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The pH dependent spectral properties of *Clostridium pasteurianum* 2[Fe_4S_4] ferredoxin

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Abstract

The recently assigned ^1H NMR hyperfine signals of *Clostridium pasteurianum* ferredoxin were investigated over the pH range 8–12 to monitor possible pH-dependent conformational changes of the protein. For very high pH values minor perturbations were detected in the chemical shifts of three signals assigned to β-CH_2 cysteine protons of cluster II, while cluster I was not affected at all. These chemical shift variations, which can be fitted to a single $pK_a \approx 10.9$, are interpreted as an effect of deprotonation of the phenolic group of Tyr-2, located reasonably close to cluster II. This hypothesis has been supported by means of other techniques such as CD and absorption spectroscopy that, on turn, are able to reveal minor pH-dependent spectral variations at high pH. Finally a UV difference experiment has provided further evidence for deprotonation of the phenolic group of Tyr-2. The possible influence of deprotonation of Tyr-2 on the redox properties of cluster II is discussed.

Key words: Ferredoxin; NMR; *Clostridium pasteurianum*; Iron sulphur protein; Circular dichroism

1. Introduction

Clostridium pasteurianum ferredoxin (CpFd hereafter) is a small iron sulfur protein (55 amino acids, MW 6,000) containing two Fe_4S_4 clusters [1–3]. The protein has been the subject of several spectroscopic investigations aimed at the determination of its solution structure [4,5] and the identification of the factors modulating the redox potential of the two clusters [6–9]. Interestingly, the two weakly paramagnetic Fe_4S_4 clusters produce a characteristic ^1H NMR spectrum with eight well-resolved hyperfine signals in the downfield region [10]. Very recently, thanks to the application of paramagnetic 2D NMR techniques, the eight hyperfine signals have been assigned specifically to the β-CH_2 protons of the cysteines coordinated to either cluster [5,6,11–13]. These assignments give us the chance to monitor in detail the perturbations that the protein experiences under different solution conditions. We report here an analysis of the effects of pH on the solution structure of the protein around the two Fe_4S_4 clusters; this is, in our opinion, an important issue in the chemistry of ferredoxins, strictly related to the extensive debate on the effect of pH on the redox potentials [14,15]. Only recently, after much controversy, two independent studies have definitely established that the reduction potential of CpFd is pH independent over the pH range 6–9 [16,17]. However, an elegant study of protein engineering by Feinberg et al. has demonstrated that the reduction potential of CpFd may be rendered pH dependent with a pK_a of about 7 by replacing Tyr-2 with a histidine [18]. It is inferred that the presence of an ionizable group, such as His, near cluster II modulates its reduction potential. A similar reasoning might hold for those groups, ionizable at high pH, that are located around the clusters in the native protein. With this in mind we have decided to explore the pH-dependent properties of CpFd in the alkaline region.

2. Materials and methods

Clostridium pasteurianum was grown and ferredoxin isolated and purified according to the method of Rabinowitz [19]. The purity of the sample was checked by absorption spectroscopy monitoring the A_{390}/A_{280} absorbance ratio [19]. For ^1H NMR experiments the protein was dissolved in 50 mM P_i deuterated buffer. The pH was then adjusted to the desired value by addition of aliquots of either HCl or NaOH. The pH values are reported as uncorrected pH meter readings.

The ^1H NMR spectra were recorded on an AMX 600 Bruker spectrometer. The chemical shift values are referred to DSS.

The CD spectra in the visible region were performed on a Jasco J500C spectropolarimeter operating at room temperature; the UV absorption spectra on a Cary 3 instrument operating at room temperature. The UV difference spectra on a Cary 17D instrument according to the reported procedure [20].

Protein concentration was about 1 mM for the ^1H NMR experiments; 3×10^{-4} M for the CD spectra and 1×10^{-5} M for the absorption and difference UV spectra.

Computer graphics analysis of the protein structure was performed on an Evans Sutherland EP 300 workstation using the SYBYL software package.

The pK_a values were determined using a standard non- linear, least squares fitting program.

