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Hypothesis

Does ferredoxin I (*Azotobacter*) represent a novel class of DNA-binding proteins that regulate gene expression in response to cellular iron(II)?

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Azotobacter vinelandii (*Av*) and *chroococcum* (*Ac*) ferredoxin I contain $[3Fe\text{-}4S]^{1+/0}$ and $[4Fe\text{-}4S]^{2+/1+}$ clusters, when isolated aerobically, which undergo one-electron redox cycles at potentials of -460 ± 10 mV (vs SHE) at pH 8.3 and -645 ± 10 mV, respectively. The X-ray structure of Fd I (*Av*) reveals that the N-terminal half of the polypeptide folds as a sandwich of β-strands which enclose the iron-sulphur clusters. The C-terminal sequence contains an amphiphilic α-helix of four turns which lies on the surface of the β-barrel. Fd I (*Av*) controls expression of an unknown protein of $M_r \sim 18000$. Fd I (*Ac*) will complex iron(II) avidly above pH ~8.0 only when the [3Fe-4S] cluster is reduced and provided that cellular nucleic acid is bound. Fd I (*Ac*) rigorously purified from nucleic acid does not undergo iron(II) uptake. These facts, together with recent evidence that the interconversion process $[3Fe\text{-}4S]^0 + Fe^{2+} \rightarrow [4Fe\text{-}4S]^{2+}$ in the iron-responsive element binding protein (IRE-BP) of eukaryotic cells is controlling protein expression at the level of mRNA [1991, Cell 64, 4771; 1991, Nucleic Acid Res. 19, 1739] leads to the following hypothesis. Fd I is a DNA-binding protein which interacts by single α-helix binding in the wide groove of DNA. The binding is regulated by iron(II) levels in the cell. The 7Fe form binds to DNA and represses gene expression. Only the DNA-bound form of the 7Fe Fd I will take up iron(II), not the form free in solution. Iron(II) becomes bound when the [3Fe-4S] cluster is reduced. The 8Fe Fd I thus generated no longer binds DNA and the gene is de-repressed. Sequence comparisons and the crystal structure suggests that the two central turns of the α-helix are important elements of the DNA-recognition process and that residues Gln^{69} and Glu^{73}, which lie on the outer surface of the helix, hydrogen-bond with specific base pairs.

Ferredoxin; DNA-binding protein; Gene expression

1. INTRODUCTION

Bacterial ferredoxins are low molecular weight acidic proteins that contain either one or two iron-sulphur clusters [1]. Examples are known which have a single [4Fe-4S], or [3Fe-4S] cluster, a pair of [4Fe-4S] clusters or one [3Fe-4S] plus a [4Fe-4S] cluster. These proteins are central to a number of biological processes but because the iron-sulphur clusters undergo redox cycling it has been widely assumed that ferredoxins function solely as soluble electron carriers in various metabolic or respiratory processes including nitrogen fixation and hydrogen uptake. Indeed electron transfer between ferredoxins and other redox active proteins can be demonstrated in vitro. However there is mounting evidence that some ferredoxins may function as DNA-binding proteins and hence control gene expression. The process may be regulated by the iron(II) levels and redox status in the cell. The evidence is examined in this article and a hypothesis of the mechanism is formulated.

One of the intriguing properties of the [3Fe-4S] cluster is its ability to undergo a redox-linked three-iron ↔ four-iron interconversion process in some proteins [2,3]. The chemistry can be written in terms of the oxidation states of the cluster cores as

$$[3FE\text{-}4S]^{1+} \underset{-e^-}{\overset{+e^-}{\rightleftharpoons}} [3Fe\text{-}4S]^0 \qquad (E^0_{3Fe})$$

$$-Fe(II)\uparrow\downarrow Fe(II)$$

$$[4Fe\text{-}4S]^{2+} \underset{-e^-}{\overset{+e^-}{\rightleftharpoons}} [4Fe\text{-}4S]^{1+} \qquad (E^0_{4Fe})$$

The $[3Fe\text{-}4S]^{1+}$ oxidation state has a low affinity whereas $[3Fe\text{-}4S]^{0+}$ has a high affinity for iron(II). The reduction potentials lie in the order $E^0_{3Fe} > E^0_{4Fe}$ [4]. Therefore iron(II) uptake or loss is tightly linked to the iron(II) concentration within the cell and the internal redox potential of the cell.

