Probing the Role of Electrostatic Forces in the Interaction of *Clostridium pasteurianum* Ferredoxin with Its Redox Partners

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ABSTRACT: The ability of several low-potential redox proteins to mediate electron transfer between *Clostridium pasteurianum* pyruvate-ferredoxin oxidoreductase and hydrogenase has been evaluated in a coupled enzymatic assay. The active electron mediators, whatever their structure, must have a reduction potential compatible with the two enzymes, but for proteins of similar potentials, a marked specificity is displayed by 2[4Fe-4S] ferredoxins of the clostridial type. Such ferredoxins are small proteins exchanging electrons with many enzymes involved in the metabolism of anaerobic bacteria. The forces underlying the interactions of ferredoxin with hydrogenase and pyruvate-ferredoxin oxidoreductase have been examined with an emphasis on electrostatics: site-directed mutagenesis experiments have been used to individually convert all conserved glutamates and aspartates of *C. pasteurianum* ferredoxin into either neutral or positively charged amino acids. Also, up to four of these residues have been replaced simultaneously. The biological activities of the resulting variants depend very little on the number and the distribution of the anionic side chains on the surface of the ferredoxin. Only those molecular forms for which the immediate environment of the clusters is perturbed, independently of the charge distribution, display variations in their catalytic properties. It is concluded that electron transfer between *C. pasteurianum* 2[4Fe-4S] ferredoxin and its partners is far less dependent on electrostatic interactions than in many other well-documented electron transfer systems.

Protein−protein recognition is one of the basic processes triggering most biological reactions. Among many relevant examples, electron transfer between two or more soluble proteins depends on efficient and specific bimolecular interactions. A case in point is provided by the electron transfer chains of bacteria, which usually thrive on diverse substrates and implement various electron pathways toward this aim (Meyer & Cusanovich, 1989). Sugars are very common substrates that are most often converted into pyruvate by heterotrophic organisms. In fermenting bacteria, pyruvate can be further metabolized by a variety of pathways leading to diverse end products. All of these reactions require electron transfer between successive enzymes of the different pathways. For instance, most clostridia (and other anaerobic organisms) first oxidize pyruvate to acetyl-CoA and CO_2, hence generating reduced 2[4Fe-4S] ferredoxin (Fd).[1]

The 2[4Fe-4S] Fd from *Clostridium pasteurianum* therefore is the major electron shuttle between the catabolic and anabolic pathways of the bacterium (Mortenson & Nakos, 1973). The ability of various low-potential electron transfer agents to couple pyruvate oxidation to proton reduction catalyzed by the enzyme hydrogenase has been assessed here in an *in vitro* enzymatic assay reconstituting part of the electron transfer chain functioning in saccharolytic clostridia. The results evidence a heretofore undetected specificity of the overall reaction toward clostridial ferredoxins. These features can easily be missed when both enzymes are studied independently and they uncover a possible regulatory effect of the electron acceptor on the activity of pyruvate-ferredoxin oxidoreductase (PFO).

Because all known enzymes interacting with *C. pasteurianum* Fd are soluble proteins, they afford a set of reactions enabling us to study the factors contributing to the tuning of electron transfer between proteins in soluble chains. The small size of *C. pasteurianum* Fd and the possibility of changing its sequence at will (Davasse & Moulis, 1992; Moulis et al., 1994) make it a protein amenable to extensive site-directed mutagenesis eliminating or reversing a given physicochemical property. This approach has been implemented here to assess the role of surface carboxylates in the interactions of *C. pasteurianum* Fd with hydrogenase and PFO from the same source. Indeed, the many 2[4Fe-4S] ferredoxins sequenced so far are characterized by a large excess of residues bearing a carboxylic group (aspartate and glutamate) over positively charged side chains (lysines and arginines). Moreover, the distribution of aspartates and glutamates is well conserved among these very acidic proteins (Figure 1). However, the involvement of these amino acids in protein−protein recognition has not yet been addressed, despite the potential role of electrostatic forces in intermolecular interactions (Davis & McCammon, 1990). The consequences of the individual replacements of all conserved carboxylic acids of *C. pasteurianum* Fd, as well

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[1] Abbreviations: Fd, ferredoxin; PFO, pyruvate-ferredoxin oxidoreductase; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)methylamine; CoASH, reduced coenzyme A; DTT, dithiothreitol; TPP, thiamine pyrophosphate; FMN, flavin mononucleotide; NAD, nicotinamide adenine dinucleotide.

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A:   AYKIADSCVSCGACASECPVNAI  SQGDSIFV IDA  DTCIDCG  NCANVCPVGAPVQE
B:   AYVINEACISCGACEPECPVNAI  SSGDDRYV IDA  DTCIDCG  ACAGVCPVDAPVQA
C:   AYVINDSCIACGACKPECPVNCI  QQG SIYA IDA  DSCIDCG  SCASVCPVGAP4X
D:   AYVINDSCISCGACEPECPVNAI  TAGDDKYV IDA  ATCIDCG  ACAGVCPVDAPQPE
E: 4XVIVDSDKCIGCGECVDVCPVEVYELQNGKAVPV NE   EECLGCE  SCIEVCPQNAIV E
F:  PATVNADECSGCGTCVDECPNDAI  TLDEEKGI AVVDNDECVECG  ACEEACPNQAI4X
G: 4XTIVNIDDCVACGACSGTCPQSVL  EVNDHVEI KNP  DDCIGCG  ACVAACPQGVLKVE
H:   AHRITEECTYCAACEPECPVNAI  SAGDEIYI VDE  SVCTDCE6XACVAVCPVDCIIKV
I:   ALYITEECTYCGACEPECPTNAI  SAGSEIYV IDA  AGCTECV6XACAAVCPAECIVQG
J:   ALMITDECINCDVCEPECPNGAI  SQGDETYV IEP  SLCTECV6XQCVEVCPVDCI24X
K: MAFKIIASQCTQCGACEFECPRGAV NFKGE KYV IDP  TKCNECK6XQCASVCPVSNT4X

