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Kinetics of the decomposition of [2Fe–2S] ferredoxin from spinach: implications for iron bioavailability and nutritional status

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Abstract

The decomposition of spinach ferredoxin has been studied, at 37°C, in the presence of: buffer (pH 7.5); water; 0.01 M hydrochloric acid; and 0.01 M hydrochloric acid with pepsin enzyme (0.09, 0.19 and 0.38%). The hydrolysis of the protein appears to occur slowly in the presence of water alone, but is greatly accelerated by the addition of acid. This hydrolysis reaction is proposed to involve the denaturation of the protein chain to the extent that the [2Fe–2S] core is released from the protein. In the presence of 0.01 M HCl, total iron release occurs. Pepsin appears to then breakdown the peptide linkages within the apoprotein. UV–visible spectroscopy has demonstrated a two stage reaction with loss of the peak at 422 nm correlating with breakdown and loss of the [2Fe–2S] cluster, and loss of the peak at 277 nm demonstrating breakdown of the protein chain. Uniphasic kinetics were observed at 422 nm with the observed pseudo-first order rate constant having a linear dependence on ferredoxin concentration.

Keywords: Spinach ferredoxin; Hydrolysis; Digestion; Stability of 2Fe–2S clusters

1. Introduction

The release of iron during digestion and, more importantly, its bioavailability, is a significant factor in the nutritional status of those following a vegetarian diet, due to the low bioavailability from plant sources. Various studies have been undertaken to demonstrate the levels of iron available from food sources (Leigh & Miller, 1983; Narasinga Rao & Prabhavathi, 1978) including spinach (Crispin & Varey, 2000; Miller, 1987; Zhang, Hendricks & Mahoney, 1989), oat bran (Rassander-Hulthén, Gleerup & Hallberg, 1990) and vegetables (Gillooly et al., 1983).

Iron, within plant sources, is found as non-haem iron. This consists of two pools: inorganic free iron and iron held within proteins. Such proteins include ferredoxins, phytoferritin and cytochromes. Ferredoxins are of particular interest because of their range of iron–sulphur cores and the fact that they are found in both vegetable and vertebrate cells. In plant cells their roles include being part of the electron transport chain of photosynthesis, nitrate reduction and other redox reactions. These iron–sulphur proteins can be broadly classified by the iron–sulphur core(s) present: those containing a single iron (Holm, Kennepohl & Solomon, 1996); those with a 2Fe–2S core (Muller, Muller, Rottmann, Bernhardt & Heinemann, 1999); a 3Fe–3S core (Hannan, Busch, James, Thomson, Moore & Davy, 2000; Macedo, Rodrigues & Goodfellow, 1999); or a 4Fe–4S core (Georgiadis, Komiya, Chakrabarti, Woo, Kornuc & Rees, 1992). Some contain more than one core with others containing mixed metal cores including that containing molybdenum in nitrogenase (Chan, Kim & Rees, 1992).

This study is focussed on (spinach) ferredoxin due to the high interest in iron bioavailability from spinach. No more than 50% of total iron appears to be released from spinach under digestion conditions (Miller, 1987), with unprocessed spinach appearing to release only ~20% under simulated digestion conditions (Crispin & Varey, 2000). This leads to the conclusion that perhaps the iron is primarily that measured from only extra- and/or intracellular fluids, with cellular breakdown and release of iron from the proteins being

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negligible. Some release of chlorophyll is observed during simulated digestion (Crispin & Varey), indicating that perhaps cellular structure is broken down, and therefore the iron-containing proteins are so stable that they do not release the iron during the time of digestion. 2Fe–2S clusters appear to be reasonably stable in isolation (Verhagen, Link & Hagen, 1995) and spinach ferredoxin containing such a cluster is utilised to study the role of enzymes (Hirasawa, Hurley, Salamon, Tollin & Knaff, 1996; Weber-Main et al., 1998) lending credence to the proposed hypothesis that they do not decompose during the digestive process. To reoxidise reduced spinach ferredoxin after experimental procedures, the samples have been reported to be left in air until fully reoxidised (Tagawa & Arnon, 1968).