The [3Fe-4S] core is liganded to the protein by three thiolate side chains of cysteine [5–8] whereas the [4Fe-4S] core requires four ligands which can all be provided by amino-acid side-chains, usually cysteine. Hence iron(II) uptake by a [3Fe-4S] cluster may lead to the binding of an additional amino-acid residue and therefore can be the trigger for conformational change.

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It is these properties which I suggest are the basis for some gene regulation in prokaryotic cells. In this article I consider the evidence for such a role for *Azotobacter* ferredoxin I.

2. *AZOBACTER* FERREDOXIN I

The ferredoxin, called Fd I, isolated from *Azotobacter vinelandii* (*Av*) [9] and *Azotobacter*

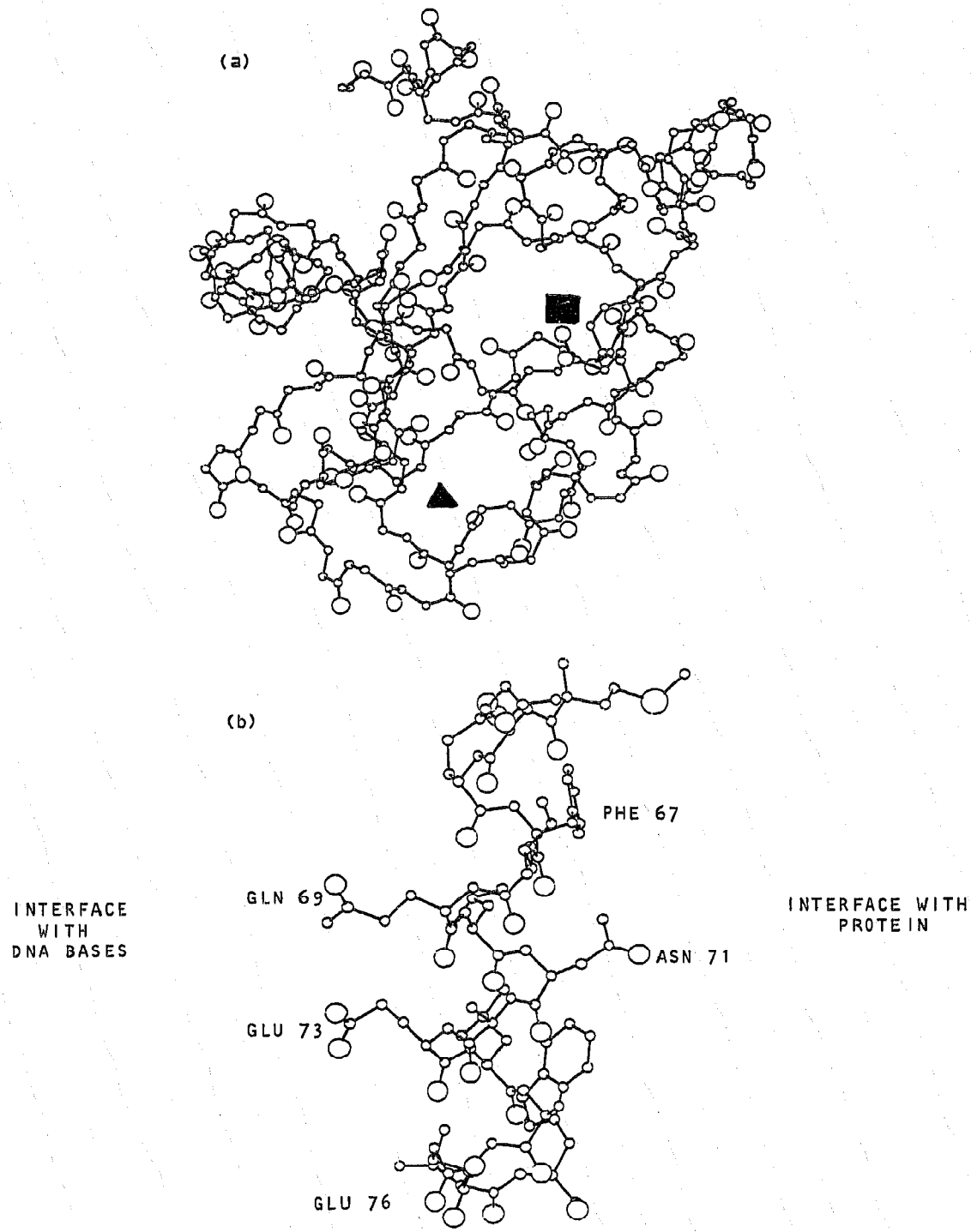

Fig. 1. (a) Polypeptide chain fold of Fd I (*Av*) with side chains omitted. The orientation provides a view along the axis of the α-helix lying on the left-hand side of the molecule. The positions of the iron-sulphur clusters are indicate by ■ and ▲. (b) The four-turns of the α-helix of Fd I (*Av*) showing the side-chain positions determined crystallographically. The right-hand side of the helix is in contact with the β-barrel of the ferredoxin. The two residues, Gln^{69} and Glu^{73}, are proposed to be the recognition residues which H-bond to base pairs of DNA. Hence the left-hand face of the helix will face into the wide groove of DNA. (Data taken from reference [6]. Coordinates were obtained from Protein Data Bank, Brookhaven National Laboratory).

chroococcum (*Ac*) [10] has been extensively studied by spectroscopic [11,12], crystallographic [5–8], and electrochemical methods [4]. Spectroscopic comparisons show the two proteins are closely similar [11,12]. Recent genetic experiments to prepare a deletion mutant of Fd I, *Av*, has given important insights into the function of this ferredoxin [13].

