Evidence for the Selective Population of FeMo Cofactor Sites in MoFe Protein and Its Molecular Recognition by the Fe Protein in Transition State Complex Analogues of Nitrogenase*

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We have collected synchrotron x-ray solution scattering data for the MoFe protein of Klebsiella pneumoniae nitrogenase and show that the molecular conformation of the protein that contains only one molybdenum per αβ2 tetramer is different from that of the protein that has full occupancy i.e. two molybdenums per molecule. This structural finding is consistent with the existence of MoFe protein molecules that contain only one FeMo cofactor site occupied and provides a rationale for the 50% loss of the specific activity of such preparations. A stable inactive transition state complex has been shown to form in the presence of MgADP and AlF4-. Gel filtration chromatography data show that the MoFe protein lacking a full complement of the cofactor forms initially a 1:1 complex before forming a low affinity 1:2 complex. A similar behavior is found for the MoFe protein with both cofactors occupied, but the high affinity 1:2 complex is formed at a lower ratio of Fe protein/MoFe protein. The 1:1 complex, MoFe protein-Fε protein (ADP-AlF4)2, formed with MoFe protein that lacks one of the cofactors, is stable. X-ray scattering studies of this complex have enabled us to obtain its low resolution structure at ~20 Å resolution, which confirms the gel filtration finding that only one molecule of the Fe protein binds the MoFe protein. By comparison with the low resolution structure of purified MoFe protein that contains only one molybdenum per tetramer, we deduce that the Fe protein interacts with the FeMo cofactor-binding α-subunit of the MoFe protein. This observation demonstrates that the conformation of the α-subunit or the αβ subunit pair that lacks the FeMo cofactor is altered and that the change is recognized by the Fe protein. The structure of the 1:1 complex reveals a similar change in the conformation of the Fe protein as has been observed in the low resolution scattering mask and the high resolution crystallographic study of the 1:2 complex where both cofactors are occupied and with the Fe protein bound to both subunits. This extensive conformational change observed for the Fe protein in the complexes is, however, not observed when MgATP or MgADP binds to the isolated Fe protein. Thus, the large scale conformational change of the Fe protein is associated with the complex formation of the two proteins.

Biological nitrogen fixation is catalyzed by nitrogenase, a two-component metalloenzyme system that couples the hydrolysis of MgATP to the reduction of dinitrogen in the reaction,

\[
N_2 + 8 H^+ + 8 e^- + 16 MgATP \rightarrow 2 NH_3 + H_2 + 16 MgADP + 16 P_i
\]

**REACTION 1**

Molybdenum-containing nitrogenases are made up of a molybdenum-containing (MoFe protein or component 1; ~230 kDa) and an iron-containing protein (Fe protein or component 2; ~60 kDa). During enzyme turnover the Fe protein functions as a specific MgATP-dependent electron donor to the MoFe protein (1-4). The x-ray crystal structures of both individual proteins isolated from Azotobacter vinelandii (Av proteins) and Clostridium pasteurianum (Cp proteins) have been determined (5-9). The x-ray structure of MoFe protein from K. pneumoniae (Kp protein) has also been determined (10). The MoFe proteins have an αβ2 subunit structure in which each subunit pair binds a unique FeS2 cluster (P cluster) positioned at the subunit interface and the active site of the enzyme, a Fe8S7 molybdenum homocitrate cluster (FeMo cofactor), within the α-subunit (11). The Fe protein is a γδ dimer that has a single Fe3S4 cluster at the subunit interface and two nucleotide binding sites, one on each subunit (6, 9). The binding of MgADP or MgATP to the isolated Fe protein results in an altered reactivity and spectroscopic properties of the Fe-S cluster, which have been well documented (see Ref. 4).

The crystal structures of Av2 and Cp2 (6, 9) display a peptide folding pattern similar to other nucleotide-binding proteins, including the ras and G-protein family, and myosin, where transient protein complexes couple nucleotide hydrolysis to signal and energy transduction processes (1). MgATP hydrolysis by nitrogenase requires the presence of both the Fe protein and the MoFe protein, and recently several groups have exploited these similarities to form stable but inactive nitrogenase complexes of A. vinelandii (12, 13) and K. pneumoniae (14, 15) using AlF4- and MgATP or MgADP. Aluminum fluoride has been extensively used as a tool to examine MgATP binding by gated proteins (16-18). It has been proposed that the ADP-AlF4- complex can be considered to be an analogue of E-ADP-Pi species in which AlF4- mimics the trigonal bipyramidal geometry of the terminal phosphate undergoing nucleophilic attack by a water molecule. More recently, Kp1 and Kp2 have been shown to form ADP-BeF4- stabilized complexes, being putative analogues of the MgATP-bound conformation (19).

