Cross-linking of Nitrogenase Components

STRUCTURE AND ACTIVITY OF THE COVALENT COMPLEX*

The nitrogenase complex from Azotobacter vinelandii is composed of the MoFe protein (Av1), an αβ2 tetramer, and the Fe protein (Av2), a γ2 dimer. During turnover of the enzyme, electrons are transferred from Av2 to Av1 in parallel with the hydrolysis of MgATP. Using the cross-linking reagent, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, we have identified some of the properties of the complex between the two components. The cross-linking reaction was highly specific yielding a single apparent Mr = 97,000 protein. The amount of cross-linked product was essentially independent of whether MgATP or MgADP were in the reaction. Also, the amount was maximum at high ratios of Av2 to Av1.

The Mr = 97,000 protein was characterized by amino acid analysis and Edman degradation and was found to be consistent with a 1:1 complex of an Av2 γ subunit and an Av1 β subunit (the amino terminal serine subunit). The complex was no longer active in the nitrogenase reaction which supports, but does not prove, the requirement for dissociation of the complex after each electron transferred. Nitrogenase activity and cross-linking were inhibited in an identical way by NaCl, which suggests that electrostatic forces are critical to the formation of the electron transfer complex.

A number of questions about the nature of the complex remain unanswered. For example, what is the symmetry of the complex, i.e. does the docking site on the MoFe protein involve both subunits? Can the complex reduce substrates without physically dissociating? Which amino acid residues are involved in the docking sites? What is the role of nucleotide binding in the docking process? Many of these questions can be evaluated by molecular model building once the crystallographic structures of the two components are known. On the other hand, model building will be greatly enhanced by having prior evidence for the docking sites. In addition some of these questions need to be considered for proteins under dynamic conditions. Towards these goals, we have used chemical cross-linking to study properties of the nitrogenase complex.

MATERIALS AND METHODS

RESULTS AND DISCUSSION

A variety of compounds have been screened as potential cross-linking agents for the nitrogenase components; only the water-soluble carbodiimide, EDC,2 which cross-links amino and carboxyl groups has proven promising. As is shown in Fig. 1, the EDC cross-linking reaction is highly specific for the nitrogenase complex. A single new protein band (apparent Mr = 97,000) was observed upon gel electrophoresis if both nitrogenase components were present in the reaction. In contrast, no cross-linking between the constituent subunits of the individual nitrogenase components was observed. When creatine phosphokinase (Mr = 45,000) was present in the reaction as part of an ATP regenerating system, it was not cross-linked either to itself or to the nitrogenase components.

Furthermore, formation of the Mr = 97,000 product appears to require the active functional complex because only nonspecific, high molecular weight cross-linked material was observed with oxygen inactivated components (data not shown). The amount of cross-linking was independent of pH between 7 and 9 and of the EDC concentration from 1 to 50 mM. At higher concentrations of EDC (>100 mM), additional very

1 Portions of this paper (including "Materials and Methods," Figs. 1-6, and Table 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviations used are: EDC, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide; Av1, component 1 or MoFe protein from Azotobacter vinelandii; Av2, component 2 or Fe protein from A. vinelandii; the Av1 α subunit is defined as the slower moving subunit on sodium dodecyl sulfate-gel electrophoresis and has threonine as its amino-terminal residue (1, 2); the Av1 β subunit is the faster moving subunit and has serine as its amino-terminal residue (1, 2); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PTH, phenylthiohydantoin.

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large molecular weight material could be detected at the top of the electrophoresis gel.

Another line of evidence that the cross-linking reaction reflects the formation of a catalytically significant complex is the time dependence of the reaction. The results shown in Fig. 1 also indicate that the cross-linking is a rapid reaction with a t<sub>80</sub> of 3 min for the protein concentrations and ratio used in the figure. The reaction had excess EDC and was not limited by this reagent. EDC added after the cessation of cross-linking (30 min) did not increase the yield of M<sub>r</sub> = 97,000 material.