2.1. *Structure*

The re-determination of the X-ray structure of the 7Fe form of Fd I (*Av*), prepared aerobically, shows the presence of one [3Fe-4S] cluster and one [4Fe-4S] cluster [5–8]. The three-iron cluster can be described as a [4Fe-4S] cube with one iron atom removed. Three cysteine residues ligate the [3Fe-4S] core to the protein. The first half of the polypeptide chain folds about the two iron-sulphur clusters in a very similar way to that of the 2[4Fe-4S] ferredoxin of *Peptococcus aerogenes*. However, Fd I (*Av*) contains an additional 56 amino acids at the C-terminus which form a single α-helix (residues 63–80) of four turns across the rear face of the protein followed by a loop wrapped around the end of the ferredoxin which contains the [3Fe-4S] cluster (Fig. 1).

2.2. *Redox properties*

The redox properties of *Azotobacter* FdI have been almost as controversial as the structure. However, by means of direct, unmediated electrochemistry at a pyrolytic graphite edge electrode the potentials of the two clusters in Fd I (*Ac*) have been determined [4]. The $[3Fe\text{-}4S]^{1+/0}$ cluster undergoes a one-electron cycle at a potential of -460 ± 10 mV (vs SHE) at pH 8.3. The potential is pH-dependent revealing a single protonation process in the $[3Fe\text{-}4S]^{0}$ state with $pK_a = 7.8$ [16]. The $[4Fe\text{-}4S]^{2+/1+}$ cluster has a reduction potential of -645 ± 10 mV, the lowest potential so far reported for a biological iron-sulphur cluster and below that attainable with solutions of sodium dithionite [4].