Extensive kinetic and modeling work has shown that follow-

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1 Throughout, we use the standard nitrogenase nomenclature, i.e. a two-letter abbreviation indicating the genus and species, followed by either 1 or 2 denoting the component type (e.g. Kp2 stands for the Fe protein of K. pneumoniae).
ing each electron transfer, the Fe protein and the MoFe protein complex dissociates in what is the rate-limiting reaction of nitrogenase turnover (see Ref. 4). It has been demonstrated that this transient complex can be stabilized in the presence of ADP and AlF₄⁻ as an inactive putative transition state complex. The structure of a 1:2 transition state complex, MoFe protein-[(Fe protein-ADP-AlF₄⁻)]₂, was recently determined at ~20 Å resolution for *K. pneumoniae* nitrogenase using x-ray solution scattering (14) and for *A. vinelandii* nitrogenase at 3 Å resolution by x-ray crystallography (20) and solution scattering (21). For both species, a 13° rotation of subunits of the Fe protein compared with that of the isolated protein was observed (21), indicating that the Fe protein undergoes a substantial conformational change either on complex formation or nucleotide binding. In the complex, the conformational change undergone by the Fe protein brings the Fe₄S₄ cluster of the Fe protein 4 Å closer to the P cluster of the MoFe protein, to a typical electron transfer distance of ~14 Å. It is unclear whether this change in conformation takes place in the complex or is a result of MgATP binding to the Fe protein. In the case of the Fe protein from Av, it has been suggested from a small angle x-ray scattering study that a substantial conformational change has already taken place upon MgATP binding (22). The hypothesis that this “priming” of the Fe protein conformation is required to allow effective docking with the MoFe protein to permit successful electron transfer (see Ref. 23 for discussion) lacks firm experimental evidence and has been questioned recently. An alternative, in which MgATP binds rapidly to a complex of Fe and MoFe proteins, followed by subsequent conformational changes in the Fe protein, has been proposed (24). Moreover, in the heterologous nitrogenase formed between the Fe protein of *C. pasteurianum* and the MoFe protein from *A. vinelandii*, MgATP is not required for complex formation (25, 26).

We have recently reported that the kinetics of the formation of the *K. pneumoniae* transition state complex are consistent with MoFe protein lacking one FeMoco center having an altered molecular conformation, which the Fe protein can recognize (15). To test this proposal experimentally, we have obtained solution x-ray scattering data of Kp1 containing one or two FeMoco centers per molecule and generated their molecular shapes at ~20 Å resolution for both forms. In addition, the 1:1 complex MoFe protein-(Fe protein-ADP-AlF₄⁻) was isolated using Kp1 with only one cofactor site occupied, and the molecular structure was determined from solution x-ray scattering data. The molecular structure of the isolated Fe protein in the absence and presence of nucleotide was also examined to establish the extent of conformational change in Fe protein on its own.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**

*Nitrogenase Component Proteins—*All manipulation of the air-sensitive nitrogenase components was done under an atmosphere of nitrogen. The nitrogenase proteins Kp1 and Kp2 were purified from *K. pneumoniae* in a anaerobic chamber (glove box) in an atmosphere of nitrogen containing a very low concentration of oxygen (~5 ppm of O₂). All samples were filtered (0.2-μm pore size) and loaded in the glove box into a brass cell (containing a Teflon ring sandwiched by two mica windows that defines the sample volume of 120 μl and a thickness of 2.5 mm). The cell was sealed with parafilm and then transferred immediately to the x-ray station. Scattering data were collected on beamline 8.2 at the Synchrotron Radiation Source (Daresbury, UK) (29) at an electron energy of 2 GeV and with beam currents between 150 and 250 mA. At the sample-to-detector distance of 3.3 m (2.5 m) and the x-ray wavelength of λ = 1.54 Å, a momentum transfer interval of 0.002 (0.004) Å⁻¹ ≤ s ≤ 0.030 (0.035) Å⁻¹ was covered on a position-sensitive quadrant multwire proportional counter (30). Values in parentheses refer to the measurements for the Fe protein only. The modulus of the momentum transfer is defined as s = 2π sin(θ)/λ, where θ is the scattering angle. The scattering pattern from an oriented specimen of wet rat tail collagen was used to calibrate the detector. Samples were measured at room temperature (~20 °C) at concentrations of 0.5 and 5 mM. To minimize anomalous effects, each data set consisted of buffer followed by protein data collection. The experimental data were recorded in frames of 100 s allowing on-line checks for changes in the scattering profiles and corrected for background scattering (subtraction of the scattering from the camera and a cell filled with buffer), sample transmission and concentration, and positional nonlinearities of the detector. Off-line data reduction was done with the OTOKO software package (31). Maximum particle dimensions Dₚmax, the radius of gyration Rg, the distance distribution function ρ(r), and the extrapolated forward scattering value I(0) were evaluated with the program GONOM (32). The latter allows the estimation of molecular mass when calibrated against the scattering from proteins with known molecular mass (apart from fully loaded Kp1, 225 kDa, as standard for an anaerobic protein sample, nitrous oxide reductase, 134 kDa, was used). The volume V of the particle can be calculated from the Porod invariant (33), including the outer part of the scattering profile. A correction factor is applied to alleviate the difficulties of the limited range of scattering data (described in Ref. 34). More details concerning data collection and reduction are given elsewhere (35).

