Biochimica et Biophysica Acta, 482 (1977) 286–300

BBA 68147

NITROGENASE OF *KLEBSIELLA PNEUMONIAE*

WATER PROTON NMR RELAXATION STUDIES ON THE BINDING OF DIVALENT METAL IONS AND NUCLEOTIDES TO THE IRON PROTEIN

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(Received November 10th, 1976)

Summary

Interactions between the iron protein, Kp2, of nitrogenase manganese ions, magnesium ions, and the nucleotides ATP or ADP, have been studied in aqueous solution by monitoring the water proton NMR relaxation rate enhancement caused by Mn^{2+}. Binding of Mn^{2+} to a molecule of Kp2 occurs at four sites, indistinguishable within experimental error, having a $K_d = 350 \pm 50$ μM. The $Mn^{2+} \cdot$ Kp2 complex has a low characteristic enhancement ($\epsilon_b = 6 \pm 0.5$). All four sites can alternatively bind Mg^{2+}, not necessarily with the same dissociation constant, but with a mean $K_d = 1.7 \pm 0.3$ mM. Ternary complexes with the configuration EMS or $_E\langle^M\rangle_S$ are formed between Kp2, Mn^{2+} and nucleotide (ATP or ADP). The ternary complexes with Mg^{2+} in place of Mn^{2+} probably have the latter configuration. A novel treatment of enhancement data (a 'high metal' approximation) is given.

Introduction

Nitrogenase (see references 1 to 3), the enzyme that reduces nitrogen to ammonia, consists of two distinct acidic metalloproteins both of which are essential for activity. The larger protein (MoFe protein, Protein 1) has a molecular weight of 220 000 and contains molybdenum, iron and acid-labile sulphur. The smaller protein (Fe protein, Protein 2), when isolated from *Klebsiella pneumoniae*, has a molecular weight of approx. 67 000 and contains four iron and four acid-labile sulphur atoms per molecule [4], probably arranged in an Fe_4S_4 cluster [5]. It consists of two apparently identical subunits with molecular weights of approx. 34 000 [4]. Both nitrogenase proteins are inactivated

by exposure to oxygen and for this reason are usually purified and handled in buffers containing $Na_2S_2O_4$.

As well as the two proteins, enzymic activity requires ATP, a divalent metal ion, a source of electrons (usually $Na_2S_2O_4$ in vitro) and an anaerobic environment [1—3]. Mg^{2+} is the most effective divalent cation but others can be used, Mn^{2+} being the best of these [4,8]. During the enzymic reaction ATP is hydrolysed to ADP and inorganic phosphate. The ADP released is an inhibitor. ATP appears to be active as its monomagnesium salt $MgATP^{2-}$, with Mg_2ATP being inactive [9] and high levels of free ATP^{4-} being inhibitory [10].

Using electron paramagnetic resonance (EPR) [3,11] and gel equilibration techniques it has been shown that $MgATP^{2-}$ and $MgADP^{-}$ bind to the Fe protein causing a conformational change and a lower redox potential [13]. The $MgATP^{2-}$ · Fe protein complex then acts as a very specific reductant for the MoFe protein [14,15] which binds reducible substrates [15]. These substrates can then be reduced by the enzyme.

Thus one role for $MgATP^{2-}$ is the 'activation' of the Fe protein; however, recent studies have indicated that there is probably more than one role for $MgATP^{2-}$ in the enzymic mechanism [16].

Water proton relaxation rate studies have been widely used to study the interaction of paramagnetic ions with proteins. Specifically, Mn^{2+} has been used to study the metal-nucleotide, metal-protein and metal-nucleotide-protein interactions in a number of kinase systems [17]. In this paper we report an investigation, using this technique, of the interactions of Mn^{2+}, Mg^{2+}, $MnATP^{2-}$ and $MnADP^{-}$ with the Fe protein from *K. pneumoniae* (Kp2 protein).

An earlier investigation [18] of a similar nature utilized unresolved nitrogenase from *Azotobacter vinelandii*. The MoFe protein: Fe protein ratio in this preparation was not determined and there is some doubt about the oxidation state of the proteins in the presence of $MnATP^{2-}$. EPR experiments have shown that in the presence of ATP the enzyme will turn over until both proteins are in their physiological oxidised forms [15]; however, the authors [18] report that addition of $Na_2S_2O_4$ did not affect the interaction of $MnATP^{2-}$ with nitrogenase. A further criticism is that it was necessary to include a creatine kinase/creatine phosphate ATP-generating system in the experiment involving ATP in order to avoid the build up of ADP. Thus the $MnATP^{2-}$ interaction was not purely with nitrogenase.

Experimental

The Kp2 protein was purified by established methods [4] and concentrated by adsorption onto a small column of DEAE 32-cellulose followed by elution with 90 mM $MgCl_2$ in 25 mM Tris · HCl buffer, pH 7.4, containing 1 mM $Na_2S_2O_4$ and 0.1 mg/ml dithiothreitol. The concentrated protein solution was then equilibrated with 25 mM Tris · HCl buffer, pH 7.4, containing 1 mM $Na_2S_2O_4$ and 0.1 mg/ml dithiothreitol by passage through a column of Sephadex G-25. Protein concentrations were determined by the method of Lowry et al. [19] which has been shown to give values within 5% of those obtained by dry-weight measurements [4]. Kp2 protein solutions were unstable and precipitated at concentrations greater than 15 mg/ml thus limiting

the range of concentrations available to us.