2.3. *Cluster interconversion*

The iron uptake properties of *Azotobacter* Fd I are unusual. The 7Fe ferredoxin, Fd III, from *Desulphovibrio africanus* avidly takes up iron(II) to generate a protein containing $2[4Fe\text{-}4S]^{2+}$ clusters and oxidative loss of iron to give the original ferredoxin takes place rapidly [2]. This is not the case for purified *Azotobacter* Fd I. Although ferredoxins are acidic proteins they bind strongly to nucleic acid which can be removed after treatment with nucleases followed by repeated chromatography [11]. Samples of Fd I (*Ac*) which have been rigorously purified from nucleic acid, as judged by the absence of a peak at 260 nm, failed to react with iron(II) after reduction of the [3Fe-4S] cluster either with dithionite or electrochemically over the pH range 6–8.5 (4). Studies on Fd I (*Av*) which had been purified by crystallisation reported only slow time-dependent conversion of the 7Fe to the 8Fe form in sodium dithionite [14,15]. The 8Fe form of Fd I (*Av*) has been made by reconstitution of the ferredoxin from apoprotein, iron(II) and sodium sulphide under rigorously anaerobic conditions [14].

However, Fd I (*Ac*) was reported to bind iron(II) readily, generating the 8Fe form, in the presence of sodium dithionite and iron(II) at pH values greater than 8.0 [16]. The protein-avidity for iron(II) when the $[3Fe\text{-}4S]^{0}$ cluster was reduced was so high that inclusion of EDTA in solution was required to prevent iron(II) uptake. The ferredoxin that avidly bound iron(II) had not been completely freed from nucleic acid [4].

The 8Fe form of Fd I (*Ac*) contains 2[4Fe-4S] only one of which can be reduced by dithionite solutions to give a $[4Fe\text{-}4S]^{1+}$ state [16]. On exposure to air, the 8Fe form of Fd I (*Av*) made by reconstitution was unstable and decomposed to a low yield, 10% of the 7Fe form [14].

The [3Fe-4S] $\leftrightarrow$ [4Fe-4S] transformation properties of *Azotobacter* Fd I require further elucidation and study. However, it seems that the 7Fe form of ferredoxin I has a low- and a high-affinity state for iron(II) and that the latter requires the presence of nucleic acid and pH conditions above ~8.0. Attempts to induce the high-affinity state with effectors such as ATP and other phosphate-containing molecules have been unsuccessful.

2.4. *Biological functions of Fd I (Av)*

The physiological function of Fd I has long been assumed to be involved in electron donation to nitrogenase. However, it has recently been demonstrated that Fd I is not required for nitrogen fixation by *Azotobacter vinelandii* [13]. By cloning and sequencing the gene for Fd I, *fdx*A, a mutant, LM100, was constructed in which the *fdx*A gene had been inactivated. This mutant grows at wild-type rates under nitrogen-fixing conditions showing that Fd I is not required for nitrogen fixation. However, an acidic protein, M_r = 18 000, which is present in wild-type cells, is overexpressed to a high level in the LM100 mutant. The nature of this protein is not yet known. This result implies that Fd I is a repressor for this protein although these experiments do not provide proof of repression by direct interaction with nucleic acid.

2.5. *Implications*

Fd I controls expression of at least one protein in *Azotobacter vinelandii*. The purified 7Fe form of the ferredoxin does not readily acquire iron(II) when free in solution even in the presence of excess iron(II) and sodium dithionite reductant. However, in the presence of nucleic acid there is ready uptake of iron(II). The X-ray structure shows a single α-helix which is exposed on the outside of the protein, a possible DNA-binding motif. These facts lead to the central proposal of this

paper that *Azotobacter* Fd I is a DNA-binding protein which regulates protein expression depending upon the iron and redox status of the cell. The [3Fe-4S] ↔ [4Fe-4S] conversion is the chemical sensor of iron(II) levels.

There is the alternative possibility that the ferredoxin acts as an mRNA-binding switch since post-translational regulation is established in prokaryotes [17]. This possibility cannot be eliminated at this stage. However, the structure of Fd I suggests a possible DNA-binding motif. Therefore this hypothesis only is explored in section 5.

