Specific Interaction of the [2Fe-2S] Ferredoxin from *Clostridium pasteurianum* with the Nitrogenase MoFe Protein

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ABSTRACT: Putative physiological partners of the [2Fe-2S] ferredoxin from *Clostridium pasteurianum* have been searched by running crude soluble extracts of this bacterium through an affinity column to which the [2Fe-2S] ferredoxin had been covalently bound. Subsequent washing of the column with buffers of increasing ionic strength revealed a strong and specific interaction of the ferredoxin with the MoFe protein of nitrogenase. This interaction was further investigated by performing cross-linking experiments with mixtures of the two purified proteins in solution. Analysis of the reactions by SDS−polyacrylamide gel electrophoresis evidenced only two covalently linked products. These were identified by N-terminal sequencing as the α and β subunits of the MoFe protein, each cross-linked to a single polypeptide chain of the ferredoxin. This result, taking into account the dimeric structure of the ferredoxin in solution, strongly suggests an interaction of the ferredoxin with the MoFe protein at a site contributed to by both subunits of the MoFe protein. The ionic strength dependence of the interaction evidenced by affinity chromatography was confirmed in the cross-linking reactions, and its specificity was assessed by showing that no cross-linking occurred when the [2Fe-2S] *C. pasteurianum* ferredoxin was denatured or replaced by spinach ferredoxin or by clostridial rubredoxin, or when the MoFe protein from *C. pasteurianum* was either inactivated or replaced by its counterpart from *Azotobacter vinelandii*. It has also been observed that the ferredoxin inhibits cross-linking between the nitrogenase Fe protein and the MoFe protein, which suggests overlapping binding sites of the ferredoxin and of the Fe protein on the MoFe protein. Cross-linking experiments implementing a number of molecular variants of the [2Fe-2S] *C. pasteurianum* ferredoxin demonstrated that glutamate residues 31, 34, and 38 are important contributors to the interaction with the MoFe protein.

The [2Fe-2S] ferredoxin (2Fe*Cp*Fd)[1] from the nitrogen-fixing saccharolytic anaerobe *Clostridium pasteurianum* is endowed with unique structural features which have been investigated by a combination of site-directed mutagenesis and spectroscopic techniques. These studies have allowed the identification of the four cysteine ligands of the [2Fe-2S] cluster (Fujinaga et al., 1993; Meyer et al., 1994; Golinelli et al., 1996; Shergill et al., 1996), and uncovered the unusual occurrence in this ferredoxin of a deletable loop on which one of the four cysteine ligands could be placed in various positions (Golinelli et al., 1996). Furthermore, mutated forms with cysteine ligands replaced by serine have afforded spectroscopic signatures of serine-ligated [2Fe-2S] clusters (Fujinaga et al., 1993; Meyer et al., 1994) and have been shown to possess unprecedented electronic and magnetic properties (Crouse et al., 1995; Achim et al., 1996). In contrast with this wealth of structural information, hardly anything is known concerning the function of the 2Fe*Cp*Fd. Early investigators noted that this protein was present in larger amounts in N_2 fixing cells than in cells grown on ammonia (Hardy et al., 1965; Meyer and Moulis, unpublished data), which suggested its possible involvement in nitrogen fixation. However, the promoter sequences upstream of the gene encoding the 2Fe*Cp*Fd did not bring forth any clearcut information on this question (Meyer, 1993).

We report here a significant progress made towards the elucidation of the function of the 2Fe*Cp*Fd, using an approach that had not been previously implemented in this case. Putative partners of the protein have first been searched by running crude soluble extracts of *C. pasteurianum* cells through an affinity chromatography column carrying covalently bound 2Fe*Cp*Fd. A specific interaction with the MoFe protein of nitrogenase was thus revealed, which was further studied by chemical cross-linking of the two purified proteins in solution. This investigation has benefited greatly from the availability of numerous mutated forms of 2Fe*Cp*Fd.

MATERIALS AND METHODS

All common DNA manipulations were as described (Ausubel et al., 1988; Meyer & Gagnon, 1991; Fujinaga & Meyer, 1993). The recombinant 2Fe*Cp*Fd and its molecular variants were prepared as previously reported (Fujinaga & Meyer, 1993; Meyer et al., 1994; Golinelli et al., 1996;

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[1] Abbreviations: *Cp, Clostridium pasteurianum;* Cp1, Av1, nitrogenase MoFe protein of *C. pasteurianum* or *Azotobacter vinelandii,* respectively; Cp2, nitrogenase Fe protein of *C. pasteurianum*; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; Fd(s), ferredoxin(s); 2Fe*Cp*Fd, [2Fe-2S] *C. pasteurianum* ferredoxin; E31K, mutated ferredoxin in which glutamate 31 has been replaced by lysine; E31K/E38K, doubly mutated ferredoxin in which both glutamate 31 and glutamate 38 have been replaced by lysine; C16/Δ19−28, variant ferredoxin in which both cysteine 14 and cystein 24 have been replaced by alanine, leucine 16 by cysteine, and in which residues 19−28 have been deleted; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; WT, wild type.