4 and 2 mM Mn^{2+} solutions were prepared by diluting an aqueous 100 mM $MnSO_4$ solution with 25 mM Tris · HCl buffer (pH 7.4, containing 1 mM $Na_2S_2O_4$). Analar $MnSO_4 \cdot 4H_2O$ was used without further purification. During the preliminary stages of the study the manganese concentration of some solutions was checked by atomic absorption spectroscopy and found to agree well with concentrations evaluated from weight data, $MnSO_4$ was used in place of the more usual $MnCl_2$ which is very hygroscopic. In one experiment it was shown that the results obtained with $MnCl_2$ were indistinguishable from those obtained with $MnSO_4$.

Standard 5-mm outer diameter NMR tubes, with or without a capillary containing $C_6{}^2H_6$ as a lock signal, were degassed and flushed with N_2 (containing less than 1 ppm O_2) several times and then sealed with degassed No. 13 Subaseals under a stream of N_2. A needle was then inserted through the subaseal and the tubes placed in a cylindrical vessel attached to the vacuum/flushing line. The tubes were then subjected to at least three more evacuation and flushing cycles, before use. When this rather elaborate procedure was used, the oxygen-sensitive Kp2 protein maintained full enzymic activity throughout the experiment.

Samples were prepared in the degassed NMR tubes by diluting various amounts of the Kp2 protein solution to 0.5 ml with 25 mM Tris · HCl buffer, pH 7.4, containing 1 mM $Na_2S_2O_4$. Buffer blanks contained 0.5 ml of this buffer. This method of preparation resulted in solutions containing differing amounts of dithiothreitol. In one experiment it was shown that variation of the dithiothreitol concentration from 0 to 0.1 mg/ml did not affect the relaxation times.

Titration with solutions of $MnSO_4$ was achieved by injection through the subaseal tube closure using a Hamilton syringe fitted with a fixed aliquot dispenser. The water proton spin-lattice relaxation time, T_1, was measured following each addition.

In those experiments involving magnesium, the metal ion was incorporated into buffer/Kp2 solutions in the NMR tubes by injecting the appropriate quantity of either 100 mM or 1 M aqueous $MgSO_4$ (nitrogen-flushed) solutions through the subaseal tube closure. For these titrations sufficient $MgSO_4$ was added to the $MnSO_4$ titrant to maintain constant Mg^{2+} concentration during titration. Analar $MgSO_4 \cdot 7H_2O$ was used without further purification.

ATP and ADP were used as disodium salts obtained from Boehringer, Mannheim GmbH, Germany.

All measurements made in this study have been on samples initially 0.5 ml in volume. Volumes substantially less than this led to poor mixing of injected solutions probably through extensive wetting of the tube walls, a phenomenon most prominent with solutions containing high concentrations of protein.

NMR measurements were made with a Jeol PFT-100 Fourier transform spectrometer operating at 100 MHz. Since samples were of the order 55 M in the observed nucleus the spectrometer had to be detuned to accommodate the signal.

Spin-lattice relaxation times were obtained by the inversion-recovery method utilizing a $180°$-τ-$90°$ pulse sequence. In the preliminary work the interpulse

delay time, τ, was varied manually and T_1 found from $\tau_{null} = T_1 \ln 2$ following a plot to establish τ_{null}. In later work an 'automatic' T_1 program was used which varies τ by computer control and evaluates T_1 from the Bloch relation; $dI_t/dt = -(I_t - I_0)/T_1$, where I_0 is the intensity of the unperturbed signal and I_t is the intensity following an interpulse delay time t. The latter method does not call for the same degree of precision in setting up a 90° pulse as the null point method. The T_1 program gives T_1 to two decimal places; further precision was obtained by reprocessing the intensity data. The automatic method required a lock signal which was provided by the inclusion of a sealed glass capillary containing deuterobenzene.

The probe temperature, monitored during experiment, was 297 K and stable to ±1 degree. In order for the sample to attain this temperature it was necessary to allow 15 min in the probe before the first measurement; subsequent measurements required less time since the volume of solution injected was small.

Results and Analysis

The binary system, Mn(II)-protein

The spin-lattice relaxation, T_1, of the water proton signal in buffered aqueous protein samples, was measured for successive additions of Mn^{2+}. This was repeated for a range of protein concentrations. The data were converted initially to plots of $1/T_1$ vs. total concentration of Mn^{2+}, to give a series of curves of which an example is shown in Fig. 1a. Such curves are closely similar to M-titrations in the nomenclature of Mildvan and Cohn [17], but the protein is diluted by 10—20% during the course of a titration (this is taken into account in later calculations). Preliminary experiments were made to establish a range of Mn^{2+} concentrations that would give curves of this form with the available protein concentrations. On each day of experiment, the 'blank' titration of Mn^{2+} against the buffer solution (Fig. 1i) was recorded at least once. All protein curves show a positive enhancement relative to the buffer curve, the upward displacement increasing with increasing protein concentration. This strongly indicates the formation of a binary complex between Mn^{2+} and Kp2 protein.

The simplest assumption that can be made is that there are 'n' independent and equivalent binding sites per protein molecule for Mn^{2+}, each having the same characteristic enhancement factor, ϵ_b, and dissociation constant K_d. Extensive data were obtained to test this assumption and to evaluate the three parameters involved. Attempts to obtain an independent check on the uncomplexed metal concentration were unsatisfactory, and the NMR enhancement data alone were used.