        D C  C  C EC          D      D   D C DC    C   C

        6         17          27     33  35  39
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FIGURE 1: Selected alignment of 2[4Fe-4S] ferredoxins. The last row emphasizes the conserved residues bearing a carboxylate (no difference has been made between D and E) with the *C. pasteurianum* numbering. X indicates any amino acid. The aligned ferredoxins are from (A) *C. pasteurianum*, (B) *C. acidurici*, (C) *Peptostreptococcus asaccharolyticus*, (D) *Clostridium sticklandii*, (E) *Desulfovibrio desulfuricans* FdII, (F) *Methanosarcina barkeri*, (G) *Entamoeba histolytica*, (H) *Chlorobium limicola* FdII, (I) *Chlorobium thiosulfatophilum*, (J) *Ch. vinosum*, and (K) *Rh. meliloti*. Sequences are from the PIR database except for *Ch. vinosum* Fd, for which the gene sequence has been used (our unpublished data).

as of the simultaneous substitution of up to four of them, on the activity have been evaluated, and these data have provided insight into the factors contributing to the interaction of the protein with its redox partners.

MATERIALS AND METHODS

All experiments, including the purification of proteins and enzymes, described in this paper have been carried out under strictly anaerobic conditions, either with argon lines or inside an anaerobic chamber (Jacomex, Livry-Gargan, France). Traces of oxygen in argon were removed by passage through a tower of BASF R3-11 catalyst.

Proteins and Enzymes. Hydrogenase (Meyer & Gagnon, 1991), [2Fe-2S] Fd (Meyer et al., 1984), and PFO (unpublished experiments) were purified from *Clostridium pasteurianum* W5 (ATCC 6013). The purifications of *Clostridium acidurici* and *Clostridium thermosaccharolyticum* Fd (Gaillard et al., 1986), *Synechocystis* sp PCC6803 Fd (Bottin & Lagoutte, 1992), spinach Fd (Meyer et al., 1986), and *Chromatium* (*Ch.*) *vinosum* Fd (Huber et al., 1995) were previously described. Recombinant *C. pasteurianum* Fd (Davasse & Moulis, 1992; Moulis et al., 1994), synthesized in *Escherichia coli* K38 containing plasmid pGP1-2 (Tabor, 1990), has been thoroughly characterized and shown to be perfectly identical to the protein isolated from *C. pasteurianum* (Davasse & Moulis, 1992; Pétillot et al., 1995).

For the preparation of *C. pasteurianum* Fd molecular variants, modular fragments of the synthetic gene encoding the protein (Davasse & Moulis, 1992) were exchanged with oligonucleotide duplexes bearing the mutation(s) (Quinkal et al., 1994). The changes introduced in the gene are summarized in Table 1. The variants used in this work and not listed in Table 1 were prepared by the exchange of DNA modules between the plasmids obtained as described in Table 1. All single-site variants of *C.pasteurianum* Fd are named as *XnY*, where residue X in position *n* of the sequence is replaced by residue Y. The presence of the required mutations on the gene was established by sequencing using the dideoxynucleotide termination method (Sanger et al., 1977). In some instances, the changes introduced were also detected on NMR spectra (Davasse et al., 1995).

Spectroscopic Methods. UV–visible and low-temperature EPR spectroscopies were implemented according to Quinkal et al. (1994) and Moulis et al. (1984), respectively. The amount of recombinant Fd present in soluble extracts of *E. coli* was determined by dithionite reduction of 3% (i.e., 150 μL, see the following) of the volume obtained from a 1 L culture and integration of the EPR spectrum against a sample of pure Fd of known concentration. It has been confirmed that similar cultures of *E. coli* lacking the plasmid encoding *C. pasteurianum* Fd did not display any EPR spectrum at this stage of fractionation.

Enzymatic Assays. The activity of *C. pasteurianum* hydrogenase with purified variants of Fd was measured as already described (Quinkal et al., 1994). The details of the coupled enzymatic assay involving both PFO and hydrogenase have been reported elsewhere (Quinkal et al., 1994). The activity of PFO has also been determined by measuring the reduction of the electron acceptor, at 425 nm for ferredoxin or at 540 nm for benzyl viologen, in a glove box, ensuring an oxygen concentration in the Ar gas phase of less than 2 ppm. The 500 μL assay contained 50 mM potassium phosphate (pH 7.5), 2 mM DTT, 140 μM CoASH, 50 μM TPP, 1 mM $MgCl_2$, and 4 μg of PFO. The reaction was started by adding 2 mM sodium pyruvate.

For evaluating the ability of modified ferredoxins to accept electrons from *C. pasteurianum* PFO, soluble extracts of *E. coli* containing the recombinant Fd were used in the coupled assay. These extracts were prepared from 1 L cultures by suspending the cell pellet in 5 mL of 20 mM potassium phosphate buffer (pH 7.5). The cell suspension was lysed by ultrasonic treatment for 3 × 45 s at 100 W (Labsonic U equipped with the 40T microprobe, B. Braun ScienceTec, Les Ulis, France) and centrifuged for 30 min at 15000*g*. The pellet was discarded and the supernatant was stored as beads in liquid nitrogen until use.