Spinach ferredoxin has been known since 1968 when the structure was elucidated by Matsubara and Sasaki (1968). Further studies have indicated that there are slight differences between the ferredoxin extracted from the leaves (Bojko & Wieckowski, 1999) and roots (Hirasawa et al., 1996) of the plant. All the ferredoxins of spinach analysed to date are similar, with the same number of amino acids, but slight variations in the amino acids 81, 83 and 141 (ExPASy, 2000). The ferredoxin is isolated as the precursor with the ferredoxin molecule structure occurring in the sequence at positions 51–147 (1–50 being transit chloroplast). Within this precursor molecule the cys residues at positions 89, 94, 97 and 127 co-ordinate the 2Fe–2S core. No 3-D model of the structure is yet available although comparison with that of *Anabaena* has been made (Muller et al., 1999).

This study has been undertaken to determine the extent that ferredoxin proteins of spinach are affected by the simulated in vitro digestion conditions to determine the extent to which these proteins could be expected to release iron in a bioavailable form.

2. Materials and methods

2.1. Terminology

In all further comparisons of amino acid sequence, reference will be made to that obtained for *Anabaena* (ExPASy: FER1_ANASP, P06543) a 98 amino acid 2Fe–2S ferredoxin, whose crystal structure has been determined (Rypniewski et al., 1991). The sequence for spinach ferredoxin commences at amino acid 51, and this will be referred to as amino acid 1 in all further discussions.

2.2. Ferredoxin

Spinach ferredoxin was purchased from Sigma. Purity was checked and a ratio of 0.35 for A_{422}/A_{276} was obtained by UV–visible spectroscopy. This compares well with those ratios of 0.49 (Tagawa & Arnon, 1968), 0.46 (Hirasawa et al., 1996) and 0.28 (Bojko & Wieckowski, 1999) reported in the literature. Concentrations were calculated using A_{422} where possible with $\epsilon = 9.7 \times 10^3$ M^{-1} cm^{-1} (Tagawa & Arnon, 1968). The stock solution of ferredoxin was reported by Sigma to be 2.2 mg ml^{-1}, correlating with 0.19 μmol ml^{-1} [$M_r = 11{,}650$ Da (Tagawa & Arnon)]. All experiments were performed at 37°C, with scan spectra run from 200 to 900 nm.

2.3. Chemicals

All reagents used were of BDH AnalaR grade or equivalent where possible. Deionised water was obtained using a Sanyo Fillstream system.

2.4. Pepsin

Pepsin solution (porcine stomach pepsin, Sigma p7000) at 4% was prepared to yield actual concentrations during digestion of 0.19%. Activity of the enzyme was studied using bovine haemoglobin with the 4% stock solution stored for no more than 3 days under acidic conditions (0.1M HCl) at 4°C.

2.5. Iron analysis

$Fe(aq)^{2+}$ was analysed by complexation with 1,10-phenanthroline (“phen”) to form $[Fe(phen)_3]^{2+}$ using visible spectroscopy ($\lambda_{max} = 510$ nm, $\epsilon = 10{,}900$ M^{-1} cm^{-1}). No interference by $Fe(aq)^{3+}$ occurs at the wavelength used.

$Fe(aq)^{3+}$ concentrations were determined via reduction to $Fe(aq)^{2+}$, using hydroxylamine hydrochloride followed by 1,10-phenanthroline complexation as described earlier.

2.6. Data analysis

Scan spectra were analysed using the MicroCal Origin™ software package to allow spectra for pepsin to be subtracted where necessary. Kinetic data were analysed using linear regression.

3. Results

3.1. Stability of ferredoxin during in vitro digestion conditions

When ferredoxin was studied in the presence of 0.01M hydrochloric acid (HCl) only, and HCl plus pepsin enzyme (0.19%) the behaviour of the protein differed. Complete release of iron from a range of ferredoxin

solutions (μM) was observed upon immediate mixing (10s) with hydrochloric acid in the absence of enzyme. The iron was analysed and determined to be present as $Fe(aq)^{2+}$ only (Table 1) with the final protein concentration determined by comparison of solutions using $\epsilon_{276} = 20{,}160\ M^{-1}\ cm^{-1}$ (Tagawa & Arnon, 1968). In the presence of enzyme, the peak at ca. 276 nm gradually decreased over time, as can be seen in Fig. 1 for a range of pepsin concentrations.