The computation of the molecular envelopes was based on the *ab initio* shape determination procedure of Svergun and Stuhrmann (36). If we assume that the scattering is caused by a globular, homogeneous molecule, one can define its molecular shape by the angular envelope function *F*(θ, ϕ) such that the particle density ρ(r) is unity inside the molecular boundary and vanishes elsewhere. *F*(θ, ϕ) can be expanded into a series of spherical harmonics *Ymn*(θ, ϕ) according to Refs. 37 and 38,

\[
F(θ, ϕ) = \sum_{l=0}^{L} \sum_{m=-l}^{l} f_{lm} Y_{lm}(θ, ϕ) \tag{1}
\]

where *f* *lm* represents complex multipole coefficients. The determination of molecular shapes directly from the scattering profile alone in a model-independent manner exploits the information inherent in the wider angle scattering data. The resolution is determined by the maximum order of included harmonics, *i.e.*, the highest value of *L*. A non-linear equations system interrelates the *f* *lm* coefficients with coefficients of the power series describing the experimental scattering curve. A reliable computational procedure was used to evaluate the multipole

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2 The abbreviations used are: FeMoco, iron-molybdenum cofactor; Kp1, Kp1 with only half the FeMo cofactors present.
coefficients by minimizing the least squares deviation ($R^2$) between experimental and calculated curves according to methods described by Svergun and Stuhrmann (36) and Svergun et al. (39). The shape calculation for the nitrogenase proteins has been performed with smoothed experimental curves (where the low angle part was adjusted to match the corresponding extrapolation to zero concentration). No molecular symmetry was assumed (except for the shape restoration of the isolated Fe protein as well as of the fully loaded MoFe protein; in both cases, a 2-fold symmetry is expected). The available experimental data range for the nitrogenase enzymes justifies the use of harmonics up to $L = 4$ (using no symmetry; i.e. 19 free parameters) and $L = 5$ (in the case of symmetry; i.e. 14 free parameters). To set the scene, atomic models have been fitted manually into the molecular shapes. The shape pictures were rendered and manipulated using a Silicon Graphics Indigo work station and the AVS graphics software (Advanced Visual Systems, Inc., Waltham, MA). The ribbon diagrams were produced using the molecular graphics program Insight II (Biosym/MSI). Crystallographic information was utilized to define the nature of the observed scattering features in structural terms. Scattering curves from atomic models of the fully loaded MoFe protein (10), a modeled MoFe protein with missing FeMo cofactor, a 1:1 complex based on the *A. vinelandii* nitrogenase complex (20), and the Fe protein in its free state (9) were evaluated using the program CRYSTOL (40). This method takes the solvent effect into account by surrounding the protein with a hydration shell that has a thickness of 3 Å and uniform density (as fit parameter) different from that of bulk solvent.

RESULTS AND DISCUSSION

**Formation of a 1:1 Complex**—In a previous study, the rate of formation of the transition state complex Kp1-Kp2-ADP-AlF$_4^-$ was monitored by the loss of nitrogenase activity as the proteins were incubated in the presence of ADP and AlF$_4^-$ (15). It was proposed that Kp1 that did not have all FeMoco and P cluster binding sites in the protein occupied had an altered conformation compared with fully loaded protein and that Kp2 reacted with these protein species at different rates to form the inhibited complex. To detect complexes formed by Kp1 preparations that are partially active due to incomplete occupancy of the FeMoco binding sites, a method was developed to monitor complex formation that was independent of activity measurements. The extent of formation of the transition state complexes was determined using gel permeation chromatography of reaction mixtures containing a range of molar ratios of Kp1 and Kp2 from 0.25 to 5 Kp2/Kp1 in the presence of MgADP and AlF$_4^-$.

Fig. 1 shows the elution profiles from a gel filtration column of the protein species present in incubation mixtures leading to the formation of the transition state complex for a preparation of Kp1 containing only 1.1 molybdenum atoms per αβ dimer. At the lowest Kp2/Kp1 ratio tested, the profile is dominated by free Kp1, which has a retention volume of 10.3 ml (bottom trace in Fig. 1). At this ratio, no peak corresponding to free Kp2 is evident, but a shoulder on the elution profile of Kp1 arising from a higher molecular weight species with a retention volume of 9.8 ml has been formed. As the Kp2/Kp1 ratio was increased to 0.5 Kp2/Kp1, this species became the dominant feature, but at higher ratios it was replaced by a peak with a retention volume of 8.9 ml. A peak corresponding to free Kp2 with a retention volume of 12.35 ml was also detectable under these conditions. The Kp2 band was first evident at a ratio of 1:1 Kp2/Kp1 and continued to grow as the ratio was increased (Fig. 1). These data are consistent with the formation of two types of stable complexes by Kp1 lacking a full complement of cofactor centers, as the Kp2/Kp1 ratio is varied. We propose that initially a 1:1 complex is formed as an intermediate on its way to the 1:2 complex, which predominates at high Kp2/Kp1 ratios. The kinetic data of Yousafzai and Eady (15) are consistent with Kp2 in this complex binding to the α$_2$β$_2$ subunit pair, which contain the metal redox centers. When similar experiments were carried out with Kp1 containing 1.9 molybdenum atoms per α$_2$β$_2$ tetramer, a similar behavior was observed, but the formation of the 1:2 complex occurred at a lower ratio of Kp2/Kp1 (data not presented), consistent with a difference in the stability of the 2:1 complexes formed by the two species of Kp1.