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Table 1: Generation of the Mutated 2Fe*Cp*Fd Genes[a]

mutation[b]	starting plasmid	mutagenic oligonucleotide[c]
E31K	Wild-type	5' GTTTCTACAATTTTAACGGAATTTTTGG 3'
E34K	Wild-type	5' CTTCCATGAATGTTTTTACAATTTCAAC 3'
E38K	Wild-type	5' GCTGTCTAACTCTTTCATGAATGTTTCTAC 3'
E31K/E38K	E38K	5' GTTTCTACAATTTTAACGGAATTTTTGG 3'
E48K/E71K[d]	Wild-type	5' GTATTATTTACCATTACTTTACTAGATAAATC 3'
W74A	Wild-type	5' CGAAGGAGTCGCGTATGGTAATGTAAC 3'
D82K	Wild-type	5' GTAACTGCTGATAAGGTTGAAGAGATTG 3'
E92K	Wild-type	5' GAGTCTCATATCAAAAACGGAGAAGTTG 3'
E88K/E92K	E92K	5' GAAGAGATTGTAAAGTCTCATATCAAAAAC 3'
E95K	Wild type	5' CGAAAACGGAAAAGTTGTAAAAAGAC 3'

[a] The mutagenesis method involved two rounds of PCR. The products of the second PCR were processed, cloned, and the mutated plasmids were characterized as described (Fujinaga et al., 1993; Meyer et al., 1994). [b] Mutations are noted with the one-letter code for amino acids: the first letter indicates the original residue, the following number refers to its position in the sequence, and the second letter indicates the substituting residue. [c] Mutagenic oligonucleotides for residues 31−48 were complementary to the coding strand. The primers used for the first round of PCR were the mutagenic oligonucleotide and an oligonucleotide complementary to the noncoding strand upstream of the gene. For the second round of PCR the primers were the product of the first round and an oligonucleotide complementary to the coding strand downstream of the gene. Mutagenic oligonucleotides for residues 74−95 were complementary to the noncoding strand. The primers used for the first round of PCR were the mutagenic oligonucleotide and an oligonucleotide complementary to the coding strand downstream of the gene. For the second round of PCR the primers were the product of the first round and an oligonucleotide complementary to the noncoding strand upstream of the gene. Mutated bases are underlined. [d] This doubly mutated gene was obtained serendipitously while preparing the E48K singly mutated one.

Shergill et al., 1996). For those variants of the 2Fe*Cp*Fd that had not yet been described, data relevant to the mutagenesis are gathered in Table 1.

Rubredoxin (Mathieu et al., 1992) and nitrogenase (Meyer, 1981) from *C. pasteurianum*, *Azotobacter vinelandii* nitrogenase (Burgess et al., 1980), and spinach ferredoxin (Meyer et al., 1986b) were purified as described.

UV−visible spectra were recorded on a Hewlett-Packard 8452 diode array spectrophotometer.

The affinity chromatography column was prepared as follows. The preparation and implementation of the column were carried out under argon in order to minimize ferredoxin damage. For the same reason, the temperature was 4 °C for the ferredoxin coupling reaction and for the storage of the column when not in use. Toyopearl AF-Formyl-650M (Tosohaas, Montgomeryville, PA) beads (0.9 mL) were washed several times, first with water and then with 5 M NaCl, and equilibrated with coupling buffer (sodium acetate, 0.1 M, pH 7.65, $MgCl_2$ 1 mM, $MnCl_2$ 1 mM, $CaCl_2$ 1 mM). A solution of 2Fe*Cp*Fd (9 mg in 0.5 mL of coupling buffer) was then added to the resin and the mixture was stirred at 300 rpm for 2 min. After addition of 15 mg (0.25 mmol) $NaBH_3CN$ (Sigma), the mixture was left to react with stirring for ca. 16 h. The resin was then washed with water to eliminate the unbound ferredoxin, and allowed to react for 2 h with 2 mL of 1 M Tris-HCl, pH 7.75, and 13 mg (0.22 mmol) of $NaBH_3CN$ in order to block the residual formyl groups. The affinity column was prepared by packing the resin in a 2 mL syringe, and equilibrated with 20 mM Tris-HCl, pH 7.4 (buffer A).

Cultures of *C. pasteurianum* (ATCC 6013) in nitrogen-fixing conditions and preparation of soluble cell extracts were as described (Meyer, 1981). Nitrogen-fixing cells were chosen because of the presumption that the [2Fe-2S] ferredoxin might be involved in nitrogen fixation (see introduction and Discussion). For the same reason, extreme care was taken to prevent inactivation of the nitrogenase protein components. Anaerobic conditions were ensured either by working with argon sparged vessels and buffers, or by carrying out the whole procedure in a glovebox maintaining the oxygen concentration in the gas phase below 2 ppm. The same results were obtained in both conditions. Also, the affinity chromatography runs were performed at room temperature (ca 25 °C) because of the cold sensitivity of the nitrogenase Fe protein (Moustafa & Mortenson, 1969). The column was loaded with 6−7 mL (ca. 100 mg of protein) of soluble cell extracts and washed first with 30 mL of buffer A and then with buffer A containing increasing concentrations of NaCl (0.1, 0.25, 0.5, and 1 M, 7−8 mL each). The eluted fractions were concentrated in an Amicon cell fitted with a PM10 membrane and analyzed by SDS−polyacrylamide gel electrophoresis.

