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SITES FOR PHOSPHATES AND IRON-SULFUR THIOLATES IN THE FIRST MEMBRANES: 3 TO 6 RESIDUE ANION-BINDING MOTIFS (NESTS)

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Abstract. Nests are common three to six amino acid residue motifs in proteins where successive main chain NH groups bind anionic atoms or groups. On average 8% of residues in proteins belong to nests. Nests form a key part of a number of phosphate binding sites, notably the P-loop, which is the commonest of the binding sites for the phosphates of ATP and GTP. They also occur regularly in sites that bind $[Fe_2S_2](RS)_4$ $[Fe_3S_4](RS)_3$and $[Fe_4S_4](RS)_4$ iron-sulfur centers, which are also anionic groups. Both phosphates and iron-sulfur complexes would have occurred in the precipitates within hydrothermal vents of moderate temperature as key components of the earliest metabolism and it is likely existing organisms emerging in this milieu would have benefited from evolving molecules binding such anions. The nest conformation is favored by high proportions of glycine residues and there is evidence for glycine being the commonest amino acid during the stage of evolution when proteins were evolving so it is likely nests would have been common features in peptides occupying the membranes at the dawn of life.

Keywords: anion, hydrothermal, inorganic membrane, iron-sulfur proteins, origin of life, pre-biotic, phosphate, protonmotive force, RNA world, thiolates

Abbreviations: ϕ, ψ: For amino acid i, phi (ϕ) is the dihedral angle between the C_{i-1}-N_i-$C\alpha_i$-C_i atoms and psi (ψ) is the dihedral angle between the N_i-$C\alpha_i$-C_i-N_{i+1} atoms (i-1 and i+1 are the preceding and succeeding residues).

1. Introduction

One model for the emergence of life proposes that the first membrane formed spontaneously as an inorganic precipitate at the interface between submarine seeps of moderate temperature and the early ocean (Russell *et al.*, 1994, 2003). This membrane, composed predominantly of mineral crystallites of mackinawite ($Fe_{1+x}S$) and minor greigite (as [SNiS][Fe_4S_4][SFeS]; Vaughan and Craig, 1978) prevented the direct mixing between the alkaline and hydrogen-bearing seepage water with this cool acidulous ocean. Nevertheless, some electron transfer from the interior of the hydrodynamically inflated FeS compartments to the photolytically generated ferric iron on the exterior augmented the ambient proton gradient. The

electron and proton activity gradients sum to a natural protonmotive force of about half a volt (Russell and Hall, 1997, 2002; Filtness *et al.,* 2003). It was presumed that protoferredoxins and phosphates were the agencies of energy transfer and expenditure. For example, clusters of $[Fe_2S_2]^{+/0}$ or $[Fe_4S_4]^{2+/+}$ that had escaped internment into mackinawite or greigite were assumed to have bonded instead to thiolate ligands in the seepage waters to form a protoferredoxin (as the anion $[Fe_4S_4][SR]_4^{2-/3-}$) in the manner demonstrated by Bonomi *et al.* (1985). The organic thiolate ligands (SR^-) would have occurred at the submarine seepage sites (Kaschke *et al.,* 1994; Heinen and Lauwers, 1996). At the same time the ambient protonmotive force could have condensed inorganic or acyl phosphates abiding in the membrane to the high energy pyrophosphate anion, a requirement for further biosynthesis (Russell *et al*., 1994). While these suggestions could be seen to follow those of Eck and Dayhoff (1966) and Hall *et al.* (1971) regarding the ancient pedigree of the ferredoxins, as well as those of Baltscheffsky *et al.,* (1966, 1999) in respect to pyrophosphate formation, just how these entities were bound in the membrane has not been addressed.