3. IRON-DEPENDENT GENE REGULATION

Iron(II) is well established as a control element in both eukaryotic and prokaryotic cells [18,19]. In eukaryotic cells iron homeostasis is achieved by the synthesis of the iron-storage protein, ferritin, and the suppression of transferrin-receptor protein synthesis when iron(II) levels in the cell rise. As the iron levels drop the opposite occurs. Regulation takes place at the mRNA level [19]. A protein binds to a common control sequence called the iron-responsive element (IRE) of the mRNA which encodes ferritin and that encoding transferrin receptors. This protein, the IRE-binding protein (IRE-BP), is prevented from binding to both mRNAs by the presence of iron(II). The sequence of IRE-BP shows a high degree of homology with that of mitochondrial aconitase especially with those residues which bind the [3Fe-4S] cluster and also with those residues lying in the pocket close to the cluster [20,20a]. The proposal has been made that the binding of iron(II) regulates mRNA binding of IRE-BP with the clear implication that [3Fe-4S] ↔ [4Fe-4S] interconversion is involved.

There are at least three bacterial genes known that appear to be regulated by DNA-binding proteins which bind iron(II) and respond directly to the oxygen tension in the cell. In *E. coli* they are the *Fur* (ferrous uptake regulation) gene and the *Fnr* gene whose product is a regulator of genes concerned with anaerobic energy metabolism. Rhizobial *nif A* gene product is an activator for the expression of symbiotic nitrogen fixation genes. The product of the *Fur* gene negatively controls a number of genes which determine function related to iron uptake. The protein is a repressor that, upon binding iron(II), blocks transcription of iron-regulated genes by binding to specific operator sequences within their promoter regions. Recent NMR studies show that the metal-binding site of FUR protein involves histidine and possibly carboxylate residues [21]. Although the protein has a high α-helical content the helix–turn–helix motif common to many prokaryotic DNA-binding regulatory proteins is apparently absent [22].

Fnr and *nif A* gene products are positive regulatory proteins that respond directly to the oxygen tension in the cell [18]. The action of both is inhibited by metal-ion chelators whose effect can be reversed by the addition of divalent metal ions of which iron(II) is the most effective. Both proteins have potential metal-binding domains. *Nif A* product has four essential cysteine residues and FNR protein also has four cysteines in the N-terminal domain of which three are proved to be essential.

These examples serve to illustrate that iron(II) is already established as a gene regulator in prokaryotic cells responsive to oxygen levels. The recent evidence on IRE-BP suggests it to be a control protein at the mRNA level employing the [3Fe-4S] ↔ [4Fe-4S] interconversion process as an iron(II) sensor. The process must also be linked to the redox status of the cell.

4. PROPOSED MECHANISM OF CONTROL

It is our hypothesis that Fd I (*Av* and *Ac*) is a DNA-binding protein which interacts by single α-helix binding in the wide groove of DNA. We further propose that the Fd I-DNA binding is regulated by iron(II) levels in the cell and that it is the conversion of the [3Fe-4S] cluster to the [4Fe-4S] form that controls DNA binding. The previously puzzling properties of this protein towards iron(II) uptake and cluster conversion become understandable. The control scheme is shown in Fig. 2. The 7Fe form of Fd I will not take up iron(II) until two conditions are fulfilled. First the Fd must bind DNA and secondly the three-iron cluster must be reduced to the $[3Fe\text{-}4S]^0$ state. The semi-reduced Fd I-DNA complex has a high affinity for iron(II). Formation of 8Fe