For N-terminal sequencing of the bands of interest, the gels were equilibrated for 5 min with blotting buffer (25 mM Tris, 200 mM glycine, 10% methanol). Transfer onto Problott (Applied Biosystems) membranes was performed with a Hoefer Scientific Transfor electrophoresis unit (Towbin et al., 1979). The membranes were washed with methanol, stained (Coomassie Brilliant Blue R-250 0.08% in methanol 40%), destained in 50% methanol, and dried. The bands were cut out and the adsorbed polypeptide chains were sequenced on a 477A (Applied Biosystems) automated sequencer equipped with an on-line phenylthiohydantoin analyzer 120A (Applied Biosystems) and run with the sequencing program recommended by the manufacturer.

Covalent cross-linking was carried out in argon-flushed stoppered tubes, in a final volume of 0.2 mL MOPS 10 mM pH 7.0, dithionite 2 mM, at 20 °C. As partial proteolysis of the β subunit of the MoFe protein was observed in reactions performed at 30 °C, the temperature was lowered to 20 °C. The ionic strength was adjusted with NaCl. The reactions were initiated by adding 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC, Sigma) at a final concentration of 10 mM and were stopped 30 min later by the addition of 2-mercaptoethanol (25 mM final concentration). Reaction products were analyzed by SDS−PAGE, transferred onto membranes, and sequenced as described above.

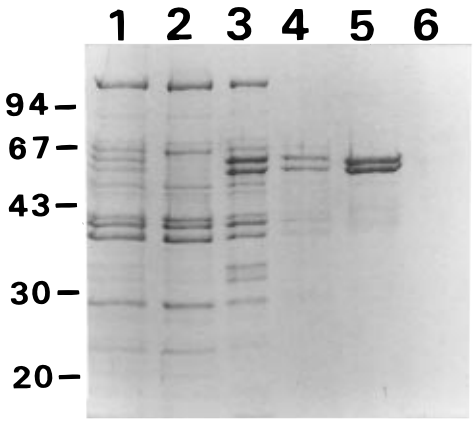

FIGURE 1: Affinity chromatography of soluble extracts of N_2-fixing *C. pasteurianum* cells on immobilized [2Fe-2S] *C. pasteurianum* ferredoxin. The column was prepared and the chromatography was run as described in Materials and Methods. The various fractions analyzed by SDS−PAGE (4% stacking, 10% separation) were the soluble cell extract (lane 1), the column flowthrough (lane 2), and the eluates with 20 mM Tris-HCl, pH 7.4 (lane 3), and with buffer containing 0.1 M NaCl (lane 4), 0.25 M NaCl (lane 5), and 0.5 M NaCl (lane 6). Molecular mass markers (in kDa) were phosphorylase *b* (94), bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), and trypsin inhibitor (20).

RESULTS

Identification of Putative Ferredoxin Partners by Affinity Chromatography

Affinity chromatography with ferredoxins immobilized on CNBr-activated Sepharose has been used for many years (Sugiyama & Yamano, 1975). Recently, mechanically more stable solid phases, namely, copolymers of ethylene glycol and methacrylate, have successfully been implemented (Sakihama et al., 1992). These resins have hydrophilic surfaces providing numerous attachment points for functional groups. In the series of available derivatives, the one carrying aldehyde groups (Toyopearl AF-Formyl-650M) has been chosen because of its specific reactivity with primary amines under mild conditions. In the conditions described in Materials and Methods, ca. 50% of the ferredoxin was covalently bound to the resin which became distinctly pink. The unbound Fd had undergone limited denaturation, as its A_{464}/A_{280} ratio had decreased from 0.47 to 0.35. At least part of this denaturation was due to the coupling agent $NaCNBH_3$. The column could be used repeatedly for several weeks, provided it was stored under argon at 4 °C. Occasionnally, irreproducible results have been obtained after prolonged storage (more than 6 months). In such cases the faded color of the resin suggested significant denaturation of the bound Fd.

The affinity chromatography of crude soluble extracts of *C. pasteurianum* grown in N_2-fixing conditions, followed by analysis of the eluted fractions by SDS−PAGE, showed that most proteins were either not bound at all, or washed out with the equilibration buffer (Figure 1, lanes 2 and 3). In contrast, the fraction eluted with 0.1 M NaCl was strongly enriched in two polypeptides having molecular masses in the 50−60 kDa range (lane 4). The bulk of these two polypeptides was eluted in the 0.25 M NaCl fraction in a nearly pure form (lane 5). Subsequent washing of the column with 0.5 M (lane 6) and 1 M NaCl (not shown) failed to elute any other proteins. The two bands eluted at 0.1−0.25 M NaCl were transferred onto membranes and sequenced. Their N-terminal sequences, determined to residue 10, identified them as subunits α (upper band) and β (lower band) of the nitrogenase MoFe protein (Wang et al., 1988). The sequencing data showed that the latter protein alone was contributing to the corresponding bands on the gels. It is worth of note that the MoFe protein subunits were clearly visible in the gel pattern obtained with the crude extract (lane 1), but were absent from the flowthrough of the column (lane 2). This indicated quantitative binding of the MoFe protein to the affinity column.