Although it seems probable that the organic thiols sequestering the iron-sulfur clusters would have been eventually replaced by the side chains of cysteine residues, cysteine itself has not been found beyond trace concentrations in hydrothermal experiments that synthesize the simple abiotic amino acids (Hennet *et al.,* 1992). Thus cysteine cannot be appealed to as a ligand at this earliest stage of evolution. Nor is it obvious just how phosphate could be held efficiently within the semipermeable iron sulfide membrane. Yet an evolutionary advantage would have been conferred upon those earliest regulated metabolists which developed molecules that bind and stabilize such anions and thereby control the distribution of electrons and protons through the membrane, so enhancing metabolism.

Here we suggest that the charges on these two anions, both indispensable to life's emergence, influenced short peptides comprising alternations of one to three glycines with other residues, in such a way as to produce a peptide nest of the type recognized recently as a common conformation in present day native proteins (Watson and Milner-White, 2002a).

2. Nests in Present Day Proteins

A novel, and surprisingly common, motif has been detected in proteins designated a nest (Watson and Milner-White, 2002a,b; Pal *et al.,* 2002). It incorporates from three to six consecutive main chain NH groups forming a concavity that binds anionic atoms or groups with a negative, or partial negative, charge. It occurs in a number of situations in proteins; the simplest, and commonest, kind has three NH groups and is illustrated in Figure 1a. The anionic atom in the nest shown is a main chain carbonyl oxygen with a partial negative charge. In most nests the anionic atom is firmly hydrogen bonded to the NH groups of residues i and i+2 and less

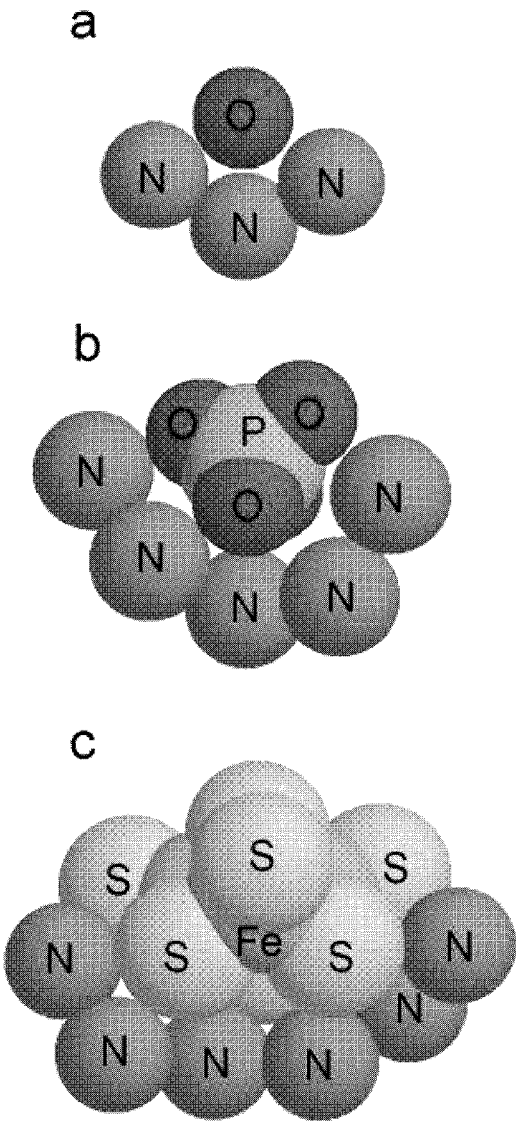

Figure 1. Nests binding anionic atoms or groups. Atom types are indicated by shading and most are labeled. For each peptide the main chain nitrogen atoms are displayed but the other main chain atoms are not. (a) A typical nest, taken from the C-terminus of an α-helix of myoglobin (Protein Data Bank code 1mbd) with a characteristic Schellmann/Paperclip loop; three successive main chain NH groups of residues 148–151 bind the carbonyl oxygen atom of residue 145 (this atom is anionic as it has a partial negative charge). (b) A compound nest from the P-loop of $p21^{ras}$ (5p21, Pai *et al*., 1990); the five successive main chain NH groups of residues 13–17 bind the β-phosphate of GTP. (c) A compound nest from the Fe_3S_4 binding loop of the ferredoxin from the archaebacterium *Sulfolobus sp.* (1xer; Fujii *et al.,* 1996). The six successive NH groups of residues 84-89 bind to the Fe_3S_4 center. The three cysteine sulfur atoms associated with the center are also shown.

