Brockman, H. L. (1984) in *Lipases* (Borgstrom, B., & Brockman, H. L., Eds.) pp 3-46, Elsevier, Amsterdam.
Foster, D. M., & Berman, M. (1981) *J. Lipid Res. 22*, 506-513.
Freed, L. M., York, C. M., Hamosh, M., Sturman, J. A., & Hamosh, P. (1986) *Biochim. Biophys. Acta 878*, 209-215.
Freudenberg, E. (1966) *Experientia 22*, 317.
Hernell, O., & Olivecrona, T. (1974) *Biochim. Biophys. Acta 369*, 234-244.
Hofman, A. F., & Small, D. M. (1967) *Annu. Rev. Med. 18*, 333-376.
Jensen, R. G., Tardiff, G. S., & Clark, R. M. (1985) *J. Pediatr. Gastroenterol. Nutr. 4*, 580-582.
Mattson, F. H., & Volpenhein, R. A. (1962) *J. Lipid Res. 3*, 281-296.
O'Connor, C. J., & Wallace, R. G. (1985) *J. Pediatr. Gastroenterol. Nutr. 4*, 587-590.
O'Connor, C. J., Walde, P., & Wallace, R. G. (1986) *J. Pediatr. Gastroenterol. Nutr. 5*, 622-629.
Olivecrona, T., & Bergtsson, G. (1984) in *Lipases* (Borgstrom, B., & Brockman, H. L., Eds.) pp 205-261, Elsevier, Amsterdam.
Ralston, M. L., & Jennrich, R. I. (1978) *Technomet. 20*, 7-14.
Schotz, M. C., Garfinkel, A. S., Huebotter, R. J., & Stewart, J. E. (1970) *J. Lipid Res. 11*, 68-69.
Segel, I. H. (1975) *Enzyme Kinetics*, pp 227-231, Wiley, New York.
Wang, C.-S. (1981) *J. Biol. Chem. 256*, 10198-10202.
Wang, C.-S., & Smith, R. L. (1975) *Anal. Biochem. 63*, 414-417.
Wang, C.-S., & Johnson, K. (1983) *Anal. Biochem. 133*, 457-461.
Wang, C.-S., & Kloer, H. U. (1983) *Biochim. Biophys. Acta 754*, 142-149.
Wang, C.-S., & Lee, D. M. (1985) *J. Lipid Res. 26*, 824-830.
Wang, C.-S., Kuksis, A., Manganaro, F., Myher, J. J., Downs, D., & Bass, H. B. (1983) *J. Biol. Chem. 258*, 9197-9202.
Wang, C.-S., Hartsuck, J. A., & Weiser, D. (1985) *Biochim. Biophys. Acta 837*, 111-118.
Wilson, E. B. (1952) in *An Introduction to Scientific Research*, pp 272-274, McGraw-Hill, New York.

^{1}H Nuclear Magnetic Resonance Studies of the Conformation of an ATP Analogue at the Active Site of Na,K-ATPase from Kidney Medulla†

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Received October 21, 1987; Revised Manuscript Received January 12, 1988

ABSTRACT: ^{1}H nuclear magnetic relaxation measurements have been used to determine the three-dimensional conformation of an ATP analogue, $Co(NH_3)_4ATP$, at the active site of sheep kidney Na,K-ATPase. Previous studies have shown that $Co(NH_3)_4ATP$ is a competitive inhibitor with respect to MnATP for the Na,K-ATPase [Klevickis, C., & Grisham, C. M. (1982) *Biochemistry 21*, 6979; Gantzer, M. L., Klevickis, C., & Grisham, C. M. (1982) *Biochemistry 21*, 4083] and that Mn^{2+} bound to a single, high-affinity site on the ATPase can be an effective paramagnetic probe for nuclear relaxation studies of the Na,K-ATPase [O'Connor, S. E., & Grisham, C. M. (1979) *Biochemistry 18*, 2315]. From the paramagnetic effect of Mn^{2+} bound to the ATPase on the longitudinal relaxation rates of the protons of $Co(NH_3)_4ATP$ at the substrate site (at 300 and 361 MHz), Mn-H distances to seven protons on the bound nucleotide were determined. Taken together with previous ^{31}P nuclear relaxation data, these measurements are consistent with a single nucleotide conformation at the active site. The nucleotide adopts a bent configuration, in which the triphosphate chain lies nearly parallel to the adenine moiety. The glycosidic torsion angle is 35°, and the conformation of the ribose ring is slightly N-type ($C_{2'}$-exo, $C_{3'}$-endo). The δ and γ torsional angles in this conformation are 100° and 178°, respectively. The bound Mn^{2+} lies above and in the plane of the adenine ring. The distances from Mn^{2+} to N_6 and N_7 are too large for first coordination sphere complexes but are appropriate for second-sphere complexes involving, for example, intervening hydrogen-bonded water molecules. The NMR data also indicate that the structure of the bound ATP analogue is independent of the conformational state of the enzyme.

Sodium and potassium ion activated adenosinetriphosphatase, or Na,K-ATPase[1] (EC 3.6.1.3, ATP phosphohydrolase), is an integral membrane protein that couples the hydrolysis of ATP to the vectorial transport of sodium and potassium ions across the plasma membrane. It is important to determine the mechanism of energy coupling in this system. Our approach to this problem has been to attempt to determine

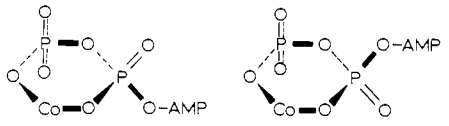
†Supported by National Institutes of Health Research Grant AM19419, a grant from the Muscular Dystrophy Association of America, and a grant from the National Science Foundation. The NMR instrumentation used in these studies was provided by grants from the National Science Foundation and the University of Virginia. C.M.G. is a Research Career Development Awardee of the U.S. Public Health Science (Grant AM00613).

[1] Abbreviations: Na,K-ATPase, sodium and potassium ion activated adenosinetriphosphatase; CoATP, β,γ-bidentate $Co(NH_3)_4ATP$; TMA, tetramethylammonium; Tes, 2-[[tris(hydroxymethyl)methyl]amino]-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

0006-2960/88/0427-4840$01.50/0

the active site structure of this enzyme and then to evaluate possible energy-coupling mechanisms within the context of the established structure. There are precious few physical techniques that can provide the kind of information needed for this type of approach. Magnetic resonance spectroscopy provides some of the most quantitative and powerful methods for such studies, and we have applied a number of these techniques to the study of the Na,K-ATPase active site structure. For example, we have characterized binding sites for sodium and potassium using $^{205}Tl^+$ and $^7Li^+$ NMR (Grisham et al., 1974; Grisham & Hutton, 1978) and a phosphate site by ^{31}P NMR (Grisham & Mildvan, 1975). We have characterized the existence and catalytic efficacy of a single divalent cation site on the enzyme in the absence of ATP and located this site with respect to the above mentioned sodium, potassium, and phosphate sites (Grisham & Mildvan, 1974; O'Connor & Grisham, 1979).

Several years ago, we showed that the β,γ-bidentate complexes of substitution-inert Co(III) and Cr(III) and ATP can be effective analogues of Mg^{2+}-ATP with this and other ATPases (O'Connor & Grisham, 1980; Grisham, 1981; Gantzer et al., 1982).



