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Linear three-iron centres are unlikely cluster degradation intermediates during unfolding of iron-sulfur proteins

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Abstract

Recent studies on the chemical alkaline degradation of ferredoxins have contributed to the hypothesis that linear three-iron centres are commonly observed as degradation intermediates of iron-sulfur clusters. In this work we assess the validity of this hypothesis. We studied different proteins containing iron-sulfur clusters, iron-sulfur centres and di-iron centres with respect to their chemical degradation kinetics at high pH, in the presence and absence of exogenous sulfide, to investigate the possible formation of linear three-iron centres during protein unfolding. Our spectroscopic and kinetic data show that in these different proteins visible absorption bands at 530 and 620 nm are formed that are identical to those suggested to arise from linear three-iron centres. Iron release and protein unfolding kinetics show that these bands result from the formation of iron sulfides at pH 10, produced by the degradation of the iron centres, and not from rearrangements leading to linear three-iron centres. Thus, at this point any relevant functional role of linear three-iron centres as cluster degradation intermediates in iron-sulfur proteins remains elusive.

Keywords: aconitase; ferredoxin; hydrogenase; iron-sulfur; linear three-iron centres; protein folding.

Introduction

Iron-sulfur clusters are ubiquitously found in all life domains and are considered to be one of the most ancient forms of biological catalysts. The versatility of functions performed by iron-sulfur proteins is directly correlated to the intrinsic structural diversity of its clusters, which ranges from the simplest rubredoxin-type iron-sulfur centres, linear and cubic clusters with higher nuclearity ([2Fe2S],[3Fe4S] and [4Fe4S]), to the very complex assemblies in which a cuboid cluster is bridged to another component, such as the cofactor cluster ([Mo7Fe9S]) and P-cluster ([8FeS7]) of nitrogenase (for a review see Beinert et al., 1997). In addition, the polypeptide chain itself plays an essential role in the fine-tuning of the cluster biological function, either by modulating the cluster reduction potential or by stabilising it. The latter has a particular importance in the non-redox functions of iron-sulfur centres, in which assembly and disassembly of the cluster provides a regulatory signal. This is the case in the fumarate-nitrate reductase regulator (FNR) protein from *Escherichia coli*, a transcription activator of genes encoding for components of anaerobic pathways, in which exposure to oxygen inactivates the protein as a result of disassembly of its [4Fe4S] cluster. Also, in the iron regulatory protein (IRP), an isoform of the enzyme aconitase, cluster rearrangements between the active [4Fe4S] and the inactive apoprotein modulate the protein function as a transcription regulator of ferritin and transferrin receptor, two proteins involved in the cellular iron metabolism. Aconitase also constitutes the clearest example so far of a protein containing a linear three-iron centre, which is formed when the inactive [3Fe4S]-containing protein is exposed to pH >9 or is partly denatured with urea (Kennedy et al., 1984). The resulting cluster has a purple colour and characteristic spectroscopic signatures, and can be re-converted to the active [4Fe4S]-containing form on lowering the pH and incubation with an iron salt and a thiol. Nevertheless, it has no known biological function and remains an interesting example of cluster interconversions within a protein environment. Recently, it has been suggested that a linear three-iron centre could be formed upon the chemical denaturation at pH 10 of a seven-iron ferredoxin (Jones et al., 2002). The original hypothesis was essentially based on spectroscopic evidence from visible spectroscopy, as a grey-purple compound was transiently formed during protein degradation and cluster dissociation. Subsequent studies in other ferredoxins, even in those containing centres with lower nuclearity (such as [2Fe2S]), have contributed to the hypothesis in the literature that linear three-iron centres are commonly observed as degradation intermediates in iron-sulfur proteins (Higgins et al., 2002; Pereira et al., 2002; Griffin et al., 2003; Mitou et al., 2003; Higgins and Wittung-Stafshede, 2004). However, recent evidence has challenged this hypothesis, as studies on the degradation pathway of a seven-iron ferredoxin indicate that the observed intermediate species are likely to result from the formation of iron sulfides (Fe_xS_y) at high pH (Leal et al., 2004). To contribute to the clarification of this issue and to clearly establish if linear three-iron centres are formed during the degradation of iron-sulfur proteins at alkaline pH, or if the transient species observed are due to non-specific reactions of the cluster degradation products, we undertook a spectroscopic and kinetic study of the degradation of several iron proteins.