strongly or hardly at all to the i+1 NH group, which often points slightly away. Simple nests such as these are extremely common in proteins especially as they are invariable components of common small hydrogen bonded motifs such as the Schellmann loop found at the C-termini of α-helices and β-bulge loops found at β-hairpin loops. The anionic atom in the nest is often a carbonyl oxygen, but other atoms are found too, such as OH or HOH oxygens or chloride ions. A few nests in protein crystal structures appear to be unoccupied.

Not uncommonly two, three or even four nests start at successive residues. They overlap in such a way that a single, deeper, concavity is formed with the four to six NH groups pointing approximately towards the center of the cavity. They are called compound nests or wide nests and are employed in several proteins for binding anionic groups, rather than single atoms. Several bind the phosphates of ligands, notably the P-loop, with its conserved GxxxxGKS/T consensus sequence, which is the commonest, and perhaps the most ancient, of the binding sites for nucleotides and other phosphate-containing molecules (Dreusicke and Schulz, 1986; Via *et al*., 2000). P-loops occur in ATPases like myosin, G-proteins like *ras*, some kinases such as adenylate kinase, and in many other proteins. The nest part of one, consisting of five successive NH groups, is illustrated in Figure 1b. It is seen to surround the β-phosphate of GTP closely. All P-loops are at the N-termini of α-helices such that the negative charge of the phosphate is bound by the non-hydrogen-bonded NH groups there. Knowing P-loops incorporate nests allows us to describe them more fully as wide nests situated at the N-termini of α-helices.

It might be supposed that nests are potentially flexible and would lose some of their concave anion-binding character when an available anion is absent. Some evidence for this comes from the wide nest in the P-loop protein family where it has been shown that the nest main chain conformations of some, though not all, of the residues in P-loops are lost (Vitagliano *et al*., 2001; Ramakrishnan *et al*., 2002) when the nest is not occupied by the ligand phosphate group.

A wide variety of proteins have iron-sulfur centers, one example of which is illustrated in Figure 2. Wide nests also occur regularly at binding sites for $[Fe_2S_2]$, $[Fe_3S_4]$ and $[Fe_4S_4]$ centers in proteins. About a half of such iron-sulfur centers are bound by one or more nests (Watson and Milner-White, 2002a). As well as the free sulfide atoms (i.e. sulfurs not bound covalently to carbon atoms) each iron atom in these iron sulfur centers (Beinert *et al*., 1997; Sticht and Rosch, 1998; Rees and Howard, 2003) usually binds the thiol of a cysteine residue. The sum of charges in the whole group, including contiguous sulfur and iron atoms, even if the iron atoms are oxidized, gives the group a net negative charge. Because sulfur atoms are less good at forming hydrogen bonds than oxygen atoms, the interactions between nest and FeS center can be said to be more electrostatic than hydrogen-bonded compared to oxygen-bound nests. A typical example, showing 6 successive NH groups bound to a Fe_3S_4center, is shown in Figure 1c.