Dipole Calculations. The dipole moments of the ferredoxins were calculated by using the QUANTA program

Table 1: Changes Introduced into the Gene Encoding *C. pasteurianum* Ferredoxin[a]

ferredoxin	restriction fragment[c]	oligonucleotides used[d]
D6A	nr	attgcgGCTagctgcgttag + GCcgcaattttataagccataag
E17A/D	*Nhe*I−*Sty*I	ctagcGMCtgcccggttaacgcgatatcc + cttgggatatcgcgttaaccgggcaGKCg
D27A/E/K	*Sty*I−*Cla*I	caaggtRMAagcatttttgttat + cgataacaaaaatgctTKYac
D33A-D35N	*Cla*I−*Nsi*I	cGCTgcgAACacatgca + tgtGTTcgcAG
D39A/T/E	nr	gcattRMAtgtggtaactgt + ARYaatgcatgtatccgcat
Δ1[b]	*Cla*I−Ns*i*I	cgctGTTacatgca + tgtAACag

[a] The gene encoding recombinant Fd has been described elsewhere (Davasse & Moulis, 1992; Moulis et al., 1994). [b] Δ1 is the form in which the ^{33}DA^{35}D tripeptide is replaced by the AV dipeptide. [c] Restriction sites used to exchange modules in the synthetic gene. nr, nonrelevant: the synthetic gene was reassembled from a series of oligonucleotides containing the pair indicated. [d] The mutagenic oligonucleotides are written in the usual 5′ to 3′ direction with the first sequence of each entry being on the coding strand and the second one corresponding to the transcribed strand. The bases encoding the mutation are in capital letters with the following code: M, C or A; K, T or G; R, A or G; Y, C or T.

(Molecular Simulations Inc., Burlington, MA). The X-ray crystal structure of *C. acidurici* Fd (Duée et al., 1994) served as a template to generate *C. pasteurianum* Fd by substituting the relevant amino acids. The similarities between the structures of these two proteins have recently been confirmed (Bertini et al., 1995). No attempts were made to minimize the resulting structure, and the site-directed derivatives were similarly modeled. Since all substitutions discussed herein involve surface residues, they are not expected to introduce major changes in the folding of the protein, as verified by NMR for some of the molecules presented (Davasse et al., 1995; unpublished results). For dipole calculations, charges of +1 and −0.5 were assigned to and locked on the amine nitrogens and carboxylic oxygens, respectively, of each molecule, and a total charge of −10 (eight carboxylates including the C-terminus, one lysine, the N-terminus, and two oxidized clusters of −2 charge) was assumed for *C. pasteurianum* Fd. The $[4Fe\text{-}4S]^{2+}$ clusters were given average charges of 0.43, −0.42, and −0.54 on irons, inorganic sulfurs, and cysteine sulfurs, respectively, in agreement with recently calculated values (Mouesca et al., 1994). Although relatively crude, such calculations are expected to approximate the changes introduced by site-directed mutagenesis experiments on *C. pasteurianum* Fd and are sufficient for the purposes of the present work.

RESULTS AND DISCUSSION

Electron Acceptors for Pyruvate-Ferredoxin Oxidoreductase. With a calculated formal potential of −520 mV at pH 7 [calculated from data in Thauer et al. (1977)], the conversion of pyruvate to acetyl-CoA by PFO can potentially provide electrons to a variety of redox agents. The methyl and benzyl viologen dyes were reduced by *C. pasteurianum* PFO. Benzyl viologen had an apparent K_m of 0.1 mM and a maximal velocity of 2.8 μmol of pyruvate oxidized (min·mg of PFO)$^{-1}$. The rates measured with NAD^+, FMN, spinach Fd, or *C. pasteurianum* [2Fe-2S] Fd were more than 100 times smaller than the rate measured with *C. pasteurianum* 2[4Fe-4S] Fd, but catalytic amounts of the latter protein strongly stimulated the reduction of the two [2Fe-2S] ferredoxins. $NADP^+$ was not reduced by *C. pasteurianum* PFO.

The reduction rate of 2[4Fe-4S] *C. pasteurianum* Fd by *C. pasteurianum* PFO with saturating amounts of pyruvate and CoASH was proportional to the concentration of Fd below ca. 5 μM. At higher concentrations of acceptor, the activity was inhibited by increasing amounts of Fd (Figure 2A). The inhibition of pyruvate−CO_2 exchange by electron acceptors of PFO has already been observed (Raeburn & Rabinowitz, 1971) and is in line with the currently accepted mechanism (Uyeda & Rabinowitz, 1971b) of this reversible reaction, in which the electron acceptor can compete with the exchange intermediate thought to be hydroxyethyl-TPP (Uyeda & Rabinowitz, 1971a). The data obtained here were analyzed using

$$v = \frac{V_{max}}{(1 + K_m/S + S/K_i)} \quad (1)$$

where, under equilibrium conditions, K_m is the apparent Michaelis constant, S is the Fd concentration, and K_i is the dissociation constant of Fd from the PFO−Fd complex. With

