# Cluster-complex mediated electron-transfer and ATP hydrolysis

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Abstract - Chemical-modeling studies of ATP-driven electron-transfer in
nitrogenase reactions show that ATP can complex with $(Fe_4S_4(SPh)_4)^{2-}$ clus-
ter (without displacing any of the thiolate ligands and without shielding
the cubane-like cluster from disruption by iron chelaters),resulting in
suppression of electronic absorption peak at 458 nm, in down-field shift-
ing of the $^{31}P$ n.m.r. peaks of $\alpha$-, $\beta$-, and $\gamma$-$PO_4$ of ATP by about 8.2 ppm,
7.9 ppm, and 10 ppm, respectively, and in shifting the polarographic half-
wave potential from -1.00 V to -1.49 V, as well as in significant enhance-
ment of the rate of redox reaction between the cubane-like cluster and in-
digo carmine, or methylene blue, with no detectable ATP hydrolysis. A
small extent of ATP hydrolysis (ca. 14%; in DMF-water, 3:2 v/v, and pH 7.0)
was observed, however, when the cubane-ATP complex was oxidized with hydro-
gen peroxide at room temperature. These results are in line with the me-
chanism of ATP-driven electron-transfer previously proposed by us.

## INTRODUCTION

Nitrogenase is a complex metallo-enzyme consisting of two metallo-protein components: com-
ponent 1, the MoFe-protein, consists of 4 subunits ($\alpha_2\beta_2$ ) and carries the substrate-binding
site; and component 2, the Fe-protein, consists of two identical subunits ($\delta_2$) with a cubane-
like 4Fe-4S cluster anchored between them probably through 4 cysteinyl-thiolate ligands, and
serves as a specific one-electron carrier to transport electrons to the MoFe-protein with the
aid of MgATP as "electron activator , each molecule of the Fe-protein being able to complex
with 2 molecules of MgATP, which are hydrolyzed into 2 MgADP and 2 Pi (inorganic phosphate),
practically concurrently with the electron-transfer from the reduced Fe-protein to the semi-
reduced MoFe-protein (ref. 1, 2). This ATP-driven electron transfer in nitrogenase catalysis
is an important example of coupled electron and energy transports in biochemical processes,
and has been the subject of extensive investigations since the late 1960s; but just how this
"electron activation" works is still an unresolved problem. For example, the site of MgATP
binding is still more or less a matter of speculation. In view of the fact that complexation
of MgATP with the reduced Fe-protein renders the 4Fe-4S center readily susceptible to disrup-
tion by batho-phenanthroline iron-chelater, most investigaters hold the views that the two
molecules of MgATP are not bound to the cubane-like center, but to some other parts of the
Fe-protein, causing a conformational change to take place with greater exposure of the 4Fe-4S
center, and that hydrolysis of the bound MgATP brings about further conformational change to
drive the electron-transfer to the MoFe-protein. However, there are some weaknesses in this
view: the nature of the bonding of MgATP with the Fe-protein is left unspecified; moreover,
it is a known experimental fact that complexation of MgATP with the reduced or oxidized Fe-
protein alone without the protein-protein electron-transfer does not lead to appreciable pro-
motion of ATP hydrolysis; so the mechanism of ATP hydrolysis, which has been shown to take
place at practically the same rate as the protein-protein electron-transfer (ref.3 ), is also
left unexplained. Since the Fe-protein is known to be sensitized by ATP, GTP, or pyrophos-
phates to inactivation and oxidation by air (ref. 1), and since these reagents are known to
be efficient iron chelaters, it seems to us that the 4Fe-4S center of the Fe-protein may be
accessible to these iron chelators.

Based upon the principles of coordination catalysis (ref. 4), a mechanism of 2-step ATP-
driven electron-transport in nitrogenase catalysis has been proposed and developed by us
(ref. 5) consisting of the following essential points: (1) coordination of MgATP (to be de-
noted by t) to the 4Fe-4S center of the reduced Fe-protein (to be denoted by [2s], where the
subscript s signifies that this species is e.p.r. active, having characteristic e.p.r. sig-
nal), to form $[2_s^{t}]$ produces a conformational change and raises the ligand field acting on the
4Fe-4S center to drive the electron-transfer to the e.p.r. active, semi-reduced MoFe-protein,