To isolate the 1:1 complex in sufficient quantity to allow structural studies, it was purified from the components formed in an incubation mixture containing Kp1 lacking a full complement of cofactor centers as described under “Experimental Procedures.” This procedure resulted in the separation of three species, which subsequent analytical gel filtration showed to have retention volumes corresponding to Kp1 and the 1:1 and 1:2 complexes.

**Solution Structure of Fully Loaded and Half-loaded MoFe Protein**—Fig. 2 compares the x-ray scattering patterns (with error bars) for the fully loaded Kp1 (i.e. with the full complement of the cofactor) and Kp1 with only half the cofactor centers present (from now on denoted as Kp1$_{1/2}$. The two scattering profiles are distinct, crossing each other at an intermediate $s$, suggesting that a significant structural difference exists. As shown in Table I, the geometrical parameters increase in the absence of a full metal cofactor complement, indicating an expansion of the overall conformation. The main differences in the $p(r)$ curves (Fig. 2, inset) occur for longer distances and in the maximum of $p(r)$, which is shifted for Kp1$_{1/2}$ to larger distances (by approximately 5 Å) to 47 Å. To evaluate possible protein aggregation, scattering patterns have been obtained in the concentration range from 0.5 to 5 mg/ml. The concentration-dependent values for radii of gyration are revealed in Fig. 3a, highlighting the difference between the two protein samples. $r_{g\text{eff}}$ values (extrapolated to infinite dilution) differ by as much as 1.5 Å, and the $R_g$ versus concentration curve shows very different slopes for Kp1 and Kp1$_{1/2}$. This may reflect a...
Fig. 2. Solution scattering profiles and distance distribution functions $p(r)$ (inset) of half-loaded (C) and fully loaded (●) MoFe protein. Smooth curves represent the scattering profiles from the restored shapes (thin lines) in Fig. 4b as well as from simulations (thick lines) using models of Kp1 and Kp1$^{1/2}$ based on crystal structure coordinates of Kp1 (10). See second section of “Results and Discussion” for further details.

Geometrical parameters of nitrogenase proteins from K. pneumoniae extracted from solution x-ray scattering data (columns 2–4) and comparison with results from scattering pattern simulations (columns 5 and 6)

| Sample                | Volume | $D_{max}$ | $R_g$ | $r_x$ | $\chi^2$ |
|-----------------------|--------|-----------|-------|-------|----------|
| Kp1 fully loaded      | 345,000| 108       | 38.0  | 38.0$^b$ | 1.7      |
| Kp1 half-loaded       | 355,000| 128       | 39.5  | 39.2$^a$ | 2.1      |
| 1:1 complex           | 470,000| 159       | 46.5  | 45.2$^{1/2}$ | 2.9     |
| 1:2 complex           | 540,000| 183       | 51.5  | 45.0$^{1/2}$ | 2.5     |
| Kp2                   | 125,000| 72        | 25.2  | 25.0$^a$ | 1.5      |
| Kp2 + MgADP           | 73     | 25.3      |       |       |          |
| Kp2 + MgATP           | 75     | 25.5      |       |       |          |

change in the electrostatic properties of the Kp1 and Kp1$^{1/2}$ surface. The difference in scattering behavior is further illustrated in Fig. 3b, where the ratio of the scattering curves for Kp1 and Kp1$^{1/2}$ are plotted (upper trace). It is clear that scattering data for the two protein samples differ over much of the range, and only beyond $s \approx 0.02 \AA^{-1}$, the ratio hovers around unity. As a control, the lower trace in Fig. 3b shows the intensity ratio of scattering profiles from half-loaded Kp1 recorded at two different concentrations. It is clear that in this case the ratio is unity over almost the whole data range. Moreover, a careful analysis of $I(0)$ did not reveal changes in molecular mass between Kp1 and Kp1$^{1/2}$ (the mass of the metal cofactor is small compared with that of the protein molecule).