Results and discussion

We investigated different metalloproteins with respect to the degradation of their iron-containing centres on chemical induction of protein unfolding under alkaline condi-

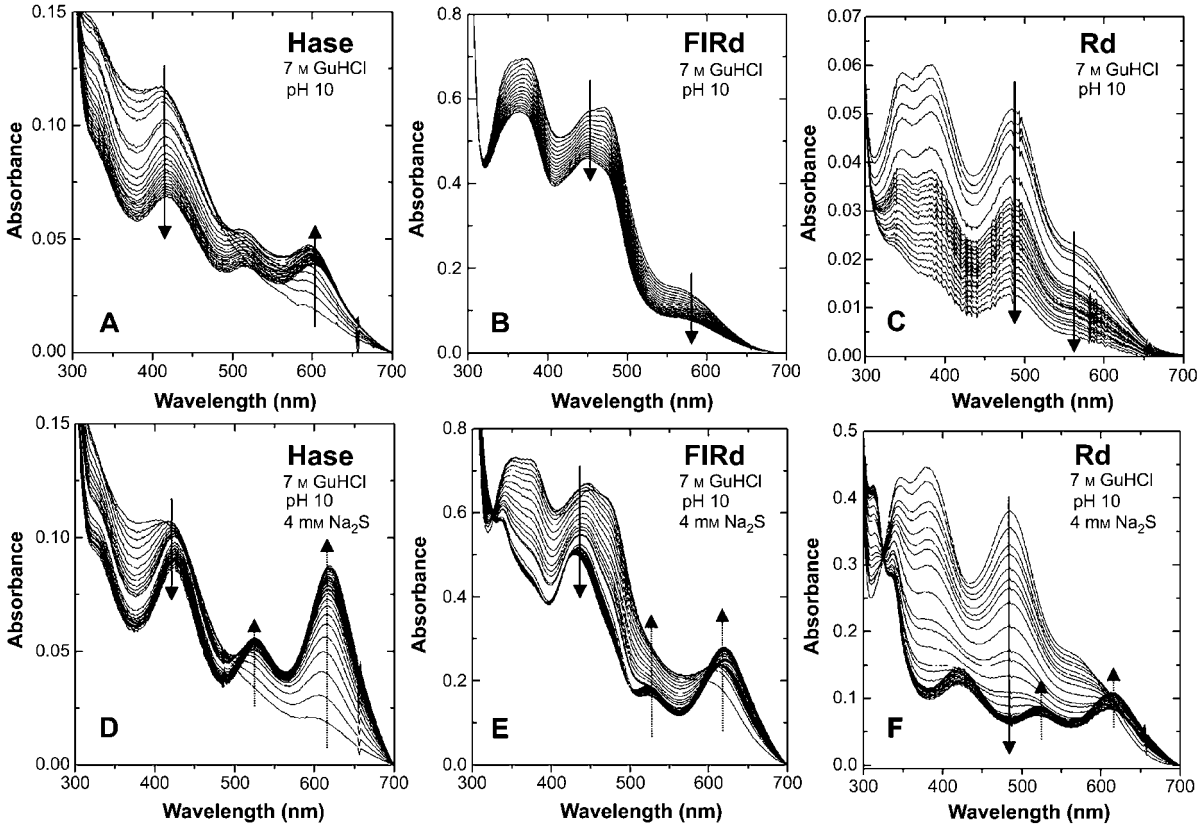

Figure 1 Absorption spectra at different times upon chemical denaturation at pH 10.
(A,D) Hydrogenase (Hase), (B,E) flavorubredoxin (FlRd) and (C,F) rubredoxin (Rd) were chemically denatured upon mixing with 7 M GuHCl, 0.2 M glycine, pH 10, at 25°C, in the absence (A–C) and presence of 4 mM sulfide (D–F). Protein concentrations used were 5.5 μM (Hase), 25 μM (FlRd) and 60 μM (Rd).