Fig. 1 shows the effects on solutions of ferredoxin (all solutions 4 μM in ferredoxin bar the 0.38% pepsin experiment which was 5 μM and is shown with absorbance reduced by 20% for comparison) with time under a range of possible in vitro digestion conditions at $\lambda = 277$ nm. The effect of acid on the peak at 277 nm appears negligible in the absence of pepsin. Studies also showed that pepsin at this wavelength did not change in absorbance over the timescale of the experiments; therefore, the absorbance due to pepsin has been removed to allow absorbance due to ferredoxin only to be considered. The experiment with pepsin present at 0.38% was the maximum possible to allow observation of the changes in the peak for ferredoxin and, as the absorbance decreased with time, data beyond 180 min were difficult to extract due to the high (masking) absorbance of the pepsin. The nature of the peak at this wavelength (absorbance due to amino acids primarily but may also contain some components due to free iron aqua ions) does not lend itself to easy kinetic analysis of initial rates; however, observation of the data shows the increasing initial rate of loss of absorbance with increasing pepsin concentration.

In general, the spectra contained no peaks at 422 or ~320 nm, indicating that the absorbances of either oxidised or reduced ferredoxin were not present. Some change in absorbance at these wavelengths was occasionally observed during 60 min when only 0.01M HCl was present (Fig. 2b); however, the initial absorbance at 422 nm was only 18% of the original "starting ferredoxin" and the value after 60 min was 7%. The absorbances at these wavelengths are not due to the amino acid sequence, but to the iron-sulphur cluster present. The lack of these peaks in general, when acid was present at $[H^+] = 0.01M$, suggested an investigation of the effects of water alone on the protein to assess the extent of the effect of hydrochloric acid.

Table 1
Release of iron [as $Fe(aq)^{2+}$] determined as $[Fe(phen)_3]^{2+}$ when spinach ferredoxin (Fd) is mixed with 0.01 M HCl under aerobic conditions at 37°C

Ferredoxin (μM)	Aqueous iron (μM) released	Fd:Fe
3.17	6.88	1:2.17
3.17	5.73	1:1.81
4.66	11.5	1:2.47
4.82	6.88	1:1.42
	Mean	1:1.97

