Mixed amino acid binding of Fe_4S_4 clusters: a ^1H NMR study

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Abstract

The cluster $[Fe_4S_4(Asp)_4]^{2-}$ (HAsp = D-aspartic acid benzyl ester) was formed in DMSO-d_6 solution by reaction of four equivalents of HAsp with $[Fe_4S_4(SBu^t)_4]^{2-}$. The new tetra-aspartate substituted cluster was characterised by ^1H NMR spectroscopy. Mixed-ligated clusters $[Fe_4S_4(Cys)_x(Asp)_{4-x}]^{2-}$ (HCys = L-cysteine ethyl ester hydrochloride; $x = 1$, 2 or 3) were prepared by a similar method. The temperature dependence of the isotropically shifted ^1H NMR signals of the α-C*H* and β-CH_2 protons was used to distinguish between the bound amino acids. Reaction of $[Fe_4S_4(SBu^t)_4]^{2-}$ with the peptides GCGGCGGCGamide (9-*mer*-CysH_3) and GSGGSGGSGamide (9-*mer*-SerH_3) formed site-differentiated clusters $[Fe_4S_4(9\text{-}mer\text{-}Cys)(SBu^t)]^{2-}$ and $[Fe_4S_4(9\text{-}mer\text{-}Ser)(SBu^t)]^{2-}$. Exchange of the remaining coordinated thiolate enabled substitution of other amino acids at the differentiated site.

Introduction

It recently has been demonstrated that cysteinyl residues are not always the ligating groups to the iron sites in naturally occurring 4Fe–4S clusters. In the active form of aconitase [1] the iron atoms of the cuboidal cluster $[Fe_4S_4]$ are differentiated in a 3:1 ratio, three iron atoms being bound by protein cysteinate residues and the fourth, labile, iron coordinated by either H_2O or OH^- [2]. *Desulfovibrio africanus* ferredoxin (Fd) III is a 7Fe-Fd [3] which contains a $[Fe_3S_4]^{1+,0}$ and a $[Fe_4S_4]^{2+,1+}$ cluster [4]. On incubation with Fe^{II} the $[Fe_3S_4]$ cluster converts to $[Fe_4S_4]$ [5]. The binding of this cluster to the protein must involve one non-cysteinate ligand as there are insufficient cysteines to bind to all of the eight irons. Thomson and co-workers [5] have proposed that the carboxylate side-chain of an aspartic acid residue is this ligand. Similar mixed ligation has been reported in the 4Fe-Fd of *Pyrococcus furiosus* [6] and is likely in the 8Fe-Fds *D. Vulgaris* [7], *Thermoplasma acidophilum* [8] and *Sulfolobus acidocaldarius* [9]. Furthermore, the larger nitrogenase protein (MoFe) contains more metal–sulfur clusters than can be accommodated by cysteinyl residues alone [10, 11], which suggests binding of the clusters by other suitable amino acid residues in addition to cysteine. It is also possible that the unusual redox and spectroscopic properties of the nitrogenase P-clusters arise from ligation, either in part or full, by amino acid residues other than cysteine [12].

It has been shown that cysteine [11, 13] and various cysteine-containing peptides [13, 14] can bind to $[Fe_4S_4]^{2+}$ model clusters. We have shown that almost any amino acid with an appropriate side-chain can bind to the cluster core [11]. Holm and co-workers [13] have demonstrated that the peptide t-BOC-GCGGCGGCGNH_2 (t-BOC = t-butoxycarbonyl) can bind to $[Fe_4S_4]^{2+}$ to generate a 3:1 site-differentiated cluster. More recently such site differentiation has been achieved by employing tripodal thiolates as ligands [15–17].

Here is reported the characterisation, in solution, of: the novel tetra-aspartate bound cluster $[Fe_4S_4(Asp)_4]^{2-}$ (HAsp = D-aspartic acid α-benzyl ester); mixed-ligand clusters such as $[Fe_4S_4(Cys)_x(Asp)_{4-x}]^{2-}$ (HCys = L-cysteine ethyl ester hydrochloride; $x = 1$, 2 or 3); and site-differentiated clusters prepared with 9-*mer* tridentate peptides, where three of the iron atoms of $[Fe_4S_4]^{2+}$ are bound by the same amino acid residue and the unique iron either by thiolate or another, different, amino acid.