The first step was to use enhancements at high protein and low metal concentrations to plot $1/(\epsilon^* - 1)$ against the reciprocal of protein concentration (Fig. 2). ϵ^* is the experimental enhancement factor relative to the buffer curve. This is called the E-titration (I) [17], and is applicable only when most of the enzyme sites are vacant. The linearity of the plot within experimental error suggests that equivalent binding sites are involved, and the absence of points close to the ordinate reflects the limited range of protein concentrations avail-

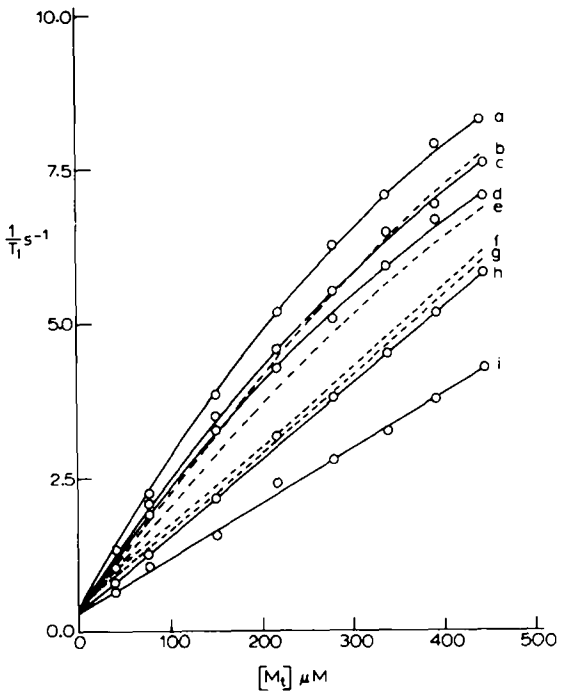


Fig. 1. Graphical representation of M-titrations as plots of $1/T_1$ vs. $[M_t]$: a, Experimental plot for 2 mM Mn^{2+} vs. 65 μM Kp2; b, calculated curve for 2 mM Mn^{2+} plus 2 mM ADP^{3-} vs. 65 μM Kp2, assuming ternary complex formation; c, experimental plot for 2 mM Mn^{2+} plus 2 mM ADP^{3-} vs. 65 μM Kp2; d, experimental plot for 2 mM Mn^{2+} plus 2 mM ATP^{4-} vs 65 μM Kp2; e, calculated curve for 2 mM Mn^{2+} plus 2 mM ATP^{4-} vs. 65 μM Kp2, assuming ternary complex formation; f, calculated curve for 2 mM Mn^{2+} plus ADP^{3-} vs. 65 μM Kp2, assuming competition between Kp2 and ADP^{3-} for the available Mn^{2+} via the formation of binary complexes only; g, calculated curve for 2 mM Mn^{2+} plus 2 mM ATP^{4-} vs. 65 μM Kp2, assuming competition between Kp2 and ATP^{4-} for available Mn^{2+} via the formation of binary complexes only; h, experimental plots for nucleotide blanks, 2 mM Mn^{2+} plus 2 mM ADP^{3-} and 2 mM Mn^{2+} plus 2 mM ATP^{4-} vs. buffer; i, manganese blank, 2 mM Mn^{2+} vs. buffer.

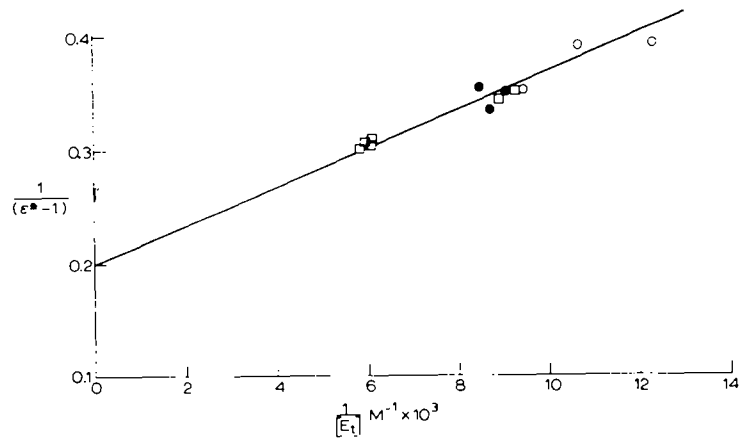


Fig. 2. The E-titration(I) as a plot of $1/(\epsilon^* - 1)$ vs. $1/[E_t]$. Data for this graph were taken from appropriate regions ($[E_f] \geq 90\%\ [E_t]$) of M-titrations of the type shown in Fig. 1. The different symbols indicate different batches of Kp2 protein: □, titrations of 4 mM Mn^{2+} vs. 117 and 175 μM Kp2; ●, titrations of 1 mM Mn^{2+} vs. 84 and 120 μM Kp2; ○, a titration of 4 mM Mn^{2+} vs. 107 μM Kp2.

able. The intercept on the ordinate is equal to $1/(\epsilon_b - 1)$ from which we infer $\epsilon_b = 6 \pm 0.5$. This is a low value for the parameter as compared with typical values for other systems in the literature [20] and dictates the use of quite high levels of protein in order to obtain usable enhancement factors. The slope of the graph in Fig. 2 is equal to $K_d/n(\epsilon_b - 1)$, giving a most probable value for K_d/n of 83 μM. The parameters finally obtained were later used to check that all points admitted to the plot in Fig. 2 satisfy the criterion $[E_f] \geqslant 90\%\ [E_t]$.