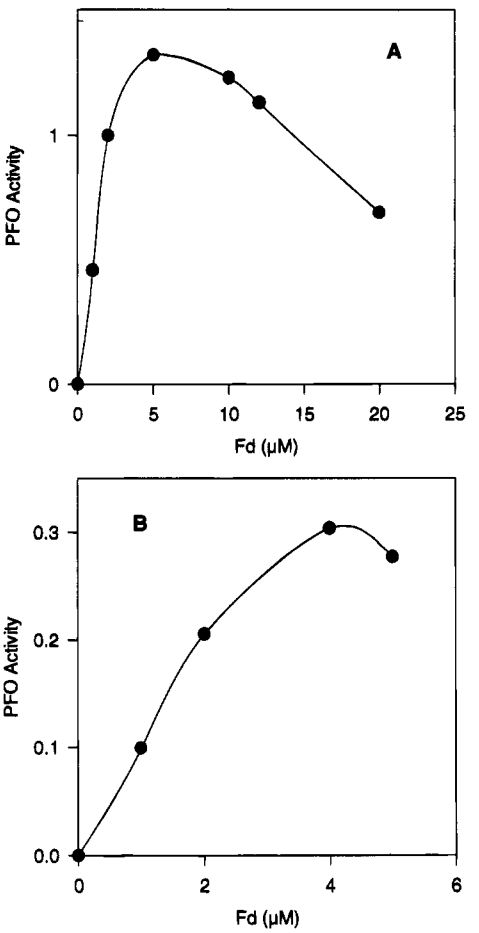

FIGURE 2: Dependence of *C. pasteurianum* PFO activity on the concentration of *C. pasteurianum* 2[4Fe-4S] ferredoxin: (A) activity measured as the decrease in absorbance at 425 nm corresponding to the reduction of ferredoxin; (b) activity measured as the production of H_2 catalyzed by the reporter enzyme, hydrogenase. Panels A and B represent two different experiments carried out with different enzyme preparations. Activities are given in micromoles of substrate (min·mg of enzyme)$^{-1}$.

such a scheme, it should be possible to obtain all kinetic constants by appropriate treatment of the data. However, in the present case, the apparent substrate (Fd) inhibition occurred at very low concentrations (Figure 2A), and no reliable constants could be determined under conditions where substrate inhibition could be neglected. Therefore, only the initial slope of the plot in Figure 2A can be estimated with confidence, which gives the V_{max}/K_m ratio. It is also relatively easy to determine the highest velocity occurring at the optimum substrate concentration from the same plot. With most preparations of PFO, values of 5 μmol of pyruvate (min·mg of PFO·μM Fd)$^{-1}$ were determined for V_{max}/K_m.

Electron Coupling between PFO and Hydrogenase. In saccharolytic clostridia, decarboxylation of pyruvate is the source of reducing power for a variety of reactions, among which the reduction of protons to hydrogen is quantitatively the most significant (Mortenson & Chen, 1974). To investigate whether the coupling of these two reactions was dependent upon the nature of the electron shuttle involved, a coupled enzymatic assay has been designed. *C. pasteurianum* hydrogenase is a far more efficient enzyme than *C. pasteurianum* PFO, since maximal activities of ca. 3000 (Mortenson & Chen, 1976) and ca. 5 μmol (min·mg of enzyme)$^{-1}$ (not shown), respectively, can be achieved with methyl viologen as the electron donor/acceptor. In an assay

Table 2: Relative Efficiency of Low-Potential Redox Proteins in Coupling *C. pasteurianum* PFO and Hydrogenase[a]

electron mediator	V_{max}/K_m (%)	S_{opt} (μM)	$E_{1/2}$ (mV)
C. pasteurianum Fd	100	4.5	−420
C. acidurici Fd	95	4.5	−430
C. thermosaccharolyticum Fd	120	4.5	−410
Synechocystis sp PCC 6803 Fd	18	20	−412
spinach Fd	5	40	−420
methyl viologen	1	nd	−440
benzyl viologen	2	nd	−350
C. pasteurianum [2Fe-2S]Fd	0		−270
Ch. vinosum Fd	0		−480

[a] Values of V_{max}/K_m are given as the percentage of the value for *C. pasteurianum* 2[4Fe-4S] ferredoxin. Assays were carried out as indicated under Materials and Methods. nd, not detected up to 1 mM. S_{opt} is the mediator concentration for which maximal activity is observed, and $E_{1/2}$ is the value (versus the normal hydrogen electrode) of the reduction potential.

where hydrogenase is added in excess over PFO (Quinkal et al., 1994), it is expected that hydrogenase acts as a reporter enzyme, affording a measurement of PFO activity through the quantitation of the hydrogen produced. This assay is more sensitive than the spectrophotometric quantitation of the reduction of the electron acceptor. The activities obtained with *C. pasteurianum* 2[4Fe-4S] Fd increased up to ca. 5 μM (Figure 2B) and decreased at higher concentrations. As expected from the design of the coupled reaction, these features are identical to those observed when the reduction of Fd is directly measured in assays without hydrogenase (Figure 2A).

Several electron transfer proteins were tested in this assay (Table 2). The ferredoxins isolated from *C. acidurici* and *C. thermosaccharolyticum* displayed the same behavior, with V_{max}/K_m ratios and S_{opt} values identical within 20% to those obtained with *C. pasteurianum* 2[4Fe-4S] Fd. In contrast, other low-potential electron carriers were far less efficient than these clostridial 2[4Fe-4S] ferredoxins. *Ch. vinosum* 2[4Fe-4S] Fd and *C. pasteurianum* [2Fe-2S] Fd did not support electron transport between *C. pasteurianum* PFO and hydrogenase. Other [2Fe-2S] ferredoxins functioning mainly in electron transport between photosystem I and Fd $NADP^+$ reductase of cyanobacteria and plants showed some activity in these assays, but with a markedly decreased ability to couple pyruvate oxidation and proton reduction (Table 2).

A general trend displayed by the proteins able to transfer electrons between PFO and hydrogenase is that the higher the V_{max}/K_m ratio, the smaller the concentration of electron shuttle at which inhibition occurs (Table 2). It is not surprising then to notice that no inhibition by excess of substrate has been observed with methyl or benzyl viologens up to concentrations of 1 mM.

The coupled assay between PFO and hydrogenase as developed here provides a more realistic view of the interactions of electron transfer agents with their redox partners than the usual assessment of their ability to receive or donate electrons to only one component of the chain. For instance, PFO was earlier shown (see above) to reduce *C. pasteurianum* [2Fe-2S] Fd with a rate at least 100 times lower than that measured for *C. pasteurianum* 2[4Fe-4S] Fd. The probably nonspecific rate with the [2Fe-2S] Fd from *C. pasteurianum* completely vanishes when the protein is analyzed in the coupled assay (Table 2).