One aspect of the nitrogenase model is that the component ratio as well as the protein concentration affect the amount of active complex and, thereby, the enzyme turnover. If the cross-linking reaction reflects the formation of the active complex, then the component ratio also might be expected to influence the rate of cross-linking. To investigate this further, the component ratio in the cross-linking reaction was varied from a 10-fold excess of Av1 to a 10-fold excess of Av2. The results are shown in Fig. 2. Throughout this 100-fold range of component ratios, only a single cross-linked protein band (M<sub>r</sub> = 97,000) was observed. As the ratio of Av2:Av1 was increased, the amount of M<sub>r</sub> = 97,000 material increased until the β subunit of Av1 was substantially depleted. Likewise, when the ratio of Av1:Av2 was increased (Fig. 2B), the amount of cross-linked material was limited by Av2 subunits. However, in contrast to the nearly complete loss of the Av1 β subunit, the Av2 subunits were decreased only 50–60%. Although we cannot exclude the possibility that 50% of our Av2 is inactivated by side reactions such that it can no longer react, it seems plausible that only one of the two subunits in each Av2 dimer is being cross-linked, whereas most of the Av1 β subunit is incorporated in the M<sub>r</sub> = 97,000 material. This is also consistent with a specific interaction leading to cross-linking.

Perhaps the most surprising result of our studies was that the cross-linking reaction is essentially independent of the presence of nucleotides (see Fig. 3). Not only was the molecular weight and composition of the cross-linked material unrelated to the presence of nucleotides, but the amount of cross-linked material was similar or even somewhat less in the presence of either MgATP or MgADP. What makes this result so striking is that numerous other probes of the Fe protein structure uniformly show significant changes upon binding of nucleotides (3, 4). Under our experimental conditions of saturating nucleotide concentration, it is unlikely that the cross-linking reaction involves the nucleotide binding site or that nucleotide binding is prevented.

Because the EDC cross-linking reaction involves closely opposed carboxylic acids and amines, it is reasonable to assume that these groups may be part of ionic (salt) interactions which, in part, are responsible for the complex formation. Indeed, patches of acidic and basic residues have been postulated for “docking” sites in other electron transfer complexes. In accordance with the cross-linking reaction, the 50% cross-linking of the Av2 subunits was observed which corresponded to the Av2 γ and Av1 β subunits. Equally importantly, no residues for the Av1 α-subunit were found. The calculated initial yields (based on M<sub>r</sub> = 89,000) were 88 pmol (15%) for the β subunit and 712 pmol (29%) for the Av2 γ subunit, with repetitive yields of 93.6 and 91.2%, respectively. The ratio of the initial yields would suggest a cross-linked complex composed of 0.5 β-subunit and 1.0 γ-subunit. However, the low percent initial yield for both amino termini, which is often observed for material isolated using denaturing buffers or material from cross-linking reactions, leaves in doubt how reliable the ratio is when determined by Edman degradation.

Another approach to characterizing the composition of the complex is the analysis of the cross-linking reaction itself. Although molecular weights determined by denaturing gel electrophoresis may give anomalous results for branched (cross-linked) proteins, the molecular weight of 97,000 seems to best fit a ratio of 1:1 (M<sub>r</sub> = 89,000). Furthermore, at saturating ratios of Av2, most of the Av1 β subunit was converted to the cross-linked complex whereas only ~50% of the Av2 subunits were cross-linked at saturating Av1. Since the Av2 subunits do not dissociate or exchange between native molecules, the 50% cross-linking of the Av2 subunits is consistent with a 1:1 complex.

Thus, taking all of the results together, we favor the M<sub>r</sub> = 97,000 cross-linked protein as being a 1:1 complex of the Av2 γ and Av1 β subunits. It should be emphasized that there is no evidence for the presence of the Av1 α subunit in the isolated cross-linked material. Identification of the specific amino acid residues involved in the cross-linking may help to determine the ratio. This is presently under way.

Our results provide several important conclusions about the interaction between the components of the nitrogenase complex, which may be related to the turnover of the enzyme during catalysis. First, at least part of the docking site for Fe

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3 T. Diets and J. B. Howard, manuscript in preparation.

4 D. Ikeda and J. B. Howard, unpublished observations.
protein on the MoFe protein is in the β subunit. Of course, we cannot exclude a docking site between the two MoFe protein subunits where only the β subunit has the chemical cross-linking site.