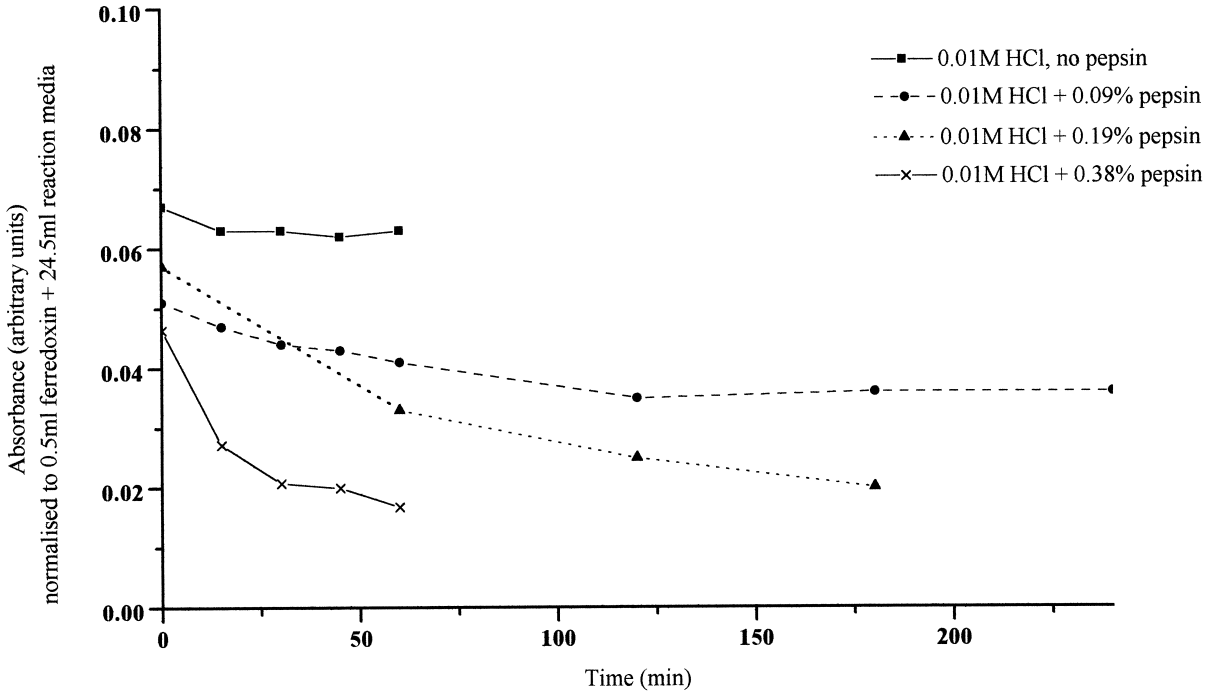

Fig. 1. Plot showing the change of absorbance (as ln), at $\lambda = 277$ nm, with time, when spinach ferredoxin is digested under a range of in vitro conditions. The calculated absorbance at 277 nm when in buffered solution with no pepsin or hydrochloric acid is 0.0766, using $\epsilon = 20.16 \times 10^3\ M^{-1}\ cm^{-1}$ (Tagawa & Arnon, 1968).

3.2. Reactivity of ferredoxin in aqueous media

3.2.1. Buffered solution

The reactivity of ferredoxin ([Fd] = 1.63 μM) in the presence of a standard buffer [20 mM TrisHCl, I = 0.01 M (NaCl), pH 7.5] was assessed and the ferredoxin found to be stable over a period of 135 min, with no apparent oxidation or degradation occurring. Buffers will to some extent prevent acidity occurring in solution; however in "unbuffered" water solutions a slight acidity will be present. To investigate this, unbuffered solutions of ferredoxin were studied using de-ionised water only (pH = 5.35, i.e. $[H^+] = 4.75$ mM). Three concentrations of ferredoxin solution plus control solutions using 0.01M HCl were studied to assess the progress of the reaction. Fig. 2a shows the scan spectra for the 3.03 μM ferredoxin solution, with those obtained in 0.01M HCl shown in Fig. 2b for the same ferredoxin concentration. The results indicate that, in the presence of water, the loss of absorbance at $\lambda \approx 422$ and 320 nm agrees with the total loss observed in the presence of 0.01M HCl. The effects (in both media), however, on the peak at 277 nm, are negligible (as expected since this peak arises from the aromatic amino acids). The slight difference in spectra shown (Fig. 2a and b) can be attributed to the different solvents, namely water and acid.

Analysis of the spectroscopic data at each wavelength, 277, 320 and 422 nm showed differing results. The peak at 422 nm is viewed as the most significant because of its complete disappearance over time, and the less likelihood of multiple interactions giving rise to the peak.

3.2.1.1. 422 nm. Uniphasic kinetics were observed for the decrease in absorbance (A) over time (t). Plots of ln absorbance versus time are shown in Fig. 3, for all three concentrations studied. A linear dependence upon ferredoxin (Fd) concentration can be observed for the resulting uniphasic rate constants (k_{obs}), leading to Eq. (1):

$$k_{obs} = k_f[Fd] + k_b \quad (1)$$

where k_f is the rate constant for the forward reaction, namely the loss of the absorbance at this wavelength, and k_b is the rate constant for the back reaction, i.e. an increase in the absorbance at this wavelength. Values obtained by data analysis indicate $k_f = (-7.72 \pm 0.27) \times 10^3$ M^{-1} min^{-1}; $k_b = (4.72 \pm 0.08) \times 10^{-2}$ min^{-1} at 422 nm.