Δ-Co(NH$_3$)$_4$ATP Λ-Co(NH$_3$)$_4$ATP

CoATP and CrATP have been shown to be linear competitive inhibitors with respect to MnATP under a variety of conditions (Gantzer et al., 1982). A unique consequence of the use of these analogues is the fact that the tight metal coordinating site on the ATP substrate is completely occupied and, at the same time, any other metal binding sites on the enzyme are left empty and can then be filled selectively by the addition of another suitable metal. Using these complexes, we have previously established the existence of two divalent cation sites at the active site of the Na,K-ATPase (Grisham, 1981) and shown that these two sites are 8.1 ± 0.5 Å apart, as measured by Mn^{2+} EPR (O'Connor & Grisham, 1980) and water proton NMR (A. O'Neal and C. Grisham, unpublished data). In these studies the Co(III) or Cr(III) of the metal–ATP analogues occupies the Mg^{2+} site on the ATP substrate, while Mn^{2+} is bound to the Mg^{2+} site on the enzyme that is involved in the activation of the ATPase (Grisham & Mildvan, 1974; O'Connor & Grisham, 1979; Robinson, 1981). This latter site binds Mn^{2+} with a very high affinity (K_D = 0.2–0.3 μM) and exists in the absence of the ATP substrate. We have previously characterized the locations of the Na^+, K^+, and phosphate sites with respect to these two divalent metal sites (Grisham et al., 1974; Grisham & Mildvan, 1975; Grisham & Hutton, 1978).

The studies summarized above are valuable in that they provide a foundation for further studies of the active site structure of the ATPase. In a series of ^{31}P NMR studies of Co(NH$_3$)$_4$ATP in solutions of the ATPase, we determined distances from Mn^{2+} at the activating metal site to all three phosphorus nuclei of the bound substrate. The obvious extension of this work would be to attempt to determine the conformation of the ribose and adenine moieties of ATP bound to the ATPase. We have recently initiated a series of ^1H NMR studies of Co(NH$_3$)$_4$ATP and the Na,K-ATPase with this in mind. In this paper, we describe the results of this work, including a comparison between experiments performed in NaCl vs KCl solutions. The results are consistent with an anti conformation (with a glycosidic torsion angle χ = 35°) and a slightly $C_{3'}$-endo ribose ring pucker for enzyme-bound Co(NH$_3$)$_4$ATP.

Experimental Procedures

Materials. The β,γ-bidentate complex of Co(NH$_3$)$_4$ATP was prepared as described by Cornelius et al. (1977). Deuterium oxide (99.8 atom %) was from the Aldrich Chemical Co., Tris-d_{11} was from MSD Isotopes, and manganese chloride was from Baker Chemicals. Adenosine 5′-triphosphate was from Sigma Chemical Co. All other reagents were of the highest purity available commercially.

Enzyme Preparation. Kidneys obtained from freshly exsanguinated sheep at Rocco Further Processing, Inc., Timberville, VA, were frozen in dry ice and stored at −20 °C. The Na,K-ATPase was purified from the outer medulla of these kidneys as previously described (O'Connor & Grisham, 1979). The ATPase activity was measured by the continuous method (Barnett, 1970), and only enzyme with a specific activity of more than 20 μmol of ATP hydrolyzed per milligram of protein per minute was used in these experiments. Protein concentration was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Solutions. In each 5-mm NMR tube, in a total volume of 600 μL, there were present 3.0 mg/mL Na,K-ATPase, 10 mM β,γ-bidentate Co(NH$_3$)$_4$ATP, 15 mM Tris-d_{11} (pH 7.5 at 5 °C and 15 mM), 150 mM KCl or NaCl, and 0–5 μM $MnCl_2$. The water present was greater than 99% D_2O. The Mn^{2+} titrations were carried out at 4 °C.

All stock solutions, except the ATPase and $MnCl_2$, were rigorously treated with Chelex 100 to remove divalent cations. The ATPase and Co(NH$_3$)$_4$ATP solutions were stored at −20 °C, while all other solutions were stored in a desiccator at 4 °C until use.

Stock D_2O–Chelex was prepared by repeated suction filtrations and dilutions of Chelex 100 with 99.8% D_2O and lyophilization. Stock D_2O was prepared by column treatment of D_2O with D_2O–Chelex.

Tris buffers for centrifugation of Na,K-ATPase were prepared by dissolving Trizma 7.0 (Sigma, T3503) into D_2O; 15 mM solutions, which had a pH of 7.5 ± 0.05 at 5 °C, were lyophilized and then stored in a desiccator at 4 °C. Tris-d_{11} buffers were similarly prepared by lyophilization. Stock Co(NH$_3$)$_4$ATP was prepared by repeated rotary evaporation, replacing lost water with D_2O.

The Na,K-ATPase was prepared for the NMR experiments by diluting 1.8 mg of enzyme in 15 mL of an ice-cold solution of 10 mM Tris, pH 7.5, in D_2O and centrifuging at 25000g for 30 min at 4 °C. The pellet was resuspended in a small volume of ice-cold D_2O, buffered by Tris-d_{11}.

Nuclear Magnetic Resonance Experiments. Proton NMR spectra were obtained at two frequencies, 361 MHz on a Nicolet Magnetics Corp. NT-360/Oxford spectrometer equipped with a 1200/293B data system and 300 MHz on a General Electric QN-300 spectrometer. The spectra were measured at 4 °C with a 5-mm proton probe and deuterium lock. The 90° pulse width was typically 7.50 μs at 361 MHz and 10.0 μs at 300 MHz. The HDO signal was decoupled during delays prior to the pulse sequences.

Longitudinal relaxation times were determined from an optimized inversion–recovery experiment (Cutnell et al., 1976; Freeman et al., 1980), and from 16 to 20 τ values were used in the 180–τ–90 sequence. T_1 values and their standard deviations were calculated by using a three-parameter fit to the data. This fitting routine corrects for inhomogeneities in the H_1 field that produce incomplete inversion during the 180° pulse (Levy & Peat, 1976).

Each point in the titration with Mn^{2+} was measured at both 300 and 361 MHz, and 16 accumulations of 8K data points were collected per τ value.

Paramagnetic contributions to the longitudinal relaxation rates of the protons of $Co(NH_3)_4ATP$ were calculated with the program NONLIN (copyright M. L. Johnson), an interactive nonlinear function minimization program that performs a weighted least-squares fit of the data points to a specified function (in this case a straight line) by a modified Gauss–Newton iteration. In the experiments involving high NaCl or high KCl concentrations, weighted averages of the slopes of the $1/T_1$ vs Mn^{2+} titrations for each proton determined the mean $1/T_{1p}$ and its uncertainty.

Conformational analysis was done on a Silicon Graphics computer using the program MMS (copyright UCSD). The basic structure of $Co(NH_3)_4ATP$ was constructed from the X-ray data of rubidium adenosine 5′-diphosphate monohydrate (Viswamitra et al., 1976) and $Co(NH_3)_4H_2P_3O_{10}\cdot H_2O$ (Merritt et al., 1978). The conformation of the enzyme-bound CoATP was then determined by using the distances calculated from the NMR data.

Theory. Calculations of Mn^{2+}–proton distances are based on the theory of Solomon and Bloembergen (Solomon, 1955; Solomon & Bloembergen, 1956; Bloembergen, 1957; Bernheim et al., 1959) for the paramagnetic effects on the nuclear spin relaxation rates of a magnetic nucleus that is bound near a paramagnetic species. In general, the paramagnetic contribution to the longitudinal relaxation rate, $1/T_{1p}$, is described by the equation

$$1/T_{1p} = pq/(T_{1M} + \tau_M) + 1/T_{1(os)} \quad (1)$$

where p is the ratio of the concentration of paramagnetic ion to ligand, q is the number of ligands in the first coordination sphere, τ_M is the residence time of the ligand in the coordination sphere of the paramagnetic ion, T_{1M} is the relaxation time in the first coordination sphere, and $1/T_{1(os)}$ is the outer-sphere contribution to the relaxation rate.