[$1_S$], resulting in the formation of the e.p.r. silent, oxidized Fe-protein and the e.p.r. silent, reduced MoFe-protein, [$1_O$]; (2) this electron outflow from the Fe-protein greatly promotes the hydrolysis of each of the two coordinated $t$ into MgADP (d) and inorganic phosphate (Pi), thus the enzyme complex is now in the state [$1_O$][$2_O^d$d, 2Pi]; (3) this enzyme complex dissociates into its two protein components for the release of the entrapped 2Pi and for successive displacement of the two coordinated $d$ by $t$, as well as for replenishment of an electron from the reductant; (4) a second step of ATP-driven electron-transfer, this time from [$1_O$] to the coordinated substrate without ATP hydrolysis, appears to be necessary since [$1_O$] alone without the Fe-protein and $t$ is known to be unable to reduce substrates; this may be accomplished by complexation of [$1_O$] with [$2_S^t$], or even with [$2_O^t$], which may also have sufficiently negative redox potential to prevent the MoFe-protein-to-Fe-protein electron-backflow; and (5) electron backflow may take place, however, whenever ]$2_O$] or [$2_O^d$], before coordinating $t$, has a chance to complex with [$1_O$]. This proposed mechanism of 2-step ATP-driven electron-transfer can explain (ref. 5-6) the redox states of the enzyme components, as revealed by the presence or absence of e.p.r. signals, at the steady state of the enzyme turnover, or at insufficient supply of reductant or ATP, as observed by Walker and Mortenson (ref. 7) with dilute solution of nitrogenase from C. pasteurianum (Cp) where the complexation of [$1_O$] with [$2_S^t$] or [$2_O^t$] may be slow enough to be rate controlling.

Some support of this proposed mechanism has been obtained by Chen et al. (ref. 8) from the observed effects of ATP on electronic absorption spectra and polarographic half-wave potential of synthetic 4Fe-4S cubane-like clusters, and on the rate of redox reaction of one of these clusters with methylene blue (M.B.).

This paper reports further support from similar chemical-modeling experiments with (Fe$_4$S$_4$(SPh)$_4$)$^{2-}$ as a model compound of the 4Fe-4S center of the oxidized Fe-protein, showing the effect of the cluster on the chemical shifts of the $^{31}$P n.m.r. peaks of MgATP, or ATP in DMF-D$_2$O solution, and the effects of ATP on the thiolate ligands, on the rates of disruption of the cluster by phenanthroline, and of oxidation by indigo carmine (In), as well as on the extent of ATP hydrolysis promoted by the oxidation of the cluster with hydrogen peroxide in DMF-water.

## EXPERIMENTAL

### Materials
All the chemicals used were of A.R. or C.P. grades. Purification of ATP (biochemical reagent from Shanghai Biochemical Research Institute of the Chinese Academy of Sciences) to an ATP content of greater than 98% was done according to known method of ion-exchange. Dimethyl formamide (DMF) of A.R. grade was freshly distilled before use. Stock solution of the redox dye indigo carmine (In) in DMF was prepared in 5.0 mM, and that of phenanthroline (phen) in DMF, 10 nM. Quarternary ammonium salts of the cubane-like cluster (e.g., the tetra-ethyl ammonium salt (Et$_4$N)$_2$Fe$_4$S$_4$(SPh)$_4$) were prepared according to known methods (ref. 9-10) and identified by their electronic absorption spectra. Non-aerobic experiments were all conducted in thoroughly deoxygenated argon atmosphere.

### Effect of (Fe$_4$S$_4$(SPh)$_4$)$^{2-}$ on $^{31}$P n.m.r. spectrum of ATP in DMF-D$_2$O
To 4.0 ml of a 15 mM solution of (Et$_4$N)$_2$Fe$_4$S$_4$(SPh)$_4$ in DMF was added 1.0 ml of 150-mM ATP in D$_2$O (the pH being adjusted to 7.0 with dilute NaOH in D$_2$O). The DMF:D$_2$O ratio (4:1 v/v) was found to be sufficient to keep the cluster complex in solution while the excess ATP largely undissolved. The mixture was shaken and allowed to stand in thoroughly deoxygenated argon atmosphere for 20 minutes, then centrifuged to remove the excess ATP; 2.5 ml of the supernatant liquid were transferred to n.m.r. cell, and the $^{31}$P n.m.r. spectrum of the sample taken with a Varian FT-80A n.m.r. spectrometer, with Na$_2$HPO$_4$ in D$_2$O as external strandard. Likewise, the $^{31}$P n.m.r. spectra of samples prepared by treating 4.0 ml of 15-mM solution of (Et$_4$N)$_2$Fe$_4$S$_4$(SPh)$_4$ in DMF with 1.0 ml of 150-mM solution of ADP (or AMP, or Pi) in D$_2$O were taken, as well as the $^{31}$P n.m.r. spectra of samples of ATP, ADP, and AMP, each in about 20-mM solution in D$_2$O, and of an ATP solultion in D$_2$O containing an equivalent amount of HSC$_2$H$_4$OH. The $^{31}$P n.m.r. peaks of the $\alpha$-, $\beta$-, and $\delta$-PO$_4$ of ATP were labelled according to the literature (ref. 11).

### Detection of any free thiophenol in (Fe$_4$S$_4$(SPh)$_4$)$^{2-}$ –ATP mixture in DMF-H$_2$O
The mixture of the cluster compound (1.0 mM) and ATP in DMF-H$_2$O (4:1 v/v, Tris HCl 25 mM, pH 7.6) was extracted with n-heptane for any free thiophenol, and the extract treated with silver dithiazon (AgDz) in carbon tetrachloride solution prepared according to standard method of silver dithiazon test (ref. 12) for the detection of trace thiophenol; (Fe$_4$S$_4$(SPh)$_4$)$^{2-}$ in DMF-H$_2$O (4:1 v/v, Tris HCl 25 mM, pH 7.6) being used as reference for the colorimetry of the thiophenol — AgDz color test at 615 mµ.