tions. For this purpose, proteins with different cofactor architectures were selected as targets: a hydrogenase, a flavorubredoxin and a rubredoxin. All these targets contain iron centres: whereas some contain a large number of iron-sulfur clusters, others also have non-cysteinyl iron centres or contain simple cysteinyl mononuclear iron centres. Specifically, the proteins used in this study were: (i) *Desulfovibrio gigas* Ni-Fe hydrogenase (Hase), which has two [4Fe-4S] centres and one [3Fe-4S] cluster, in addition to a binuclear Ni-Fe site (total of 12 mol Fe/mol protein) (Volbeda et al., 1995); (ii) *Escherichia coli* flavorubredoxin (FlRd), having a non-cysteinyl diiron site and a rubredoxin-type centre (3 mol Fe/mol protein) (Gomes et al., 2000); and (iii) the rubredoxin (Rd) domain resulting from truncation of FlRd (1 mol Fe/mol protein) (Gomes et al., 2000). The rationale for this choice was to include iron-sulfur (Hase) and non-iron-sulfur proteins (FlRd and Rd) as targets for investigation of the possible transient formation of linear three-iron centres upon protein degradation at high pH. Moreover, structural information is available for these proteins, either obtained directly by X-ray crystallography for *D. gigas* Hase (Volbeda et al., 1995) or via homology molecular modelling for *E. coli* FlRd from the crystal structure of the homologous protein rubredoxin:oxygen oxidoreductase (Frazão et al., 2000). This allows the framing of eventual cluster rearrangements or interconversions in a structural perspective. In fact, in the targets chosen, iron is inserted in a variety of metallic centres with different geometries (cubic, cubane, μ-oxo diiron and mononuclear Fe) and involving distinct types of ligands (Cys, His).

Kinetics of iron centre degradation at pH 10

The kinetics of chemical unfolding promoted by guanidinium hydrochloride (GuHCl) at pH 10 was monitored by UV-visible spectroscopy, which allows direct probing of the metal site integrity and inspection of the formation of bands in the 500–620-nm region, which have been attributed to the transient formation of linear three-iron centres (Wittung-Stafshede et al., 2000; Jones et al., 2002; Higgins and Wittung-Stafshede, 2004). Anaerobic conditions were used to protect possible reaction intermediates from degradative oxidation by molecular oxygen. The results obtained show that during unfolding of Hase, new bands formed at 530 and 620 nm (Figure 1A). On the other hand, during FlRd and Rd unfolding (Figure 1B, C), these new features were not observed; instead, there was bleaching of the spectrum corresponding to the native protein form, which is compatible with the ongoing denaturation process. Of these proteins, hydrogenase is the only one for which there is release of sulfide (S^{2-}) as a result of iron-sulfur cluster decomposition during unfolding. A different scenario occurs during degradation of the FlRd and Rd iron centres, which simply result in iron release. Similar results were obtained for all

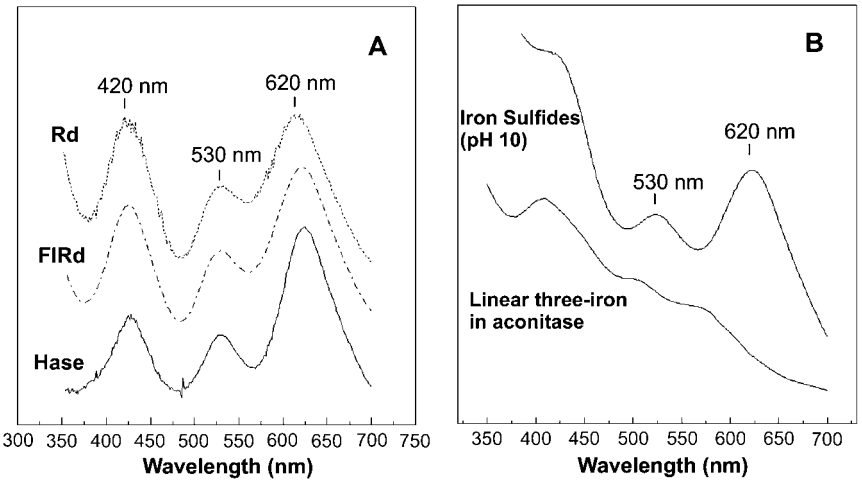

Figure 2 Comparison of the decomposed spectra with those of iron sulfides at pH 10 and purple aconitase (linear three-iron cluster). (A) Decomposition spectra of the chemical species formed upon unfolding of hydrogenase (Hase), flavorubredoxin (FlRd) and rubredoxin (Rd) in 7 M GuHCl, 4 mM Na_2S, pH 10. (B) Absorption spectra of the linear three-iron centre in purple aconitase [in 0.1 M glycine, pH 10.5, replotted from Kennedy et al. (1984), and of iron sulfides (Fe_xS_y) obtained by mixing excess sulfide and ferrous iron in 0.1 M glycine, pH 10].

proteins when the experiment was carried out under aerobic conditions, with the difference that the intensity of the 620- and 530-nm absorption bands for Hase was lower.