Control experiments were performed in order to assess that the binding of the MoFe protein to the affinity column was due to a specific interaction with the 2Fe*Cp*Fd. Untreated resin did not bind any of the proteins of the cell extract (not shown). When the resin was treated with Tris, in order to block the surface formyl groups, several proteins remained weakly bound to the column and were eluted with 0−0.25 M NaCl. None of these migrated like the subunits of the MoFe protein. A similar behavior was observed when apoferredoxin (acid denatured) rather than holoferredoxin was bound to the column (not shown). These controls demonstrate that the strong binding of the MoFe protein to the Fd-modified column is due to a specific interaction with 2Fe*Cp*Fd and, furthermore, that this interaction requires the Fd to be in its native structure.

The specificity of the affinity column was further assessed by loading it with purified nitrogenase proteins. It was found to bind the purified nitrogenase MoFe protein from *C. pasteurianum* but not the MoFe protein from *A. vinelandii* or the nitrogenase Fe protein from *C. pasteurianum*.

Covalent Cross-Linking of 2FeCpFd to the Nitrogenase MoFe Protein

The ionic-strength dependence of the interaction between the 2Fe*Cp*Fd and the nitrogenase MoFe protein (Figure 1) points to a strong contribution of electrostatic forces. Investigation of this interaction has therefore been carried out by covalently cross-linking the two purified proteins using a reagent, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), that catalyzes the cross-linking of carboxylates with neighboring primary amines, i.e., the very residues that are likely to be involved in the electrostatic interaction (Poulos & Kraut, 1980).

When the Fd or the MoFe protein were individually incubated with EDC, no cross-linking was observed, as previously reported for the MoFe protein from *A. vinelandii* (Willing et al., 1989). When the two proteins were incubated together in the presence of EDC, the electrophoresis gels displayed two bands running slower than the MoFe protein subunits, and having apparent molecular masses larger than those of the latter by ca. 10 kDa (Figure 2). The products present in these bands were identified by blotting and N-terminal sequencing, carried out on a sample obtained in the conditions described in Figure 2A, lane 3. Initial sequencing yields calculated from the first twenty cycles showed that the upper band contained 12.8 pmol of Fd and 14.9 pmol of the α subunit of the MoFe protein. The lower band contained 7.4 pmol of Fd and 8.2 pmol of the β subunit. These data show that the α and β subunits of the MoFe protein were each cross-linked to one subunit of the Fd, which is known to be dimeric in solution (Meyer et al., 1984;

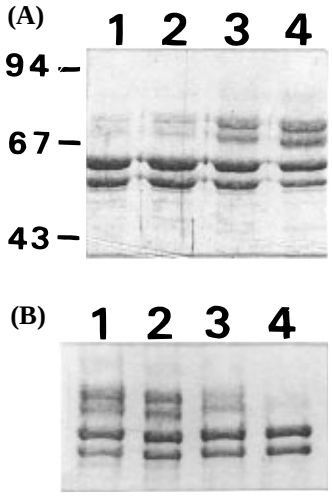

FIGURE 2: Cross-linking reactions between the 2Fe*Cp*Fd and the nitrogenase MoFe protein from *C. pasteurianum*. Reactions were carried out as described in Materials and Methods and analyzed by SDS−PAGE (3% stacking, 7% separation). (A) Dependence on the ratio of the two proteins. The reaction buffer was 20 mM Tris-HCl, pH 7.4, 0.12 M NaCl. The MoFe protein concentration was 5 μM in all cases. The 2Fe*Cp*Fd concentration was 1.7 μM (lane 1), 5 μM (lane 2), 15 μM (lane 3), or 35 μM (lane 4). (B) Ionic strength dependence. MoFe protein concentration was 5 μM, 2Fe*Cp*Fd concentration was 35 μM. The reaction buffer (20 mM Tris-HCl, pH 7.4) contained 0.05 M (lane 1), 0.1 M (lane 2), 0.2 M (lane 3), or 0.5 M NaCl (lane 4). Molecular mass markers (in kDa) were as in Figure 1.

Pétillot et al., 1995). The sequencing yields might suggest that cross-linking of Fd with the α subunit is more efficient than that with the β subunit. However, it was also noted that in these reaction conditions the β subunit was significantly less stable than the α subunit and was observed to be degraded at 30 °C in preliminary experiments (see Materials and Methods). Therefore, the lower amount of cross-linked β subunit is possibly an artifact resulting from the lower stability of this subunit in the conditions of the cross-linking reaction. In any event, the cross-linking of the 2Fe*Cp*Fd with both the α and β subunits of Cp1 does not imply that the cross-linking sites are symmetry related.

The amount of cross-linked material increased when the ratio of Fd to MoFe protein was increased (Figure 2A). However, even in the presence of a 7-fold molar excess of Fd (Figure 2A, lane 4), no more than approximately one half of the total amount of MoFe protein underwent cross-linking. This may result from kinetic limitations which would not allow the cross-linking reaction to go to completion. Alternatively, the covalent binding of Fd to one αβ pair of subunits may modify the structure of the MoFe protein in a way that would forbid Fd binding to the remaining αβ pair. Another possibility is that at any given binding site, cross-linking could take place on only one, either α or β, of the MoFe protein subunits. Then, even if the reaction went to completion, only half of the MoFe protein subunits would be cross-linked.