### Effect of ATP (ADP, AMP, or Pi) on rate of disruption of (Fe$_4$S$_4$(SPh)$_4$)$^{2-}$ by phenanthroline in DMF-H$_2$O (3:2 v/v)
In a 0.5-cm spectrophotometric cell closely fitted with stopper were placed 1.5 ml of DMF-H$_2$O (3:2 v/v, Tris HCl 25 mM, pH 7.5) containing 0.15 µmol (Fe$_4$S$_4$(SPh)$_4$)$^{2-}$ and 1.5 µmol ATP;

then 0.3 ml of 10-mM phen in DMF was quickly added and the electronic absorption at 510 mμ recorded with time. Similar spectrophotometric experiments were performed with samples containing, separately, 1.5 μmol ADP, 1.5 μmol AMP, and 1.5 μmol Pi, instead of the 1.5 μmol ATP; and with a reference containing only the cluster and phen in the same medium.

## Effect of ATP (ADP, AMP, or Pi) on rate of redox reaction between the cluster and indigo carmine in DMF-H₂O

Into 1-cm spectrophotometric cells with closely fitted stoppers were introduced 2.0 ml of 0.05-mM $(Fe_4S_4(SPh)_4)^{2-}$ in DMF and 0.50 ml H₂O containing 1.0 umol of ATP, or ADP, or Pi (each adjusted to pH 7.0 with 50-mM Tris HCl buffer). After standing for 20 minutes, 0.15ml of 0.50-mM indigo carmine in DMF was quickly added, and the rate change in optical density at 610 mμ was recorded spectrophotometrically.

## Determination of extent of ATP hydrolysis promoted by reactions between the cluster and redox dye or hydrogen peroxide

Phosphate ions (Pi) liberated from ATP hydrolysis may be determined by a modification of Baginski's molybdenum blue method (ref. 13). With MgATP, or ATP-oxidant (M.B.) as reference, the amount of Pi liberated from $(Fe_4S_4(SPh)_4)^{2-}$-MgATP, and from $(Fe_4S_4(SPh)_4)^{2-}$-MgATP-M.B. may be determined sparately by the modified molybdenum blue method by subtracting the amount of Pi determined in the reference from the total amount of Pi determined in each of the test systems.

Since both methylene blue and the cluster interfere seriously with the molybdenum blue colorimetry, these must be removed by extraction before the additon of the molybdenum blue color developing reagents. Methylene blue cation and perclorate anion can from chloroform-soluble ion-pair; so, in the presence of $ClO_4^-$ M.B. can be extracted with chloroform. The actual procedure adopted was, after the addition of 5.0 ml of 10-mM NaClO₄ and 3-M NaCl to 3.0 ml of each of the samples, the mixture was extracted with 2.0 ml cloroform with vigorous shaking for 4 minutes, and then centrifuged; 5.0 ml of the clear upper layer (aqueous) were extracted twice with 2 ml chloroform. Finally, the clear aqueous layer was used for the determination of Pi by the modified molybdenum blue method (ref. 13).

For the determination of Pi liberated from ATP in the redox reaction between the cluster and hydrogen peroxide in the presence of ATP, in DMF-H₂O (3:2 v/v), the following procedure was used. To 3.0 ml of the sample containing 30 μmol ATP and 30 μmol $(Fe_4S_4(SPh)_4)2-$ reacted with 60 μmol H₂O₂ in DMF-H₂O (60:40 v/v) were added 0.30 ml of 1.2-M n-Bu₄NI in methanol solution and 10 ml water; the mixture was shaken and then centrifuged to remove the insoluble $(Bu_4N)_2Fe_4S_4(SPh)_4$ in the form of voluminous precipitate. The supernatant liquid was filtered and the pH adjusted to about 9 with dilute NaOH. The solution was allowed to pass slowly through an anion-exchange column (i.d. 0.6 cm, filled to a volume of 2.0 ml with a strong-base type #717 anion-exchange resin in the chlorids form). The column was rinsed with 60 ml of distilled water made slightly alkaline (pH 9) with dilute NaOH. It was then eluted with 1.0 M KCl acidified with dilute HCl to pH 2, and the eluted solution allowed to run into a receiver containing a small amount of solid NaOH sufficient to make the solution slightly alkaline, in which ATP hydrolysis was found to be very slow. The Pi content was then determined by the modified Baginski's molybdenum blue method (ref. 13). The reference sample containing the same amounts of ATP and the cluster, but without the hydrogen peroxide, was similarly treated, and the amount of Pi determined was used as blank correction. Each experiment was run in duplicates. Three series of experiments were performed with three different ratios of DMF: H₂O since the extent of ATP hydrolysis was found to vary with the composition of the mixed solvents.

