Cross-linking Site in Azotobacter vinelandii Complex*

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The Fe-protein and the MoFe-protein of the Azotobacter vinelandii nitrogenase complex can be chemically cross-linked by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Willing, A., Georgiadis, M. M., Rees, D. C., and Howard, J. B. (1989) J. Biol. Chem. 264, 8499–8503). In this reaction, one of the identical subunits of the Fe-protein dimer is linked by an isopeptide bond to each β-subunit of the MoFe-protein tetramer. The reaction has been found to be highly specific with >85% of amino acid residues Glu-112 (Fe-protein) and Lys-399 (MoFe-protein) cross-linked to each other. Although Glu-112 is located in a highly conserved amino acid sequence, it is found in only half of the known Fe-protein sequences. Likewise, Lys-399 is not a conserved residue in the MoFe-protein. Glu-112 appears to be part of an anionic cluster of nine carboxylic acids which is located between the proposed thiol ligands for the FeS center. In contrast, the basic residue cluster which includes Lys-399 has been found in only a few of the known MoFe-proteins. Thus, this cross-linking reaction either is unique to Azotobacter nitrogenase or must involve other residues in the MoFe-protein of other species. Because Lys-399 and Glu-112 form a specific cross-link, it is probable that they are part of the interaction site leading to productive complex formation. This information should be useful for the model building of the complex from the crystallographic structures of the individual components.

Dinitrogen reduction is catalyzed by the two-component complex, nitrogenase, which is composed of the MoFe- and Fe-proteins (1, 2). As part of the process, ATP is bound by the Fe-protein and hydrolyzed during the electron transfer from the Fe-protein to the MoFe-protein. Substrates (e.g., protons, acetylene, or dinitrogen) are reduced at the active protein. Because only single electrons are transferred between two or more electrons, the complex must dissociate and reform marked “aduertisement” in accordance with 18 U.S.C. Section 1734 sequence (8). For the Azotobacter proteins, the amino-terminal residue (7, 8). Numbering of the amino acid residues is based defined as the slower moving subunit on sodium dodecyl sulfate gel electrophoresis and has Thr as its amino-terminal residue (7, 8); the Avl β-subunit is the faster moving subunit and has Ser as its amino-terminal residue (7, 8). Numbering of the amino acid residues is based on the protein sequences which are less than the DNA-sequenced sequence (8). For the Azotobacter proteins, the amino-terminal methionines have been removed in vivo by processing (7, 8).

multiple times during catalysis (3, 4). The formation of the complex is highly specific. For example, the Fe-protein is the obligate electron donor for nitrogenase turnover; other low potential electron donors cannot replace Fe-protein. Likewise, the Fe-proteins isolated from various microbial species have different rates of nitrogenase turnover when used with a common MoFe-protein (5, 6). Thus, the interaction sites must provide the correct orientation between the components for efficient electron transfer and coupled ATP hydrolysis.

Recently, we reported that the two components from Azotobacter vinelandii could be covalently cross-linked by the water-soluble carbodiimide EDC (10). The cross-linking reaction was dependent upon several factors known to affect the enzyme activity levels and, therefore, was considered to occur during the formation of the active complex. The reaction was observed to be highly specific with only one of the two identical Av2 subunits cross-linked to each Av1 β-subunit (Av1 is an αβ4 tetramer). One important conclusion from the proposed stoichiometry of the cross-linking reaction was that once Av2 was bound to Av1, the two Av2 subunits had different points of contact with the Avl β-subunit. That is, the Av2 binding site is asymmetric with respect to the two subunits. Because EDC cross-linking involves carboxyl and amino groups, and because the enzyme activity is significantly inhibited by moderate salt concentrations, ionic interactions are likely to be important elements of the protein-protein binding site (10, 11). In this communication, the specific amino acid residues involved in the EDC cross-linking reaction are identified.