A few reports have considered the activity of electron carriers in comparable coupled assays. A mixture of Fd has been qualitatively shown to transfer electrons between PFO and nitrogenase from Rhodospirillum rubrum (Brostedt & Nordlund, 1991). More informative is the observation that 6 μM *C. pasteurianum* 2[4Fe-4S] Fd supports the reduction of ca. 4 times more molecular nitrogen into ammonia with pyruvate as the electron donor than the same concentration of flavodoxin isolated from the same organism (Knight & Hardy, 1966). These data correlate with flavodoxin being 30% and 70% as efficient as Fd as an electron donor to nitrogenase and hydrogenase, respectively (Knight & Hardy, 1966). A variety of electron transfer agents have also been analyzed in several coupled reactions, including one involving *C. pasteurianum* PFO and hydrogenase, but in this case crude extracts rather than purified proteins were the source of enzyme activities (Fitzgerald et al., 1980). Although apparently only one concentration of electron carrier was tested, *C. pasteurianum* 2[4Fe-4S] Fd proved to be more active than many other Fd and flavodoxins, suggesting that the affinity of the mediator for the enzymes mainly determines its activity in such reconstituted systems (Fitzgerald et al., 1980). However, no further quantitative evaluation has been provided in any of these studies.

The data of Table 2 indicate that the electron carrier must fulfill a number of criteria to efficiently couple PFO and hydrogenase. First, H_2 production has been observed only for electron transfer agents having a reduction potential between ca. −350 and −450 mV. No reaction has been detected with *C. pasteurianum* [2Fe-2S] Fd and *Ch. vinosum* 2[4Fe-4S] Fd, whose potentials fall outside this range (Table 2). While such a result was expected for *C. pasteurianum* [2Fe-2S] Fd, which has a potential ca. 150 mV less negative than the H_2/H^+ couple, it is more surprising for *Ch. vinosum* 2[4Fe-4S] Fd. Indeed, the latter protein contains two active sites that are structurally very similar to those of *C. pasteurianum* 2[4Fe-4S] Fd (Huber et al., 1995; unpublished experiments), and its redox potential (Smith & Feinberg, 1990) would, in principle, allow its reduction by PFO, although with a small driving force. On the basis of thermodynamic considerations only, *Ch. vinosum* Fd should efficiently reduce hydrogenase. However, it is worth noting that, despite its similarities with other 2[4Fe-4S] Fd the low-potential Fd from *Ch. vinosum* displays a slow electron transfer rate between its two clusters (Huber et al., 1995).

The value of the reduction potential of the mediator is not the only factor influencing the reaction, as further witnessed by the comparison between *C. pasteurianum* 2[4Fe-4S] Fd, *Synechocystis* PCC6803 Fd, and spinach [2Fe-2S] Fd, which all display almost equal redox potentials (Table 2). The physiological electron carrier is ca. 5 times more efficient than the cyanobacterial Fd, as judged by the V_{max}/K_m ratio and the optimal electron shuttle concentration (Table 2). *Synechocystis* Fd itself is between about 2 and 4 times more efficient than spinach Fd, following the same criteria. These results, in addition to those previously reported (Fitzgerald et al., 1980), show that 2[4Fe-4S] Fd of the clostridial type, i.e., short polypeptides of ca. 55 amino acids, are the most efficient mediators among low-potential electron transfer proteins between *C. pasteurianum* PFO and hydrogenase.

Thus, it may be that some features in the tertiary structure of 2[4Fe-4S] Fd allow them to efficiently and specifically interact with the electron exchanging sites of PFO and hydrogenase. The ability to reconstitute *in vitro* this

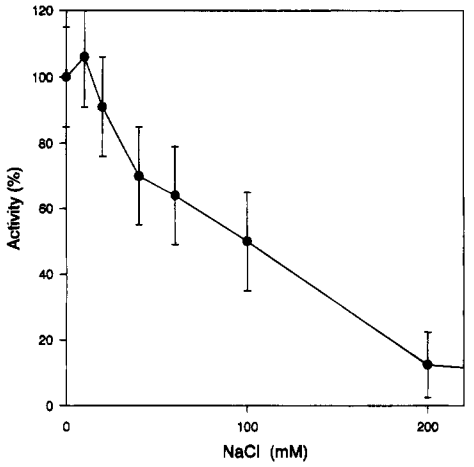

FIGURE 3: Effect of NaCl on the activity of *C. pasteurianum* PFO with ferredoxin as the electron acceptor. PFO activity was measured in the coupled enzymatic assay using hydrogenase as a reporter enzyme (Materials and Methods) with 4 μM Fd as electron mediator.

important part of the electron transfer chain of many anaerobic organisms provides an opportunity to study the molecular basis for recognition and electron transfer between soluble redox partners, as exemplified in the following.