Experimental

The iron–sulfur cluster $[NMe_4]_2[Fe_4S_4(SBu^t)_4]$ was prepared as previously described [18]. L-Cysteine ethyl ester hydrochloride and D-aspartic acid α-benzyl ester were purchased from Aldrich Chemical Co. Ltd. (UK) and Sigma Chemical Co. Ltd. (UK), respectively. The 9-*mer* peptides GCGGCGGCGamide (9-*mer*-CysH_3) and GSGGSGGSGamide (9-*mer*-SerH_3) (G = glycine; C = cysteine; S = serine) were purchased from Multiple Peptide Systems Inc. (USA). All chemicals were used

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without further purification. Hexadeuterodimethylsulfoxide (DMSO-d_6) was dried over molecular sieve 4A (BDH) and degassed before use.

^{1}H NMR spectra were recorded on a Jeol GSX270 spectrometer as DMSO-d_6 solutions and chemical shifts were referenced against $SiMe_4$. Typically NMR solutions were prepared by adding a solution of $[NMe_4]_2[Fe_4S_4(SBu^t)_4]$ (15 mg, 1.75×10^{-5} mol) to the appropriate amount of solid amino acid ester, amino acid ester mixture, or peptide, under an atmosphere of dinitrogen. The mixtures were shaken under dynamic vacuum for 5 min then transferred to NMR tubes.

Results and discussion

The metal–sulfur cluster $[Fe_4S_4(SBu^t)_4]^{2-}$ reacts in DMSO-d_6 solution with four equivalents of HAsp by metathesis, eqn. (1), to give the tetra(amino acid) substituted cluster $[Fe_4S_4(Asp)_4]^{2-}$ (**1**).

$$[Fe_4S_4(SBu^t)_4]^{2-} + 4HAsp \rightleftharpoons [Fe_4S_4(Asp)_4]^{2-} + 4HSBu^t \quad (1)$$

The reaction is driven by removal of liberated thiol *in vacuo*. The ^{1}H NMR spectrum of the final solution shows no signals from coordinated t-butylthiolate but does show new signals arising from coordinated Asp. The α-C*H* (6.4 ppm) and β-CH_2 (8.0 ppm) resonances are isotropically shifted downfield compared to those of the corresponding diamagnetic amino acid. This is consistent with the coordination of Asp to $[Fe_4S_4]^{2+}$. The magnitude of the shift is less than that previously observed in cysteinate, serinate and tyrosinate analogues [11], Table 1. The α-carboxylate is esterified to prevent its binding to the cluster and we have previously shown [11] that the amino-NH_2 does not coordinate to $[Fe_4S_4]^{2+}$. The aspartate is, therefore, bound to the cluster core through the β-, or side-chain, carboxylate. The same β-carboxylate binding might be expected to occur in natural systems.

TABLE 1. ^{1}H NMR parameters of some amino acid ligated $[Fe_4S_4]^{2+}$ clusters in DMSO-d_6 at 298 K

Compound	Chemical shift (ppm)		Notes
	α-C*H*	β-CH_2	
$[NMe_4]_2[Fe_4S_4(Asp)_4]$	6.4	8.0	a, b
$[NMe_4]_2[Fe_4S_4(Cys)_4]$	5.3	13.2	a
$[NMe_4]_2[Fe_4S_4(Tyr)_4]$	12.5	20.7	c
$[NMe_4]_2[Fe_4S_4(Ser)_4]$	10.0	24.5	c, d

[a]This work. [b]Benzyl ester resonances at 7.34 ($C_6H_5CH_2$) and 5.12 ($C_6H_5CH_2$) ppm. [c]Ref. 11. [d]HSer = L-serine methyl ester hydrochloride.