MATERIALS AND METHODS

Nitrogenase components from A. vinelandii, Av1 and Av2, were isolated and characterized as described previously (12, 13). The cross-linked complex was prepared by the reaction of Av2 (22.7 μM) and Av1 (3.7 μM) with 12 mM EDC in 25 mM Hepes/KOH buffer at pH 8.0 under anaerobic atmosphere of argon (10). This ratio of components results in the nearly complete cross-linking of the Av1 β-subunit to Av2 (10). The reaction was terminated after 20 min by the addition of sodium acetate (100 mM, final concentration) followed by rapid gel filtration. The Mₙ = 97,000 cross-linked protein complex was isolated, as described previously, by gel chromatography on a 4 × 150-cm column of Sepharose 6-300 equilibrated with 0.1% sodium dodecyl sulfate, 1 mM dithiothreitol, 50 mM Tris/HCl buffer at pH 8.0 (10). The complex and Av2 were reduced and carboxymethylated using 2-[3H]iodoacetate and 2-[14C]iodoacetic, respectively (7). The isolated 2-[14C]-carboxymethylated α- and β-subunits of Av1 were the generous gift of Dr. D. Lummel, Shearing-Plough, Inc. (7). The purity and identity of the complex and of the individual subunits were characterized by amino acid analysis and by repetitive Edman degradation. Methods of amino acid analysis and protein sequencing have been previously reported (10). Ion exchange chromatography was performed using fast protein liquid chromatography (Pharmacia LKB Biotechnology Inc.) and a 0.5 × 5 cm MonoQ column at pH 9.15, with 5 mM Tris/HCl buffer. A gradient to 1 M NaCl was used.

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1 Av1, Component 1 or MoFe protein from A. 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 Thr as its amino-terminal residue (7, 8); the Avl β-subunit is the faster moving subunit and has Ser as its amino-terminal residue (7, 8). Numbering of the amino acid residues is based on the protein sequences which are less than the DNA-sequenced sequence (8). For the Azotobacter proteins, the amino-terminal methionines have been removed in vivo by processing (7, 8).

‡‡ The abbreviations used are: EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
to elute the peptides. Reverse phase chromatography was performed on either C-4 or C-8 resin using a buffer of 0.1% trifluoroacetic acid and a gradient of acetonitrile.

RESULTS AND DISCUSSION

The EDC cross-linking of the nitrogenase complex is most likely between one of the two identical Av2 subunits and each of the Av1 β-subunits (9). However, the experimental approach used in our earlier report could not exclude the possibilities that both Av2 subunits were cross-linked or that, in addition, the cross-linking involved the α-subunit which was blocked at its amino terminus. To resolve these questions, mixed peptide maps were prepared which included 2-H-carboxymethylated complex and either 2-14C-carboxymethylated Av2, α-Av1, or β-Av1 subunits. Because the tryptic digests of the carboxymethylated subunits of Av1 and Av2 are well characterized, the cysteyl peptides derived from the individual components can be identified readily in the peptide map of the complex (7,12). The tryptic peptide maps for those mixtures are shown in Fig. 1.

Three important conclusions were obtained from inspection of the maps: First, only the cysteine-containing tryptic peptides from Av2 and β-Av1 were found in the tryptic map of the cross-linked complex. That is, the complex was composed only of Av2 and β-Av1. This confirms our earlier results. Second, from the specific radioactivity of the individual peptides in the map, the ratio of the two subunits in the complex was calculated to be 1:1. Thus, as previously suggested, only one of the two identical Av2 subunits was cross-linked to the β-Av1 subunits. Third, except for one peptide in each subunit, all of the cysteine-containing tryptic peptides from β-Av1 and Av2 were accounted for in the map of the complex. The missing peptide in the β-subunit, designated α-6 (residues 381–400 of the β subunit), contains 2 cysteines (7,8). Because the peptide terminates with the sequence, -Lys-Arg, there are two trypsin cleavage sites, both of which were observed in the map of the subunit (indicated by arrows in Fig. 1C) but were missing in the map of the complex. Likewise, five of six cysteine-containing tryptic peptides from Av2 were present in the complex. The only cysteyl peptide missing was T-11 (residues 101–140 of Av2, indicated by the arrow in Fig. 1A).

However, two new radioactive peptides were identified in the maps. The peptides (a major and a minor peak, >85 and <15% of the radioactivity in this region of the map, respectively) are identified as A and B in Fig. 1C and together they contained the amount of radioactivity expected for the missing α-6 and T-11 peptides (2.85/3.00, based upon the specific radioactivity for 1 cysteine). Thus, we conclude that Av1 and Av2 were cross-linked through the regions of peptides T-11 and α-6 and that the resulting peptides were eluted later in the solvent gradient.