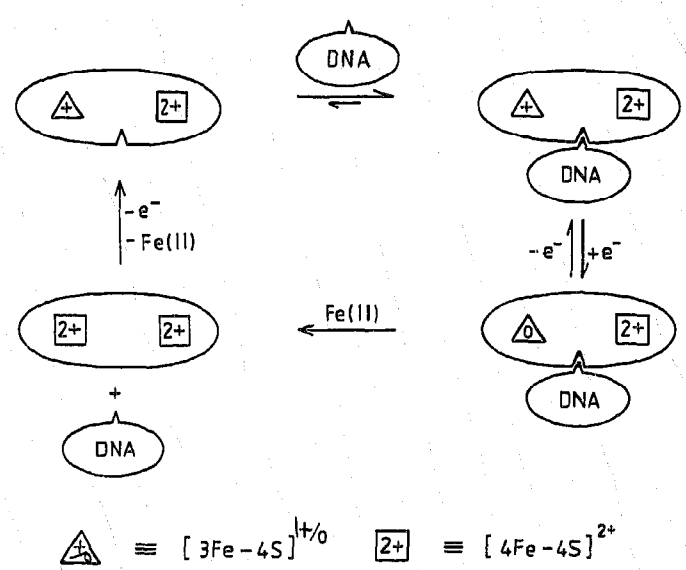

Fig. 2. Proposed reaction scheme for the binding of the 7Fe form of Fd I (*Azotobacter*) to DNA and the loss of binding following reduction of the [3Fe-4S] cluster and uptake of iron(II) to generate the 8Fe form of Fd I. Recovery of the 7Fe form takes place when the iron(II) level drops and when the redox potential rises. Gene transcription is repressed when the 7Fe form is bound to DNA.

Fd I leads to a lowering of its affinity for DNA, the Fd is unbound and the gene de-repressed. The 7Fe Fd is regenerated by loss of iron(II). This will only occur when the redox potential of the cell is high enough and also when the free iron(II) level within the cell has dropped. It is important that the 7Fe form of Fd I cannot convert to the 8Fe Fd until it has bound DNA, otherwise 7Fe-8Fe conversion would take place in the cytoplasm without the involvement of DNA binding.

The role of the other cluster, [4Fe-4S], which does not exchange iron is unclear. The E_0 value of this cluster in the 7Fe form is very low, -640 mV. Its value in the 8Fe Fd and in the DNA-Fd complex are not known. It may be that this cluster has a redox role in passing electrons from a donor to the $[3Fe\text{-}4S]^{1+}$ cluster when the 7Fe Fd is bound to DNA. This device would separate the role of interconversion from the E_0 value of the sensing cluster. The E_0 value of the [4Fe-4S] could be tuned over a relatively wide range in different organisms to provide control at different redox values without altering the mechanism of the trigger, the [3Fe-4S] cluster. Hence the electron transfer role of the clusters is a necessary one. These will be interesting mechanistic questions to pursue.

5. MODE OF DNA BINDING

A common structural motif for the binding of bacterial repressor proteins such as cro, lac and trp is a helix-turn-helix pattern [23]. The second helix, called the recognition helix, consists of two turns which bind in the wide groove of DNA. Contacts consist of hydrogen bonds between hydrophilic amino-acid side chains such as glutamine, arginine, lysine, glutamic acid and the nitrogen and oxygen atoms of base pairs in the major groove. Interactions with the phosphate groups of the DNA backbone are also important. A comparison between the sequences of the recognition helix of DNA repressor-binding proteins reveals an alternation of hydrophobic and hydrophilic residues so that the recognition residues lie on the outer face of the helix in order to interact with the base pairs. The hydrophobic residues lie on the helix surface facing into the protein.