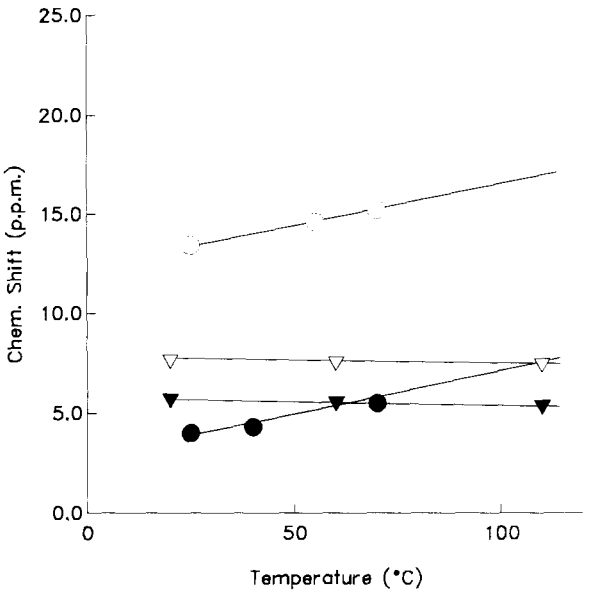

Fig. 1. Variation of chemical shifts with temperature for $[NMe_4]_2[Fe_4S_4(Cys)_4]$. (○) β-CH_2, (●) α-C*H* and $[NMe_4]_2[Fe_4S_4(Asp)_4]$, (▽) β-CH_2, (▼) α-C*H*, in DMSO-d_6.

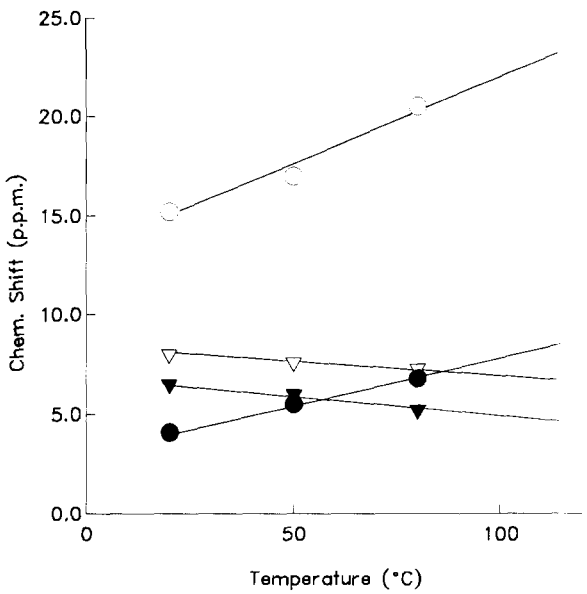

Fig. 2. Temperature dependence of chemical shift in the mixed-ligand cluster $[Fe_4S_4(Cys)_x(Asp)_{4-x}]^{2-}$ ($x=1$, 2 or 3). (○) β-CH_2 Cys, (●) α-C*H* Cys, (▽) β-CH_2 Asp, (▼) α-C*H* Asp, in DMSO-d_6.

The temperature dependence of the α-C*H* and β-CH_2 ^{1}H NMR resonances of $[Fe_4S_4(Cys)_4]^{2-}$ are compared with those of **1** in Fig. 1. An inverse temperature dependence of the shifts is observed for **1**. Therefore, although the room temperature NMR spectrum is consistent with the dominant contact mechanism determining the shifts [19], this particular temperature dependence, in a temperature range below the Néel

TABLE 2. ^{1}H NMR parameters (ppm) for peptide-bound site-differentiated $[Fe_4S_4]^{2+}$ clusters in DMSO-d_6 at 298 K [a]