To confirm the identity of the peptides and to determine which specific residues composed the cross-linking site(s), a preparative scale separation of the peptides was performed. For this purpose, only the cross-linked complex was used (the marker peptides from purified subunits were omitted). Peptides comparable to those indicated by bars A and B in Fig. 1C were pooled. The combined yield of the major and minor radioactive peaks was >60% based upon the assumption of 3 cysteines in the cross-linked peptides. A single radioactive peptide (55% yield) from pool B was separated from small quantities of other, nonradioactive peptides by reverse phase chromatography on a C-4 resin. The nonradioactive peptides were identified by amino acid sequencing as predominantly tryptic peptides T-8 (residues 53–77) and T-27 (residues 251–984) of Av2 and residues 481–503 of the β-Av1 subunit (data not shown) (8,12). These peptides are known to elute in this

FIG. 1. Mixed peptide maps of the cross-linked complex and isolated nitrogenase subunits. For each map, the complex, labeled by reductions and carboxymethylations with [3H]iodoacetic acid, was mixed with either Av2, Ser-Av1 subunit, or Thr-Av1 subunit, labeled with [14C]iodoacetic acid. The mixtures were digested with trypsin and the resulting peptides were separated as described under "Materials and Methods." 1.0-ml fractions were collected. The linear salt gradient was begun at fraction 15. A, complex and Av2 subunit. B, complex and Av1 α-subunit (Thr-Av1). C, complex and Av1 β-subunit (Ser-Av1).
region of the ion exchange chromatogram. Because a single, unique sequence was found for these peptides, they did not appear to contain the sites of cross-linking.

The amino acid composition for the major radioactive peptide, B, is given in Table I and the results of Edman degradation are given in Table II. The composition was that expected for a 1:1 mixture of T-11 and a-6. For the first 20 cycles, except for cycles 12 and 19, each cycle of Edman degradation contained 2 residues of nearly equal yield. These residues exactly matched the sequences for T-11 and a-6. For cycle 12, the expected glutamic acid in T-11 was absent; likewise, the expected lysine at cycle 19 for a-6 was absent. It should be noted that none of the other acidic residues in either peptide was below the expected yield given the overall sequencing repetitive yield. The absence of the penultimate lysine is significant because the carboxyl-terminal arginine was found. In addition, there is only 1 lysine in the composition of the putatively cross-linked peptide. Therefore, the missing lysine at cycle 19 must be one of the two moieties of the isopeptide bond. Beyond cycle 20 only the sequence of T-11 was observed because of the difference in length between a-6 and T-11. The positions of the 3 carboxymethylcysteines were confirmed by their radioactivity.

The sequence and composition for the minor radioactive peptide, A, was less clear due to the substantially higher concentration of the non-cysteinyl peptides and smaller quantities of the radioactive peptide for purification. To find the cross-linking site in this material, a portion of pool A was digested with chymotrypsin and the resulting peptides were separated by reverse phase chromatography on C-8 resin. One of the isolated peptides had the amino-terminal sequences expected for a cross-linked peptide between T-11 and a-6 (data not shown). Although this peptide is consistent with peptide B, the limited amount of material available was insufficient to determine unambiguously which glutamyl residue between 110 and 112 was in the isopeptide bond. The elution of peptide A at lower ionic strength than B suggests that A has a more positive charge than B. A tentative explanation is that peptide B contains an extension at the carboxyl terminus due to incomplete trypsin hydrolysis at the arginine next to Glu (Table I).

Table I

| Residue | Amino acid composition of peptide B |
|---------|------------------------------------|
| Ax   | 8.0 (8) |
| Thr  | 1.0 (1) |
| Ser  | 0.2 (0) |
| Glx  | 6.5 (6) |
| Pro  | 2.1 (9) |
| Gly  | 7.0 (7) |
| Ala  | 3.2 (3) |
| Cys  | 2.8 (3) |
| Val  | 5.9 (6) |
| Met  | 1.1 (1) |
| Ile  | 3.8 (4) |
| Leu  | 7.0 (7) |
| Tyr  | 1.9 (2) |
| Phe  | 4.7 (5) |
| His  | 1.8 (2) |
| Lys  | 1.1 (0) |
| Arg  | 2.0 (2) |

* Asx and Glx are the sum of the acid and amide forms. Cysteine was determined as carboxymethyl derivative.