Inspection of the amino-acid sequence and crystal structure of Fd I (*Av*) shows that the 4-turn α-helix runs between residues 63 and 80, Fig. 1b. The surface of the helix facing the solvent contains two hydrophilic residues, Gln^{69} and Glu^{73}, ideal for interaction with bases in the wide groove of DNA. Asn^{71} faces into the protein. However, on either side of this residue hydrophobic amino acids are present. A four-turn α-helix is slightly too long to fit into the wide groove of DNA. It should curve to follow the DNA helix. Asn^{71} is conserved in the sequences of seven ferredoxins of this class (see below). It lies between the two recognition

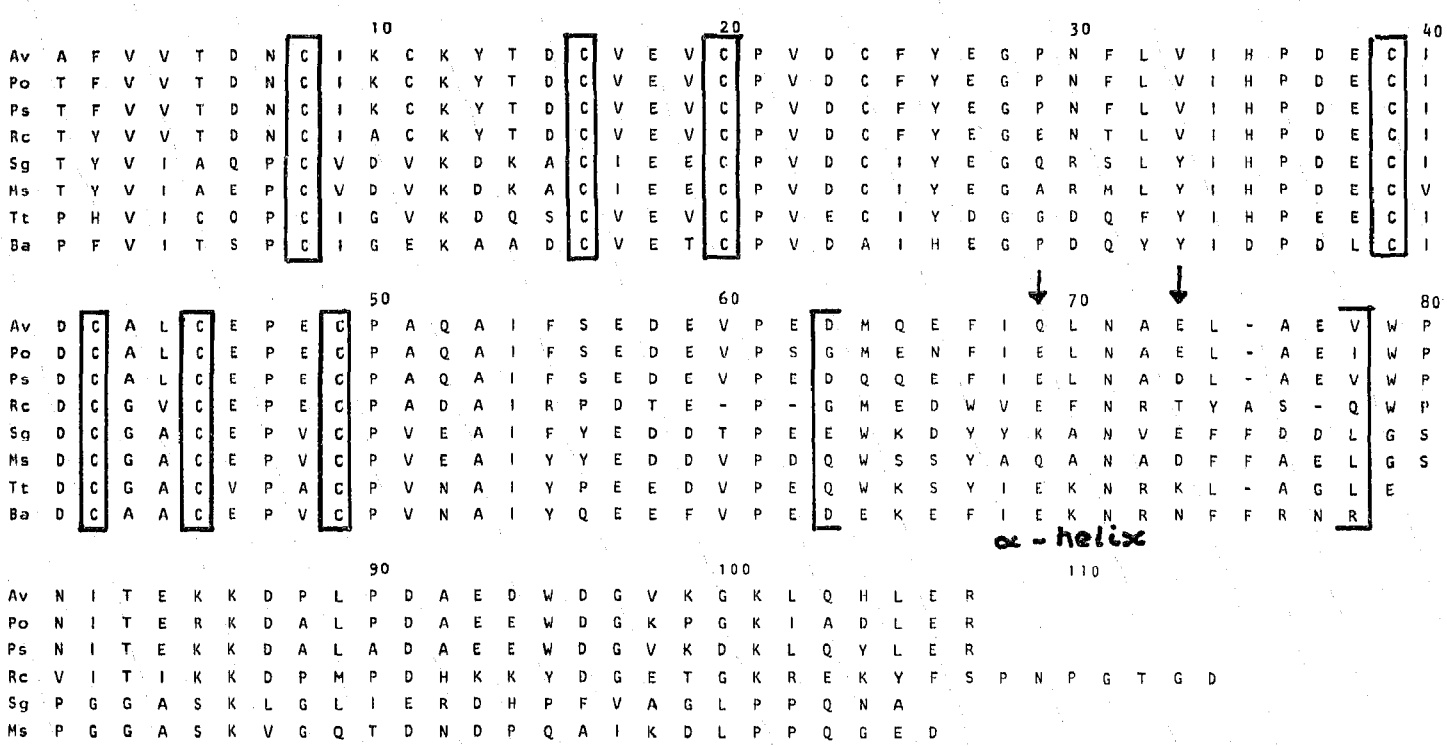

Fig. 3. Sequence comparisons of the 7Fe ferredoxins which are homologous to Fd I (*Av*). C8, C16 and C49 bind the three-iron cluster and C20, C39, C42 and C45 bind the four-iron cluster. The α-helix runs from residues 63–80. Key: Av, *Azotobacter vinelandii* [24]; Po, *Pseudomonas ovalis* [25]; Ps, *Pseudomonas stutzeri* [26]; Rc, *Rhodobacter capsulatus* [27]; Sg, *Streptomyces griseus* [28]; Ms, *Mycobacterium smegmatis* [29]; Tt, *Thermus thermophilus* [30]; Ba, *Bacillus acidocaldarius* [31].