3.2.1.2. 320 nm. Uniphasic kinetics were again observed for the decrease in absorbance at this wavelength over time. Plots of ln absorbance versus time are shown in Fig. 4. Data analysis indicates an inverse dependence

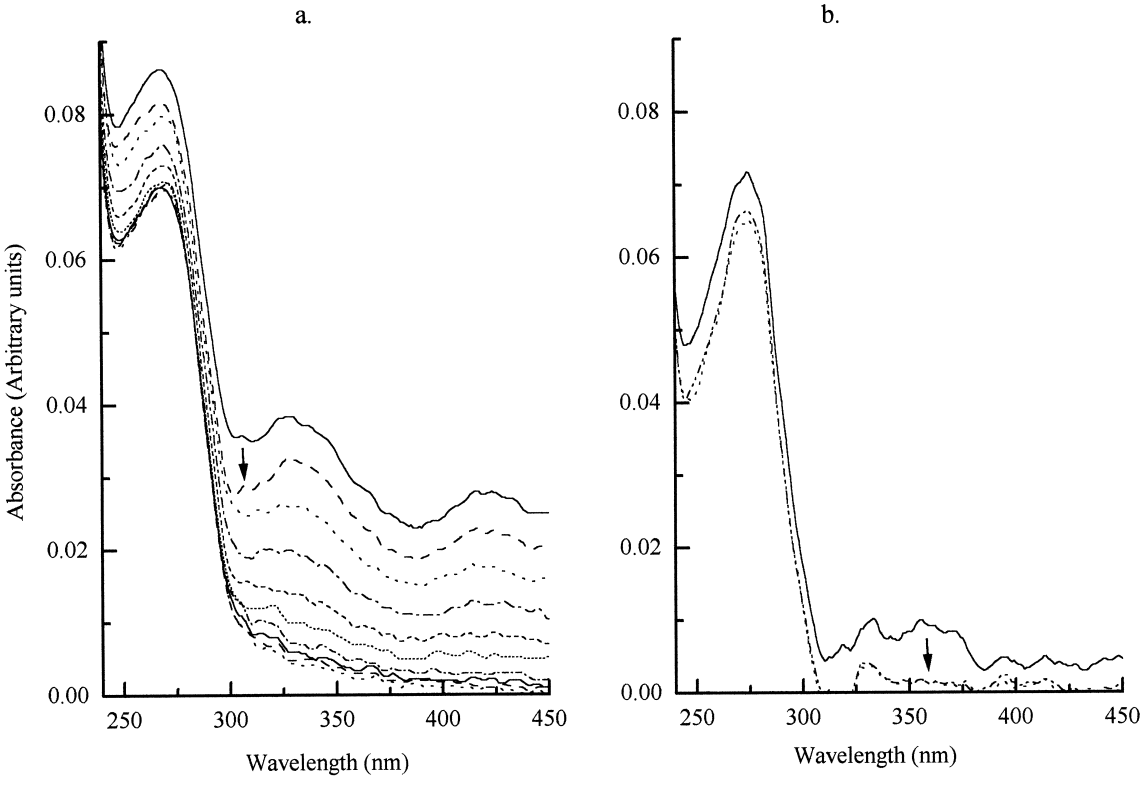

Fig. 2. Scan spectra showing the changes in absorbance with time when spinach ferredoxin is placed a. in water, and b. in 0.01 M HCl. Initial scan spectra at $t = 0$ min (——) and spectra at 60 min (— - —) and 135 min (- - -) are shown on both. Arrows indicate the direction of the absorbance change with time.