In the limit of fast exchange, when the rate of chemical exchange of the ligand between the coordination sphere and the bulk solvent is faster than the rate of relaxation in the first coordination sphere (i.e., when $1/\tau_M \gg 1/T_{1M}$), and assuming outer-sphere contributions are negligible, eq 1 reduces to

$$1/T_{1p} = pq/T_{1M} \quad (2)$$

The Solomon–Bloembergen equation that describes the dipolar Mn^{2+}–proton interaction is

$$r^6 = 815T_{1M}f(\tau_c) \quad (3)$$

where r is the Mn^{2+}–proton internuclear distance and

$$f(\tau_c) = \frac{3\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{7\tau_c}{1 + \omega_S^2\tau_c^2} \quad (4)$$

where ω_I and ω_S are the nuclear and electronic Larmor frequencies, respectively. τ_c is known as the dipolar correlation time and characterizes the process that modulates the dipolar interaction. In turn, it is described as a function of three time constants by

$$1/\tau_c = 1/\tau_r + 1/\tau_s + 1/\tau_m \quad (5)$$

τ_r characterizes the rotation of the internuclear ion–nucleus radius vector. τ_s is the electron spin relaxation time. τ_m is the residence time of the nuclear species in the first coordination sphere of the paramagnet. The smallest time constant determines the value of τ_c.

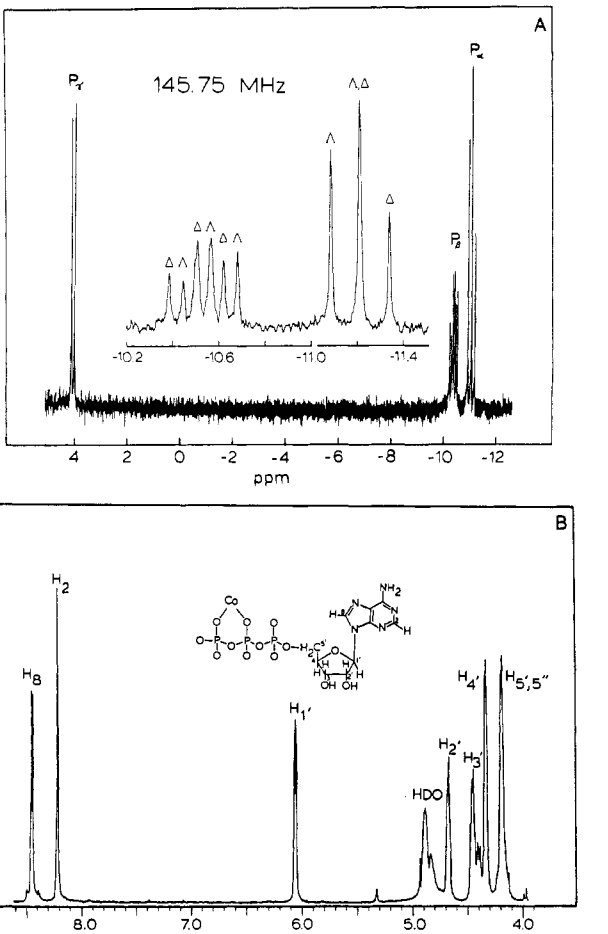


FIGURE 1: (A) Phosphorus-31 nuclear magnetic resonance spectrum of $Co(NH_3)_4ATP$ at 145.75 MHz, pH 7.5, 4 °C. Chemical shifts are relative to 85% phosphoric acid. The solution contained D_2O (40%) for field/frequency locking. (B) Hydrogen-1 NMR spectrum of $Co(NH_3)_4ATP$ at 361 MHz, pH 7.5, 4 °C. Chemical shifts are relative to DSS. The solution contained D_2O (99+%), and the residual water signal was irradiated to reduce its intensity.

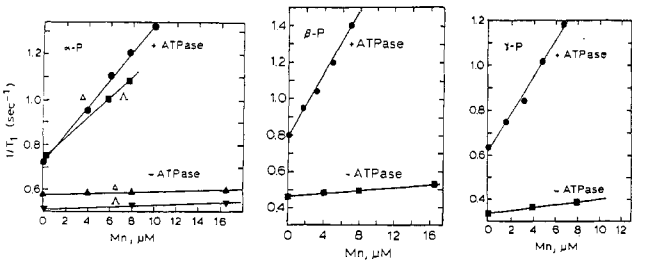


FIGURE 2: Effect of Mn^{2+} on longitudinal relaxation rates of the α-, β-, and γ-phosphorus nuclei of $Co(NH_3)_4ATP$ in the presence and absence of Na,K-ATPase. The solution contained 20 mM $Co(NH_3)_4ATP$, 82 mM Tris, pH 7.4, 100 mM NaCl, 10 mM KCl, and 10 μM ATPase.

RESULTS

^{31}P NMR Studies of the Ternary Mn^{2+}–ATPase–$Co(NH_3)_4ATP$ Complex. ^{31}P nuclear relaxation rates of $Co(NH_3)_4ATP$ were measured in solutions of Mn^{2+} and Na,K-ATPase at 145 MHz. The ^{31}P spectrum of $Co(NH_3)_4ATP$ is shown in Figure 1A. The spectrum consists of superimposed resonances for both the Δ and Λ diastereomers of $Co(NH_3)_4ATP$. The correct assignments of the resonances of the diastereomers in Figure 1A were determined by two-di-

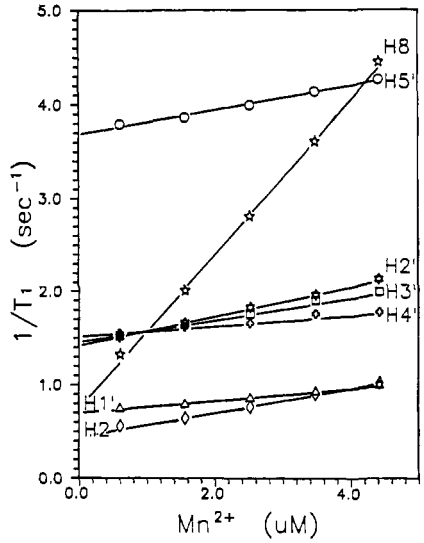


FIGURE 3: Effect of Mn^{2+} on the longitudinal relaxation rates of the protons of $Co(NH_3)_4ATP$ in the presence of Na,K-ATPase. The solution contained 15 mM $Co(NH_3)_4ATP$, 15 mM Tris, pH 7.5, 150 mM NaCl, and 8–10 μM ATPase in 99+% D_2O. The data represent the results of a single experiment. The data of Table I are averages of multiple experiments of this type.

mensional homonuclear chemical shift correlation maps obtained with a Jeener pulse sequence. As shown in Figure 2 and in previous, preliminary studies (Klevickis & Grisham, 1982), the presence of the Na,K-ATPase causes a 6–10-fold enhancement of the effect of Mn^{2+} on the ^{31}P relaxation rates of $Co(NH_3)_4ATP$. The increases in $1/T_1$ relaxation rate for the three phosphorus nuclei of the substrate analogue are not observed in the absence of enzyme and are not observed when diamagnetic Mg^{2+} is added to the enzyme in the place of Mn^{2+}. The dissociation constant for Mn^{2+} ion from the Na,K-ATPase, determined by Mn^{2+} EPR (O'Connor & Grisham, 1979), is 0.2 μM. Given a dissociation constant for the binary Mn^{2+}–$Co(NH_3)_4ATP$ complex of 15 mM (Armstrong et al., 1979), it is easy to show that, under the conditions of these experiments, at least 97% of the added Mn^{2+} was enzyme-bound. Thus, no corrections must be made for the small contribution of the binary complex to the measured paramagnetic relaxation rates (T_{1p}^{-1}). The inescapable conclusion from these data is that Mn^{2+} and $Co(NH_3)_4ATP$ are bound simultaneously in a ternary complex with the Na,K-ATPase. The observed increase in $1/T_1$ could not occur in any other way.