To obtain the best evaluation of n and K_d, experimental data were now processed graphically by both the Hughes-Klotz [20,21] and Scatchard [22] methods. The latter was found to give a better spread of points and was used alone in the later stages. In this method, ϵ_b and the observed enhancement were used to calculate concentrations of free metal $[M_f]$, bound metal $[M_b]$, and thence R, the degree of binding ($R = [M_b]$/total concentration of protein). A computer programme was written to perform this calculation on all experimental data, and to fit the best straight line to each Scatchard plot by a least squares method. A number of superimposed Scatchard plots, $R/[M_f]$ vs. R, for several titrations are shown in Fig. 3. In the initial stages of the study we pursued the possibility of two types of binding sites which would have been indicated by non-linear Scatchard plots. After much investigation we con-

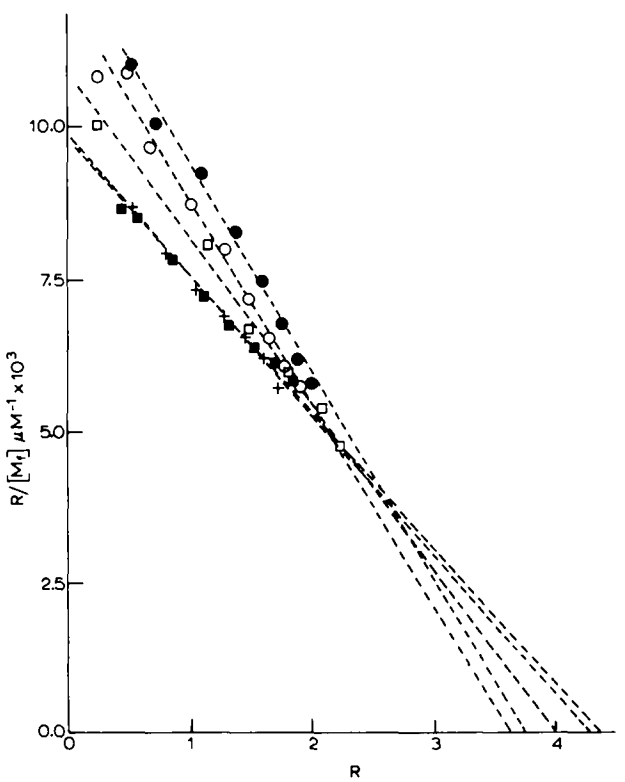


Fig. 3. Some Scatchard plots $[R/[M_f]$ vs. R $(= [M_b]/[E_t])$, taking $\epsilon_b = 6.5]$ for titrations of: +, 2 mM Mn^{2+} vs. 80 μM Kp2; ■ and ●, 2 mM Mn^{2+} vs. 65 μM Kp2; □, 2 mM Mn^{2+} vs. 50 μM Kp2; ○, 2 mM Mn^{2+} vs. 40 μM Kp2. The dashed lines are best least squares straight lines through individual titrations.

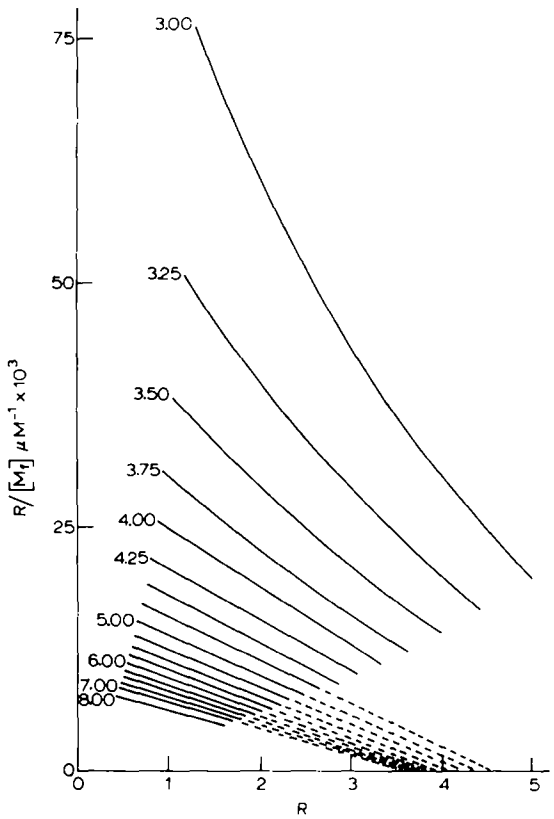


Fig. 4. A series of Scatchard plots for the titration of 2 mM Mn^{2+} vs. 40 μM Kp2 (Fig. 3) for a range of values of the characteristic enhancement, ϵ_b, indicating those for which the intercept on the R axis is 4 ± 0.5.

sidered that, within experimental error, the data indicated equivalent binding sites.

Assessment of the probable errors is important in interpreting the results. Probably the largest contributions to random error are the syringe injection, mixing procedures and assay of the protein. We consider the scatter of points in Fig. 3 to be indicative of all sources of error except the uncertainty in ϵ_b.

The curves of Fig. 4 have been calculated to illustrate the dependence of Scatchard plots on the value of ϵ_b. Each curve, calculated from one of the titrations of Fig. 3, corresponds to a different value in the range 3.0—8.0 for ϵ_b and all other parameters have been held constant. The parameter of greatest interest is the number of binding sites, n. The value of n, found from the intercept on the R axis, was 4 to the nearest integer for the curves with ϵ_b = 4.50—7.00. This allows a greater uncertainty in ϵ_b than our experimentally determined 6 ± 0.5 and strongly indicates $n = 4$. The other titrations of Fig. 3 have intercepts of 4 ± 0.5 and we conclude that there are four binding sites for Mn^{2+} on Kp2 protein.