Influence of Ionic Strength on Hydrogenase and Pyruvate-Ferredoxin Oxidoreductase Activities. Electrostatic interactions are sensitive to the shielding of charges by added ions. In order to examine whether PFO and hydrogenase interact with 2[4Fe-4S] Fd with a contribution from such interactions, the variations in the activity of both enzymes have been measured as a function of NaCl concentration. The apparent K_m and k_{cat} for the reaction where Fd is the electron donor to hydrogenase were multiplied and divided, respectively, by a factor of about 3 when 0.1 M NaCl was added to the assay in 0.1 M Tris Cl (pH 7). In the case of PFO, NaCl up to ca. 20 mM had little effect on the activity, but increasing ionic strengths induced a significant decrease (Figure 3); at 0.5 M NaCl, the activity was annulled. Although hydrogenase was used as a reporter enzyme in this experiment, the decrease in the activity of the coupled enzymatic reaction cannot be fully ascribed to the gradual decrease in its activity. Indeed, a 0.1 M increase in ionic strength corresponds to a ca. 10-fold lower activity for hydrogenase (see above), which should remain undetected, at least up to 0.1 M NaCl, in the coupled assay in which hydrogenase activity is in large excess over PFO. Therefore, both hydrogenase and PFO activities for which 2[4Fe-4S] Fd serves as electron donor/acceptor were found to be sensitive to ionic strength. Moreover, these results are not restricted to the effects of Na^+ or Cl^- since variations in the potassium phosphate concentration used as a buffer had similar consequences. Such behavior of multimolecular complexes is very often associated with the involvement of electrostatic interactions at the interface (e.g., Meyer et al., 1993), and it justifies the search for the Fd residues implicated in these interactions.

Measurement of the Electron Mediator Ability of Recombinant Ferredoxin in Extracts of E. coli. The most sensitive assay for studying the interaction between *C. pasteurianum* PFO and Fd involves the use of hydrogenase in a coupled enzymatic reaction. To be able to conveniently test the many molecular forms of Fd prepared in the present work, a procedure avoiding the numerous and potentially deleterious

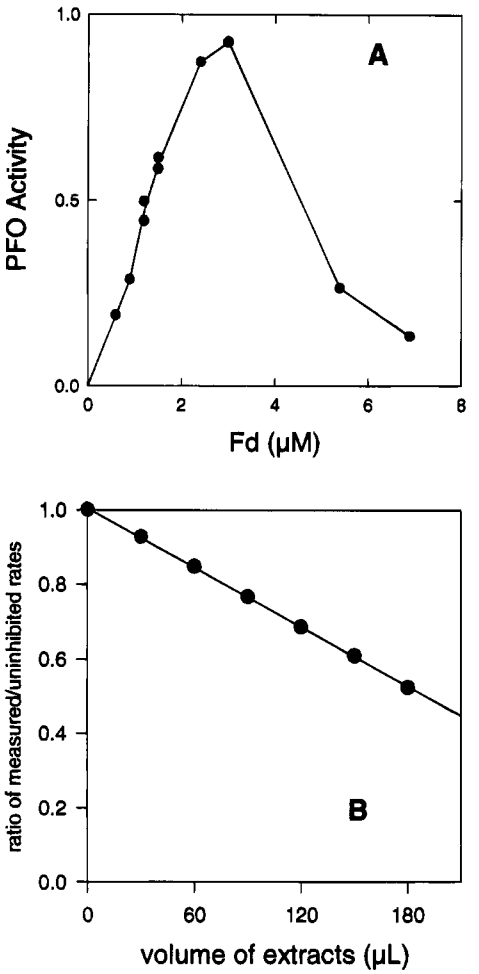

FIGURE 4: Recombinant 2[4Fe-4S] ferredoxin in soluble *E. coli* extracts as the electron mediator between *C. pasteurianum* PFO and hydrogenase. (A) Hydrogen evolution activity [in micromoles $(min \cdot mg)^{-1}$] as a function of the amount of Fd added. The Fd concentration in soluble extracts was determined by EPR (Materials and Methods). (B) Correction of the inhibition induced by the *E. coli* soluble extract. This relationship was determined at different concentrations of purified Fd by comparing rates obtained with and without Fd-deprived *E. coli* soluble extract added; these extracts were prepared as described under Materials and Methods.

steps required for protein purification has been sought. It was first found that the recombinant Fd concentration could be conveniently measured by EPR in soluble extracts of *E. coli* K38/pGP1-2 (Materials and Methods). These extracts have thus been tried as coupling agents between *C. pasteurianum* PFO and hydrogenase (Figure 4A), and they have been found to sustain PFO activity in much the same way as does purified Fd (Figure 2). The only difference is that the decrease in activity for Fd concentrations higher than optimum was sharper when *E. coli* extracts were used than with the purified protein. Also, the Fd concentration for which the highest activity is observed was shifted to slightly lower values than in the assay involving pure Fd (ca. 3.5–4 vs 4.5 μM).

These results prompted us to examine the effect Fd-deprived *E. coli* extracts have on the assay. The *E. coli* soluble extract acted as an inhibitor of the coupled enzymatic assay linking *C. pasteurianum* PFO to hydrogenase through Fd. However, the concentration of Fd is usually large enough to overcome the inhibitory effect of the extracts. The measured reaction rates can thus be corrected for the marginal inhibition, as shown in Figure 4B, and afford values

corresponding to what they would be if purified Fd was used. Therefore, even unstable variants of 2[4Fe-4S] Fd could be studied in this assay as electron acceptors for *C. pasteurianum* PFO.

Ferredoxin Variants with Substituted Acidic Residues as Electron Acceptors for PFO. A noteworthy feature of 2[4Fe-4S] ferredoxins isolated from clostridia and other anaerobic bacteria is the high conservation of many acidic residues, which constitute the vast majority of the charged amino acids in these sequences (Figure 1). The available data from X-ray crystallographic studies (Adman et al., 1973, 1976; Duée et al., 1994) show that all of these residues lie on the surface of the molecule. In view of their distribution and the sensitivity of the enzymatic reactions involving *C. pasteurianum* Fd toward salt effects, acidic residues are primary targets for mutagenesis experiments aiming to probe their role in the interaction with PFO and hydrogenase.