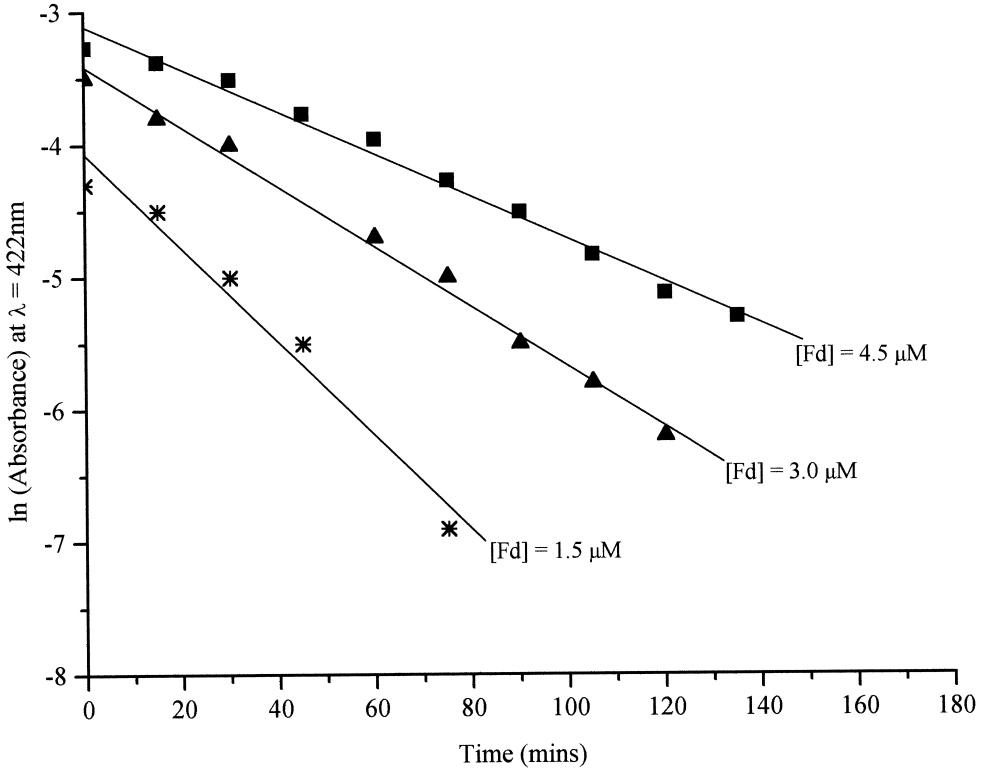

Fig 3. Kinetic plot showing the uniphasic, pseudo first-order decay of the absorbance at 422 nm with time for ferredoxin in water, over a range of initial concentrations of ferredoxin.