Previously, we have reported the molecular shape of the fully loaded Kp1 (41) and shown this to be in good agreement with the overlaid crystal structure of the MoFe protein; i.e. essentially flexible polypeptide segments appear outside the molecular envelope (see also Fig. 4a). In these calculations, a 2-fold symmetry was assumed for the α$\beta_2$ tetramer; thus, shape restoration up to harmonics $L = 6$ was justified. To assess if there are differences in the molecular structure due to the absence of one of the cofactors, we have undertaken shape restoration without assuming a 2-fold symmetry (Fig. 4b). As a control, a shape restoration of Kp1 containing both cofactors was attempted, where no symmetry is assumed. In this case, spherical harmonics of only up to $L = 4$ are permissible (19 free parameters). Fig. 4b (left panel, yellow envelope) shows two views of the molecular shape of Kp1 restored with $L = 4$. A comparison with Fig. 4a shows that the two shapes resemble each other closely, demonstrating that shape restoration with $L = 4$ is sufficient to recognize the characteristic features of the molecule such as the presence of a 2-fold symmetry. Fig. 4b (right panel, pink envelope) provides two views of the Kp1$^{1/2}$ shape at $L = 4$, where again no symmetry was assumed (fits to the experimental data with final residual $R = 2.1$ and 2.4% for Kp1 and Kp1$^{1/2}$, respectively, are shown in Fig. 2). The shape for Kp1$^{1/2}$ differs significantly from Kp1; an extension or bulge appears that breaks the familiar view of 2-fold symmetry. An assessment of the differences of both molecular envelopes (Kp1 versus Kp1$^{1/2}$ at $L = 4$) demonstrates that the left half of the molecule remains essentially the same in the two cases (see Fig. 4b), but significant expansion is observed on the right half of the molecule in the absence of the cofactor. These shapes indicate that the missing FeMo cofactor in Kp1 results in a significantly less compact structure, which is underlined by the geometrical parameters given in Table I. This structural expansion may also explain the slightly larger volume of Kp1$_2$, as a result of water filling the created cavities and clefts. Although the molecular shape for both Kp1 and Kp1$^{1/2}$ (represented by an average envelope deduced from several shape reconstruction runs using different starting conditions) offers a qualitative insight into the structural change as a result of FeMoco ab-
sence, the use of available crystallographic information allows us to assess the experimental findings more accurately. For that reason, we attempted to model the structure of Kp1$_{1/2}$ based on the following rationale. The FeMo cofactor is entirely contained in the α-subunit, at the boundary between three domains. Inspection of the protein environment around the FeMo cofactor shows primarily hydrophilic residues forming a shallow cavity for metal cofactor anchoring. This interdomain location and the distinctive collection of polar and charged groups are likely to play an important role in stabilizing the protein's native conformation. Significant conformational rearrangements and destabilization would be expected in the absence of the metal cluster. Consequently, as a result of the missing FeMoco center, an opened α-subunit is conceivable in which domain III (domains are labeled according to Ref. 5) is rotated against domains I and II around a hinge comprising the residues α54 and α299 (a rotation of up to 22° was applied). Apart from a few contacts between the N-terminal residues of the α-subunit and domain I of the β-subunit (see below), the hinge movement was considered to be a rigid body rotation of the α-subunit alone with minor effects on the β-subunit (Fig. 4c), the latter playing a major role in tetramerization. However, since some of the helices in domain III of the α-subunit help to stabilize the tetramer interface (5), an influence on the arrangement of the two αβ dimer pairs cannot be excluded. Furthermore, due to the likely nature of a flexible hinge, the possibility of multiple conformers cannot be ruled out. The result from the scattering pattern simulation for the model of Kp1$_{1/2}$ (together with the result for Kp1) is given in Fig. 2. The simulated profile based on the Kp1 crystal structure (10) agrees very well with the scattering curve of fully loaded Kp1 in solution. The profile from the theoretical model of Kp1$_{1/2}$ effectively reproduces the characteristic features of the experimental curve (this is also reflected in the goodness of fit ($\chi^2$ value; see Table I). Deviations for 0.015 Å$^{-1} \leq s \leq 0.022$ Å$^{-1}$ may be rationalized, given that this only represents one (i.e. a model that has been obtained by rigid body movement of domain III of one of the α-subunits only) of several possible conformations. Besides, parts of the surrounding environment and even the tetrameric conformation are probably affected. Ribbon drawings representing the structure of Kp1 and the modeled structure of Kp1$_{1/2}$ have been superimposed on the shapes displayed in Fig. 4b.