residues but points into the centre of the protein. Tension on Asn^{71} would cause the axis of the α-helix to bow away from the protein and thereby to improve the fit to the curvature of the DNA-helix. Aspargine is known to be important in causing a bend in the middle of the seven-turn α-helix of the leucine zipper proteins which bind to DNA [38]. In this case Asn is totally conserved in the sequence. It H-bonds to a residue further down the helix to form a helix cap and hence to bend the helix. The mechanism proposed to be present in Fd I is different. It could be that relaxation of the tension on Asn^{71} would be a plausible mechanism of lowering the affinity of the Fd for DNA. Hence the α-helix of Fd I has characteristics appropriate to be a recognition helix with residues 69, 71 and 73 playing an important role.

The amino-acid sequences of seven ferredoxins of this class are known. They are highly homologous to Fd I (*Av*) (Fig. 3). The high degree of homology in the first 60 N-terminus residues which bind the iron-sulphur clusters show that the β-barrel core structure is unchanged. The C-terminal region also contains a good deal of homology. The alignment shown in Fig. 3 allows inspection of the four-turn α-helix and enables the recognition residues at positions 69 and 73 to be identified. Although they are not conserved the substitutions maintain the pattern of amino-acid residues in these positions which have hydrophilic side chains suitable for base-pair interaction. The alternation between hydrophobic and hydrophilic residues is largely but not perfectly maintained.

Helix-turn-helix repressors are invariably dimeric in the form which binds to DNA since the operators are usually inverted repeats. The molecular weight determination of Fd I (*Av*) has been problematical. Although generally accepted to be a monomeric protein [32] analysis by size exclusion HPLC and PAGE gave a dimeric molecular weight [24]. A dimeric form has also been reported for the 7Fe Fd from *Streptomyces griseus*. However, inclusion of 1.0 M NaCl in the eluting buffer reduced the dimer to a monomer [28]. *Pseudonomas ovalis* Fd I is also reported to be dimeric [33]. Hence there are certain conditions of ionic strength and pH which cause some ferredoxins of this class to dimerize. The possibility that Fd I can bind as a dimer to DNA cannot be excluded.

6. IMPLICATIONS

The amino-acid sequence data suggests that ferredoxin I is a widespread repressor in bacterial species including those which do not fix nitrogen. The identiy of the protein controlled is not yet known. The M_r value of ~18 000 reported compares with an M_r of 17 000 of the subunits of *Azotobacter* bacterioferritin [34]. It could be that Fd I controls bacterioferritin expression. This would be consistent with its role as an iron(II)-dependent repressor. Bacterioferritins have been identified in some but not all of the bacteria in which this 7Fe Fd has been found.

The hypothesis presented here suggests a search for other ferredoxins which possess a surface α-helix capable of DNA recognition. Indeed there are two such classes, namely *Desulphovibrio gigas* Fd II [7] and *Bacillus thermoproteolyticus* Fd [35]. *D. gigas* Fd II contains only a single [3Fe-4S] cluster. It is a dimer which will transform its [3Fe-4S] cluster into a [4Fe-4S] cluster [36]. Little work has been reported of the mechanism and rate of this process. This protein is a candidate to be regarded as a DNA-binding transcription factor. *Bacillus* ferredoxin is isolated containing one [4Fe-4S] cluster which can be converted into a [3Fe-4S] cluster with oxidising agents such as $[Fe(CN)_6]^{3-}$ [34]. The hypothesis presented here suggests the re-examination of the roles of a number of bacterial ferredoxins which contain either [4Fe-4S] or [3Fe-4S] clusters.

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