Nests have a bearing on the oxidation-reduction potential of iron-sulfur centers. By stabilizing the reduced, somewhat more than the oxidized forms of iron-sulfur

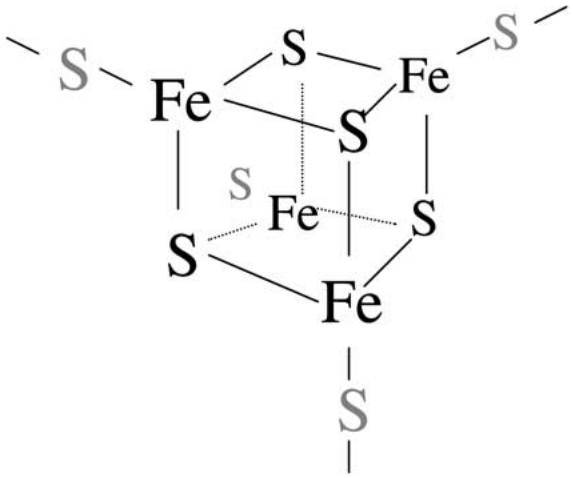

Figure 2. An iron-sulfur cube-shaped Fe_4S_4 module. The Fe_4S_4 center, as found in proteins, includes four sulfide sulfur atoms and is normally accompanied by four extra cysteine sulfur atoms, shown here in lighter shading.

centers, nests are expected to encourage the reduced forms and thereby reduce the Eh values.

Nests have approximately enantiomeric (mirror image) pairs of main chain torsion angles for successive amino acid residues: if $\phi = 90°$, $\psi = 0°$ for one residue, the next residue is $\phi = -90°$, $\psi = 0°$ and so on. Such conformations are favored by particular sequences. In present day native proteins an alternation of L-amino acids and glycines is typical of nest sequences. Since glycines are achiral, they are equally comfortable adopting the $\phi = 90°$, $\psi = 0°$ conformation favored by D-amino acids as the $\phi = -90°$, $\psi = 0°$ one favored by L-amino acids.

3. Nest Function in Early Peptides

The ability to bind phosphates and iron-sulfur centers would have had huge benefits for the earliest regulated metabolists. Both phosphates (inorganic and organic) and iron-sulfur modules will have featured in association with submarine hydrothermal precipitates of moderate temperature (Russell *et al*., 1994, 2003; Martin and Russell, 2003). It has been proposed (Baltscheffsky *et al*., 1999) that inorganic di- or triphosphates acted as high energy compounds in early metabolism so the effect of having nest peptides that bind them would be both to allow an increase in their availability and render them more stable, both enhancing metabolism. Alternatively, if the high energy compounds were nucleotide di- and triphosphates, as in present day metabolism, nest peptides are observed to bind their phosphate groups effectively, as in the P-loop proteins.

Some iron-sulfur centers would have existed both as components of minerals such as greigite; others would have occurred in solution as part of [4Fe-4S]4SR)$^{3-/2-}$ iron-sulfur clusters, where SR denotes organic thiols likely to occur at submarine seepage sites (Kaschke *et al*., 1994; Russell *et al*., 1994; Heinen and Lauwers, 1996; and see Bonomi *et al*., 1985). The organic thiols would be expected to take the place of the cysteine residues observed in present day iron-sulfur centers in proteins. During evolution it seems probable that the organic thiols would have been gradually replaced by the side chains of cysteine residues. Another possibility is the [2Fe-2S]4SR)$^{-/0}$center, but, being considerably less reduced, these may have been less common initially.

Iron-sulfur centers can catalyze a range of chemical reactions (Baymann *et al.,* 2003). Their most obvious property is that they can exist in oxidized or reduced forms at a wide range of Eh potentials so are part of dehydrogenases and oxidoreductases. They can also gain or lose one electron at a time and can also catalyze proton transfer (Chen *et al*., 2000). Hence they are useful for obtaining and transmitting the energy from electrons at various free energy levels (Cammack, 1996). In addition, FeS centers, acting as Lewis acids, can catalyze other categories of biologically useful reactions (Flint and Allen, 1996), especially those involving dehydration. They can also act as oxygen sensors (Allen, 1993). In association with other metals such as nickel and molybdenum the range of activities is extended further to include those of nitrogenase, carbon monoxide dehydrogenase and hydrogenase (Johnson, 1996).