Table II

| Cycle | Residue (picomoles) | Initial yield, 79 pmol | Repetitive yield, 94.8% |
|-------|---------------------|------------------------|-------------------------|
|       | Gly (67), Phe (88)  | Gly, Phe               |                         |
| 2     | Val (72), Leu (78)  | Val, Leu               |                         |
| 3     | Ile (70), Leu (82)  | Ile, Leu               |                         |
| 4     | Thr (42), Glu (62)  | Thr, Glu               |                         |
| 5     | Ala (60), Leu (53)  | Ala, Leu               |                         |
| 6     | Ile (41), Gly (41)  | Ile, Gly               |                         |
| 7     | Asn (61), Cys (3)   | Asn, Cys               |                         |
| 8     | Phe (59), Glu (54)  | Phe, Glu               |                         |
| 9     | Leu (50), Pro (49)  | Leu, Pro               |                         |
| 10    | Glu (47), Val (37)  | Glu, Val               |                         |
| 11    | Glu (51), His (22)  | Glu, His               |                         |
| 12    | As (55)             | Glu, Ile               |                         |
| 13    | Gly (39), Leu (34)  | Gly, Leu               |                         |
| 14    | Ala (38), Cys (3)   | Ala, Cys               |                         |
| 15    | Tyr (34), His (19)  | Tyr, His               |                         |
| 16    | Glu (36), Asn (14)  | Glu, Asn               |                         |
| 17    | Asp (37), Gly (10)  | Asp, Gly               |                         |
| 18    | Asp (41), Asn (7)   | Asp, Asn               |                         |
| 19    | Leu (30), 1'        | Leu, Lys               |                         |
| 20    | Asp (27), Arg (4)   | Asp, Arg               |                         |
| 21    | Phe (19)            | Phe                    |                         |
| 22    | Val (14)            | Val                    |                         |
| 23    | Phe (14)            | Phe                    |                         |
| 24    | Tyr (12)            | Tyr                    |                         |
| 25    | Asp (12)            | Asp                    |                         |
| 26    | Val (8)             | Val                    |                         |
| 27    | Leu (6)             | Leu                    |                         |
| 28    | Gly (6)             | Gly                    |                         |
| 29    | Asp (7)             | Asp                    |                         |
| 30    | Val (5)             | Val                    |                         |
| 31    | Val (7)             | Val                    |                         |
| 32    | Cys                 | Cys                    |                         |
| 33    | Gly (3)             | Gly                    |                         |
| 34    | Gly (4)             | Gly                    |                         |
| 35    | Phe (3)             | Phe                    |                         |
| 36    | Ala (3)             | Ala                    |                         |
| 37    | Met (2)             | Met                    |                         |
| 38    | Pro (2)             | Pro                    |                         |
| 39    | Ile (4)             | Ile                    |                         |
| 40    | Arg (1)             | Arg                    |                         |

* Values for a 1:1 mixture. Calculated from the known sequences (7-9).
FIG. 2. Summary of the amino acid sequences surrounding the proposed site of EDC cross-linking. *, invariant residues; parentheses indicate alternate residues commonly found; ?, variable residues, •, putative ligands to the Av2 Fe:S center (14-17). T-11 is residues 101-140 of Av2 and a-6 is residues 381-400 of Av1.

cross-linked residue is invariant among the known sequences of the nitrogenase components (see Fig. 2) (14-16). Although Glu-112 is found in only half of the species, it is located in one of the most conserved sequences of the Fe-protein. Indeed, when the 10 sequences of eubacterial Fe-proteins are compared, this region has 30 of 40 residues that are invariant. An additional 6 residues are highly conserved or are found in 9 of the 10 species. In contrast, Lys-399 is not found in any of the other five MoFe-proteins which have been sequenced and is in a region with substantial variation. Indeed, even the cysteines at residues 387 and 394 are not conserved.

It is tempting to speculate that the region around the Av2 Glu-112 may be part of a general ionic interaction site. For example, 7 or more carboxylic acid residues are found in this region of most Fe-proteins; 4 carboxyl residues are invariant. In addition, the region is located between the probable Fe:S cluster thiol ligands (residues Cys-97 and Cys-132) and, therefore, is likely to interact with the MoFe-protein for efficient electron transfer (17).

It is less clear how the sequence around Lys-399 participates in the complex formation. Although 4 of the 5 residues in this region of the Av1 β-subunit (residues 399-403) are positively charged, these residues are not conserved (8, 15, 16). Indeed, the comparable region in five other MoFe-proteins is as likely to have carboxylic acids as cationic residues. However, the differences in residues 399-403 did not correlate with the activity level of these MoFe-proteins when assayed with heterologous Fe-proteins (Fe-protein from other species) (5, 6). For example, the MoFe-protein from *Rhsobiella pneumoniae* is fully active with Av2, yet only Lys-403 is common in the sequence from 399-403. Thus, if there is a conserved motif of a cationic site, the basic residues must be separated in the linear sequence. Alternatively, the interaction sites on the MoFe-protein are unique. In either case, the cross-linking reaction may be restricted to the *Azotobacter* system and the fortuitous location of Lys-399. The role of these residues in the formation of productive complexes leading to nitrogenase activity is presently being investigated by chemical modification and by site-specific mutagenesis.

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