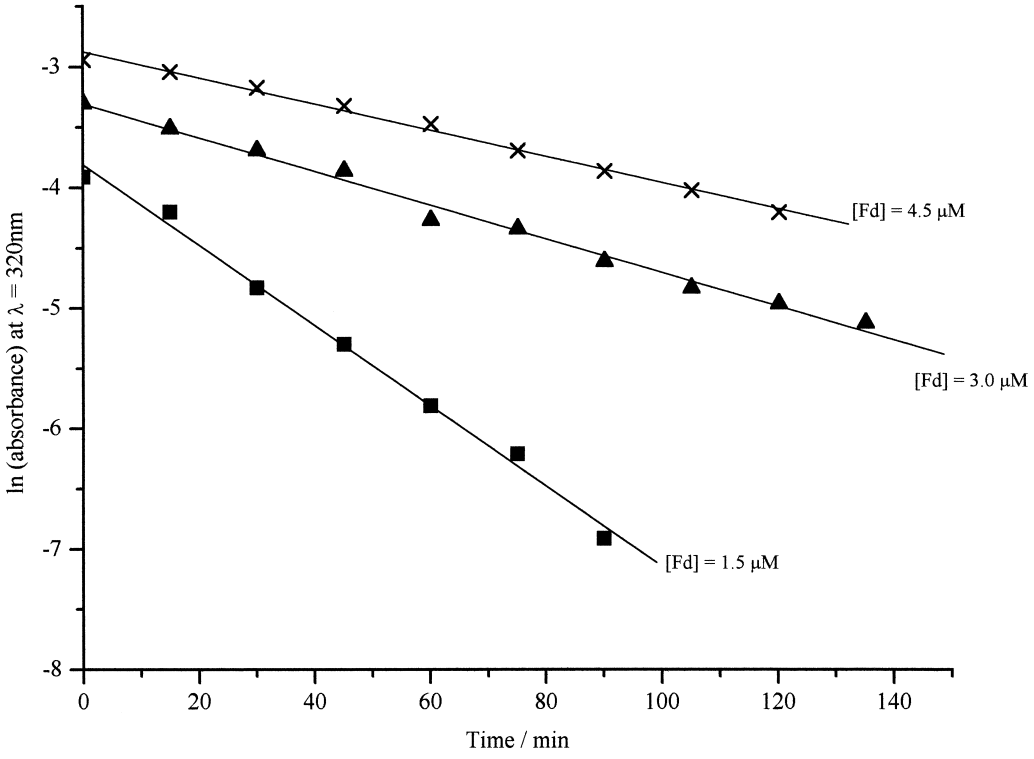

Fig. 4. Kinetic plot showing the uniphasic, pseudo first-order decay of the absorbance at 320 nm with time for ferredoxin in water, over a range of initial concentrations of ferredoxin.

upon initial ferredoxin concentration [Fd], of the resulting uniphasic rate constant ($k_{obs'}$) with $k_{f'}$ ($=2.0\times10^7$ M^{-2} min^{-1}) as the rate constant for the loss of the absorbance at this wavelength, and $k_{b'}$ ($=0.597$ min^{-1}) as the rate constant for an increase in the absorbance at this wavelength. However, it is more likely that a linear dependence upon initial ferredoxin concentration (as at 420 nm) is occurring, based upon the possibility of a range of species absorbing at this wavelength (see discussion) and analysis yields values of $k_{f'}=(-9.27\pm2.58)\times10^3$ M^{-1} min^{-1} and $k_{b'}=(4.45\pm0.78)\times10^{-2}$ min^{-1}.

For both wavelengths, fitting of data for exponential decay of absorbance with time indicated single stage reactions. No evidence was found to indicate two stage reactions.

4. Discussion

Ferredoxin proteins containing a [2Fe–2S] core are generally well-studied, with crystal structures known (Holden et al., 1994; Hurley et al., 1997; Muller et al., 1999). A 3-D structure for spinach ferredoxin is not available at present, but homology between plant and vertebrate ferredoxins is common, allowing the assumption that the structure is similar to the well-studied vegetative *Anabaena* ferredoxin. This assumption is based upon the analogy between the amino acid sequences as shown in Fig. 5.

The underlined cysteinyl residues in Fig. 5 are known to co-ordinate the 2Fe–2S core with C41 and C46 of *Anabaena* vegetative ferredoxin (AFd) coordinating to the Fe^{2+} (generally referred to as Fe1) and C49 and C79 co-ordinating to the Fe^{3+} (referred to as Fe2) (Skjeldal et al., 1991). More interestingly, when the four cysteinyl residues were replaced by serine groups, visible absorption bands between 300 and 600 nm were still observed [due to the dipole-allowed sulfur/oxygen to iron charge transfer (Noodleman & Baerends, 1984)], except when C79 was replaced by serine. In this case, no peak at ca. 400 nm was observed (Cheng, Xia, Reed & Markley, 1994). This is evidence that this residue is important for the absorbance at 422 nm, with the remaining iron–sulfur absorbances occurring perhaps in the region of 320–360 nm. The possibility that the absorbance at 422 nm is due to only one charge transfer, allows the possibility of relatively uncomplicated kinetics to be measured. This is apparent when the absorbance over time is measured at this wavelength and then compared with the results obtained at 320 nm. Whilst uniphasic reactions were observed at both wavelengths, if three charge transfer losses are occurring at very similar rates, as would be expected, it would be very difficult to distinguish between them. In analogue clusters, where molybdenum and tungsten replace iron, no difference has been observed in reactivities at molybdenum or tungsten centres when two or more atoms of each element are present (Varey & Sykes, 1993).

The rate of decomposition of the ferredoxin clusters in both water and acid lead to the conclusion that, under digestion conditions alone, all iron will be released if exposed to stomach acid, or for those suffering from achlorydia during the digestion process, since food can be expected to be retained in the digestive tract in general for greater than 135 min. The general drawback for iron bioavailability would appear therefore to be release of the ferredoxin clusters from the cell and/or chloroplast in the first instance.