The six conserved carboxylates present at positions 6, 17, 27, 33, 35, and 39 (all aspartates, except glutamate 17) in the *C. pasteurianum* Fd sequence have individually been changed to neutral (A), polar (T, N), or positively charged (K) residues. All variants were produced in amounts similar to native recombinant Fd. The EPR spectra of all *C. pasteurianum* Fd variants reported herein were similar to that of wild-type *C. pasteurianum* Fd, except for minor differences assigned to slight changes in the magnetic interaction between the two clusters (not shown). Also, singly substituted Fd molecules displayed values of the measured reduction potential within 20 mV of the one from wild-type *C. pasteurianum* Fd.

The Fd variants have been examined in the coupled assay linking pyruvate oxidation to proton reduction. In view of the differences evidenced among several electron transfer proteins of similar reduction potentials in this assay (Table 2), it is anticipated that the interaction between PFO and Fd significantly contributes to the rate-limiting step of the reaction according to

$$PFO_{red} + Fd_{ox} \overset{K_s}{\leftrightarrow} (PFO_{red},Fd_{ox}) \xrightarrow{k_{et}} PFO_{ox} + Fd_{red} \quad (2)$$

Since it was shown earlier (see above) that the only reliable measurements in the reaction were the rates at low Fd concentrations, i.e. V_{max}/K_m, the following relationship has to be used as an estimate of the specificity of the electron mediator toward the enzyme:

$$V_{max}/K_M = (k_{et}/K_s)e$$

where e is the enzyme concentration. Therefore, any change in either the dissociation constant between PFO and Fd or the electron transfer rate constant should translate into different hydrogen evolution activities.

As can be seen in the upper part of Table 3, none of the variants for which one carboxylic group was substituted displayed a change in V_{max}/K_m larger than 40% of the ratio exhibited by wild-type recombinant Fd. The same conclusion was reached previously with a molecular form in which two carboxylic acids were added in positions 19 and 21 (Quinkal et al., 1994; Table 3). The only exception to this trend is D39E, which displayed a 60% increase in V_{max}/K_m, but the charge of the residue is not responsible for the better activity as D and E both bear an acidic group. It should instead be recalled that the carboxylic group of D39 is involved in a hydrogen bond with the N-terminus (Duée et al., 1994). The disruption of this interaction apparently has no consequence on the activity, as witnessed by the D39A variant. However, keeping this hydrogen bond with a longer side chain in D39E may induce strains on the nearby C40 and on the [4Fe-4S] center to which C40 is bound. An earlier report established that the D35E form of *Rhizobium meliloti* FdxN protein (Figure 1) can rescue the Fix$^-$ (nitrogen fixation) phenotype of an *fdxN*$^-$ mutant as efficiently as the native protein (Masepohl et al., 1992).

Table 3: Dipole and Activity of the Molecular Variants of *C. pasteurianum* Ferredoxin

ferredoxin	net charge	dipole moment[a] (D)	relative V_{max}/K_m (% variant/native)
native	−10	197	100 ± 10
D6A	−9	179	74 ± 20
E17A	−9	226	105 ± 10
E17D	−10	190	110 ± 10
D27A	−9	156	96 ± 20
D27E	−10	214	122 ± 20
D27K	−8	124	142 ± 20
D33A−D35N	−8	124	70 ± 20
D39A	−9	197	83 ± 10
D39T	−9	200	91 ± 10
D39E	−10	195	158 ± 10
Δ1	−8	nc	42 ± 20
E17A−D39A	−8	224	140 ± 20
D33A−D35N−D39A	−7	147	98 ± 10
E17A−D33A−D35N−D39A	−6	170	72 ± 20
P19D−N21D	−12	221	78 ± 20[b]

[a] Calculated as described under Materials and Methods. nc, not calculated. [b] Data from Quinkal et al. (1994).

The results obtained with molecular forms of 2[4Fe-4S] *C. pasteurianum* Fd in which a single negative charge (two in the case of D33A−D35N) has been neutralized show that the building of even one salt bridge between Fd and hydrogenase or PFO can be excluded. Indeed, in the many cases of reactive complexes stabilized by salt bridges, the removal of a single charged side chain involved in the interaction is enough to induce dramatic changes. Among recent examples dealing with electron transfer complexes, the replacement of D76 or D79 of human [2Fe-2S] Fd almost completely abolished cholesterol side chain cleavage by cytochrome $P450_{scc}$ (Coghlan & Vickery, 1991, 1992; Brandt & Vickery, 1993). Also, the reversal of the E94 charge by K in *Anabaena* [2Fe-2S] Fd decreased the second-order rate constant of Fd-$NADP^+$ reductase reduction by a factor of more than 2×10^4 (Hurley et al., 1993, 1994). Such a prominent role for the acidic residues of 2[4Fe-4S] Fd is clearly to be excluded.

The building of salt bridges or the local charge distribution is not the only force available for a charged protein to interact with a partner via electrostatic interactions. Indeed, two charged molecules may orient favorably relative to each other (i.e., steer) through Coulombic forces to form a competent encounter (Davis & McCammon, 1990; Northrup et al., 1988; 1993). To experimentally test the importance of electrostatically driven diffusional motions in the reactions of *C. pasteurianum* Fd with the PFO and hydrogenase of the same bacterium, additional variants in which several acidic residues were substituted at the same time with neutral amino acids have been examined (lower part of Table 3). These molecular forms were also produced in amounts similar to that of native recombinant Fd, except for the triply, D33A−D35N−D39A, and quadruply, E17A−D33A−D35N−D39A,

modified molecules, which yielded only 60% and 30%, respectively, of this amount. All variants exhibited kinetic properties very similar to those of native Fd and singly substituted variants, although the absolute value of the total dipole moment was varied by a factor of almost 2 (Table 3) and its direction spanned an angle of almost 170°.