4. Sequences and Encoding

To form nests one possibility is a sequence with alternating L- and D- amino acids. Another is alternating L-amino acids and glycines, or, of course, D-amino acids and glycines. Three observations favor alternating glycines and L-amino acids forming nests in early peptides. One is that the present day genetic code has codons for glycine but not for D-amino acids. A second comes from a reconstruction of the earliest codons by sequence analysis (Trifonov *et al*., 2001), suggesting that glycine and alanine were the first of the 20 codons to evolve and would thus have been the main amino acids in polypeptides at the earliest stages of evolution. Finally, it has been inferred (Hennet *et al*., 1992; Marshall, 1994) that glycines were by far the commonest amino acid occurring naturally during early evolution and that alanines were next most represented. For example, in hydrothermal experiments designed to simulate mixing at submarine springs of moderate temperature, Hennet *et al.* (1992) recorded ∼12mM of glycine and 1.3mM of alanine in experiments buffered/catalysed with FeS/FeS_2,Fe_3O_4, TiO_2 and Pt. Minor to trace concentrations of other amino acids were seen.

Another point about nests binding iron-sulfur centers or phosphates is the minimal requirement for ‘encoding’ such peptides. Although we do not know precisely

what molecules might have carried out such encoding, it seems plausible that some molecule, perhaps a polyribonucleotide, might have encouraged their formation. A four to six residue sequence with alternating glycines and any L-amino acid is all that is needed. The idea of alternating amino acids being favored in early peptides is not new, having been proposed by Fox (1959).

Once the module for iron-sulfur and phosphate binding evolved it is likely that genetic duplications would have generated molecules bringing multiple phosphate and/or iron-sulfur centers to be bound by a single polypeptide, not unlike present day polyferredoxins (e.g., Steigerwald *et al.* 1990). These would have been useful for electron transport systems. Also phosphate and iron-sulfur centers with polypeptide extensions acting as handles to allow them to be appropriately bound could have been another functional adaptation.

5. Discussion and Conclusions

A model for the origin of life formulated previously is that it emerged in structured iron monosulfide precipitates at submarine alkaline seepage sites from a redox, pH, and temperature gradient between, on the inside, sulfide-rich hydrothermal fluid, and, outside, the $Fe^{III/2+}$-containing acidulous waters comprising the earliest ocean (Russell *et al.*, 1994; Martin and Russell, 2003). Iron-sulfur minerals commonly occur in hydrothermal ore deposits and iron-sulfur centers are ubiquitous in living matter. Thus we have argued that the first membrane comprised iron monosulfide (FeS), with a rather more oxidised entity, greigite (as $[SNiS][Fe_4S_4][SFeS]$), its subsidiary. Although such a membrane would have kept the two aqueous fluids far from electrochemical equilibrium, left unaddressed was the manner in which the electron transfer iron-sulfur modules, as well as the phosphate/pyrophosphate moieties, were bound within the membrane (Russell *et al.*, 1994). Here we offer a solution.

The capacity to bind iron-sulfur centers and phosphates would have greatly increased the control of the distribution of electrons and protons through the membrane and thereby conferred benefits to a regulated metabolist operating in the confines of an iron sulfide membrane. Short peptides comprising alternations of two or three glycines with other residues would have been capable of binding both thiolates and phosphates. The interaction between these anionic groups with main chain NH groups would produce an assemblage in which the peptide would adopt a concave conformation or nest to accommodate the anion (Watson and Milner-White, 2002a). $[Fe_4S_4]$ clusters not incarcerated in the iron sulfide crystallites comprising the putative membrane may have been thiolated. Such entities would have been sequestered by the peptide nests. The resulting complexes would partition into the membrane where they might space themselves in arrays suitable to transfer electrons and interact with protons. The nest conformation is favored by high proportions of glycine residues and there is evidence for glycine being the

commonest amino acid during the stage of evolution when proteins were evolving (Hennet *et al.,* 1992). So it is likely nests would have been common features of the earliest peptides.

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