K_d as found from the slope ($=-K_d^{-1}$) within individual titrations has values in the range 300—400 μM. As compared to the value of approx. 350 μM deduced

by both the E-titration (I) (assuming $n = 4$) and the 'high metal' method, the agreement is acceptable. A cautious inference from all available data is that $K_d = 350 \pm 50$ μM. It should be noted that Figs. 1, 2 and 3 involve three largely independent sets of experimental points.

The binary system, Mg(II)-protein

The discovery that the Fe protein has four binding sites for Mn^{2+} was unexpected since $MgATP^{2-}$ is reported [12] to bind at only two sites. We have therefore investigated the binding of Mg^{2+} to Kp2 protein by monitoring its effect on the proton relaxation enhancement caused by Mn^{2+} binding.

Some buffered aqueous Kp2 solutions each containing a known concentration of $MgSO_4$ were titrated against $MnSO_4$ solutions. Titrations of buffer and buffered Kp2 against $MnSO_4$ were carried out as reference titrations. A separate experiment showed that the presence of Mg^{2+} did not affect the buffer titration. The range of magnesium concentrations examined was 0.5—50 mM. It was found (Fig. 5a) that at the higher concentrations of Mg^{2+} proton relaxation enhancement in the $Mn^{2+} \cdot$ Kp2 protein system was almost completely suppressed.

To assist in the interpretation of these results, a computer programme was written to simulate the M-plots of Fig. 5a for several possible models involving competition between Mg^{2+} and Mn^{2+} for binding sites on the protein. For each model, the M-plots were calculated using $K_d = 350$ μM for $Mn^{2+} \cdot$ Kp2 dissociation and a trial value (see caption to Fig. 5) for $Mg^{2+} \cdot$ Kp2 dissociation. These parameters were combined with total concentrations of metals and protein in iterative calculations to yield $Mn^{2+} \cdot$ Kp2 complex concentrations, from which $1/T_1$ data were calculated from the previously obtained value of 6 for ϵ_b. Examples of such calculated M-plots are shown in Fig. 5 for cases of competitive binding of Mg^{2+} to all four of the Mn^{2+} sites (Fig. 5b) and to two only of the Mn^{2+} sites (Fig. 5c). In the latter case, the enhancement is never reduced to less than 50% of that in the presence of Mn^{2+} alone, and is clearly

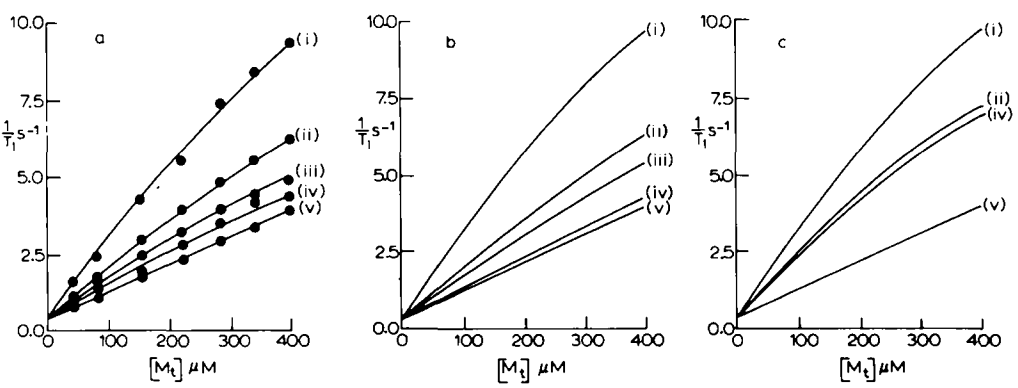


Fig. 5. The effect of added Mg^{2+} on M-titrations (see Fig. 1) for (a) experimental data; (b) an example of calculated curves for a model with four equivalent Mg^{2+} sites having $K_d = 1400$ μM; (c) an example of calculated curves for a model with two equivalent Mg^{2+} sites having $K_d = 350$ μM. i, reference titration of 2 mM Mn^{2+} vs. 90 μM Kp2; ii, as i plus 5 mM Mg^{2+}; iii, as i plus 10 mM Mg^{2+}; iv, as i plus 50 mM Mg^{2+}; v, buffer blank.

incompatible with the experimental result (Fig. 5a). However, the similarity of Fig. 5a and Fig. 5b strongly indicates the involvement of all four Mn^{2+} sites on Kp2 in binding Mg^{2+}. Furthermore, since the concentrations of Mg^{2+} are one or two orders of magnitude greater than the Mn^{2+} concentrations (approx. 400 μM at the end of the titration) the binding of Mg^{2+} is a weaker interaction.

A further model investigated was that of four sites for Mg^{2+} of which two were characterised by one dissociation constant and the remaining two by a different constant. The results from this model were very similar to those for four equivalent sites even when the two K_d values differed by a factor of 2. Thus, while we conclude that there are four sites on Kp2 for Mg^{2+}, these are not necessarily equivalent. However, since the data can be explained adequately in terms of equivalent sites for Mg^{2+}, this has been the basis for further calculations performed on all data obtained, to estimate the mean dissociation constant for the Mg^{2+} · Kp2 complex. The calculation involves using the enhancement data to obtain the concentration of Mn^{2+} bound to Kp2, and then using this and K_d for the Mn^{2+} · Kp2 complex to evaluate the concentration of bound Mg^{2+} by taking a mass balance over the enzyme sites. Once the concentration of Mg^{2+} bound to Kp2 is known, then K_d for Mg^{2+} · Kp2 follows directly with the assumption that there are four available sites. Using this method we estimate K_d for the Mg^{2+} · Kp2 complex to be 1.7 ± 0.3 mM.