^1H NMR Studies of the Ternary Mn^{2+}–ATPase–Co$(NH_3)_4$ATP Complex. ^1H nuclear relaxation rates of the protons of $Co(NH_3)_4ATP$ were measured in solutions of Mn^{2+} (or Mg^{2+}) and Na,K-ATPase at 361 and 300 MHz. The 361-MHz ^1H NMR spectrum of $Co(NH_3)_4ATP$ is shown in Figure 1B. The resonances are labeled in the conventional manner. In this particular sample, $Co(NH_3)_4ATP$ was dissolved in D_2O (99.8%). The residual HDO signal, which is partially irradiated to reduce its intensity, appears at 4.99 ppm relative to DSS at 4 °C. At 23 °C, it appears at 4.79 ppm relative to DSS. All the NMR experiments described here were performed at 4 °C to ensure enzyme stability. An unexpected benefit of the lower temperature is the downfield shift of the HDO resonance that is sufficient to expose a well-resolved $H_{2'}$ resonance at 361 MHz, which is obscured by the water signal at temperatures above 15 °C. The NH_3 protons of $Co(NH_3)_4ATP$ were exchanged for deuterons by raising the solution to pD 9.0 for a few minutes. Nevertheless, there remains in this spectrum a small signal (the doublet at 4.52 ppm) from the *cis*-NH_3, which exchange more slowly than do the *trans*-NH_3.

As shown in Figure 3, addition of Mn^{2+} to solutions of ATPase and $Co(NH_3)_4ATP$ results in linear increases in $1/T_1$ of all seven proton resonances of the nucleotide analogue. The relative magnitudes of these slopes reflect the degree of interaction of each of the protons of $Co(NH_3)_4ATP$ with bound Mn^{2+}. Thus, it is clear from the data in Figure 3 that the H_8 proton of the nucleotide experiences the greatest interaction with the Mn^{2+} at its site on the enzyme.

In order that these paramagnetic probe experiments might be used to determine internuclear distances at the active site, the assumption of fast exchange must be quantitatively justified. It has been shown (Klevickis & Grisham, 1982) that the paramagnetic contribution to the transverse relaxation rate ($1/T_{2p}$) of the β-phosphorus nucleus of $Co(NH_3)_4ATP$ in the presence of Na,K-ATPase and Mn^{2+} is 23.4×10^4 s^{-1}. In theory, this value sets a lower limit on $1/\tau_M$ that is much greater than the $1/T_{1M}$ values of all the protons of Co$(NH_3)_4ATP$, consistent with fast exchange of $Co(NH_3)_4ATP$ between its site on the enzyme and the solvent. However, it has been argued and successfully demonstrated (Jarori et al., 1985) that $1/T_{2p}$ information is unreliable in establishing limits on the exchange rate, $1/\tau_m$. Temperature studies could shed light on the exchange rate (Mildvan & Cohn, 1970). However, at elevated temperatures, phosphorylation of Na,K-ATPase by CoATP (Scheiner-Bobis et al., 1987) and/or breakdown of CoATP both occur (McClaugherty & Grisham, 1982). In addition, it is physically impossible at present to make very high concentrations (1 mM) of Na,K-ATPase, so that these experiments must necessarily be conducted at high CoATP/ATPase ratios. Jarori et al. (1985) indicate that such conditions will likely be detrimental to a correct interpretation of temperature dependence data. Another possibility is to use a different, weaker paramagnetic probe in place of Mn^{2+}. Attempts were made in these studies to use Co^{2+} for such purposes, but the results were highly ambiguous, because Co^{2+} binds very weakly to the high-affinity divalent cation site on the ATPase and complications arise from binding of Co^{2+} to other sites (including metal binding sites on the lipid membrane). Clearly, the most straightforward method for determining the exchange regime of the experiment is to analyze the frequency dependence of the paramagnetic relaxation by Mn^{2+}.

As can be seen in eq 3 and 4 above, T_{1M} depends on the frequency at which the paramagnetic effect is measured. However, the rate of chemical exchange, $1/\tau_m$, is independent of the frequency at which the experiment is performed.

In what follows, the ratio of the paramagnetic effects for a given resonance at two frequencies will be described by the symbol η. We shall use A to describe the same ratio, but in the condition of an infinite-exchange rate. At frequencies of 300 and 361 MHz, η and A are defined mathematically by

$$\eta \equiv (1/T_{1p})^{300}/(1/T_{1p})^{361} \tag{6}$$

and, assuming the second term of eq 4 to be negligible

$$A \equiv \frac{(1/T_{1M})^{300}}{(1/T_{1M})^{361}} \equiv \frac{1 + (\omega_{361}\tau_c)^2}{1 + (\omega_{300}\tau_c)^2} \tag{7}$$

Note that, for these frequencies, $1 \leq A \leq 1.44$, and the exact value of A is determined by τ_c.

There are only three possible outcomes for this experiment: (1) $\eta = 1$, and therefore the resonance is totally exchange limited ($\tau_m \gg T_{1m}$; see eq 1); (2) $\eta = A$ and $A > 1$ for all measured resonances of the substrate, and therefore the sub-

Table I: Paramagnetic Contributions to Relaxation Rates at 300 and 361 MHz for Protons of $Co(NH_3)_4ATP$ and Mn^{2+}–H Distances in the ATPase-Bound Mn^{2+}–$Co(NH_3)_4ATP$ Complex

	H_8	H_2	$H_{1'}$	$H_{2'}$	$H_{3'}$	$H_{4'}$	$H_{5',5''}$
$[1/f(T_{1p})]^{300}$ (s^{-1})	8522 ± 104	1323 ± 20	708 ± 11	1530 ± 33	1198 ± 35	623 ± 27	715 ± 29
$[1/f(T_{1p})]^{361}$ (s^{-1})	8060 ± 62	1103 ± 13	565 ± 19	1170 ± 36	929 ± 11	517 ± 15	615 ± 18
$(1/T_{1p})^{300}/(1/T_{1p})^{361}$	1.092 ± 0.036	1.202 ± 0.023	1.249 ± 0.053	1.308 ± 0.100	1.207 ± 0.048	1.252 ± 0.083	1.235 ± 0.068
$1/T_{1M}$[a] (s^{-1})	17000–37600	1410–1530	730–770	1650–1810	1250–1390	620–690	710–800
r_{Mn-H}[b] (Å)	4.3–4.9	7.4 ± 0.1	8.2 ± 0.1	7.2 ± 0.1	7.5 ± 0.1	8.4 ± 0.1	8.2[c]

[a] Using $1/\tau_M = 1.395\ (\pm 0.275) \times 10^4\ s^{-1}$, as determined in graph; reported by range of values. [b] Using $\tau_c = 5.47\ (\pm 0.40) \times 10^{-10}$ s, as determined in graph. [c] Only an approximate distance due to orientational uncertainties.

Table II: Comparison of Paramagnetic Relaxation Rates of CoATP in Solutions of Na,K-ATPase and NaCl or KCl

	Na^+		K^+		av	
	$1/T_{1p}$[a] (s^{-1})	η[b]	$1/T_{1p}$ (s^{-1})	η	$1/T_{1p}$ (s^{-1})	η
H_8	8634 ± 117	1.104 ± 0.059	8085 ± 230	1.084 ± 0.045	8522 ± 104	1.092 ± 0.036
H_2	1380 ± 35	1.170 ± 0.032	1274 ± 26	1.232 ± 0.032	1323 ± 20	1.202 ± 0.023
$H_{1'}$	753 ± 25	1.266 ± 0.140	696 ± 12	1.242 ± 0.058	708 ± 11	1.249 ± 0.053
$H_{3'}$	1287 ± 66	1.149 ± 0.066	1164 ± 41	1.268 ± 0.068	1198 ± 35	1.207 ± 0.048
$H_{4'}$	640 ± 36	1.273 ± 0.102	600 ± 42	1.212 ± 0.142	623 ± 27	1.252 ± 0.083
$H_{5',5''}$	810 ± 68	1.310 ± 0.130	681 ± 34	1.207 ± 0.080	715 ± 29	1.235 ± 0.068

[a] Value at 300 MHz. [b] η is defined in eq 6 (text).

strate is in fast exchange ($\tau_m \ll T_{1m}$); (3) $1 < \eta < A$, for some resonances, and therefore the substrate is partially exchange limited ($\tau_m \approx T_{1m}$).