The electrostatic interactions between two charged molecules are the sum of terms involving both monopolar and dipolar contributions. The exact expressions for these terms somewhat depend on the model used to represent the charged molecules and their interaction surfaces, but they are all expected to contribute to the reaction rate when the building of the active complex is driven by electrostatic interactions (e.g., Van Leeuwen, 1983; Watkins et al., 1994). Numerous relevant examples for electron transfer complexes have been evidenced, either by studying the rate dependence as a function of the ionic strength (e.g., Tollin & Hazzard, 1991; Zhou & Kostic, 1993; Davidson & Jones, 1995) or by using recombinant (Guillemette et al., 1994) or naturally homologous proteins (Tiede et al., 1993) with different physicochemical properties, including electrostatic potential surfaces. In the present case, large variations in the total dipole, as well as in the electrostatic potential at various loci on the surface of the 2[4Fe-4S] Fd (Table 3), failed to reveal an electrostatically driven, diffusion-controlled, rate-limiting process in the interaction between Fd and at least PFO.

Another variant in which the ^{33}DA^{35}D tripeptide was replaced by the neutral AV dipeptide (Δ1) displayed a ca. 2.5-fold decrease in activity compared to native Fd (Table 3). Although not very large, this change is, together with the results obtained with D39E (see above), the largest observed in the present studies. As in this former case, a structural perturbation is expected from the substitution of the ^{33}DAD^{36}T turn by a shorter peptide. Indeed, the crystallographic model of *C. acidurici* Fd reveals that this part of the molecule is highly structured, with mean *B* values lower than average especially for the main chain, through the building of a network of hydrogen bonds (Duée et al., 1994). It thus may be expected that shortening of the sequence in this region disturbs the local folding, including the C37 ligand and the nearby cluster. The high sensitivity of 2[4Fe-4S] ferredoxins to relatively minor structural changes is borne out further by our attempts to replace the ^{33}DAD^{36}T sequence by a single amino acid (A or V), a three-residue deletion; genes bearing such changes failed to give proteins stable enough to be produced in *E. coli*.

Taken together, the results of Table 3 indicate that many kinds of substitutions involving all carboxylates of *C. pasteurianum* Fd do not abolish nor strongly modify the activity of the protein as an electron acceptor from PFO. Due to the characteristics of the enzymatic assay used, a similar conclusion may be drawn for the reaction with hydrogenase, but in order to detect more subtle changes in the latter interaction, a few variants have been studied further as electron donors to hydrogenase.

Ferredoxin Variants with Substituted Acidic Residues as Electron Donors to Hydrogenase. The assay of Fd as an electron donor to hydrogenase is not biased by the substrate inhibition evidenced with PFO. Three different variants on the ^{33}D and ^{35}D charged residues have been studied, namely, D33A−D35N, D33A−D35K, and D35K. They all displayed apparent K_m and V_{max} kinetic constants in this reaction within 20% of those of native Fd. In contrast, Δ1 (see above) showed a Michaelis constant larger than that of native Fd by a factor of about 2. These observations agree with those made with the same molecular forms in the reaction with PFO (Table 3). Cancellation or reversal of the charges on ^{33}D and ^{35}D has little effect on the interaction between Fd and both of its redox partners, while Δ1 exhibits small but clear differences in both cases.

CONCLUSIONS

Despite the wide occurrence of the electron transfer chain linking pyruvate oxidation to proton reduction in anaerobic organisms, the details of the interactions among its components have received little attention so far. The 2[4Fe-4S] Fd is a ubiquitous central protein in such systems, and it is the most specific electron transfer agent found among various other redox proteins (Table 2). In all instances, 2[4Fe-4S] Fd are highly charged molecules with many conserved carboxylic residues (Figure 1).

It thus may come as a surprise that the removal and even reversal of these charges have very little influence on the steady state kinetics of the interaction of the protein with its two major redox partners. It should be noted, however, that crystal structures of redox bimolecular complexes often reveal a largely hydrophobic interacting surface (Pelletier & Kraut, 1992; Chen et al., 1992) with little evidence for the involvement of extensive electrostatic forces. This conclusion is not restricted to protein−protein recognition between electron transfer molecules (Young et al., 1994). Obviously, crystallizable, long-lived redox complexes may not exactly represent the active encounter(s) between the proteins involved, but these structures show that short-range electrostatic interactions (i.e., salt bridges) are not mandatory components of redox complexes. Indeed, trapping of stable intermediates between redox partners through salt bridges may decrease the reaction rate if the release of reactants after electron transfer becomes limiting.

A consequence of the present findings is that the salt effect evidenced earlier (see above) for *C. pasteurianum* PFO and hydrogenase (Figure 3) does not seem to directly involve the interaction with Fd. In the case of PFO, the dimeric active form of the enzyme may be stabilized by electrostatic interactions, which can be disrupted by high ionic strengths. However, this explanation cannot hold for hydrogenase, and the molecular basis for the salt effects of opposite signs observed with different electron donors for *Megasphera elsdenii* hydrogenase (Van Dijk et al., 1979) will require further work.

The experiments reported herein shed light on the way *C. pasteurianum* 2[4Fe-4S] Fd may interact with its redox partners. The fact that charges on the surface of Fd have no important role in the rate-limiting processes of the reactions in which the protein is involved may indicate that these reactions are not diffusion controlled. Rather it is once the bimolecular complex is formed that electron transfer depends on the exact fitting of the two molecules. In this respect, the surface residues of Fd studied here have no major roles, but any change suspected to modify the immediate environment of the clusters (in the D39E and Δ1 variants) is more likely to perturb the reactions, as observed here and before (Gaillard et al., 1993; Quinkal et al., 1994). These observations lead the path to further experiments aimed at

mapping the structural requirements for the efficient reaction of this kind of protein with its partners.

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