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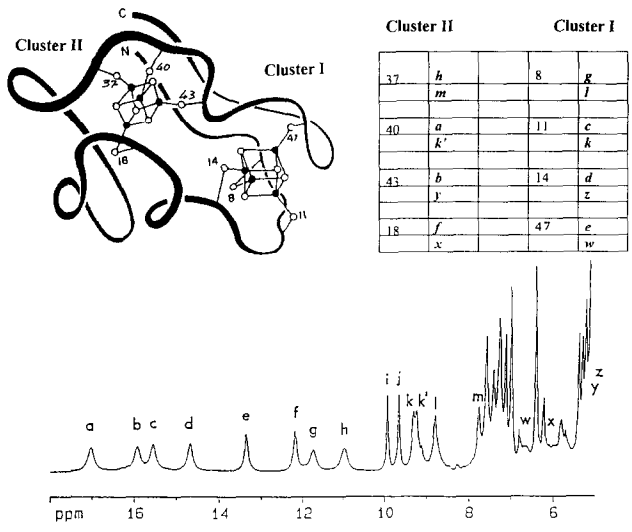

Fig. 1. ^{1}H NMR spectrum of oxidized CpFd. The hyperfine shifted resonances are labeled as previously reported [6]. A schematic drawing of the protein and the two Fe_4S_4 clusters is shown. The sequence-specific assignments of the hyperfine signals to Cys β-CH_2 protons are also reported according to [12]. Conditions are the following: CpFd 1 mM, 50 mM phosphate buffer, pH 8.0, 300 K.

3. Results

3.1. ^{1}H NMR results

The ^{1}H NMR spectrum of oxidized CpFd at pH 8 is shown in Fig. 1. The spectrum is characterized by several well-resolved isotropically shifted signals lying downfield in the 20–10 ppm range. Recent 2D NMR studies permitted the identification of the geminal connectivities of these signals and their sequence-specific assignment to the β-CH_2 protons of the cluster-coordinated cysteines [6,12]. More precisely, signal pairs **g-l, c-k, d-z**, and **e-w** were assigned to the eight β-CH_2 protons of the four cysteines bound to cluster I – Cys-8, -11, -14 and -47, respectively – whereas signal pairs **h-m, a-k′**, **b-y**, and **f-x** were assigned to the cysteines bound to Cluster II, Cys-37, -40, -43 and -18, respectively. The sequence specific assignments of the Cys β-CH_2 signals of the two clusters according to [12] are shown in Fig. 1.

A series of ^{1}H NMR spectra recorded on oxidized CpFd at increasing pH values are shown in Fig. 2. It can be noticed that the spectra do not show meaningful changes over the pH interval 8–9.5. However, for very high pH values, three of the β-CH_2 signals belonging to cluster II, namely signals **a, b** and **h** exhibit small but significant changes of their chemical shifts (the total variations are of the order of 0.5 ppm) whereas the remaining hyperfine signals are not perturbed. The chemical shift values of the hyperfine signals versus pH in the alkaline region are reported in Fig. 3; the chemical shift variations of signals **a, b** and **h** can be fitted to a single pK_a value around 10.9. The latter value, however, has to be used with some caution since the occurence of protein denaturation for pH ≈ 12 prevents analyzing the whole pH-dependency profile. The observed spectral variations are reversible; indeed, if a CpFd sample, previously brought to pH 11.2, is acidified down to pH 8, the typical spectrum of Fig. 1 is obtained again.

3.2. CD results

The above ^{1}H NMR changes may be interpreted as a consequence of deprotonation at high pH of an acid group located in close proximity of cluster II; we thought