The data from these experiments are summarized in Table I. By the criteria outlined above, the results of Table I clearly indicate that the proton resonances of CoATP are partially exchange limited in the presence of Na,K-ATPase.

By combining eq 1, 4, 6, and 7 and assuming no field-dependent outer-sphere contributions, an equation can be derived that proves to be very useful in situations where the resonances of interest are partially exchange limited.

$$\eta = (1 - A)\tau_m(1/pqT_{1p})^{300} + A \qquad (8)$$

In a plot of η vs $(1/pqT_{1p})^{300}$, the y intercept is A, from which τ_c can be easily calculated from eq 7. The exchange rate is the value of x when y is set equal to 1. Values of both τ_c and τ_m are essential for distance calculations.

The data of Table I are plotted in Figure 4, representing averaged data from five experiments. It should be noted that, whereas eq 8 describes a linear relationship between η and $(1/pqT_{1p})^{300}$, the only "points" which may be plotted in Figure 4 correspond to the individual nuclei of $Co(NH_3)_4ATP$. Since the H_8 proton is the only proton that gives evidence of being exchange limited, the plot has the appearance of being heavily dependent on the placement of the point for H_8. On the other hand, the data for the H_8 proton were highly reproducible, and the plot in Figure 4 may thus be regarded as providing at least a useful estimate of the exchange rate and dipolar correlation time. The line fitted by NONLIN yields a value for A of 1.231 ± 0.013, from which a correlation time is calculated to be 5.47 (±0.40) × 10^{-10} s. The exchange rate is determined to be 1.395 (±0.275) × 10^4 s^{-1}.

Knowing τ_m, it is possible to calculate T_{1M} from eq 1. Knowing τ_c and T_{1M}, it is possible to calculate Mn^{2+}–H distances at the active site of the Na,K-ATPase for the seven resonances of $Co(NH_3)_4ATP$. These results are also shown in Table I.

Comparison of Conformational States of the Na,K-ATPase. It is well established that, in the absence of phosphorylation, the E_1 conformation of the enzyme predominates in solutions high in Na^+ ions, while E_2 is the primary conformation in the presence of high levels of K^+ ions. We have performed paramagnetic relaxation studies in solutions that differ only in the particular monovalent cation present in an attempt to distinguish between Na^+ and K^+ conformations of the enzyme. The data of the three Na^+ and two K^+ experiments are shown in Table II. The results indicate no differences in the conformation of bound CoATP or in its rate of exchange on and off Na,K-ATPase when 150 mM K^+ is replaced by 150 mM Na^+. Thus, the structure of ATP bound to the Na,K-ATPase is independent of the conformational state of the enzyme, at least at the resolution of these NMR experiments.

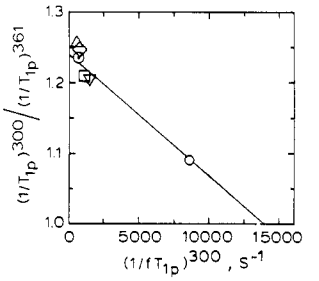


FIGURE 4: Plot of the quantity $(1/T_{1p})^{300}/(1/T_{1p})^{361}$ vs $[1/f(T_{1p})]^{300}$ for the relaxation data of Table I. The exchange rate of CoATP on and off the ATPase is obtained from the x intercept according to eq 8 in the text. Data are shown for the H_8 (○), H_2 (▽), $H_{1'}$ (◇), $H_{3'}$ (□), $H_{4'}$ (△), and $H_{5',5''}$ (⊙) protons.

Table III: Reevaluation of Phosphorus-31 Paramagnetic Relaxation Data for CoATP Bound to Na,K-ATPase

	$1/f(T_{1p})$[a] (s^{-1})	$1/T_{1M}$[b] (s^{-1})	r[c]
P_α (Δ)	820 ± 60	800–960	6.4 ± 0.1
P_α (Λ)	500 ± 40	470–570	7.0 ± 0.1
P_β (Δ, Λ)	1670 ± 100	1750–2100	5.6 ± 0.1
P_γ (Δ, Λ)	1670 ± 80	1760–2070	5.6 ± 0.1

[a] Klevickis and Grisham (1982). [b] Using $1/\tau_M = 1.395\ (\pm 0.275) \times 10^4\ s^{-1}$. [c] Using $\tau_c = 5.47\ (\pm 0.40) \times 10^{-10}$ s.

DISCUSSION

The data of Figure 2 provide compelling evidence that a ternary Mn^{2+}–$Co(NH_3)_4ATP$–enzyme complex is formed under the conditions of these NMR experiments. Previous kinetic studies (Grisham et al., 1974; Robinson, 1981; Gantzer et al., 1982) and spectroscopic data (O'Connor & Grisham, 1979, 1980; Klevickis & Grisham, 1982) are consistent with the premise that Mn^{2+} and $Co(NH_3)_4ATP$ bind to relevant catalytic sites in this complex and that the Mn^{2+} site is in fact close to the nucleotide site. In such a case, the data of Table I should provide information on the conformation of the ATP

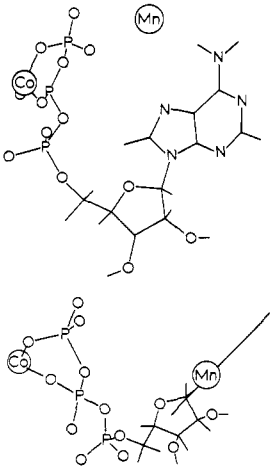


FIGURE 5: Conformation of $Co(NH_3)_4ATP$ and the enzyme-bound Mn^{2+} ion at the active site of kidney Na,K-ATPase. The structure was derived on a Silicon Graphics work station using MMS software with the distances determined from the magnetic resonance experiments. The Mn^{2+}–Co^{3+} distance is taken as that obtained with Cr^{3+} by Mn^{2+} EPR (O'Connor & Grisham, 1980). All other distances were determined from the paramagnetic relaxation data presented here. These latter distances were calculated by using a dipolar correlation time of 5.47×10^{-10} s.

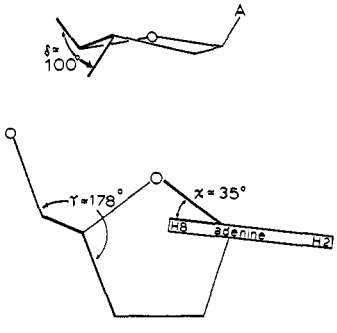


FIGURE 6: Two views of the ribose ring of CoATP bound to the Na,K-ATPase. The structure is derived from the distances calculated from the paramagnetic relaxation measurements in Table I. The torsional angles χ, δ, and γ are shown for the bound CoATP.

site at the active site of the Na,K-ATPase. The data of Table I, together with the previous ^{31}P NMR data (Klevickis & Grisham, 1982) presented in Table III (with new values for $1/T_{1M}$ and distances, r, based on the exchange rate determined herein), are consistent with a model for the active site in which the nucleotide adopts a bent or folded conformation and the Mn^{2+} is above and approximately planar with the adenine ring. This model, developed on a Silicon Graphics graphic work station using the distances calculated in the present study, is shown in Figure 5. The conformation of the $Co(NH_3)_4ATP$ has the glycosidic bond linking the adenine base to the ribose sugar in the anti conformation. The sequence of atoms defining the angle is $O_{4'}$–$C_{1'}$–N_9–C_8. The torsional angle about the $C_{1'}$–N_9 bond that links base to sugar is denoted by χ. The term anti refers to the values of χ in the range of $0 \pm 90°$. Syn refers to angles of $180 \pm 90°$. The value of χ determined from the model built from the data of Table I is 35°. The anti configuration is much more generally found in purine nucleotides and their complexes in the crystalline state as well as in solution. As shown in Figure 6, the conformation of the ribose ring is slightly N-type (in which $C_{2'}$ is exo and $C_{3'}$ is endo). In solution, purine ribosides normally show a small conformation preference for the S-type conformer (in which $C_{2'}$ is endo and $C_{3'}$ is exo) (Altona & Sundaralingam, 1973). The torsional angle δ is indicative of the puckering in the ribose and is defined by the atom sequence $C_{5'}$–$C_{4'}$–$C_{3'}$–$O_{3'}$ (Saenger, 1984). The orientation of $O_{5'}$ relative to the ribose ring is determined by the torsional angle γ, defined by the sequence $O_{5'}$–$C_{5'}$–$C_{4'}$–$C_{3'}$ (Saenger, 1984). The value measured in the present model is 178°, a typical value for protein-bound MgATP (Fry et al., 1985). The conformation of ATPase-bound CoATP in terms of χ, δ, and γ is described in Figure 6.