Compound[b]	Cys		Tyr		Asp		Ser		SBu^t
	α-CH	β-CH_2	α-CH	β-CH_2	α-CH	β-CH_2	α-CH	β-CH_2	
$[Fe_4S_4$(9-*mer*-Cys)(SBu^t)$]^{2-}$ (**2**)	7.0	10.0, 11.3 11.9, 12.7 13.2							2.8
$[Fe_4S_4$(t-BOC-9-*mer*-Cys)(SBu^t)$]^{2-}$ [c]	6.48 6.69	9.9, 11.1 11.5, 12.6 13.6							2.8
$[Fe_4S_4$(9-*mer*-Cys)(Tyr)$]^{2-}$ (**3**)	4.4	10-14[d]	8.2	19.9[e]					
$[Fe_4S_4$(9-*mer*-Cys)(Asp)$]^{2-}$ (**4**)	4.6	13[f]			6.0	~8.0			
$[Fe_4S_4$(9-*mer*-Ser)(SBu^t)$]^{2-}$							13	19[f] 24[f]	2.8
$[Fe_4S_4$(9-*mer*-Ser)(Cys)$]^{2-}$	5.6	14					13	19[f] 24[f]	

[a]Cation and ester signals excluded. [b]Cation = $[NMe_4]^+$. [c]Ref. 13. [d]Broad, partially resolved. [e]Tyrosine *m*-H, 6.5 ppm, *o*-H not observed. [f]Broad, unresolved.

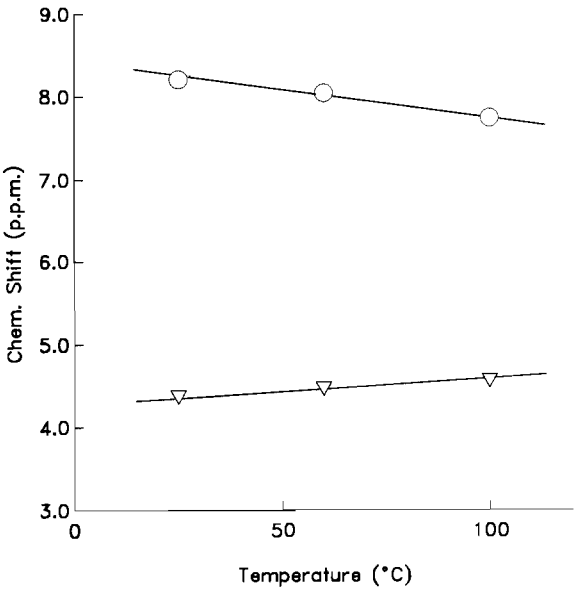

Fig. 3. Temperature dependence of α-CH resonance for $[Fe_4S_4$(9-*mer*-Cys)(Tyr)$]^{2-}$ (○) Tyr, (▽) Cys, in DMSO-d_6 solution.

temperature [20] is not. We have previously shown that serinate and tyrosinate analogues, $[Fe_4S_4\{OCH_2CH(COOMe)(NH_3Cl)\}_4]^{2-}$ and $[Fe_4S_4\{OC_6H_4CH_2CH(COOMe)(NH_3Cl)\}_4]^{2-}$, also have resonances with a temperature dependence inconsistent with the theory [11]. Further refinement of the theory is required to explain the shifts and dependencies in detail.

Mixed amino acid ligated clusters $[Fe_4S_4(Cys)_x(Asp)_{4-x}]^{2-}$ ($x = 1$, 2 or 3) were easily generated in solution, by thiolate exchange, from $[Fe_4S_4(SBu^t)_4]^{2-}$ and appropriate mixtures of HCys with HAsp. Liberated thiol was again removed by pumping. The isotropically shifted signals of the coordinated amino acids were broad, hence the value of x could be determined neither from the position nor integration of the signals. However, the α-CH and β-CH_2 resonances of Cys and Asp were easily distinguished by the temperature dependence of their NMR spectra. A plot of chemical shift against temperature, Fig. 2, was very similar to that observed above (Fig. 1) for the parent tetra(amino acid) substituted clusters. However, the magnitude of change in chemical shift with temperature of both cysteinate and aspartate α-CH and β-CH_2 was greater in the mixed-ligand system.