The efficiency of cross-linking was strongly dependent on the ionic strength (Figure 2B). The amount of cross-linked material was constant up to 0.1 M NaCl (lane 2), decreased at 0.2 M NaCl (lane 3), and was nil at 0.4 M NaCl (lane 4). This was consistent with the affinity chromatography results (Figure 1) and confirmed the strong contribution of electrostatic forces to the interaction.

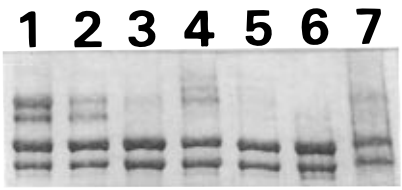

FIGURE 3: Cross-linking reactions between the *C. pasteurianum* nitrogenase MoFe protein (Cp1) and various other proteins. Conditions were as described in Materials and Methods. Reactions were analyzed by SDS−PAGE (3% stacking, 7% separation). Cp1 concentration was 5 μM, other proteins were 35 μM. The reaction buffer was 20 mM Tris-HCl, pH 7.4, with added NaCl as indicated. lane 1: 2Fe*Cp*Fd, 0.1 M NaCl. lane 2: 2Fe*Cp*Fd, 0.2 M NaCl. lane 3: 2Fe*Cp*Fd apoprotein (acid denatured), 0.1 M NaCl. lane 4: spinach Fd, 0.1 M NaCl. lane 5: spinach Fd, 0.2 M NaCl. lane 6: *C. pasteurianum* Rd, 0.1 M NaCl. lane 7: Cp1 (air-inactivated for 24 h) and 2Fe*Cp*Fd, 0.1 M NaCl.

The experimental conditions for both the affinity chromatography and the cross-linking reactions have been devised in order to prevent inactivation of even the most oxygen-sensitive proteins present in *C. pasteurianum*. Therefore, all experiments were performed under argon, and dithionite (2 mM) was added to the cross-linking reactions. Under such conditions the 2Fe*Cp*Fd was reduced, and all heretofore described interactions with the MoFe protein involved the Fd in the reduced level. Control cross-linking experiments with oxidized Fd were run in dithionite-free buffer and with a MoFe protein stock solution in which the dithionite concentration had been decreased to less than 25 μM by serial dilution/concentration in an Amicon cell. Thus, the dithionite concentration in the cross-linking reaction medium was less than 2 μM, and therefore more than 95% of the Fd was oxidized. These conditions did not modify the cross-linking pattern, which showed that the interaction between the two proteins is not dependent on the redox level of the Fd.

The specificity of the interaction of the Fd with the MoFe protein was assessed in a number of ways (Figure 3). No cross-linked products were obtained when 2Fe*Cp*Fd was replaced by apoferredoxin, even in the presence of only 0.1 M NaCl (Lane 3). When the MoFe protein was inactivated by exposure to air for 24 h, a weak and fuzzy band arising from cross-linked material was observed when the reaction was performed in 0.1 M NaCl (lane 7) but not in 0.2 M NaCl (not shown). Thus the interaction between the 2Fe*Cp*Fd and the MoFe protein requires both partners to assume their native structure. When the 2Fe*Cp*Fd was replaced by spinach Fd, weak and blurred bands were observed with 0.1 M NaCl (lane 4), and no cross-linking took place in 0.2 M NaCl (lane 5). Thus, the interaction of the MoFe protein is much weaker with spinach Fd than with 2Fe*Cp*Fd in the same reaction conditions (lanes 1 and 2). Spinach Fd shares some features with 2Fe*Cp*Fd, namely its [2Fe-2S] cluster, its low isoelectric point, and the length of the polypeptide chain. However, spinach Fd is a monomer in solution and its sequence is unrelated to that of 2Fe*Cp*Fd (Meyer et al., 1986a). Therefore the distribution of charges on its surface is most likely to differ from that of 2Fe*Cp*Fd and to be less suitable for specific electrostatic interactions with the MoFe protein. The rubredoxin from *C. pasteurianum*, a small (6 kDa) acidic iron−sulfur protein (Mathieu et al., 1992) yielded no cross-linked products with the MoFe protein (Figure 3, lane 6). When the MoFe protein from *C. pasteurianum* was replaced by its counterpart from *A.*

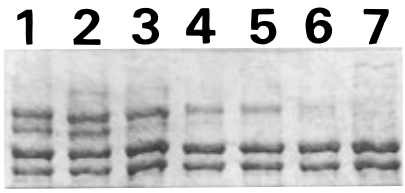

FIGURE 4: Cross-linking reactions between the nitrogenase MoFe protein from *C. pasteurianum* (Cp1) and molecular variants of 2Fe*Cp*Fd. Conditions were as described in Materials and Methods. Reactions were analyzed by SDS−PAGE (3% stacking, 7% separation). Cp1 concentration was 5 μM, 2Fe*Cp*Fd was 35 μM. The reaction buffer was 20 mM Tris-HCl, pH 7.4, 0.1 M NaCl. Lane 1: wild type 2Fe*Cp*Fd. lane 2: C16/Δ19−28. lane 3: C16/Δ19−30. lane 4: E31K. lane 5: E34K. lane 6: E38K. lane 7: E31K/E38K.

vinelandii, the bands arising from cross-linked material on the gels were fuzzy and could be observed only with reactions performed in 0.1 M NaCl, not in 0.2 M NaCl (not shown). Thus, as inferred above from the affinity chromatography experiments, the interaction of the 2Fe*Cp*Fd was much weaker and less specific with Av1 than with Cp1.