Second, the docking site has some element of asymmetry in that only one of the two identical subunits of the Fe protein is cross-linked. Furthermore, because there is nearly complete incorporation of the β subunit, we conclude that the 2-fold symmetry of the MoFe protein (17) provides two separate binding sites and that each binding site interacts with a Fe protein dimer.

Third, a primary component of the docking recognition and binding energy comes from ionic interactions, at least some of which are between amino and carboxyl functions. The lack of cross-linking between subunits of the individual components, i.e., Av1 and Av2, suggests that ionic interactions may be more important in component docking than in subunit stabilization.

Fourth, the cross-linked complex appears to be inactive which supports the earlier hypothesis that the components must dissociate after each electron transferred.

Fifth, the individual components were neither inactivated nor cross-linked, whereas the complex was both rapidly cross-linked and inactivated. This is evidence for a specific interaction in the docking process.

Last, the docking of the two components is independent of the conformational changes induced by nucleotides bound to the Fe protein. This is quite significant, because the general belief is that these conformational changes are large if not global. However, wherever the scope of the structural change, it does not alter the region involved in binding to the MoFe protein. Since the docking site and the FeS cluster in the Fe protein might be expected to be contiguous for efficient electron transfer, an alternate model for the effect of ATP binding can be suggested. Namely, nucleotide binding could cause reorientation of the subunits with respect to each other without large intrasubunit conformational changes. In addition, if the nucleotide-dependent conformational change is a gating mechanism for electron transfer, then the component docking and electron transfer steps must be uncoupled to some degree.

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Figure 1: Specificity and time dependence of the cross-linking reaction between nitrogenase components. A: SDS-detergent gel electrophoresis of samples from cross-linking reaction (see Methods). A: Specificity of the cross-linking reaction. The proteins band at Mr=30,000 is cyanide phosphokinase from the AIF repressing system. Lane 1: mixture of apo (1.4 µM) and mnl (4 µM) incubated in presence of 12.5 mM EDC for 30 min at 25°C; Lane 1: apo without EDC. Lane 3: apo incubated with EDC. Lane 4: apo without EDC. Lane 5: apo incubated with EDC. B: Time dependence of the cross-linking reaction. Lanes 1-4: apo and mnl (1.4 µM and 12.5 µM, respectively) were incubated with 12.5 mM EDC. Samples were removed from the reaction mixture at 0, 0.1, 1, 2, 3, 5, 10, and 20 min., and were analyzed by SDS-detergent gel electrophoresis.

Figure 2: Component ratio dependence of the cross-linking reaction between apo and mnl. Both mnl-detergent gel electrophoresis of samples from cross-linking reaction. A: Reaction in the presence of an excess of apo under standard conditions. Lanes 1-6: 1.4 µM apo was reacted for 1 minutes with 0.7 µM, 1.5 µM, 3.0 µM, 12.5 µM, 22.5 µM, 29.8 µM, and 31.5 µM apo, respectively. B: Reaction in the presence of an excess of mnl under standard conditions. Lanes 1-4: 2 µM mnl was reacted for 10 minutes with 2.5 µM, 4.5 µM, 8.0 µM, and 12.0 µM mnl. Lane 5: reaction mixture as in lane 4, but without EDC. Due to the large amount of mnl protein used in this experiment, minor bands (c45) become detectable, but can be seen by comparison of lanes 4 and 5 the Mr=37,000 band is the only cross-linked product.

Figure 3: Cross-linking of nitrogenase components in presence and absence of nucleotides. 1.4 µM apo and 2.5 µM mnl were incubated for 3 min. with EDC under standard conditions. Samples of the reaction mixture were analyzed by SDS-detergent gel electrophoresis. Lane 1: cross-linking without added nucleotide. Lane 2: cross-linking in presence of 50 µM ATP. Lane 3: cross-linking in presence of 1 mM ATP.