**Solution Structure of 1:1 Complex and Changes in the Fe Protein**—The X-ray solution scattering curve and pair distribution function for the isolated 1:1 complex, purified as described under “Experimental Procedures,” is compared with the 1:2 complex data (21) in Fig. 5a. The scattering results for both complexes are considerably different (see also Table I). Analysis of the I(0) intensities of 1:2 and 1:1 complex revealed a ratio of 1.3, confirming the proposed stoichiometry for both complexes, since an intensity ratio of 1.21 would be expected based on the difference in molecular masses of the two complexes. The distance distribution function (Fig. 5a, inset) of 1:1 complex computed for infinite dilution shows a decrease of approximately 30 Å in long distances compared with the 1:2 complex being consistent with only one Kp2 bound to Kp1. For shape calculations, again no symmetry was assumed, and an envelope with harmonics up to $L = 4$ could be restored. The fits to the experimental data are superimposed in Fig. 5a and yielded R factors of 2.2% (1:1 complex) and 1.8% (1:2 complex). Two views of the molecular shape thus obtained for the 1:1 complex are shown in Fig. 5b. This is superimposed with the model built for Kp1$_{1/2}$ (see above) and one Fe protein. Although this comparison clearly demonstrates that only one Fe protein can be included in the restored shape for the 1:1 complex, a scattering pattern simulation (distinguishing between the two known structural states of the Fe protein) confirms that the Fe protein undergoes a very similar conformational change (see simulated curves in Fig. 5a and $\chi^2$ values given in Table I) as that documented for the 1:2 complex (20, 21). Interestingly, in all simulations the goodness of fit improves when the Fe protein from the Av1-Av2 complex (20) is considered (Table I). This is also emphasized visually by a better agreement with the restored molecular envelope (see Fig. 5b) overlaying the compact conformation for the Fe protein when in complex with the MoFe protein (20) rather than the less tight conformation of the free Fe protein as suggested in the original docking model (5). Deviations between experimental and simulated scattering results for the 1:1 complex (in particular concerning the scattering range 0.018 Å$^{-1} \leq s \leq 0.023$ Å$^{-1}$ (see Fig. 5a) as well as the $R_v$ value (Table I) indicate, however, that the missing cofactor in Kp1$_{1/2}$, together with the formation of the 1:1 complex, causes further structural rearrangements compared with the model presented here (based solely on an open α-subunit in one of the αβ subunit pairs of Kp1$_{1/2}$). Additional structural modifications regarding the αβ subunit pairs offer an attractive option also in
Fig. 4. a, two orientations of the shape of fully loaded Kp1 restored with symmetry (L = 5). Superimposed is a ribbon model of the Kp1 crystal structure (10) highlighting α- and β-subunits in red and blue, respectively. Metal cofactors are depicted as ball and stick models (P clusters in black and FeMo cofactors in magenta). b, The left panel (yellow envelope) represents the shape of fully loaded Kp1 restored without symmetry (L = 4); the panel on the right gives the molecular envelope for the half-loaded Kp1 (in pink, restored with harmonics up to L = 4). The extension of the shape of Kp1½ with regard to Kp1 (i.e. on the right of the pink envelope) is also highlighted by superimposed ribbon drawings of the Kp1 crystal structure (onto the yellow envelope) and a modelled structure of Kp1½ (onto the pink envelope). In the latter model, domain III of the right-hand α-subunit (green colored ribbon segments) has been moved by a 22° hinge rotation. As indicated, the corresponding views on the top and bottom are related by a 90° rotation around the horizontal axis. c, ribbon model of an αβ subunit pair from the structure of Kp1 with FeMo cofactor buried in the α-subunit (left) and from the modeled structure of Kp1½ in which the missing FeMo cofactor causes an opening of the α-subunit (right). The same color code as in b is used. The view shows the approximate pseudo-2-fold symmetry. In addition, the two helices involved in docking the Fe protein are indicated.
view of the recent evidence for long range conformational changes in the MoFe protein upon Fe protein binding (26).

The absence of the second Fe protein from the opposite side of the molecule that lacks the cofactor provides support to the idea that the Fe protein is able to recognize the altered conformation due to the missing cofactor from the MoFe protein (Fig. 4b). At this point, it has to be mentioned that the "open" conformation of Kp1₁⁄₂ (here modeled simply as rigid body rotation of domain III in one of the α-subunits) does not directly affect the surface area implicated in Fe protein docking. However, as a result of certain contacts between the N-terminal residues (α₁–α₅₆) in the α-subunit (forming part of domain III) and residues β₁₁₁–β₁₄₀ in domain I of the β-subunit, this particular region of the αβ-interface is likely to be modified. Most importantly, the latter polypeptide segment of the β-subunit contains one of the two helices for Fe protein docking (see Ref. 20; see also Fig. 4c). It is therefore most plausible to assume that destabilization or reorientation of this docking helix in the β-subunit (as a consequence of a hinge movement in the α-subunit due to the missing FeMo cofactor) leads to rearrangements of a considerable section in the interaction surface with the Fe protein. It is assumed that the other docking helix (located in domain I) in the α-subunit is unaffected due to stabilizing effects of the bound P cluster. This is an appealing structural scenario, considering that the gel permeation data presented above show the binding of a second Fe protein to Kp1₁⁄₂, albeit with lower affinity. Interestingly, besides binding the Fe protein, additional functional roles of the β docking helix have been inferred from the structure of Kp1 (10). Our examination may even suggest that this helix is able to sense the absence of the FeMo cofactor.

Conformational Change of the Isolated Fe Protein upon Nucleotide Binding—The conformational changes observed for both Av2 and Kp2 in the transition state complexes are of significant functional importance, since they enable the Fe₄S₄ cluster of Fe protein and MoFe protein to approach significantly closer together to typical electron transfer distances. This is likely to result in an efficient electron transfer between the Av2/Kp2 Fe₄S₄ cluster to the Av1/Kp1 P cluster, which in the complex is some distance away from the FeMo cofactor, the site of nitrogen reduction.

There is a body of experimental data indicating that the binding of nucleotides to the Fe protein results in changes in the spectroscopic properties and reactivity of the Fe₄S₄ center (see Ref. 4) and in the sensing of the redox level of the cluster by bound nucleotide (42). Both MgATP and MgADP, competitive inhibitors of electron transfer, are expected to bind at the same site, which is located some ~20 Å away from the Fe₄S₄ cluster, thus their effect on the properties of the cluster has been rationalized to result from a conformational change in the Fe protein. It is of interest to see if this conformational change in the isolated protein is similar to that observed for Kp2 or Av2 in the transition state complex analogues. A preliminary x-ray scattering study has been reported on the effects of nucleotide binding on Av2, where the radius of gyration (Rg), deduced from the diffraction region alone has been determined (22). Recent advances in the x-ray scattering technique, particularly the ability to use a wider data range, have proved very powerful in studying conformational changes in proteins (14, 35, 43).