Interaction of Molecular Variants of 2FeCpFd with MoFe Protein

Since both the affinity chromatography (Figure 1) and the cross-linking reactions (Figure 2) pointed to a strong contribution of electrostatic forces in the interaction between the 2Fe*Cp*Fd and the MoFe protein, charged residues were considered as privileged targets of site-directed mutagenesis. The 2Fe*Cp*Fd includes seven basic residues and fifteen acidic ones (Meyer et al., 1986a). Five of the former are scattered in the N-terminal half of the sequence, the two remaining ones are very near to the C-terminus. As to the acidic residues, most of them (fourteen out of fifteen) are clustered in the 31−48 and 81−95 segments of the sequence. A number of charge inverting mutations have therefore been introduced in these two regions (see Table 1). The behavior of the E31K, E34K, E38K, E31K/E38K, E48K/E71K, D82K, E92K, E88K/E92K, and E95K variants in the cross-linking reactions will be considered below. The chromophores of these mutated forms were identical to that of the wild type, and their stability was not significantly impaired. Additional double or triple mutations involving these residues have been performed, but the corresponding proteins were not stable enough to be satisfactorily implemented in cross-linking reactions. The E48K/E71K, D82K, E92K, E88K/E92K, and E95K mutated forms produced cross-linking patterns with the MoFe protein that were identical with that of the wild type protein (not shown). In contrast, the E31K, E34K, E38K, and E31K/E38K variants displayed lower affinities (Figure 4). With the E31K (lane 4) and E34K (lane 5) proteins, the cross-linking with subunit β was barely detectable, and that with subunit α was visible but weaker than with the wild type. The E38K (lane 6) variant showed an even weaker cross-linking with subunit α, and none at all with subunit β. With the E31K/E38K doubly mutated Fd (lane 7), no cross-linked products were observed whatsoever. In all cases the cross-linking reactions were apparently less efficient with the β than with the α subunit (see in particular lanes 3−6). Whereas this may reflect a selective weakening of the interaction of the mutated ferredoxins with the β subunit of the MoFe protein, it should be kept in mind that even for the wild type Fd more cross-linked material was obtained with the α than with the β subunit, an observation that might be rationalized by the lower stability of the latter subunit in the reaction conditions (see above).

Numerous other molecular variants of the 2Fe*Cp*Fd have been prepared by site-directed mutagenesis for various purposes and were previously described (Meyer et al., 1994; Shergill et al., 1996; Golinelli et al., 1996). Most of these mutated ferredoxins were found to display cross-linking patterns with the MoFe protein identical with that of the wild type 2Fe*Cp*Fd (not shown). For instance, substitutions of histidine residues (H7A; Shergill et al., 1996), or replacement of the active site ligand C24 by a cysteine introduced in position 16 (C16; Golinelli et al., 1996), did not alter the interaction with the MoFe protein. It has previously been shown that the stability of the 2Fe*Cp*Fd was not severely impaired by various deletions in the region of residues 19−30, which was therefore predicted to be a flexible loop (Golinelli et al., 1996). This putative loop is unlikely to be involved in the interaction with the MoFe protein, since the C16/Δ19−23 (deletion of residues 19 to 23) and C16/Δ19−28 (deletion of residues 19−28) variants (Golinelli et al., 1996) behaved identically to the WT in cross-linking experiments (Figure 4, lane 2). A significant weakening of the interaction with the MoFe protein was observed only upon extending the deletion to residue 30 (C16/Δ19−30 variant; Figure 4, lane 3). A previously unreported point mutation, W74A, has been performed to check whether this tryptophan residue was involved in hydrophobic interactions. The W74A variant was as stable as the wild type, in line with the suspected near-surface location of W74, it had a [2Fe-2S] chromophore identical with that of the wild type, and its reactivity with the MoFe protein did not differ from that of the wild type (not shown).