Figure 4: Salt dependence of nitrogenase activity and of the cross-linking reaction. Activity assays were one at a 10-fold molar excess of edd (27 µg apo) and 500 µg apo) in presence of the indicated concentrations of NaCl in 1x4 standard assays. In a separate experiment the nitrogenase components were incubated with 12.5 mM EDC for 5 min. in presence of various concentrations of NaCl. Samples of the reaction gels were analyzed by SDS-detergent gel electrophoresis. The results are shown in the insert. Lane 1: 2.5 µM holo-protein and 2.5 µM Fe-protein were cross-linked for 25 min. under standard conditions in presence of 0 mM, 100 mM, 200 mM, 300 mM, 400 mM, 500 mM, 600 mM, and 700 mM NaCl, respectively.

Figure 5: Component ratio dependence of nitrogenase activity under cross-linking conditions. 1.1 µM apo was incubated with 12.5 mM EDC and either a 3-fold molar excess (1:3) or a 6-fold molar excess (1:6) of mnl under standard conditions. At the indicated time points, samples were assayed and assayed for nitrogenase activity in 1x4 standard by evolution assays as described under Methods. As a comparison the unmodified components apo, mnl were also assayed under identical conditions. In these cases samples of the reaction mixture were assayed in presence of saturating concentrations of the other component.

Figure 6: Amino terminal sequence of the crosslinked complex. Edman degradation for 20 cycles was performed as indicated in Methods. For comparison, the amino terminal sequences for the isolated apo and mnl subunits are given.
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### TABLE 1

| Amine Acid | $\delta_{1}$ - Composition | $\delta_{2}$ - Composition | $\delta_{1,2}$ - Composition |
|------------|-----------------------------|-----------------------------|-----------------------------|
|            | Fixed | Calculated Difference | Fixed | Calculated Difference | Fixed | Calculated Difference |
| Arg        | 86.3  | 87 + 0.8  | 116.7 | 117 + 0.8  | 82.4  | 79 + 3.9  |
| Thr        | 44.6  | 44 + 1.48 | 60.3  | 56 + 3.76  | 42.5  | 32 + 10.0  |
| Ser        | 34.3  | 35 + 2.06 | 46.5  | 45 + 2.06  | 37.7  | 41 + 20.7  |
| Ala        | 56.4  | 58 + 2.18 | 68.1  | 64 + 1.76  | 33.6  | 32 + 1.16  |
| Glu        | 34.6  | 34 + 2.18 | 46.6  | 42 + 1.16  | 30.9  | 30 + 0.18  |
| Gly        | 59.3  | 61 + 2.48 | 62.7  | 62 + 0.16  | 59.1  | 73 + 9.5   |
| Lys        | 59.6  | 64 + 6.48 | 83.4  | 80 + 5.16  | 59.9  | 63 + 3.2   |
| Cys        | 34.1  | 35 + 3.76 | 51.2  | 53 + 2.5   |
| Val        | 35.4  | 56 + 1.26 | 56.4  | 56 + 1.26  |
| Met        | 52.3  | 54 + 3.9  | 93.3  | 93 + 3.9   |
| Ile        | 47.9  | 45 + 2.48 | 89.4  | 88 + 1.18  | 61.8  | 68 + 6.8   |
| Leu        | 46.3  | 68 + 0.38 | 89.2  | 87 + 3.18  | 61.3  | 69 + 20.0  |
| Tyr        | 26.7  | 25 + 1.06 | 36.8  | 35 + 0.60  | 24.5  | 30 + 15.7  |
| Phe        | 36.7  | 36 + 4.76 | 46.2  | 42 + 2.04  | 30.7  | 27 + 30.0  |
| His        | 17.7  | 18 + 1.48 | 22.2  | 22 + 1.48  | 10.4  | 12 + 20.0  |
| Trp        | 56.2  | 56 + 0.48 | 75.0  | 75 + 0.48  |
| Arg        | 28.6  | 28 + 5.00 | 48.7  | 47 + 1.00  | 24.4  | 20 + 14.0  |

**Differences, not been squared**

- Arg, Val, Cys, Leu, Trp: +
- Thr, Ser, Ala, Met, Ile, Tyr, Phe, His, Lys, Arg: -

### Molecular Weight

| Amino Acid | Calculated Molecular Weight |
|------------|-----------------------------|
| Arg        | 130,442                     |
| Thr        | 84,950                      |

**Notes:**
- Values are extrapolated to initial time of hydrolysis to account for deamination
- Cystine is determined as cysteic acid/cysteine