The ternary system, Mn(II)-protein-nucleotide

$MgATP^{2-}$ is known to be essential to the enzymic action of nitrogenase, so preliminary experiments were carried out to establish whether or not ATP or ADP could participate in complex formation with Kp2 in presence of Mn^{2+}. Syrtsova et al. [18] have indicated that this was the case with unresolved nitrogenase in the presence of an ATP-generating system.

In the present study buffer and buffered Kp2 solutions were each titrated against freshly prepared solutions containing $MnSO_4$ and buffered nucleotide (ATP or ADP) in 1 : 1 molar ratio. Typical experimental curves are shown as solid lines in Fig. 1. Both Mn^{2+} plus nucleotide vs. protein curves show an enhancement which is intermediate between that for Mn^{2+} plus nucleotide vs. buffer and that for Mn^{2+} vs. protein. In the case of ATP this behaviour is very similar to that reported by Syrtsova et al. [18] with unresolved nitrogenase. In the case of ADP they observed greater enhancements for Mn^{2+} plus nucleotide vs. protein than for Mn^{2+} vs. protein, which is contrary to our result using Kp2 protein.

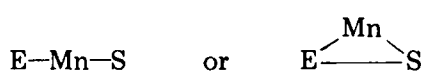
We have not attempted a full quantitative analysis of our data on the ternary systems since the limited range of protein concentrations available, and the low characteristic enhancements of the complexes relative to the experimental errors, make such an analysis dubious. Qualitatively our observations are open to two possible interpretations: (a) competition between nucleotide and protein for the available Mn^{2+} via formation of binary complexes only; or (b) formation of a ternary complex between protein, Mn^{2+}, and nucleotide. The broken curves in Fig. 1 for Mn^{2+} plus nucleotide vs. protein were computed to differentiate between these possibilities. The calculated curves for competitive binding were computed using published stability constants [23,24] for Mn^{2+} · ATP^{4-} and Mn^{2+} · ADP^{3-} complexes, corresponding to dissociation constants

of, respectively, 17 and 33 μM, and a value of 350 μM for the Mn^{2+} · Kp2 protein dissociation constant. The equilibrium concentrations of binary complexes and free Mn^{2+} were calculated for a series of total metal values, and weighted mean enhancement factors found in each case. Besides ϵ_b established already, this required values of the characteristic enhancement ϵ'_b for the Mn^{2+} · ATP^{4-} and Mn^{2+} · ADP^{3-} complexes. A value of 1.55 was calculated for the former and 1.65 for the latter, from the Mn^{2+} plus nucleotide vs. buffer titration data. These values agree with the qualitative observation that since the binding in Mn^{2+} · nucleotide complexes is strong, then the characteristic enhancements for these are approximately the ratio of the slope for Mn^{2+} plus nucleotide vs. buffer curve to that for Mn^{2+} vs. buffer (approx. 1.4).

Since the Mn^{2+}-nucleotide interactions are an order of magnitude stronger than the Mn^{2+}-protein interaction, the calculated curves for competitive binding are depressed close to that for Mn^{2+} plus nucleotide vs. buffer, and are nearly linear over the given range of Mn^{2+} concentrations. The large differences between the experimental (Figs. 1c and 1d) and these calculated curves (Figs. 1f and 1g) are good evidence that the former cannot be explained in terms of binary complexes alone.

The estimate of curves for ternary complex formaion was made with simplifying assumptions, and is intended to be very approximate only. The first assumption is that the dissociation constants for Mn^{2+}-nucleotide and for Mn^{2+}-protein binding are unaffected by binding to the third component, i.e. that dissociation constants for the assumed ternary complexes are the same as those for the corresponding binary complexes. With the further assumption that the number of protein binding sites for the ternary complex is four, as obtained for the binary complex, the equilibrium concentrations of all species can be estimated. A weighted mean enhancement factor can then be found if the characteristic enhancements ϵ_b, ϵ'_b, and ϵ_t are known. We have as yet no direct information on ϵ_t, for the assumed ternary complexes, but an order of magnitude estimate (see Appendix) suggests a value of about 6 in the case of the complex with ATP, and about 8 for that with ADP.

The curves obtained (Figs. 1b and 1e) are quite similar to those found experimentally for titrations of Mn^{2+} plus nucleotide against protein (Figs. 1c and 1d), although the agreement must be partly fortuitous in view of the assumptions made. However, it does demonstrate that formation of ternary complexes, with a reasonable set of assumed properties, adequately explains experimental behaviour that cannot be accounted for otherwise. Moreover, the assumed properties are appropriate to the configurations,



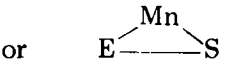
in which the metal ion is bound both to protein and nucleotide. These constitute the 'Type II' classification of Dwek [20], in which there is a reduction of metal ion · protein enhancement in the presence of substrate (here nucleotide). We infer qualitatively that ternary complexes of this type are formed in the case of Mn^{2+}, both with ATP or ADP, but do not claim that the number of binding sites is necessarily the same as that in the metal ion · protein binary complex. Syrtsova et al. [18] came to the same conclusion in the case

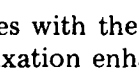
of the complete enzyme, although they had to invoke a change of enzyme conformation to explain their maximal enhancement for the ternary system enzyme-Mn^{2+}-ADP.

Discussion

We have shown that Kp2 protein forms binary complexes with Mg^{2+} and Mn^{2+} and ternary complexes with $MnATP^{2-}$ and $MnADP^{-}$. Within experimental error, we have found four equivalent binding sites for Mn^{2+} (K_d = 350 ± 50 μM) or Mg^{2+} (K_d = 1.7 ± 0.3 mM) although we cannot exclude the possibility that there are two types of site with similar binding constants. These are the first quantitative measurements of divalent ion binding to either nitrogenase protein.