Effect of exogenous sulfide

The same experiments were performed in the presence of exogenous sulfide (Figure 1D–F). In this case, not only were the 620- and 530-nm absorption bands observed for all proteins, but the spectra were also identical for all proteins, suggesting that a common chemical species was formed upon degradation of the iron sites (see below). Furthermore, the species formed that has absorption bands at 530 and 620 nm is not transient, but is rather stable in solution over a very long time scale (tens of minutes). Interestingly, adding sulfide to the fully degraded FlRd and Rd obtained from unfolding experiments in the absence of exogenous sulfide (Figure 1B, C) resulted in the immediate formation of intense bands at 530 and 620 nm, identical to those observed when sulfide was present during unfolding. Thus, it is clear that the bands at 530 and 620 nm are only formed when iron and sulfide are available in solution.

Spectral decomposition of the species with bands at 530 and 620 nm

Spectra of the species with bands at 530 and 620 nm observed during unfolding of Hase, FlRd and Rd in the presence of sulfide were obtained by two different analysis techniques, which gave identical results (Figure 2A). Difference spectroscopy consisted of subtracting the final spectra for protein denatured in the absence of sulfide from those for protein obtained in its presence. Singular value decomposition analysis (SVD) allowed deconvolution of the progression of the unfolding process on the basis of its spectral components and their relative occupancy during the reaction.

This analysis clearly shows that identical bands were observed in all proteins, with features at 420, 530 and 620 nm. These are identical to those observed during the unfolding of several ferredoxins at high pH (Higgins et al., 2002; Jones et al., 2002; Griffin et al., 2003; Mitou et al., 2003), which have been suggested to arise from linear three-iron centres on the basis of spectral similarity (Table 1). A comparison of these spectra with those of the linear three-iron centre from aconitase and iron sulfides (Fe_xS_y) produced at pH 10 is shown in Figure 2B. It is clear that

Table 1 Iron-sulfur proteins exhibiting the appearance of absorption bands at 530 and 620 nm during chemical denaturation, assigned to linear three-iron centres.

Protein (organism)	Denaturation conditions	Reference
[2Fe2S] (*Aquifex aeolicus*)	6–8 M GuHCl, pH 10	Higgins and Wittung-Stafshede, 2004
[2Fe2S][a] (*Bos taurus*)	6 M GuHCl, pH 8.5, 10 mM Na_2S	Burova et al., 1996
[3Fe4S] (*Rhodothermus marinus*)	5.5 M GuHCl, pH 7	Pereira et al., 2002
[3Fe4S][4Fe4S] (*Acidianus ambivalens*)	7 M GuHCl, pH 10	Wittung-Stafshede et al., 2000
[3Fe4S][4Fe4S] (*Thermus thermophilus*)	6–7.9 M GuHCl, pH 10	Griffin et al., 2003
[3Fe4S][4Fe4S] (*Sulfolobus acidocaldarius*)	6–7.5 M GuHCl pH 10, 4–6 M GuSCN, pH 11	Jones et al., 2002

[a]In this case the observed bands were not interpreted as resulting from linear three-iron centres.