Mixed-ligated $[Fe_4S_4]^{2+}$ systems were also prepared by using tridentate, in this case peptide, ligands. Site-differentiated products were obtained; one iron is bound to a group different to that binding the other three irons of the cluster. An analogue of Holm's [13] 9-*mer*-peptide cysteinate-bound cluster, $[Fe_4S_4$(t-BOC-GCGGCGGCGamide)(SBu^t)$]^{2-}$, was prepared first. Reaction of $[Fe_4S_4(SBu^t)_4]^{2-}$ with one equivalent of GCGGCGGCGamide in DMSO-d_6 gave the site-differentiated cluster $[Fe_4S_4$(9-*mer*-Cys)(SBu^t)$]^{2-}$ (**2**). ^{1}H NMR spectroscopy showed α-CH and β-CH_2 resonances (Table 2) shifted downfield. The coordinated butylthiolate signal remains but in decreased intensity, consistent with its partial replacement. A total of twelve inequivalent methylene protons is expected [13] due to effects of the adjacent chiral centres and the chelating properties of the peptide. Here only five β-CH_2 signals are resolved. A similar result was previously observed by Holm and co-workers [13]. Addition of a solution of **2** to one equivalent of either HTyr (L-tyrosine methyl ester hydrochloride) or HAsp results in liberation of the remaining bound thiolate and substitution at the

differentiated site to give the new mixed-ligand species $[Fe_4S_4(9\text{-}mer\text{-}Cys)(Tyr)]^{2-}$ (**3**) and $[Fe_4S_4(9\text{-}mer\text{-}Cys)(Asp)]^{2-}$ (**4**). ^1H NMR parameters are collected in Table 2. The magnitude of shift for the α-C*H* and β-CH_2 protons of Tyr and Asp in **3** and **4** are of the same order as observed in the corresponding tetra-substituted clusters [11]. The cysteinate signals move away from the diamagnetic on increasing temperature, as in $[Fe_4S_4(Cys)_4]^{2-}$, and those of both Tyr and Asp shift in the opposite sense as found for$[Fe_4S_4(Tyr)_4]^{2-}$ [11] and $[Fe_4S_4(Asp)_4]^{2-}$. The temperature dependencies of the peptide cysteinate α-C*H* and tyrosinate α-C*H*, as an example, are shown in Fig. 3. The species $[Fe_4S_4(9\text{-}mer\text{-}Cys)(Asp)]^{2-}$ is interesting as it is a potential model for naturally occurring cysteinate/aspartate bound $[Fe_4S_4]$ clusters.

The cluster $[Fe_4S_4(9\text{-}mer\text{-}Ser)(SBu^t)]^{2-}$ can also be generated in solution by the general exchange-reaction method. In this case three iron vertices of the iron–sulfur cubane are bound by serinato groups. Substitution of butylthiolate at the differentiated site by Cys gives $[Fe_4S_4(9\text{-}mer\text{-}Ser)(Cys)]^{2-}$. ^1H NMR parameters are listed in Table 2; both the magnitude and temperature dependence of the shifted signals were as expected. The temperature dependence aided in the assignment of the resonances.

In the reaction of tridentate peptide ligands with $[Fe_4S_4]^{2+}$ there is always the possibility that the ligand will link several clusters rather than closing around just one. The formation of such polymers in the systems described here cannot be excluded but is unlikely as the products remain soluble even at quite high concentrations.

Addition of a slight excess (5–6 equiv.) of thiophenol to solutions of all new cluster species prepared above gave easily recognised $[Fe_4S_4(SPh)_4]^{2-}$ [21]; this suggests that the $[Fe_4S_4]^{2+}$ core has remained intact.

Conclusions

The novel tetra-aspartate bound cluster $[Fe_4S_4(Asp)_4]^{2-}$ has been prepared in solution as have mixed amino acid ligated systems $[Fe_4S_4(Cys)_x(Asp)_{4-x}]^{2-}$. The products possess normal NMR parameters, though some temperature dependencies are inconsistent with current theory. This anomalous temperature dependence provides a means of distinguishing those amino acids which are bound. Site-differentiated clusters have been generated by reaction of tridentate 9-*mer*-peptides with $[Fe_4S_4]^{2+}$; substitution at the unique iron site gives products, such as $[Fe_4S_4(9\text{-}mer\text{-}Cys)(Asp)]^{2-}$, which may be analogues of naturally occurring mixed-ligated clusters.

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