Qualitatively our results with the ternary complexes involving Mn^{2+} are consistent with the configurations

E—Mn—S or 

in which the metal is bound to both protein and nucleotide. Our value of K_d = 1.7 ± 0.3 mM for the binding of Mg^{2+} to the Fe protein is different from the apparent Michaelis constant (K_m) of about 400 μM [9] for $MgATP^{2-}$ in the enzymic reaction. This unfortunately gives us no information on the nature of $MgATP^{2-}$ binding to the enzyme since the Michaelis constant for a substrate of a multisubstrate enzyme can be greater, equal to or less than its dissociation constant [7]. However, since Mg^{2+} does exhibit specific binding to the Fe protein and nitrogenase exhibits nucleotide specificity (GTP, CTP and UTP do not induce enzymic activity [26]) then it seems probable that both the metal ion and the nucleotide are important in $MgATP^{2-}$ binding to the Fe protein and that ternary complexes with the configuration  are formed. Unfortunately the proton relaxation enhancement method is not ideal for the study of these ternary systems since the available range of protein concentrations is limited and the observed specific enhancements are small. Thus an unambiguous quantitative analysis of the results is very difficult.

Studies on the Fe protein of *Clostridium pasteurianum* nitrogenase (Cp2 protein) showed two $MgATP^{2-}$ binding sites on that protein. In addition steady-state H_2 evolution assays with the enzyme from *K. pneumoniae* [27] gave a Hill coefficient of 2 for $MgATP^{2-}$ and $MgADP^{-}$. These observations apparently conflict with our result of four Mg^{2+} binding sites on Kp2 protein. Two possible explanations to reconcile these results are (a) that the binding of two $MgATP^{2-}$ molecules (one to each subunit) sterically hinders binding of $MgATP^{2-}$ at the other two sites, or (b) that the binding of two $MgATP^{2-}$ molecules causes a conformational change [1,2] in the protein and weakens the Mg^{2+} binding constant at the other two sites. At high levels (>30 mM), free Mg^{2+} inhibits nitrogenase from *K. pneumoniae* [9]. This observation would be consistent with postulate (b); however, EPR studies [15] (Smith, B.E., Lowe, D.J. and Bray, R.C., unpublished) have shown Mg^{2+} binding to Kp2 protein at high Mg^{2+} concentrations (>20 mM) in the absence of ATP, indicating that the inhibitory site is present in the unmodified protein. Thorneley and Willison [9]

also observed unexpectedly low activities at very low levels of ATP^{4-} in the presence of 2.5 mM Mg^{2+}. This could indicate inhibition by free Mg^{2+} at concentrations commensurate with our dissociation constant of 1.7 mM. One of us (Smith, B.E. and Pitts, J.) has also observed weak (approx. 10%) inhibition of activity in C_2H_2 reduction assays in which the total Mg^{2+} concentration was approx. 2 mM above that of ATP at 5 or 10 mM. Thus on balance we favour postulate (a) with two sites sterically blocked for $MgATP^{2-}$ but not for Mg^{2+} binding after binding two molecules of $MgATP^{2-}$. The physiological function of the extra two sites is not clear.

The results of Syrtsova et al. [18] on unresolved and stoichiometrically uncharacterised nitrogenase from *A. vinelandii* are qualitatively similar to our results with purified Kp2 protein except for $MnADP^-$ binding. In contrast to us they found greater enhancements with $MnADP^-$ than with Mn^{2+} alone and postulated an enzyme conformational change which rendered the Mn^{2+} more accessible to water in the ternary complex. Since the major difference between the two sets of data is the presence of the MoFe protein in their experiments, it is possible that their results reflect the specific inhibitory function of $MgADP^-$ which is only fully apparent with the whole enzyme. This hypothesis cannot be tested without further studies of this type on the isolated MoFe protein as well as the combined proteins.

Appendix

The 'high metal' approximation

This depends on the approximation $[M_t] \approx [M_f]$, i.e. $[M_t] \gg [M_b]$, where suffixes t, b, and f denote total, bound and free metal, respectively. The experimental conditions are therefore low protein concentration and high metal concentration, the converse of those for the 'E(I)-plot' of Fig. 2.

Then

$$K_d = \frac{(n[E_t] - [M_b])([M_t] - [M_b])}{[M_b]} \approx \frac{(n[E_t] - [M_b])[M_t]}{[M_b]}$$

Rearranging,

$$\frac{[M_b]}{[M_t]} \approx \frac{n[E_t]}{K_d + [M_t]}$$

In general,

$$\frac{[M_b]}{[M_t]} = \frac{\epsilon^* - 1}{\epsilon_b - 1}$$

Equating the two expressions for $[M_b]/[M_t]$ and rearranging, we have,

$$\frac{[E_t]}{\epsilon^* - 1} \approx \frac{1}{n(\epsilon_b - 1)} ([M_t] + K_d)$$

Hence plotting $[M_t]$ against $[E_t]/(\epsilon^* - 1)$, when the approximation is valid, should give a straight line of slope $n(\epsilon_b - 1)$, and an intercept on the $[M_t]$ axis of $-K_d$. The method is quick to apply, like the 'E(I)-plot', and with ϵ_b from the latter, will give approximate values for K_d and n separately if the binding