Fig. 6a shows the calculated scattering profiles for Av2 obtained using the crystallographic structures of Av2 on its own (6, 9) and that observed in the Av1-(ADP·AlF₄)₂–Av2) complex (20). The compact nature of Av2 in the complex is reflected in the change in radius of gyration (Rg) (ΔRg = 1.9 Å) and D₃₁₅₅ (ΔD₃₁₅₅ = 6 Å). The changes in the scattering profile over the extended scattering range are similar in nature to those observed in the half-molecule of transferrin upon binding of iron (35) and thus should be easily accessible by x-ray scattering. Fig. 6b illustrates the experimental x-ray scattering data for Kp2 on its own, with MgADP and with MgATP. The profiles are practically indistinguishable. The lack of change compared with the one seen in Fig. 6a is apparent. This experiment has been repeated for the Fe protein of the vanadium nitrogenase system (see Ref. 44), and identical results were obtained (data not
changes in Av2 to accommodate specific interactions between the two proteins (20).

The excellent agreement between structures in solution and crystalline state is illustrated when the scattering data for Kp2 are compared (Fig. 6c, solid line) with the scattering profile calculated from the crystallographic structure of free Av2 (9) surrounded by a water layer (40). The $R_g$ from the crystal structure increases from 23.9 to 25.5 Å when hydration effects are taken into account and agrees neatly with the experimental $R_g$ value. The inclusion of a hydration shell improves the fit to the experimental x-ray scattering pattern considerably (45, 46). In fact, the value for the hydrated structure of free Av2 is equivalent to what was observed in an earlier study by Chen et al. (22) for Av2 with MgATP but is substantially different from their values for native Av2 and Av2 plus MgADP. Their $R_g$ values (>27 Å) for the latter two states would suggest an Fe protein structure even less compact compared with the structures reported for the free Fe protein (6, 9).

**REFERENCES**

1. Howard, J. B., and Rees, D. C. (1994) Annu. Rev. Biochem. 63, 235–264
2. Howard, J. B., and Rees, D. C. (1996) Chem. Rev. 96, 2985–2982
3. Peters, J. W., Fisher, K., and Dean, D. R. (1995) Annu. Rev. Microbiol. 49, 335–368
4. Burgess, B. K., and Lowe, D. J. (1996) Chem. Rev. 96, 2983–3011
5. Kim, J., and Rees, D. C. (1992) Nature 360, 553–560
6. Georgiadis, M. M., Komiya, H., Chakrabarti, P., Woo, D., Kornuc, J. J., and Rees, D. C. (1992) Science 257, 1653–1659
7. Bolin, J. T., Campobasso, N., Muchmore, S. W., Morgan, T. V., and Mortenson, L. E. (1993) ACS Symp. Ser. 535, 186–195
8. Kim, J., Woo, D., and Rees, D. C. (1993) Biochemistry 32, 7104–7115
9. Schlesman, J. L., Woo, D., Joshua-Tor, L., Howard, J. B., and Rees, D. C. (1998) J. Mol. Biol. 286, 669–685
10. Mayer, S. M., Lawson, D. M., Gornall, C. A., Roe, S. M., and Smith, B. E. (1999) J. Mol. Biol. 292, 871–891
11. Chan, M. K., Kim, J., and Rees, D. C. (1993) Science 260, 792–794
12. Duyvis, M. G., Wassinck, H., and Haaker, H. (1996) FEBS Lett. 380, 233–236
13. Benner, K. A., and Howard, J. B. (1996) Biochemistry 35, 5353–5358
14. Grossmann, J. G., Hainain, S. S., Youssafzii, F. K., Smith, B. E., and Eady, R. R. (1997) J. Mol. Biol. 266, 642–648
15. Youssafzii, F. K., and Eady, R. R. (1997) Biochem. J. 326, 635–640
16. Coleman, D. E., Berghuis, A. M., Lee, K., Linder, M. E., Gilman, A. G., and Sprang, S. R. (1994) Science 265, 1405–1412
17. Sondek, J., Lambricht, D. G., Noel, J. P., Hamm, H. E., and Sigler, P. B. (1994) Nature 372, 276–279

Fig. 6. Predicted (a) and experimental (b) scattering patterns and $p(r)$ functions (inset) of free Kp2 with and without bound nucleotide. Experiments have been carefully performed so as to make sure that protein samples were either in the nucleotide free or nucleotide state. The improved fits to the experimental x-ray scattering pattern considerably (45, 46). In fact, the value for the hydrated structure of free Av2 is equivalent to what was observed in an earlier study by Chen et al. (22) for Av2 with MgATP but is substantially different from their values for native Av2 and Av2 plus MgADP. Their $R_g$ values (>27 Å) for the latter two states would suggest an Fe protein structure even less compact compared with the structures reported for the free Fe protein (6, 9).
Transition State Complex Analogue of Nitrogenase