The general denaturing of the protein leads to the decomposition of the amino acid residue chains by pepsin and can be observed at 276 nm. A very gradual decay occurs only in the presence of acid or water, indicating slight changes in the absorbance at 276 nm upon denaturation (expected due to changes in the hydrogen bonding between aromatic and other amino

Spinach (from a.a. 51) AAYKVTLV TPTGNVEFQC PDDVYILDAA

Anabaena ATFKVTLINE AEGTKHEIEV PDDEYILDAA

EEEGIDLPYS CRAGSCSSCA GKLKTGSLNQ DDQSFLDDDQ IDEGWVLTCA

EEQGYDLPFS CRAGACSTCA GKLVSGTVDQ SDQSFLDDDQ IEAGYVLTCV

AYPVSDVTIE THKEEELTA

AYPTSDVVIQ THKEEDLY

Fig. 5. Primary sequences for the ferredoxin molecules from spinach (aa 51–147) and vegetative *Anabaena* (aa 1–107). All sequences were obtained from the ExPASy database.

acids). A greater rate of decay is observed in the presence of pepsin (Fig. 1), but its effect appears to be dependent on the level of pepsin present, indicating some inhibition of the enzyme either by the apoprotein, released amino acids or possibly the released soluble iron and/or sulphur species present in solution.

A loss of the charge transfer between C77 (i.e. C79 of AFd) and iron would appear, by comparison with other studies, to be the process being observed overall for the decay in absorbance at 422 nm. Uniphasic kinetics were obtained that showed a dependence on ferredoxin concentration. Due to the wide range of possible reactions occurring, any of which could be rate-determining, a possible sequence of events could be postulated as: protonation of the cysteinyl ligand, followed by water ligation at the iron atom, and then the gradual release of the core due to the change of bond lengths throughout the cluster. This is based on the observation that buffered solutions do not destabilise the protein, whilst water (weak acid) does so slowly, and hydrochloric acid, at 0.01 M, does so immediately. The step measured as the uniphasic kinetics is believed to be the initial protonation of the cysteinyl residue since both forward and backward reaction kinetics have been measured. Reaction at 320 nm was also observed and appeared to be slightly slower, indicating that reactivity at 422 nm (i.e. presumably C77) may be the fastest.

This is surprising given that Fe1 is known to be closer to the surface of the protein, to have more hydrogen bonds than Fe2, and to be more polar (Muller et al., 1999). The lower number of hydrogen bonds to Fe2, however, may increase its reactivity in the presence of acid. Water itself was not expected to react significantly when buffered, due to the established fact that [2Fe–2S] plant ferredoxins have a funnel of five solvent (water) molecules leading from the protein surface near the C-terminus into the protein, and coming close to the iron–sulfur cluster (Muller et al.). The cluster is held to the alpha helix of the C-terminus and the hydrogen bonding involved within this solvent funnel appears to stabilise the flexible C-terminus of the protein (Hurley et al., 1997) No water molecules have been found to be located closer than ~6 Å to the [2Fe–2S] cluster itself (Holden et al., 1994). Interestingly, however, the cysteinyl residues all appear (in AFd) to have potential hydrogen bonds to backbone amide hydrogens and the sulfur atoms of the cluster also have such hydrogen bonds. Only C79 of AFd does not have such amide hydrogen bonds, again indicating its relatively likely assignment as the initial site of reactivity, and hence extrapolation to C77 of spinach ferredoxin. It should be noted, however, that until further studies have clarified this, the authors do not discount the possibility that the reactivity could be attributed to removal of this hydrogen bonding between the solvent molecules and the protein within the solvent funnel and the resultant effect on the protein as it denatures and the iron–sulfur cluster "drops out" of the ferredoxin, leaving the apoprotein.

5. Conclusions

The release of iron from isolated spinach ferredoxins is expected to be complete under in vivo digestion conditions, based upon the in vitro studies. However, the well-documented low bioavailability of iron from spinach leads to the conclusion that the cellular components are not released during the digestion process, either in vivo or in vitro. Food processing techniques which allow release of cellular components and breakdown of chloroplasts and mitochondria should enhance bioavailability; however, the reaction of the iron with other cellular components then affects uptake and hence nutritional status of the body.

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