## RESULTS AND DISCUSSIONS

As shown in Fig. 1, treatment of ATP with $(Fe_4S_4(SPh)_4)2-$ in DMF-H₂O (4:1 v/v) caused the 31P n.m.r. peaks of the α-, β-, and ɣ-PO₄ of ATP to shift downfield by about 8.2 ppm, 7.9 ppm, and 10 ppm, respectively, with the β-³¹P n.m.r. peak very much broadened and the ɣ-31P n.m.r. peak greatly suppressed. This appeared to be quite similar to the downfield shifts of the α-, β-, and ɣ-³¹P n.m.r. peaks of MgATP by about 8.7 ppm, 9.0 ppm, and 7.7 ppm, respectively, caused by the additon of the reduced Fe-protein to MgATP, as observed by Mortenson and Upchurch (ref. 11). Note that additon of MgCl₂ to the ATP, or to the ATP-$(Fe_4S_4(SPh)_4)2-$ system in DMF-H₂O made no appreciable difference to the 31P n.m.r. spectra, and that additon of an equivent amount of HSC₂H₄OH to ATP in DMF-H₂O did not produce any shifting in the 31P n.m.r. peaks.

Chen et al. (ref. 8) have shown that addition of ATP to $(Fe_4S_4(SPh)_4)2-$ suppressed the polarographic half-wave of the cluster at -1.00 V and produced a new half-wave at -1.49 V (Fig. 2). This again is qualitatively analogous to the shifting of the redox potential of the reduced Fe-protein by about -0.1 to -0.2 V due to the addition of MgATP (or MgADP) (rev. in ref. 1,2). They have also observed that the electronic absorption peak of the cluster $(Fe_4S_4(SPh)_4)2-$ at 458 nm was suppressed by the addition of ATP (Fig. 3), and that ATP (ADP, Pi, AMP) promoted the redox reaction between the cubane-like cluster and methylene blue, the

observed order of decreasing extent of rate promotion being ATP ≯ ADP ~ P$_i$ ≯ AMP.
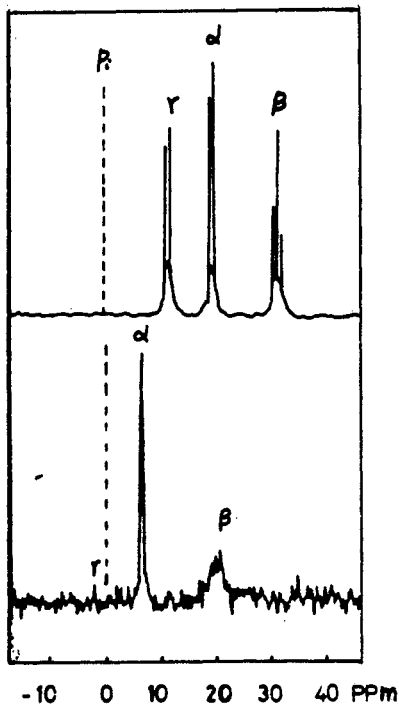


Fig. 1.  Shifting of 31P n.m.r.
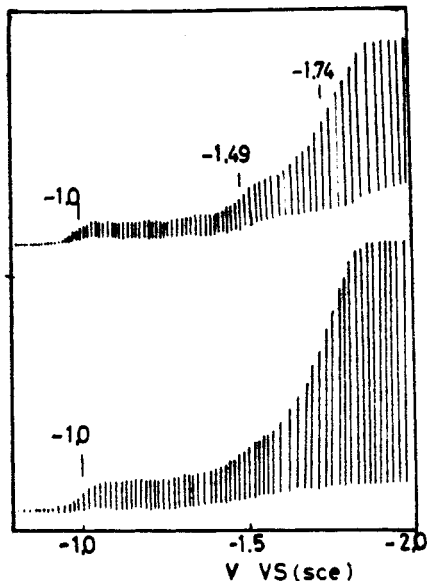peaks of ATP due to addition
of (Fe$_4$S$_4$(SPh)$_4$)$^{2-}$



Fig. 2.  Shifting of polarographic
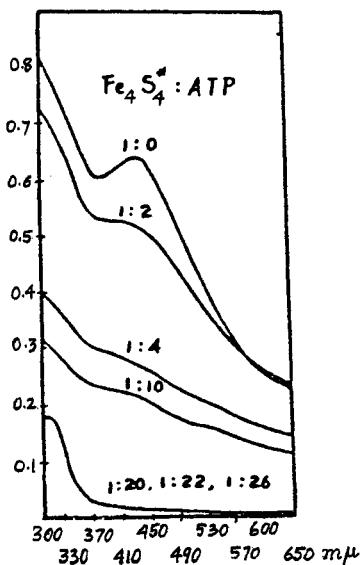half-wave of (Fe$_4$S$_4$(SPh)$_4$)$^{2-}$ due to
addition of ATP, or MgATP



Fig. 3. Effect of ATP on electronic
absorption of Fe$_4$S$_4$(SPh)$_4$)$^{2-}$

These experimental results of chemical modeling
strongly indicate that ATP can complex with (Fe$_4$S$_4$-
(SPh)$_4$)$^{2-}$, and that MgATP is most probably coordi-
nated directly to the 4Fe-4S center of the Fe-
protein.

