound Mn^{2+} is in a rhombic environment, more detailed analyses are not possible with the present data.

An estimate of the dissociation constant for ATP at the site involved in the effects on the Mn^{2+} spectrum would facilitate comparisons of structural and mechanistic phenomena in this system. Various studies have provided evidence for both high and low affinity sites for ATP on the $(Na^+ + K^+)$-ATPase (3,8). In all cases the apparent binding constant for the high affintiy site is approximately 1×10^{-6} M, while that of the low affinity site is 500-1000 times higher. While the titration behavior of the ATP effects on the Mn^{2+} spectrum of this enzyme will be described more completely in future communications, it should be pointed out that no appreciable change in the

spectrum is detected at levels of ATP as high as 80 x 10^{-6} M. This observation essentially rules out high affinity sites for ATP as the source of the Mn^{2+} EPR spectral changes.

Two conclusions can be drawn from the spectra of Figure 2 at this point. First, the terminal or γ-phosphate of ATP must be intimately related to the large distortion of the Mn^{2+} geometry observed at 35 GHz with ATP, since AMP-PNP shows the same effect as ATP, while ADP leaves the Mn^{2+} 35 GHz spectrum relatively unaffected. AMP-PNP and ADP have both been shown to be competitive inhibitors of the $(Na^+ + K^+)$-ATPase(8), AMP-PNP being a nonhydrolyzable substrate analogue, while ADP is a product of the ATPase reaction. Since both inhibitors would be expected to interact with binding sites on the enzyme in a similar fashion, the simplest conclusion would be that the γ-phosphate of ATP is essential to the distortion of the Mn^{2+} site on the enzyme.

The second conclusion which can be drawn from Figure 2 is that, whatever the nature of the ATPase-Mn^{2+}-ATP complex (or the ATPase-Mn^{2+}-(AMP-PNP) complex, the Mn^{2+} ion has not been drawn into a M^{2+}-ATP complex of the type observed for enzymes which use M^{2+}-ATP as a substrate and which form enzyme-ATP-M^{2+} bridge complexes. For these latter enzymes, the EPR spectrum of the ternary complex is usually identical (or nearly identical) with that of the binary Mn^{2+}-nucleotide complex (9,10). Our EPR results do not rule out the possibility that a second divalent cation may interact with the nucleotide in an ATPase-M^{2+}-ATP-M^{2+} complex. Rather, the Mn^{2+} EPR data at 9.1 GHz and 35 GHz suggest that the single, tight divalent cation enzyme is preserved in the ternary complex, and probably enters into an ATPase-Mn^{2+}-ATP bridge complex. In order to more fully

characterize this complex, we have employed the Co(III) and Cr(III) complexes of ATP in a series of kinetic and magnetic resonance studies with $(Na^+ + K^+)$-ATPase. This work will be the subject of future communications.

In view of the similarity of these spectral changes (induced in this case by diamagnetic substrate analogues) and the decreases in signal intensity which can be caused by adjacent paramagnetic species (the so-called Leigh effect (11)), it is worth pointing out that proper control experiments must be pursued by workers using the Leigh effect to estimate metal-metal or metal-substrate distances in macromolecular systems.

This work was supported by NIH Grants AM19419 and AM00613, Petroleum Research Fund Grant 8757G-4, administered by the American Chemical Society, and grants from the Research Corporation, the Muscular Dystrophy Association of America and the University of Virginia, as well as a grant from the National Science Foundation for the purchase of an EPR spectrometer and computer system.

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