It should be noted that the absolute Mn^{2+}–proton distances depend on knowledge of the dipolar correlation time (see eq 3 and 4). The dipolar correlation time determined in this experiment is $5.47 (\pm 0.40) \times 10^{-10}$ s. The 8% error in τ_c, determined from the uncertainty in the y intercept of Figure 4, becomes negligible in the determination of $f(\tau_c)$, the correlation function, since $1 \gg \omega^2\tau^2$. Accurate knowledge of the correlation time permits the calculation of very accurate distances, since r is inversely proportional to the sixth root of the correlation function. Several correlation times have previously been reported for this system (Klevickis & Grisham, 1982). The present value is very close to the value of 4.0×10^{-10} s, calculated from the frequency dependence, measured at 40 and 145.8 MHz, of the paramagnetic effect on the phosphorus nuclei of CoATP, under the assumption of no frequency dependence of τ_c itself (Klevickis & Grisham, 1982). The agreement between these two correlation times and the narrow range of frequency used in the present work permit accurate determination of proton to Mn^{2+} distances, assuming accurate values for T_{1M}.

In the limit of fast exchange, the measurement of T_{1M} is simple. Unfortunately, Na,K-ATPase does not exchange CoATP fast enough for a straightforward analysis. However, it is the partially exchange-limited nature of the system that enables us to measure the exchange rate, by eq 8, and the value of τ_M thus obtained facilitates the calculation of T_{1M}. Uncertainties in τ_M affect the determination of T_{1M}, especially for small values of T_{1M}. This is clearly seen in Table I in the case of the H_8 proton. Because τ_M affects determination of short distances preferentially, uncertainty in τ_M affects the relative proton–Mn^{2+} distances, whereas uncertainty in τ_c affects the absolute distances.

An important assumption made in the previous phosphorus-31 NMR studies of this enzyme was that the Δ and Λ isomers of CoATP, present in equal amounts, bind to Na,K-ATPase with equal affinity. That both bind is demonstrated by the effect of protein-bound Mn^{2+} ion on the relaxation of the resolvable Δ and Λ resonances of the α-phosphorus. In fact, a significant difference in $1/T_{1p}$ was observed. That difference, however, cannot be ascribed to different binding affinities for Δ and Λ isomers. The exchange rate determined in this experiment, which must represent an average of the residence times of the isomers, is much too small to account for the differences in the relaxation behavior of the P_α nuclei. A recalculation of the ^{31}P NMR data, based on the value of τ_c determined in the present work, is shown in Table III. The new calculated distances are not significantly different from those previously reported (Klevickis & Grisham, 1982). It is clear, in any case, that the Mn–P distance differs for P_α of the two diastereomers of CoATP. It may thus be the case that the other Mn–nucleus distances reported here represent averages of conformations for the two diastereomers of CoATP bound to the ATPase.

With the value of τ_m determined here, it is possible to estimate a dissociation constant for the bound CoATP. Since

$$K_D = k_{off}/k_{on}$$

where k_{off} is the off-rate constant and k_{on} is the on-rate constant, assuming an on-rate constant of 10^8 M^{-1} s^{-1} and an

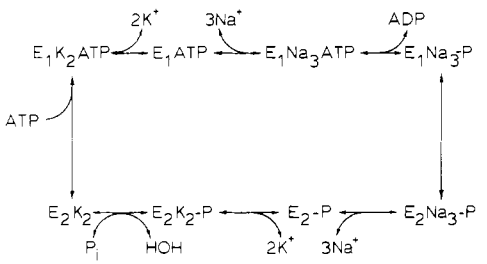


FIGURE 7: Simple mechanism for the Na,K-ATPase similar to several that have been proposed (Jørgensen, 1982).

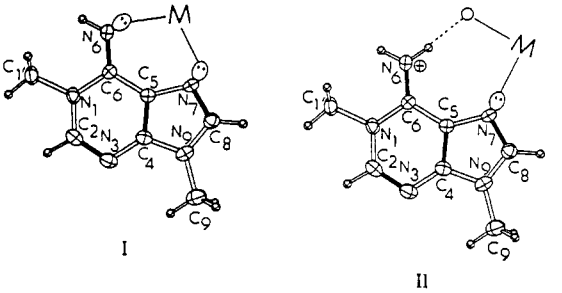


FIGURE 8: Structures described by Chiang et al. (1979) in crystallographic studies of Co(III) complexes of the 1,9-dimethyladeninium cation.

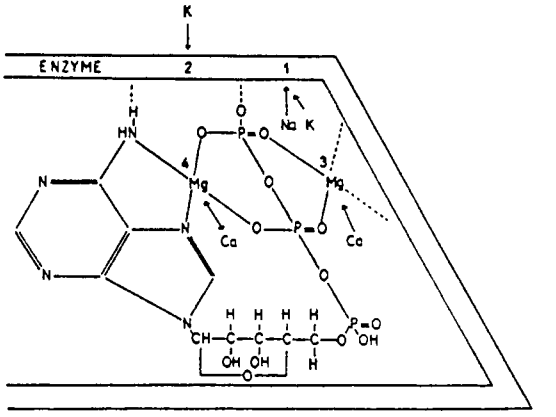


FIGURE 9: Model for the active site structure originally proposed on the basis of kinetic data by Skou (1960).

off-rate constant equal to $1/\tau_M$, a dissociation constant for the "weak site" of 140 μM is calculated for the protein–CoATP complex at 4 °C. Pauls et al. (1986) measured a dissociation constant for the "tight site" Na,K-ATPase–CoATP complex of 500 μM at 37 °C. Gantzer et al. (1982) determined the K_i of CoATP at the tight site to be 10 μM and at the weak site to be 1.6 mM. This is a considerably weaker interaction than for the MgATP (1 and 480 μM) or CrATP complexes (8 and 330 μM) (Post et al., 1965, 1972; Pauls et al., 1980, 1986; Gantzer et al., 1982). The value of 140 μM estimated here agrees well with that determined by Pauls et al. and lies between the other estimates. Of course, an accurate value for K_D for CoATP would require a carefully determined value for k_{on}, rather than the estimate used here.

There is a growing body of evidence supporting the relevance of Co(III) and Cr(III) nucleotide complexes and their usefulness as structural probes for the Na,K-ATPase. Recently, it was shown by Pauls et al. (1986) that Na,K-ATPase is phosphorylated by CrATP and that this phosphorylation supports Na^+–Na^+ and Rb^+–Rb^+ exchange. Vilsen et al. (1987) show CrATP phosphorylation in the same stoichiometry as $^{48}VO_4$ and [^{3}H]ouabain binding, with occlusion of $^{22}Na^+$ and $^{86}Rb^+$ in membranous and in solubilized Na,K-ATPase. Scheiner-Bobis et al. (1987) show that CoATP inactivates at the weak site and it, too, phosphorylates the enzyme, but only at 37 °C. It is clear, however, from both our own work (Stewart, 1987; Gantzer et al., 1982; Klevickis & Grisham, 1982) and that of others (Pauls et al., 1986; Scheiner-Bobis et al., 1987) that CoATP in complexes of the ATPase at 0–4 °C is uniformly and reliably stable for times substantially longer than those required for the NMR experiments. No enzyme inactivation or phosphorylation is observed under these conditions.