No thiophenol was detected after the cubane-like
cluster (Fe$_4$S$_4$(SPh)$_4$)(NEt$_4$)$_2$ in DMF-H$_2$O was treated
with ATP, showing that complexation with ATP did not
displace any of the thiolate ligands on the cluster.
Thus ligation of ATP to any of the Fe(II,III) pro-
bably took place simply by changing the tetrahydral
coordination of the metal ion into trigonal-bipyri-
midal coordination, resulting in an increase in
ligand field acting on the cluster anion to exert
additional driving force for the electron outflow.

It is astonishing to find that complexation of ATP
with the cubane-like cluster did not appear to
shield the cluster from disruption by the  iron
chelater, phenanthroline in DMF-H$_2$O (3:2 v/v, pH
7.5); instead, it enhanced the rate of development
of the characteristic absorption peak of Fe(II)-phen
complex at 510 nm (Fig. 4), as did ADP to a smaller
extent.  Again this is qualitatively analogous to
the dramatic sensitization,by the addition of MgATP,
of the 4Fe-4S center of Fe-protein to disruption by
iron chelaters (ref.1,2).

A plausible explanation is as follows: In either case the complexation of 2ATP, or 2MgATP in
the case of the Fe-protein, with the 4Fe-4S cubane-like cluster is fast and reversible, as
indicated by the n.m.r. 31P peaks broadening; thus at certain instances only one face of the
cubane-like 4Fe-4S cluster is ligated with ATP, or MgATP in the case of the Fe-protein, and
the valence dislocalization of Fe(II,III) in the cluster is broken up, with the ATP, or
MgATP, preferentially coordinated to the Fe(III), leaving the 2 Fe(II) on the opposite face
of the 4Fe-4S cubane-like cluster coordinatively unsaturated and open to attack by the Fe(II)

chelater phenanthroline. In the case of the Fe-protein, complexation of MgATP to the 4Fe-4S center on one face might also produce some conformational change with the two protein sub-units open up a little so that the opposite face the 4Fe-4S center is rendered more accessible to phen, or reductant. It is to be noted that, with higher proportion of DMF in DMF-$H_2O$ (e.g., 4:1 v/v), some inhibition of the cubane-like cluster $(Fe_4S_4(SPh)_4)2-$ by ATP or ADP, from attack by phen was observed; probably here the Fe(II) of the opposite face of the cluster is partially protected by solvation with DMF.

As shown in Fig. 5, the rate of redox reaction between the $(Fe_4S_4(SPh)_4)2-$ and indigo car-mine was found to be significantly enhanced by the addition of ATP, and to a smaller extent by the addition of ADP or Pi, while AMP is almost ineffective. This is in the order of in-creasing steric hindrance if ATP, ADP, and AMP are all coordinated to the Fe(II,III) of the 4Fe-4S center through their terminal $PO_4$. Similar order of   decreasing   rate enhance-ment in the redox reaction between the cluster and methylene blue has been reported by Chen et al. (ref. 8).
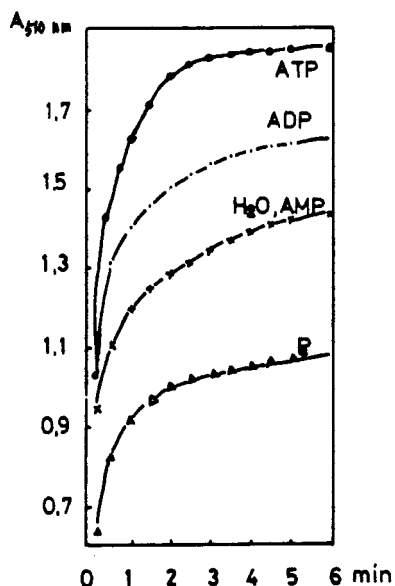


Fig. 4. Sensitization of cluster disruption by phen due to addi-tion of ATP or ADP in DMF-$H_2O$ (3:2 v/v, pH 7.5)
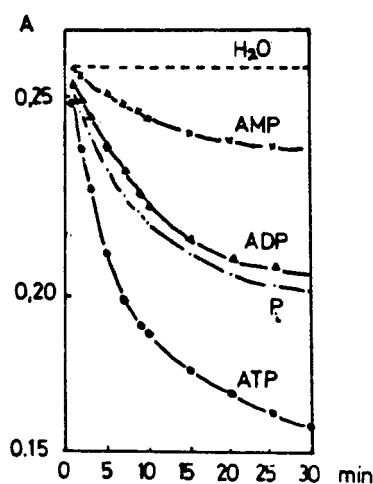
Fig. 5. Promotion of redox reaction between $(Fe_4S_4(SPh)_4)2-$ and indigo carmine by ATP (ADP, $P_i$, AMP) in DMF-$H_2O$

However, no ATP hydrolysis was detected in the sample after the redox reaction and removal of the dye and the cluster by extraction with chloroform.