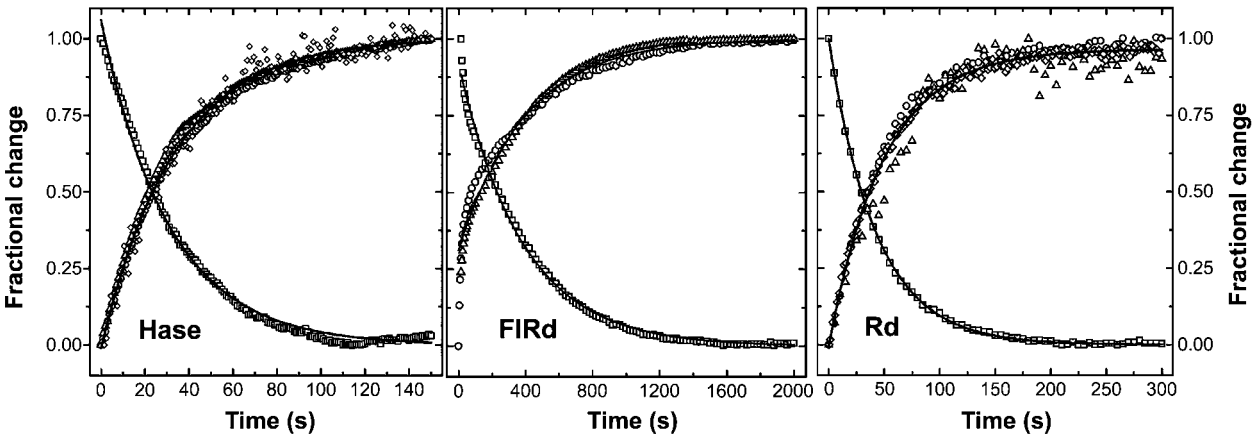

Figure 3 Kinetic traces for protein unfolding, metal centre degradation, iron release and variation in the 620-nm band.
The kinetics of protein unfolding was measured after mixing with 7 M GuHCl, 0.2 M glycine, pH 10, 4 mM Na_2S. Symbol key for all panels: (□) metal centre degradation; (◇) protein unfolding (tryptophan fluorescence emission); (○) iron release; (△) formation of iron sulfides (increase in 620-nm band). Protein concentrations used in the assays were 1.5 μM (Hase), 5 μM (FIRd) and 5 μM (Rd). Released iron corresponded to the total expected (ca. 11 Fe/mol for Hase, 2.8 Fe/mol for FIRd and 0.9 Fe/mol for Rd). Solid lines correspond to best fits and the observed rate constants are given in Table 2.

the bands observed during unfolding of iron-containing proteins are the result of the formation of iron sulfides and not of linear three-iron centres, as previously postulated.

Protein unfolding and iron release kinetics

To correlate the formation of the bands at 530 and 620 nm with protein unfolding and metal centre dissociation, the protein unfolding kinetics during GuHCl denaturation at pH 10 in the presence of sulfide was also investigated by intrinsic tryptophan emission and iron release measurements (Figure 3). The results show that protein unfolding, iron centre degradation, iron release and formation of the 530- and 620-nm bands are simultaneous events (Table 2). These events also occurred at identical rates during the unfolding of a seven-iron ferredoxin (Leal et al., 2004). In *E. coli* FIRd, protein unfolding proceeds via a more complex mechanism because the protein is a fusion of three distinct structural domains: a β-lactamase-like, a flaxodoxin-like and a rubredoxin-like fold (Frazão et al., 2000; Gomes et al., 2000).

Overall, these results show that protein unfolding leads to simultaneous metal centre degradation, without the formation of any detectable protein unfolding intermediate, and that released iron readily reacts with available sulfide, leading to the formation of the iron sulfides responsible for the bands at 530 and 620 nm. Taken together, all the evidence agrees well with a re-interpretation of events occurring during the alkaline unfolding of iron-sulfur proteins that excludes the formation of intermediate linear three-iron centres.

Conclusions

In this study we investigated possible iron-centre interconversions upon metalloprotein unfolding in alkaline conditions. The pioneer work by Kennedy et al. (1984) showed that the cubic iron-sulfur centre from beef heart aconitase, upon protein incubation at pH>9.5 or treatment with 4–8 M urea, undergoes a reversible conversion to a linear three-iron centre. The chemical nature of this new type of centre was then clearly established, both by comparative studies using model compounds and Mössbauer spectroscopy (Kennedy et al., 1984). More recently, it has been postulated that identical linear iron-sulfur centres could be formed during ferredoxin chemical unfolding at high pH (Jones et al., 2002), although this hypothesis was subsequently revised (Leal et al., 2004). To contribute to the understanding of metal centre transformations during protein unfolding, we studied several iron-proteins containing a variety of different iron centres. The issue of the biological synthesis of iron centres is of ultimate interest to the field of protein folding and catalysis, and in this respect the investigation of cluster

Table 2 Observed kinetic rates for protein unfolding, metal centre dissociation, iron release and iron sulfide formation.

	k_{obs} (s^{-1})			
	Hydrogenase	Flavorubredoxin	Rubredoxin	Ferredoxin[a]
Metal centre dissociation	2.24×10^{-2}	1.85×10^{-3}	1.63×10^{-2}	ND
Protein unfolding	1.82×10^{-2}	ND	1.21×10^{-2}	39×10^{-4}
Fe_xS_y formation	2.09×10^{-2}	1.98×10^{-3}	1.11×10^{-2}	12×10^{-4}
Iron release	1.95×10^{-2}	1.80×10^{-3}	1.37×10^{-2}	12×10^{-4}

See legend of Figure 3 for conditions. ND, not determined.
[a]Data taken from Leal et al. (2004).

assembly and eventual inter-conversions is particularly challenging.