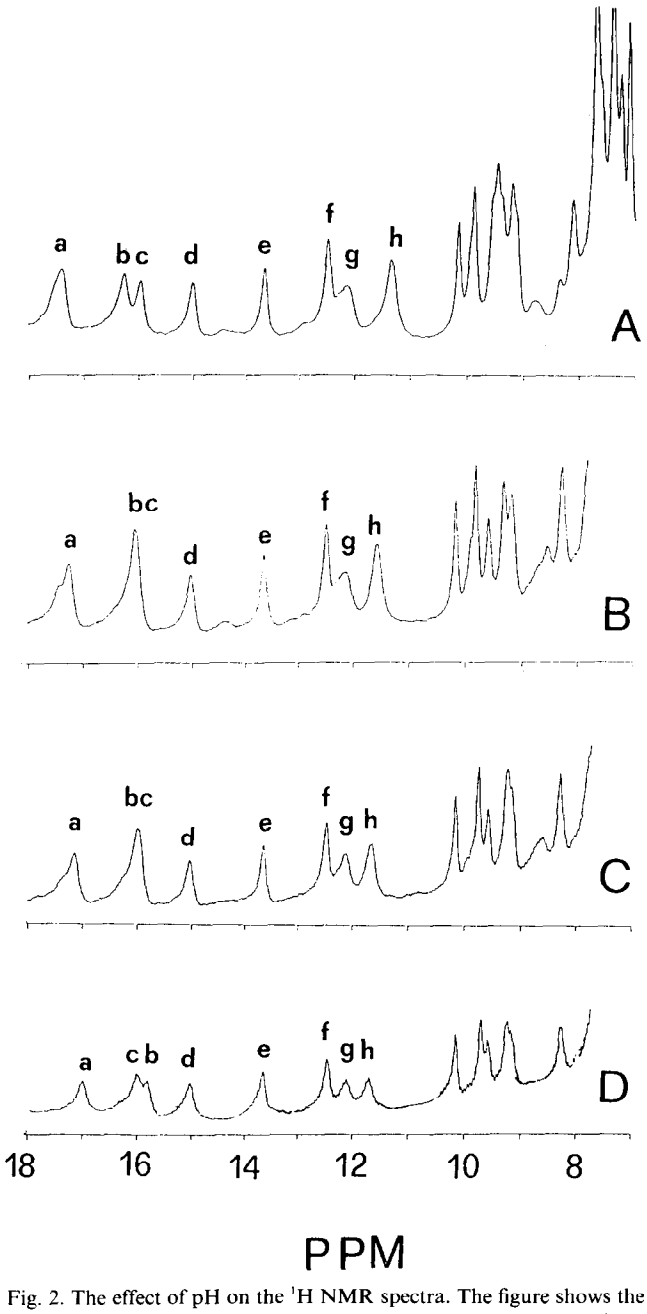

Fig. 2. The effect of pH on the ^{1}H NMR spectra. The figure shows the 600 MHz ^{1}H NMR spectra of *Clostridium pasteurianum* ferredoxin at the following pH values: (A) 9.3; (B) 10.8; (C) 11.4; (D) 11.8. CpFd 1 mM, phosphate buffer 50 mM, 300 K.

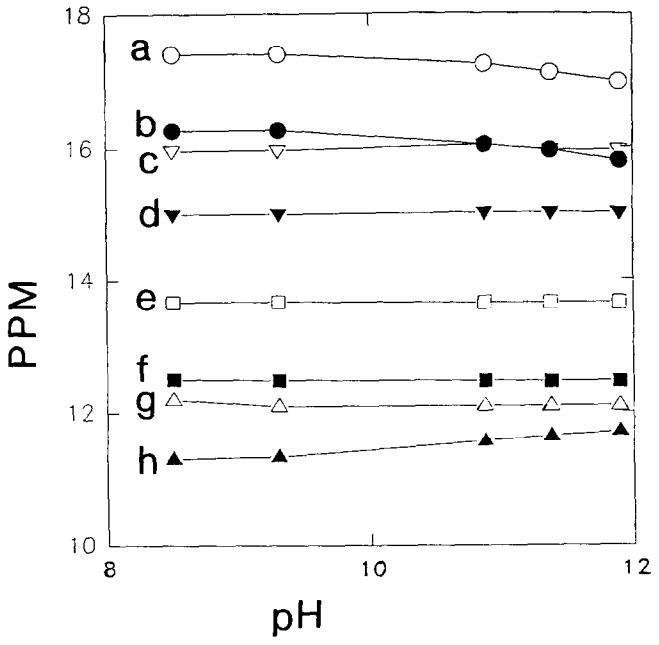

Fig. 3. pH dependence of the hyperfine signals. The chemical shifts of the downfield shifted ^{1}H NMR hyperfine signals are reported vs. pH over the pH range 8–12.