Cross-Linking Reactions Involving the Nitrogenase Fe Protein

The 2Fe*Cp*Fd affinity column did not bind the nitrogenase Fe protein, neither when present in *C. pasteurianum* crude extracts (Figure 1) nor in its purified state (not shown). The absence of significant interactions between the two proteins was confirmed by cross-linking experiments similar to those described above with the MoFe protein: no bands arising from covalent cross-linking between the Fe protein and the 2Fe*Cp*Fd were detectable on gel electrophoreses in the various conditions implemented above with the MoFe protein (not shown). While the Fd appeared not to interact directly with the Fe protein, the posssibility that it might interfere with the interaction between the two nitrogenase protein components has been considered, and investigated by cross-linking with EDC. When reacted on its own with EDC, the Fe protein, like the MoFe protein, underwent no covalent cross-linking. This again was in line with previous observations with *A. vinelandii* nitrogenase (Willing et al., 1989). Incubation of mixtures of the two nitrogenase components with EDC resulted in the production of only one major band, of which the apparent size suggested the cross-linking of one subunit of the Fe protein with one subunit of the MoFe protein (Figure 5, lanes 1−3). Blotting and N-terminal sequencing of the band of interest identified it as arising from the cross-linking of one subunit of the Fe protein with one subunit α of the MoFe protein. The initial sequencing yields, extrapolated from the first fifteen cycles were 7.3 pmol for the Fe protein subunit and 6.5 pmol for the α subunit,

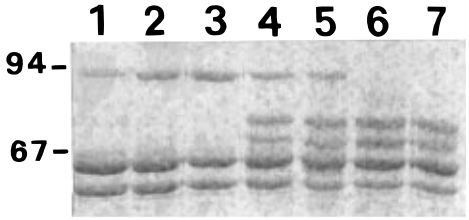

FIGURE 5: Cross-linking reactions involving the two nitrogenase components and the 2Fe*Cp*Fd. Conditions were as described in Materials and Methods. The reactions were analyzed by SDS−PAGE (3% stacking, 7% separation). lanes 1−3: Cp1 concentration was 5 μM, Cp2 was 25 μM. The reaction buffer was 20 mM Tris-HCl, pH 7.4, containing 0.2 M NaCl (lane 1), 0.1 M NaCl (lane 2), or 0.05 M NaCl (lane 3). lanes 4−7: The reaction buffer was 20 mM Tris-HCl, pH 7.4, 0.1 M NaCl. Cp1 concentration was 5 μM in all reactions. Cp2 concentration was 25 μM (lanes 4−6) or 0 μM (lane 7). 2Fe*Cp*Fd concentration was 12.5 μM (lane 4), 25 μM (lane 5), 50 μM (lane 6), or 35 μM (lane 7). Molecular mass markers (in kDa) were as in Figure 1.

confirming the stoichiometry of one to one. With *A. vinelandii* nitrogenase a single cross-linked product has previously been described, but then it involved one subunit of the Fe protein and one β subunit of the MoFe protein (Willing et al., 1989). This discrepancy was not unexpected since the sequences of the α and β subunits differ between Cp1 and Av1. Also, these two nitrogenases differ by the respective contributions of the α and β subunits to the Fe protein−MoFe protein interface (Kim et al., 1993). The amount of cross-linked material was found to increase upon increasing the ratio of Cp2 to Cp1, at least up to a value of 5 to 1 (not shown). This ratio was implemented in the investigation of the effects of the ionic strength on the coupling (Figure 5, lanes 1−3). The highest amount of cross-linked polypeptides was obtained with 0.05 M NaCl (lane 3). It decreased slightly with 0.1 M NaCl (lane 2) and considerably with 0.2 M NaCl (lane 1). This indicated that electrostatic forces are involved here as well, and that the interaction is probably weaker between the nitrogenase components than between the MoFe protein and the 2Fe*Cp*Fd (Figure 2B).

When the cross-linking reactions between the two nitrogenase components were performed in the presence of increasing amounts of 2Fe*Cp*Fd, the band arising from the covalent reaction between the two nitrogenase proteins weakened and eventually disappeared. It was replaced by the bands arising from the reaction between the Fd and the MoFe protein (Figure 5, lanes 4−6). Thus, the binding of the 2Fe*Cp*Fd to the MoFe protein appears to prevent the interaction of the two nitrogenase component proteins. It was noted that no bands attributable to cross-linked ternary complexes, i.e., with masses higher than 100 kDa, were observed (Figure 5, lanes 4 to 6).

DISCUSSION

Both the affinity chromatography and the cross-linking experiments have afforded compelling evidence for a strong and specific interaction of the 2Fe*Cp*Fd with the nitrogenase MoFe protein. In both occurrences the interaction was inhibited by increasing salt concentrations, which suggests an important contribution of electrostatic forces. Cross-linking reactions implementing a number of molecular variants of the Fd have pointed to three negatively charged residues, E31, E34, and E38 as significant agents in the interaction with the MoFe protein. The effects of charge inversions on residues 31 and 38 were found to be additive (Figure 4). Although the three-dimensional structure of the Fd is still unknown, the segment encompassing residues 30−40 can be predicted to form an alpha helix. Among all other mutated forms of the Fd, the only one that differed from the WT with respect to its interaction with the MoFe protein was the C16/Δ19−30 variant, which has a deletion (residues 19−30) located in a flexible region of the molecule (Golinelli et al., 1996). In contrast, a deletion shorter by only two residues, as in C16/Δ19−28, did not impair binding to the MoFe protein. Since residues 29 and 30 are serine and valine, respectively, they are unlikely to be directly involved in the interaction. Instead, their deletion possibly displaces the presumptive 30−40 helical region in a way that weakens the contribution of the charged residues 31, 34, and 38 to the interaction. A number of other negatively charged residues are not involved in electrostatic interactions with the MoFe protein, since the E48K/E71K, D82K, E88K/E92K, and E95K mutations had no effect. The positively charged residues, though fewer than the acidic ones (seven versus fifteen), are more scattered along the sequence, and therefore have the potential of providing valuable information for the mapping of the interface between the Fd and the MoFe protein. Residues K19 and K27 are most unlikely to be involved since they have been deleted in the C16/Δ19−28 variant which is wild type-like as to its interaction with the MoFe protein. The effects of mutations on residues K5, R15, R43, K98, and R99 are currently being investigated.