Small extents of ATP hydrolysis were observed, however, by oxidation of the cubane-like clus-ter $(Fe_4S_4(SPh)_4)2-$ with hydrogen peroxide in the presence of ATP in DMF-$H_2O$, and the extent of ATP hydrolysis was found to decrease with increasing proportion of DMF in the mixed sol-vents. Thus with DMF-$H_2O$ (3:2 v/v) containing 30 μmol of the cluster, 30 μmol ATP and 60 μmol $H_2O_2$ in 30 ml of solution, the extent of ATP hydrolysis after correction for blank (1.26 μmol Pi liberated) was 2.44 μmol, i.e., ca 8.1% of the total ATP; and with DMF-$H_2O$ (3:2 v/v) con-taining 15 μmol of the cluster, and 60 μmol of ATP, and 30 μmol of $H_2O_2$, the extent of hydro-lysis was found to be 14.4% of the cluster as the limiting factor; while with 3.0 ml of DMF-$H_2O$ (4:1 v/v) containing 30 μmol of the cluster, 30 μmol ATP, and 60 μmol $H_2O_2$, the extent of ATP hydrolysis at 30°C was only about 4.2% of the toal ATP after correction for blank, ion-exchange method being used in each determination for the isolation of the liberated Pi.

According to Haight et al. (ref. 14), hydrolysis of ATP or triphosphate coordinated in cer-tain mode of chelation on $VO2+$ or $Mn2+$ was greatly enhanced by oxidizing the $VO2+$ or $Mn2+$ with hydrogen peroxide; and rate enhancement of the order of $10^4$ to $10^6$ times for the hydro-lysis of ATP or triphosphate could be obtained simply by complexation with $VO3+$ or $Mn3+$. Haight et al. (ref. 15) have also found that triphosphate coordinated as tridentate ligand on Co(III) was hydrolyzed by attack from adjacent nucleophile at pH 7.3 about $10^6$ times faster than the uncoordinated triphosphate. However, no work has been reported in the literature on the hydrolysis of ATP or triphosphate coordinated on iron-sulfur clusters. With the cubane-like $(Fe_4S_4(SPh)_4)2-,3-$ clusters each with 6 M-M bonds and formal number of electrons in the

coordination sphere equal to 66, or 67, respectively, each of the Fe(II,III) can easily accommodate an additional monodentate (but not bidentate!) ligand to assume a trigonal-bipyrimidal coordination with only a slight readjustment of the position of the thiolate ligand. Thus ATP (or MgATP), or ADP (or MgADP) can easily coordinate to it as a monodentate ligand through the $\gamma$- or $\beta$-PO$_4$; but the $\alpha$-PO$_4$ appears to be ineffective due to the proximity of the bulky adenyl group. The internuclear distance of each pair of adjacent Fe(II,III) of the cubane-like cluster (ref. 9,10) is about 2.70 to 2.76 Å; so, ATP or MgATP molecule may also be coordinated as a bridge-ligand with the o- and B-phosphonyl oxygen atoms spanning two Fe (II,III) at about 2.85 Å internuclear distance with relaxation of the M-M bond. Thus the formal total numbers of electrons in the coordination spheres of the 4Fe(II,III) will be increased to 70 and 71, respectively, comparable with [Fe$_4$S$_4$(S$_2$C$_2$(CF$_3$)$_2$)$_4$]$^{2-}$ with 4 M-M bonds (ref. 16) and formally 70 electrons, and with [Fe$_4$S$_4$(SPh)$_2$(dtc)$_2$]$^{2-}$ with 5 M-M bonds (ref. 17) and formally 68 electrons (for each M-M bond, the electron pair being counted twice). Thus these iron-sulfur clusters are still coordinationally unsaturated in the sence that the formal total number of electrons in the coordination sphere of the 4 Fe(II,III) is still less than 72. In DMF-H$_2$O, ATP appears to coordinate predominantly as a monodentate ligand, and only a small part as bridge ligand, probably due to strong competition from DMF for the coordination site. Conceivably, this part of ATP coordinated as bridge-ligand is much more susceptible to hydrolysis by nucleophilic attack, especially when the 4Fe-4S core becomes more positively charged due to the loss of an electron in the redox reaction. This may be the reason why only a small extent of ATP hydrolysis was observed with the model system in DMF-H$_2$O. In the case of Fe-protein-2MgATP complex, there is no competition from strongly ligating solvent molecules; furthermore, the 2t may be constrained by the micro-environment inside the Fe-protein to coordinate only as bridge-ligands through the $\gamma$- and $\beta$-PO$_4$, so that hydrolysis of the anhydride linkage by nucleophilic attack from adjacent nucleophile inside the protein micro-environment is greatly promoted attending the electron outflow from the 4Fe-4S center. This large rate enhancement makes the ATP hydrolysis and the protein-protein electron-transfer appear as practically concurrent events, both being limited by the rate of protein-protein complexation in the 40-45 ms range. A probable mode of bridge-type coordination of the 2t in [2$_s^{tt}$] is shown in Fig. 6. Note that a strong support of this bridge-type coordination is as follows: considering only the two O$_3$POPO$_3$ and the 4Fe-4S core with 4 thiolate ligands, we see a near S$_4$ local symmetry of the cluster-2MgATP complex, in accord with the near axial symmetry indicated by the e.p.r. signal of [2$_s^{tt}$], as observed Mortenson and Walker (rev. in ref. 1). A probable mode of coordination of MgADP as monodentate ligand is shown in Fig. 7.
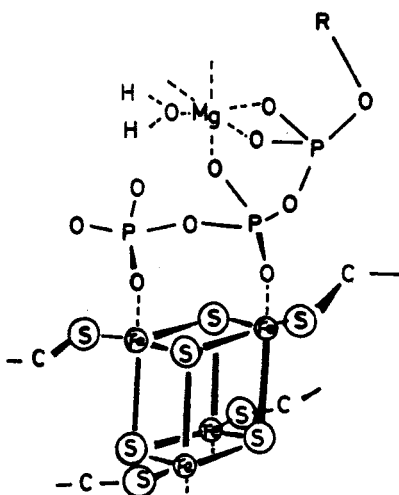