To investigate these processes, however, it is often necessary to induce non-native protein states that correspond to partly misfolded protein forms, which somehow may represent facilitated intermediate states in the cluster assembly or rearrangement mechanisms. This can be achieved using non-physiological pH conditions, namely high pH. Therefore, we studied the kinetics of the chemical unfolding of hydrogenase, flavorubredoxin and rubredoxin at pH 10, as linear three-iron-sulfur centres are known to be preferentially formed under these conditions. Our results clearly show that in all these different proteins, visible absorption bands at 530 and 620 nm are observed upon chemical unfolding, but that these result from iron sulfides, and not from the transient formation of linear three-iron centres. These spectroscopic bands are identical to those observed during the unfolding of several ferredoxins suggested to arise from linear three-iron centres (Jones et al., 2002). In fact, we observed that iron sulfides are formed from reaction of the iron released from iron centre degradation when sulfide is available in solution. This is clearly shown by the experiments with exogenous sulfide, in which the intensity of these spectral bands increased. In fact, as demonstrated, the observed transient spectral species are due to the formation of iron sulfides at pH 10. Providing that exogenous sulfides are available, these are even observed in iron proteins that contain no iron-sulfur centres. Thus, our work provides a new appraisal disproving the view that these centres are general cluster degradation intermediates that are transiently formed during degradation of iron-sulfur clusters, although the possibility of residual cluster structures associated with the polypeptide chain during unfolding cannot be completely excluded. At this point, any relevant biological function for linear three-iron centres remains unclear.

Materials and methods

Chemicals

Guanidinium hydrochloride (GuHCl) and Tiron ($C_6H_4Na_2O_8S_2$), were purchased from Promega (Lisbon, Portugal) and Riedel-de Haën (Sintra, Portugal), respectively. Sodium sulfide (Na_2S) and iron(III) nitrate [$Fe(NO_3)_3$] were obtained from Sigma (Sintra, Portugal). All reagents were of the highest purity grade commercially available.

Proteins

Proteins used in this study were purified as described elsewhere (see references below) and were kind gifts of M. Teixeira [*Escherichia coli* flavorubredoxin and the rubredoxin domain (Gomes et al., 2000) and I. Pereira (*Desulfovibrio gigas* hydrogenase (Teixeira et al., 1985; Volbeda et al., 1995)], both at Instituto Tecnologia Química e Biológica, Universidade Nova de Lisboa, Portugal.

Spectroscopic methods

Visible absorption measurements were performed on a Shimadzu MultiSpec-1501 diode-array spectrophotometer equipped with cell stirring and temperature control via a computer-controlled water bath (Julabo, Seelbach, Germany). The integrity of iron-sulfur clusters and iron centres was monitored by following the decay at 420–700 nm for hydrogenase, at 480–700 nm for the rubredoxin domain, and at 440–700 nm for flavorubredoxin. Fluorescence spectroscopy was carried out on a Cary Varian Eclipse spectrofluorimeter equipped with cell stirring and Peltier temperature control. Intrinsic tryptophan fluorescence emission was monitored between 300 and 450 nm (slit width 5 nm), after excitation at 280 nm (slit width 5 nm). All experiments were performed at room temperature.

Iron quantification

Quantification of the iron released during unfolding was performed using 1,2-dihydroxy-3,5-benzenedisulfonic acid (Tiron; Berger and McKay, 1974) and bathophenanthroline disulfonic acid (BPS; Cowart et al., 1993). The time course of iron release was obtained by continuously monitoring the formation of the Fe(III)-Tiron complex at 480–700 nm during protein unfolding. A control experiment, performed in the absence of Tiron, was used to subtract the contribution owing to protein variation at this wavelength. Iron was quantified from calibration curves made with accurately titrated $Fe(NO_3)_3$ solutions (a kind gift from Rita Delgado, ITQB/UNL).

Kinetic experiments

The chemical denaturant GuHCl was used to promote protein unfolding at 25°C and pH 10. A stock solution of GuHCl at pH 10 was prepared in 200 mM glycine buffer and its accurate concentration was confirmed by refractive index measurements (Shirley, 1995). The reaction mixture was allowed to stabilise at 25°C and the reaction was started with the addition of a concentrated protein solution.

Data analysis

Optical decomposition was performed in Matlab, using the built-in singular value decomposition (SVD) algorithm. Briefly, in this procedure a data matrix of time-resolved spectra (A) is decomposed by SVD in the product $A=U\times S\times V^T$, in which U is the basis spectra matrix, S the matrix of relative occupancies and V the time dependence of the basis spectra (Henry and Hofrichter, 1992). The kinetic experimental data were processed and fitted using the program Origin (OriginLab, Northampton, USA).

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