that the most probable candidate for this deprotonation might be the phenolic group of Tyr-2, which is within 6 Å from cluster II and is usually expected to exhibit a pK_a value around 10.9 [21]. Deprotonation of such a bulky group as tyrosine is believed to bring about significant conformational variations of the protein around the cluster; we therefore decided to monitor the high pH transition of CpFd by CD spectroscopy, a technique particularly sensitive to conformational rearrangements at the active site of metalloproteins. As reported, the visible CD spectrum of oxidized CpFd is characterized by two intense positive bands, respectively, located at 410 and 565 nm, plus a broad negative band around 700 nm [22,23]. The spectrum is pH independent from pH 7 to 9 (see also [14]). Upon further raising the pH we detected significant changes in the relative intensity of the two main bands at 410 and 565 nm: the former increases with increasing pH whereas the latter decreases. For pH values around 12 extensive denaturation of the sample starts occuring and both bands drastically decrease in intensity; at pH 13 the CD spectrum is almost totally abolished. The spectral changes of the band at 565 nm as a function of pH, together with the best fitting analysis, are shown in Fig. 4. A pK_a of about 10.7 has been estimated which matches reasonably well with the pK_a value obtained from the NMR spectra.

3.3. *Absorption spectroscopy results*

To support the hypothesis of Tyr-2 deprotonation we recorded the absorption spectra in the UV-visible region of a diluted ferredoxin sample (1×10^{-5} M) at increasing pH values. The UV-visible absorption spectrum of CpFd is predominantly characterized by an intense LMCT transition at 390 nm from the iron sulfur cluster, plus a bigger, composite band around 280 nm originating from the aromatic residues [19]. We noted that upon increasing the pH from 8 to 11.4 the LMCT band at 390 nm is virtually unaffected; conversely the bands in the near UV region, assigned to the aromatic residues, undergo significant perturbations upon passing from pH 9.3 to pH 11.4 (see Fig. 5). Interestingly, the UV difference spectrum obtained by subtracting the spectrum of CpFd at pH 9.3 from that at pH 11.4 (see Fig. 5, inset) consists of two bands, respectively located at 290 and 235 nm, that are an index of perturbation of the π to π^* absorption bands of the phenolic ring following deprotonation. Similar UV difference spectra are usually observed when metal ions bind apotransferrin, a process that is accompanied by the deprotonation of two tyrosines in each metal binding site of the protein [20].

4. Discussion

The application of ^{1}H NMR spectroscopy of paramagnetic systems to 2-Fe_4S_4 ferredoxins has proved extremely useful to obtain detailed information on the solution structure and the reactivity of these small and interesting proteins. In particular the sequence-specific assignments of the hyperfine signals permit the observed variations in shape and position of these signals to be correlated with specific perturbations of well-defined portions of the protein [6,12]. For instance, we have

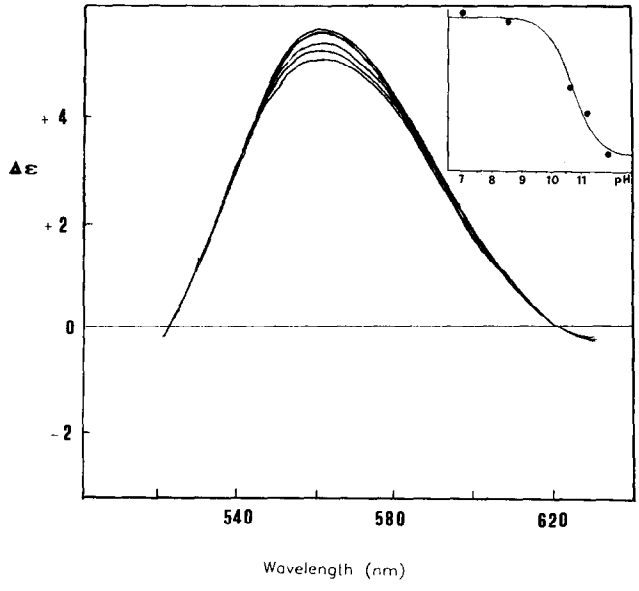

Fig. 4. pH dependence of the 565 nm CD transition. The changes of the CD band at 565 nm as a function of pH are shown over the pH range 7–11.9. The pH values of each spectrum are the following (from the top): 7.0, 8.5, 10.6, 11.2, 11.9. The inset shows the best-fitting analysis of the variations in intensity of the 565 nm band vs. pH. Conditions: CpFd 3×10^{-4} M, phosphate buffer 50 mM.