Although crystal structures are available for MoFe proteins from both *A. vinelandii* (Av1) (Kim & Rees, 1992) and *C. pasteurianum* (Cp1) (Kim et al., 1993), no information on the three-dimensional structure of the 2Fe*Cp*Fd has been obtained yet. Thus, the interaction between Cp1 and 2Fe*Cp*Fd cannot at this point be discussed in any structural detail. However, from the fact that the Fd is a dimer in solution, and therefore must possess a 2-fold symmetry element, it may be inferred that its interaction with Cp1 most likely takes place on a 2-fold symmetry element of the latter protein. The $\alpha_2\beta_2$ Cp1 tetramer is formed of a pair of $\alpha\beta$ dimers that are related by a 2-fold rotation axis (Kim et al., 1993). Either the upper or the lower face of Cp1, near this 2-fold axis, could thus provide a docking site for the Fd. However, the structures of Cp1 and Av1 in these regions are very conserved (Kim et al., 1993), which would seem to disagree with the different affinities of the Fd for Cp1 and Av1 (see Results). MoFe proteins also possess pseudo 2-fold symmetry elements relating the α and β subunits within each $\alpha\beta$ pair (Kim & Rees, 1992). These provide potential binding sites for the Fd which would be consistent with both the different affinities of the Fd towards Av1 and Cp1, and with its competing with the Fe protein for the binding to Cp1. Indeed, the surface regions near the pseudo 2-fold axes differ significantly between Av1 and Cp1 (Kim et al., 1993; Howard & Rees, 1994; Peters et al., 1995) and have been proposed to encompass the surface of interaction with the Fe protein (Kim & Rees, 1992; Howard & Rees, 1996).

Previous investigations have given indications that the 2Fe*Cp*Fd might be involved in nitrogen fixation. It was first observed to be present in higher abundance in N_2-fixing than in NH_3-grown cells (Hardy et al., 1965). The amounts of 2Fe*Cp*Fd present in cells under these two growth conditions

were subsequently estimated, on the basis of purification yields (J. Meyer and J.-M. Moulis, unpublished) and rocket immunoelectrophoresis (J. Meyer, unpublished), to differ by a factor of 5−10. The present demonstration of a strong and highly specific electrostatic interaction of the 2Fe*Cp*Fd with the nitrogenase MoFe protein indicates that this protein must in some way be involved in nitrogen fixation. Various possibilities may be considered at this stage, including an electron donating function analogous to that normally assumed by the 2[4Fe-4S] Fd (Mortenson, 1964), a role in the assembly of the metal clusters of the MoFe protein (Dean et al., 1993), or a short term regulation of nitrogenase activity. In preliminary experiments, the 2Fe*Cp*Fd was found to display no significant effect on nitrogenase activity *in vitro*.

Other clues to the function of the 2Fe*Cp*Fd may be drawn from comparisons with structurally related proteins. Of particular interest are two [2Fe-2S] proteins purified from the aerobic nitrogen fixer *A. vinelandii* (Shethna et al., 1968). The better characterized of the two, designated FeSII, has been shown to associate with the two protein components of nitrogenase, thus forming a ternary complex protecting nitrogenase against oxygen damage (Haaker & Veeger, 1977; Robson,1979; Moshiri et al., 1994). It shares some features with the 2Fe*Cp*Fd, with respect to its encoding gene (Meyer, 1993; Moshiri et al., 1994), to its increased synthesis in nitrogen-fixing conditions, to its size and dimeric structure with one [2Fe-2S] cluster per subunit (Moshiri et al., 1995). However, the two proteins differ by the properties of their chromophores and by the positions in their sequences of the cysteine ligands of the cluster (Meyer et al., 1984, 1986a; Moshiri et al., 1994, 1995). In the light of the present work, even more significant discrepancies appear to exist between their interactions with nitrogenase. Indeed, the evidence reported here that the 2Fe*Cp*Fd interacts only with the MoFe protein and that in this interaction it competes with the Fe protein, argues strongly against the formation of a ternary complex. Also, if such associations had the same function in *C. pasteurianum* and in *A. vinelandii*, i.e. protection against oxidative damage, it would be difficult to rationalize the privileged binding of the 2Fe*Cp*Fd to the more stable MoFe protein. Therefore it seems unlikely that the 2Fe*Cp*Fd has a function similar to that of the FeSII protein from *A. vinelandii*. In contrast, the FeSI protein from the same organism (Shethna et al., 1968), of which the function is unknown, appears to have a chromophore intriguingly similar to that of the 2Fe*Cp*Fd. Thus, biochemical and genetic investigations on both proteins in their respective hosts should be expected to provide complementary information on their possibly related functions.

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