It is well-known that cation binding in the absence of other ligands affects the conformation of the nucleotide binding domain and phosphorylation site of the Na,K-ATPase peptide (Jørgensen et al., 1982). The affinity of the ATPase for ATP is approximately 100-fold greater in the presence of Na^+ than K^+. It is also known that in the presence of K^+ increasing nucleotide concentration favors the E_1KATP conformation over E_2K. This is the potassium–nucleotide antagonism as described for example by Jørgensen and Petersen (1985). A kinetic scheme for the Na,K-ATPase is shown in Figure 7. At the concentration of nucleotide analogue used in this experiment, E_1KATP must certainly be the predominant conformation of the Na,K-ATPase in the potassium solution. That E_1KATP is a conformation distinct from E_1NaATP is plausible considering the many conformations demonstrated by the extrinsic fluorescent probe fluorescein isothiocyanate (Hegyvary & Jørgensen, 1981; Karlish, 1979, 1980) that are undetectable by intrinsic tryptopan fluorescence (Karlish & Yates, 1978; Jørgensen & Karlish, 1980). However, we are unable to detect any differences between E_1KATP and E_1-NaATP. The NMR experiments described here should be very sensitive to differences in distance, since the relaxation enhancements are proportional to the sixth power of the Mn^{2+}–proton distance. Yet no such differences are seen, implying that the protein binds the same conformation of CoATP in the presence of high K^+ as in high Na^+.

It is instructive to compare the Mn^{2+}–CoATP–ATPase complex described here to similar structures from the chemical literature. The enzyme-bound Mn^{2+} ion is close enough to the adenine moiety of CoATP to permit some type of binding interaction with the N_7- and N_6-positions of adenine. There is in fact substantial precedent for metal coordination to nucleotides of the type shown in Figure 5. Thus, Chiang et al. (1979) describe two possible chelation models for a metal interacting with the N_7- and N_6-positions of adenine. In their neutral imino form I (Figure 8), adenine nucleotides are capable of acting either as bidentate chelating agents, employing the lone-pair density at the endocyclic N_7-site and the exocyclic imino group N_6H, or in a monodentate mode with N_7 as the sole binding site and with the possible utilization of the imino proton as a hydrogen-bond donor. In the protonated form II, N_7 is the primary metal binding site, and the exocyclic amino group acts as a hydrogen-bond donor to acceptor sites on the ligands (H_2O, for example) in the primary coordination sphere about the metal center. In this same report, Chiang et al. describe X-ray crystal studies of a complex of Co(III) with a 1,9-disubstituted adenine derivative, which exhibits a structure similar to II in Figure 8. In the structure of the ATPase-bound CoATP, Mn^{2+} appears to be approximately planar with the adenine and above N_7 and N_6. The Mn–N distances are too large for first coordination sphere complexes. On the other hand, Mn^{2+} coordination to either N_7 or N_6 in second-sphere complexes involving intervening hydrogen-bonded water molecules is possible and reasonable.

In one of the first papers ever published on the Na,K-ATPase, Skou (1960) suggested on the basis of kinetic evidence

and chemical intuition that the bound ATP substrate interacted with two Mg^{2+} ions, one coordinated directly to the β- and γ-phosphorus nuclei of the triphosphate chain and one coordinated to the N_7- and N_6-positions of the adenine moiety. Skou's model, shown in Figure 9, also suggested back-binding of the adenine-coordinated metal to the phosphoryl groups of ATP. While direct binding of this metal to the triphosphate chain appears to be excluded by the structure in Figure 5, it is interesting to consider the precise nature of the interaction between the enzyme-bound Mn^{2+} and the phosphoryl groups of bound ATP. The distances in Table III are too large for an inner-sphere complex. From the results of small-molecule crystallography and molecular model studies (Mildvan & Grisham, 1974), the Mn^{2+} to phosphorus distance is 2.8–3.0 Å for an inner-sphere complex of tetrahedral phosphate. On the other hand, a second-sphere complex in which an inner-sphere water or a ligand of comparable size intervenes between the Mn^{2+} and phosphate typically gives Mn^{2+}–P distances of 6.1 ± 0.5 Å. The distances calculated in Table III are thus consistent with a second-sphere complex between Mn^{2+} and the γ- and β-phosphates of CoATP. In support of this model, we have previously shown that Mn^{2+} at the high-affinity site of this enzyme coordinates four rapidly exchanging water protons (e.g., two water molecules), one of which is lost (either displaced or "frozen" in place) upon addition of phosphate (Grisham & Mildvan, 1974). ^{31}P NMR studies subsequently established the existence of a phosphate site 6.9 Å from the enzyme-bound Mn^{2+} (Grisham & Mildvan, 1975). These results were interpreted in terms of a second-sphere complex of Mn^{2+} and phosphate with an intervening water molecule. The previously characterized phosphate site is quite possibly distinct from the sites involved in coordination of CoATP, but this point has yet to be established with certainty. [It should be noted, however, that phosphate and ATP can bind simultaneously to the ATPase (Grisham, 1979).] In any case, except for the difference regarding possible back-binding of the second metal, Skou's model bears an interesting resemblance to the structure determined in the NMR studies presented here.

ACKNOWLEDGMENTS

We acknowledge the generous and invaluable help of Nancy Cook, Rocco Further Processing, Timberville, VA, the excellent technical assistance of Jaymee P. Girard, and helpful conversations with Drs. N. S. Rao and Bruce Ray on the analysis of exchange rates.

REFERENCES

Altona, A., & Sundaralingam, M. (1973) *J. Am. Chem. Soc. 95*, 2333.

Armstrong, R., Kondo, H., Granot, J., Kaiser, E., & Mildvan, A. (1979) *Biochemistry 18*, 1230.

Barnett, R. E. (1970) *Biochemistry 9*, 4644.

Bernheim, R., Brown, T., Gutkowsky, H., & Woessner, D. (1959) *J. Chem. Phys. 30*, 950.

Bloembergen, N. (1957) *J. Chem. Phys. 27*, 572.

Chiang, C., Epps, L., Marzilli, L., & Kistenmacher, T. (1979) *Inorg. Chem. 18*, 791.

Cornelius, P., Hart, P., & Cleland, W. (1977) *Inorg. Chem. 16*, 2799.

Cutnell, J. D., Bliech, H. E., & Glasel, J. A. (1976) *J. Magn. Reson. 21*, 43.

Davies, D. B. (1978) *Prog. Nucl. Magn. Reson. Spectrosc. 12*, 135.

DePont, J., Schoot, B., Van Prooijen-VanEeden, A., & Bonting, S. (1977) *Biochim. Biophys. Acta 482*, 213.

Freeman, R., Kempsell, S. P., & Levitt, M. H. (1980) *J. Magn. Reson. 38*, 453.

Gantzer, M. L., Klevickis, C., & Grisham, C. M. (1982) *Biochemistry 21*, 4083.

Grisham, C. M. (1979) *Adv. Inorg. Biochem. 1*, 193.

Grisham, C. M. (1981) *J. Inorg. Biochem. 14*, 45.

Grisham, C. M., & Mildvan, A. (1974) *J. Biol. Chem. 249*, 3187.

Grisham, C. M., & Mildvan, A. (1975) *J. Supramol. Struct. 3*, 304.

Grisham, C. M., & Hutton, W. (1978) *Biochem. Biophys. Res. Commun. 81*, 1406.

Grisham, C. M., Gupta, R., Barnett, R., & Mildvan, A. (1974) *J. Biol. Chem. 249*, 6738.

Hegyvary, C., & Post, R. (1971) *J. Biol. Chem. 246*, 5234.

Hegyvary, C., & Jørgensen, P. L. (1980) *J. Biol. Chem. 256*, 6296.