Fig. 6.  Probable mode of
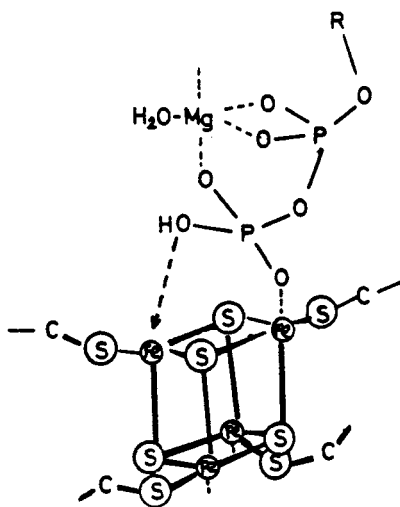coordination of 2t in [2$_s^{tt}$]
with local $\bar{4}$ axial symmetry

Fig. 7.  Probable mode of
coordination of 2d in [2$_s^{dd}$]

Fig. 8 is a diagram showing the nitrogenase-catalyzed reaction pathways for a mechanism of 2-step ATP-driven electron-transfer, which takes into account the following points: (a0 ATP hydrolysis takes place only in the electron outflow from the reduced Fe-protein to MoFe-protein (ref. 18); (b) with more concentrated solution of nitrogenase from K. pneumoniae (Kp), the dissociation of the enzyme complex [1$_o$][2$_o^{dd}$, 2$^{Pi}$] after the protein-protein electron-transfer may be the rate controlling step of the enzyme turnover (ref. 19); and (c) there is the possibility that [2$_s^{td}$] might also have sufficiently negative redox potential to drive the protein-protein electron-transfer in order to account for the observation by Mortenson Upchurch (ref. 11) that, with appropriate ratio of d/t (in the range of 0.3 to 0.5) and with dilute Cp nitrogenase, the ATP/2e ratio could be as low as 2.