A273, 773–777

Boulain, C., Kempf, R., Koch, M. H. J., and McLaughlin, S. M. (1986) Nucl. Instrum. Methods Phys. Res. A A249, 399–407

Semenyuk, A. V., and Svergun, D. I. (1991) J. Appl. Crystallogr. 24, 537–540

Porod, G. (1951) Koloid Zeitschrift 124, 83–114

Feigin, L. A., and Svergun, D. I. (1987) Structure Analysis by Small-angle X-Ray and Neutron Scattering, pp. 79–81, Plenum Press, New York

Grossmann, J. G., Crawley, J. B., Strange, R. W., Patel, K. J., Murphy, L. M., Neu, M., Evans, R. W., and Hasnain, S. S. (1988) J. Mol. Biol. 279, 461–472

Svergun, D. I., and Stuhrmann, H. B. (1991) Acta Crystallogr. A 47, 736–744

Stuhrmann, H. B. (1970) Acta Crystallogr. A 26, 297–306

Stuhrmann, H. B. (1970) Z. fur Phys. Chem. 72, 177–184, 185–198

Svergun, D. I., Volkov, V. V., Kozin, M. B., and Stuhrmann, H. B. (1996) Acta Crystallogr. A 52, 419–426

Svergun, D., Barberato, C., and Koch, M. H. J. (1995) J. Appl. Crystallogr. 28, 768–773

Grossmann, J. G., Murphy, L. M., Haldane, S. A. T, Hasnain, S. S., Eady, R. R., Yousefzai, F. K., and Smith, B. E. (1997) in Daresbury Annual Report 1996/97: Scientific Reports, pp. 246–247, CCLRC Daresbury Laboratory, Warrington, United Kingdom

Miller, R. W., Eady, R. R., Germain, C., Fuhlerst, S. A., and Smith, B. E. (1998) Biochem. J. 334, 601–607

Grossmann, J. G., and Hasnain, S. S. (1997) J. Appl. Crystallogr. 30, 770–775

Eady, R. R. (1996) Chem. Rev. 96, 3013–3030

Grossmann, J. G., Abraham, Z. H. L., Adman, E. T., Neu, M., Eady, R. R., Smith, B. E., and Hasnain, S. S. (1993) Biochemistry 32, 7360–7366

Svergun, D. I., Richard, S., Koch, M. H. J., Sayers, Z., Kuprin, S., and Zuccaro, G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2267–2272

18. Fisher, A. J., Smith, C. A., Thoden, J., Smith, R., Sutoh, K., Holden, H. M., and Rayment, I. (1995) Biochemistry 34, 8960–8972
19. Clarke, T. A., Yousafzai, F. K., and Eady, R. R. (1999) Biochemistry 38, 9906–9913
20. Schindelin, H., Kisker, C., Schlessman, J. L., Howard, J. B., and Rees, D. C. (1997) Nature 387, 370–376
21. Grossmann, J. G., Hasnain, S. S., Yousefzai, F. K., Smith, B. E., Eady, R. R., Schindelin, H., Kisker, C., Howard, J. B., Tsuruta, H., Muller, J., and Rees, D. C. (1999) Acta Crystallogr. B 55, 727–728
22. Chen, L., Gavini, N., Tsuruta, H., Eliezer, D., Burgess, B. K., Doniach, S., and Hodgson, K. O. (1994) J. Biol. Chem. 269, 3290–3294
23. Seefeldt, L. C., Ryle, M. J., Chan, J. M., and Lanzilotta, W. N. (1998) in Biological Nitrogen Fixation for the 21st Century (Elmerich, C., Kondorosi, A., and Newton, W. E., eds) pp. 39–42, Kluwer Academic Publishers, Dordrecht, The Netherlands
24. Duyvis, M. G., Wassink, H., and Haaker, H. (1998) Biochemistry 37, 17345–17354
25. Chan, J. M., Ryle, M. J., and Seefeldt, L. C. (1999) J. Biol. Chem. 274, 17593–17958
26. Clarke, T. A., Maritano, S., and Eady, R. R. (2000) Biochemistry 39, 11434–11440
27. Yousefzai, F. K., Buck, M., and Smith, B. E. (1996) Biochem. J. 318, 111–118
28. Eady, R. R., Smith, B. E., Cook, K. A., and Postgate, J. R. (1972) Biochem. J. 133, 655–675
29. Bras, W., Derbyshire, G. E., Ryan, A. J., Mant, G. R., Felton, A., Lewis, R. A., Hall, C. J., and Greaves, G. N. (1993) Nucl. Instrum. Methods Phys. Res. A A325, 587–591
30. Lewis, R., Sumner, I., Berry, A., Bordas, J., Gabriel, A., Mant, G., Parker, G., Roberts, K., and Worgan, J. (1988) Nucl. Instrum. Methods Phys. Res. A A273, 773–777
Evidence for the Selective Population of FeMo Cofactor Sites in MoFe Protein and Its Molecular Recognition by the Fe Protein in Transition State Complex Analogues of Nitrogenase

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