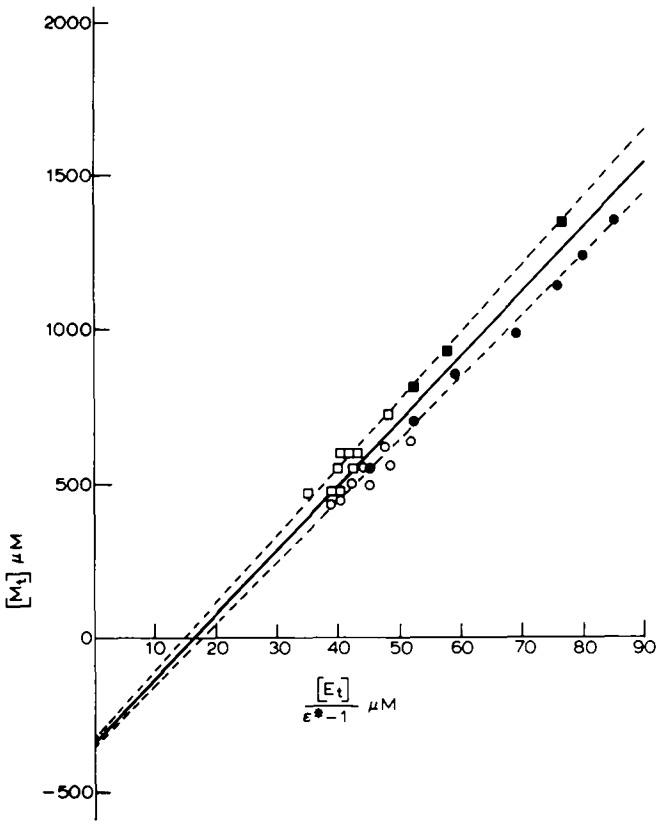


Fig. 6. "High metal" plot of $[M_t]$ vs. $[E_t]/(\epsilon^* - 1)$ for which data have been taken from appropriate regions ($[M_f] \geqslant 90\%\ [M_t]$) of M-titrations of the type shown in Fig. 1. The different symbols indicate different batches of Kp2 protein: ○, titrations of 4 mM Mn^{2+} vs. 36 μM Kp2; □, titrations of 4 mM Mn^{2+} vs. 23, 35, and 47 μM Kp2; ■, titrations of 10 mM Mn^{2+} vs. 23 μM Kp2; ●, a titration of 10 mM Mn^{2+} vs. 25 μM Kp2.

sites are the same in each case. Applied to the present system, the graph obtained is shown in Fig. 6, in which the parameters obtained were used to check that all points admitted satisfy the criterion $[M_f] \geqslant 90\%\ [M_t]$. The slope of 22.1 indicates $n = 4$ if $\epsilon_b = 6.5$, and the intercept yields $K_d = 330$ μM, in satisfactory agreement with the values given earlier. The accuracy of the 'high metal' method will be limited in this particular case since very short relaxation times approaching the limit of the instrument were measured to obtain an adequate range of points. The method should be more reliable in cases of weaker complex formation.

The estimation of ϵ_t

The characteristic enhancement, ϵ_b, for metal ion · protein binary complexes is related, in the limit of fast chemical exchange, to hydration numbers and the spin-lattice relaxation times of bound sites by the equation

$$\epsilon_b = \frac{q^*}{q} \cdot \frac{T_{1,M}}{T_{1^*,M}}$$

where q is the number of coordination sites on the metal occupied by water molecules, and $T_{1,M}$ is the relaxation time of bound water protons. Asterisks denote the presence of the protein.

In order to estimate the characteristic enhancements for ternary complexes it is sufficient to write equations, analagous to that above, for the binary metal ion · nucleotide complexes (a prime is used to denote the presence of nucleotide) and for the ternary complexes (the superscript t is used to denote the presence of ternary complexes):

$$\epsilon'_b = \frac{q'}{q} \cdot \frac{T_{1,M}}{T'_{1,M}} \qquad \epsilon_t = \frac{q^t}{q} \cdot \frac{T_{1,M}}{T_1{}^t{}_{,M}}$$

Hence,

$$\frac{\epsilon_t}{\epsilon'_b} = \frac{q^t}{q'} \cdot \frac{T'_{1,M}}{T_1{}^t{}_{,M}}$$

(If fast exchange conditions do not apply, $T_{1,M}$ should be replaced by $T_{1,M} + \tau_M$ in the equations and this discussion). We have no direct information on the ratio $T'_{1,M}/T^t_{1,M}$, but would expect similarity, and assume equality, to the ratio $T_{1,M}/T^*_{1,M}$ for the binary metal ion · protein complex. The latter ratio is evaluated as 6.5 × 6/5, using the experimental value of ϵ_b, the value $q = 6$ for the hexahydrated Mn^{2+} in aqueous solution, and the likely value $q^* = 5$ for the binary metal ion · protein complex. Binding of Mn^{2+} to ATP [25] or ADP occurs via one nucleotide nitrogen and, respectively, 3 or 2 phosphate oxygens. This gives $q' = 2$, $q^t = 1$ for the complex with ATP and $q' = 3$, $q^t = 2$ for that with ADP. Substituting these values, with $\epsilon'_b = 1.55$ for ATP and 1.65 for ADP, gives ϵ_t the values 6 and 8 for the ternary complexes with ATP and ADP, respectively.

Acknowledgements

We thank Mr. T.M. Siverns for help with the NMR measurements, Mrs. Anne Funnell for protein preparation and Mr. K. Baker for growing the bacteria. Also, our thanks to the Science Research Council for research studentships (to D.O. and M.D.L.) and for financial support in the purchase of the Jeol PFT-100 spectrometer.

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