For an enzyme system $[1_s]:[2_s]$ with sufficient supplies of reductant and ATP, the initial step is the complexation of $[2_s]$ with $2t$ followed by complexation with $[1_s]$ to form the enzyme complex $[1_s][2_s^t t]$. After that the main reaction pathway proceeds in cycle along the steps $(2)-(3)-(4')-(4'')-(5)-(6)-(7)-(2)$, and a secondary reaction pathway along the steps $(2)-(3)-(4')-(4'')-(12)-(13)-(14)-(15)-(1)-(2)$, with the step $(3)!$ as the rate-determining step for either pathway; so most of the enzyme system under this steady state conditions of the enzyme turnover will be in the e.p.r. silent state $[1_0][2_0^{dd}]$, as observed by Smith et al. (ref. 19). Two other secondary reaction pathways will be of minor importance, or practically negligible, if the relative concentrations of $t$, $d$, ($t/d$ ratio large), and of reductant (small compared with $t$) are such that step $(5')$ and step $(12)$ are both very slow compared with step $(4'')$. If this is the case and only these four reaction pathways are considered, then the ATP/2e ratio will be practically equal to 4, or slightly less than 4. However, there is alway some chance for the reductant independent ATP-hydrolysis reaction-cycle $(3)-(8)-(9)-(10)-(11)-(1')-(2')-(3)$ to occour, especially when $[1_0]$ is about qual to, or large than $[2_0]$ in molar concentration, or when the two protein components complex with each other unusally firmly (as in the case of certain cross components, like Kp1:Av2),; so, the overall ATP/2e ratio is usually greater than 4. For an enzyme system $[1_s]:[2_s]$ with sufficient supply of reductant but limited supply of ATP, step $(8)$ will become more and more important compared with step $(4')$ when $t$ is being used up; and when $t$ is exhausted, steps $(4')$ and $(11)$ can no longer proceed, so the enzyme system will end up with the state $[1_s][2_s^d]$, or its dissociated state $[1_s]:[2_s^d]$, or $[1_s]:[2_s^{dd}]$ with both components e.p.r. active. On the other hand, if $t$ is sufficient, but reductant exhausted, then steps $(5)$, $(5')$, and $(15')$ can not proceed, so the enzyme system will end up with the state $[1_s][2_s^t t]$, or $[1_s][2_s^{td}]$, $[1_s]:[2_s^{td}]$ states, showing only the e.p.r. signals of the semi-reduced MoFe-protein, in accord with the observations of Mortenson and Walker (ref. 7) and Smith et al. (ref. 19). From Fig. 8 it can be inferred that, the appropriate conditions for observing an ATP/2e ratio small than 4 (this, however, has not been reported elsewhere) would seem to be the use of very dilute enzyme solution with a fairly large excess of the Fe-protein, very high concentration of reductant, and high concentrations of $t$ and $d$ with appropriate ratio of $t/d$ (0.3 to 0.5), so that the enzyme reaction proceeds predominantly by the pathway $(2')-(3)-(4')-(5')-(6')!-(7')-(2')$, and the secondary pathway $(3)-(4)-(12')!-(13')-(14')-(15')-(1')-(2')-(3)$, with compara-
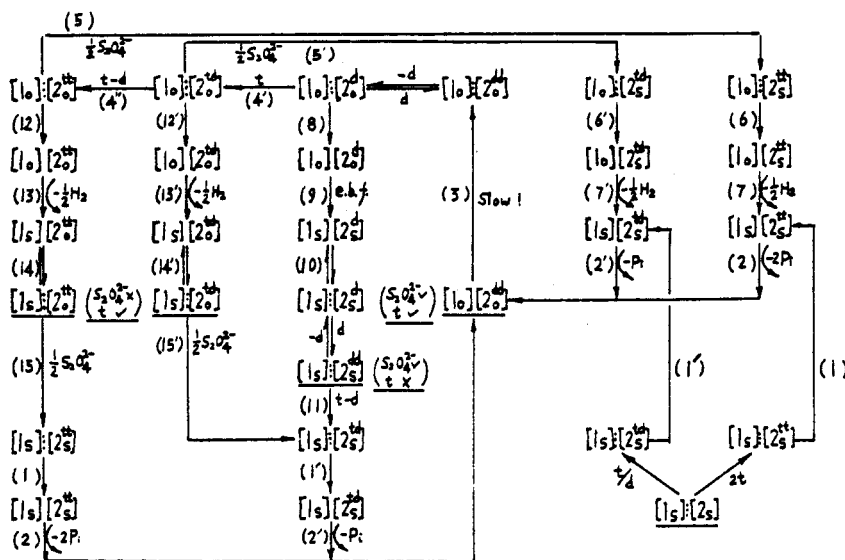


Fig. 8. Diagram showing reaction pathways for a mechanism of 2-step ATP-driven electron transfer. $S_2O_4 \vee$ or $\chi$, and/or $t \vee$ or $\chi$ denote sufficient or insufficient supply of reductant and/or $t$. For other notation see text.

tively small probability for electron backflow because step $(8)$ will be very slow compared with $(4')$ under the above conditions with $S_2O_4 > t \sim 2d \gg [2] \gg [1]$. Finally the reductant-independent, nitrogenase-catalyzed ATP-hydrolysis reaction pathway is easily seen from Fig. 8 to be the cyclic sequence of setp: $(3)-(8)-(9)-(10)-(11)-(1')-(2')-(3)$, with ATP/2e $\to \infty$. This will take place to a greater extent if $[1_0] > [2_0]$, or if $[1_0]$ has a strong affinity to bind $[2_0]$, as in the case of the tight-binding cross components Av1 and Cp2 (ref. 20).

## CONCLUDING REMARKS

Chemical modeling experiments show that ATP, or MgATP, can complex with the cubane-like

cluster $(Fe_4S_4(SPh)_4)^{2-}$ (without displacing any of the thiolate ligands), resulting in down-field shifting of the $^{31}P$ n.m.r. peaks of ATP by about 8-10 ppm, in sensitizing the cluster to disruption by phenanthroline in DMF-$H_2O$ (3:2 v/v), and in shifting the redox potential of the cluster to more negative value to promote electron outflow. All these are analogous to the effects of complexation of MgATP with the Fe-protein, indicating direct coordination of MgATP to the 4Fe-4S center of the Fe-protein to drive the electron-transfer. Oxidation of the $(Fe_4S_4(SPh)_4)^{2-}$ with hydrogen peroxide in DMF-$H_2O$ (3:2 v/v) promotes ATP hydrolysis to a small extent of about 8-14%. In this medium, ATP appears to coordinate to the cluster predominantly as a monodentate ligand through the terminal $PO_4$; whereas in the Fe-protein, the 2 MgATP are most probably constrained by the miro-environment inside the protein to coordinate to the 4Fe-4S center only as bridge-ligands through the $\delta$ - and $\beta$-$PO_4$, thus they are conceivably much more susceptible to hydrolysis by nucleophilic attack from adjacent nucleophile in the electron outflow from the reduced Fe-protein.

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