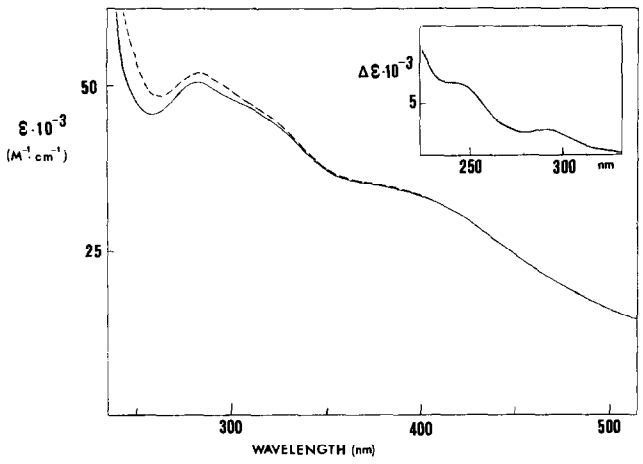

Fig. 5. Electronic absorption spectra of CpFd in the UV region. The figure shows the UV-visible absorption spectra of CpFd at the following pH values: 9.3 (dashed line); 11.4 (solid line). The inset shows the UV difference spectrum obtained upon subtracting the spectrum at pH 9.3 from the spectrum of the same sample at pH 11.4. All spectra were recorded at room temperature on a 1 × 10–5 M CpFd sample in a 50 mM phosphate buffer.

investigated the effects of addition of ferricyanide to oxidized ferredoxin and unambiguously shown that cluster I is converted into a Fe_3S_4 cluster while the hyperfine signals of cluster II are virtually unaffected [23]. In addition, the paramagnetic ^{1}H NMR spectra of two site-directed mutants of CpFd, respectively at Pro-19 (P19K) and Pro-48 (P48K), have been reported by Gaillard et al. [9]; in the light of the now available sequence-specific assignments the observed ^{1}H NMR spectral perturbations may be re-interpreted and reasonably accounted for on the basis of local and symmetrical rearrangements of the two clusters.

Here, we have extended the paramagnetic NMR approach to the investigation of the pH-dependent properties of CpFd and definitely shown that the hyperfine signals are pH insensitive over the pH interval 8–9.5. Only for pH values higher than 10 are small but significant variations observed in the position of three signals belonging to cluster II, variations that correspond reasonably well to a single pK_a of ≈10.9. Analysis of the primary sequence of CpFd permits it to be establish that the protein does not possess any acid-base group with pK_a values in the range 7–9. There are, however, some groups for which deprotonation at higher pH values is expected; these groups are the N-terminal amino group of Ala-1 (expected pK_a around 9.9), the phenolic group of Tyr-2 (expected pK_a around 10.9) and the ε-amino group of Lys-3 (expected pK_a around 9.2). Inspection of a computer graphic model of the protein structure, derived from the X-ray structure of *Peptococcus aerogenes* ferredoxin [4] after the appropriate amino acid replacements, permits to state that the ε-amino group of Lys-3 is far apart from both clusters and, in principle, unable to affect their spectral properties. In contrast both the N-terminal amino group of Ala-1 and the phenolic group of Tyr-2 are relatively close to cluster II so that they might influence its spectroscopic properties. Since the estimated pK_a for the observed ^{1}H NMR effects is around 10.9, a value very close to that expected for a phenolic group, we propose that the group responsible for the spectral changes at high pH is indeed Tyr-2. Further observations support our hypothesis. From inspection of the computer graphic model, it appears that Tyr-2 is within 6 Å from three Cys β-CH_2 groups of cluster II (namely Cys-37, -40 and -43) but very far away (more than 9 Å) from the β-CH_2 group of Cys-18, the fourth cysteine of cluster II. In good agreement with this observation we noticed that the hyperfine signals **a**, **b** and **h**, experiencing the largest pH-dependent shift variations, belong indeed to Cys-40, -43 and -37 whereas signal **f**, assigned to Cys 18, the one far away from Tyr-2, does not change its chemical shift with increasing pH. The additional CD data reported in this work, revealing minor but significant spectral changes of the bands of the iron clusters in the high pH region, lend further support to our hypothesis. Finally the reported UV difference experiment, revealing the difference bands typical of phenolate, provides, in our opinion, conclusive evidence for tyrosine deprotonation. Given the relative proximity of Tyr-2 to cluster II, we may expect that variations in charge distribution around cluster II, following Tyr deprotonation at high pH, do affect its reduction potential.

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