Jarori, G. K., Ray, B. D., & Rao, B. D. N. (1985) *Biochemistry 24*, 3487.

Jensen, J., & Nørby, J. (1971) *Biochim. Biophys. Acta 233*, 395.

Jørgensen, P. L. (1982) *Biochim. Biophys. Acta 694*, 27.

Jørgensen, P. L., & Karlish, S. J. D. (1980) *Biochim. Biophys. Acta 597*, 305.

Jørgensen, P. L., & Petersen, J. (1985) *Biochim. Biophys. Acta 821*, 319.

Jørgensen, P. L., Skriver, E., Hebert, H., & Maunsbach, A. (1984) *Ann. N.Y. Acad. Sci. 402*, 207.

Kahlenberg, A., Galsworthy, P., & Hokin, L. (1968) *Arch. Biochem. Biophys. 126*, 331.

Karlish, S. J. D. (1979) in *Na,K-ATPase Structure and Kinetics* (Skou, J. C., & Nørby, J. G., Eds.) pp. 115–128, Academic, New York.

Karlish, S. J. D. (1980) *J. Bioenerg. Biomembr. 12*, 111.

Karlish, S. J. D., & Yates, D. W. (1980) *Biochim. Biophys. Acta 527*, 115.

Klevickis, C., & Grisham, C. M. (1982) *Biochemistry 21*, 6979.

Levy, G., & Peat, I. (1975) *J. Magn. Reson. 18*, 500.

Lowry, O., Rosebrough, N., Farr, A., & Randall, R. (1951) *J. Biol. Chem. 193*, 265.

McClaugherty, S. H., & Grisham, C. M. (1982) *Inorg. Chem. 21*, 4133.

Merritt, E. A., Sundaralingam, M., Cornelius, R. D., & Cleland, W. W. (1978) *Biochemistry 17*, 3274.

Mildvan, A. S., & Cohn, M. (1970) *Adv. Enzymol. Relat. Areas Mol. Biol. 33*, 1.

Mildvan, A. S., & Grisham, C. M. (1974) *Struct. Bonding (Berlin) 20*, 1.

Nishigaki, I., Chen, F., & Hokin, L. (1974) *J. Biol. Chem. 249*, 4911.

O'Connor, S., & Grisham, C. (1979) *Biochemistry 18*, 2315.

O'Connor, S., & Grisham, C. (1980) *FEBS Lett. 118*, 303.

Patzelt-Wenczler, R., & Schoner, W. (1975) *Biochim. Biophys. Acta 403*, 538.

Patzelt-Wenczler, R., Pauls, H., Erdmann, E., & Schoner, W. (1975) *Eur. J. Biochem. 53*, 301.

Pauls, H., Bredenbrocker, B., & Schoner, W. (1980) *Eur. J. Biochem. 109*, 523.

Pauls, H., Serpersu, E. H., Kirch, U., & Schoner, W. (1986) *Eur. J. Biochem. 157*, 585.

Post, R., & Kume, S. (1973) *J. Biol. Chem. 248*, 6993.

Post, R. L., Sen, A., & Rosenthal, A. (1965) *J. Biol. Chem. 240*, 1437.

Post, R. L., Hegyvary, C., & Kume, S. (1972) *J. Biol. Chem. 247*, 6530.
Robinson, J. (1981) *Biochim. Biophys. Acta 642*, 405.
Saenger, W. (1984) *Principles of Nucleic Acid Structure*, Springer-Verlag, New York.
Scheiner-Bobis, G., Buxbaum, E., & Schoner, W. (1987) Proceedings of the Fifth International Conference on Na,K-ATPase, Aarhus, Denmark, Abstract 66a.
Shull, G. E., Schwartz, A., & Lingrel, J. B. (1985) *Nature (London) 316*, 691.
Skou, J. C. (1960) *Biochim. Biophys. Acta 42*, 6.
Skou, J., & Hilberg, C. (1965) *Biochim. Biophys. Acta 110*, 359.
Solomon, I. (1955) *Phys. Rev. 99*, 599.
Solomon, I., & Bloembergen, N. (1956) *J. Chem. Phys. 25*, 261.
Stewart, J. M. MacD. (1987) Ph.D. Dissertation, University of Virginia.
Vilsen, B., Andersen, J. P., Petersen, J., & Jørgensen, P. L. (1987) *J. Biol. Chem.* (in press).
Viswamitra, M. A., Hosur, M. V., Shakked, Z., & Kennard, O. (1976) *Cryst. Struct. Commun. 5*, 819.

The Membrane as an Environment of Minimal Interconversion. A Circular Dichroism Study on the Solvent Dependence of the Conformational Behavior of Gramicidin in Diacylphosphatidylcholine Model Membranes†

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Received October 21, 1987; Revised Manuscript Received February 8, 1988

ABSTRACT: The conformation of gramicidin in diacylphosphatidylcholine model membranes was investigated as a function of the solvent in which peptide and lipid are initially codissolved. By use of circular dichroism it is demonstrated that, upon removal of the solvent and hydration of the mixed gramicidin/lipid film, it is the conformational behavior of the peptide in the organic solvent that determines its final conformation in dimyristoylphosphatidylcholine model membranes. As a consequence, parameters that influence the conformation of the peptide in the solvent also play an essential role, such as the gramicidin concentration and the rate of interconversion between different conformations. Of the various solvents investigated, only with trifluoroethanol is it possible directly to incorporate gramicidin entirely in the $\beta^{6.3}$-helical (channel) configuration. It is also shown that the conformation of gramicidin in the membrane varies with the peptide/lipid ratio, most likely as a result of intermolecular gramicidin–gramicidin interactions at higher peptide/lipid ratios, and that heat incubation leads to a conformational change in the direction of the $\beta^{6.3}$-helical conformation. Using lipids with an acyl chain length varying from 12 carbon atoms in dilauroylphosphatidylcholine to 22 carbon atoms in dierucoylphosphatidylcholine, it was possible to investigate the acyl chain length dependence of the gramicidin conformation in model membranes prepared from these lipids with the use of different solvent systems. It is demonstrated for each solvent system that the distribution between different conformations is relatively independent of the acyl chain length but that the rate at which the conformation converts toward the $\beta^{6.3}$-helical configuration upon heating of the samples is affected by the length of the acyl chain. The conversion to the $\beta^{6.3}$-helical configuration is fastest in the short-chained dilauroylphosphatidylcholine. Finally, the effect of chemical modification of gramicidin on its conformational behavior was investigated by using the N-terminal-modified derivatives desformylgramicidin and *N*-acetylprolyldesformylgramicidin, the tryptophan-substituted analogue 9-phenylalanylgramicidin, and tryptophan-formylated gramicidin. It is shown that, by codissolving peptide and lipid in trifluoroethanol, all these analogues can be incorporated in the $\beta^{6.3}$-helical conformation but that they differ in their interaction with Na ions, as determined by ^{23}Na NMR measurements.

The peptide antibiotic gramicidin is a hydrophobic linear pentadecapeptide produced by *Bacillus brevis*, strain ATCC 8185 (Katz & Demain, 1977). Although it is a relatively simple peptide, gramicidin exhibits a complex conformational behavior. Among the various conformations that have been proposed are single-stranded helices, which can be left- or right-handed and may vary in pitch (Urry et al., 1971; Ramachandran & Chandrasekaran, 1972), and double-stranded helices, which can also be left- or right-handed and vary in pitch and which in addition can run either parallel or antiparallel (Veatch et al., 1974; Sychev et al., 1980). Hybrid structures, consisting of partially intertwined helices, may also occur (Heitz et al., 1986), and interconversion via hybrid structures has been suggested (Urry et al., 1975). In organic solvents the distribution of conformations, as well as the kinetics of interconversion from one conformation to another,

† This work was supported in part by NIH Grant GM26898 to D. W.U. J.A.K. is a recipient of a stipend from the Netherlands Organization of Pure Research (ZWO).
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0006-2960/88/0427-4848$01.50/0